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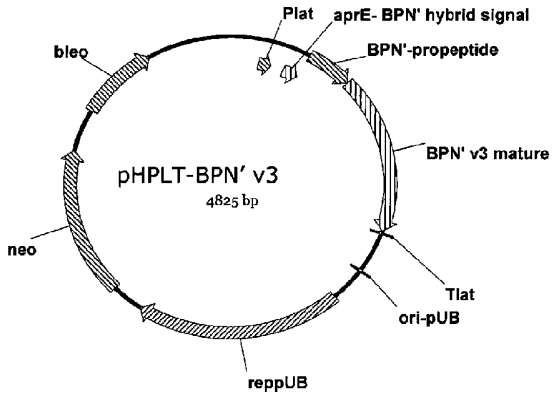
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[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS COMPRISING PROTEASE VARIANTS

FIGURE 1



(57) Abstract: The present invention provides protease variants, compositions comprising protease variants, and methods of using such protease variants and compositions.

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COMPOSITIONS AND METHODS COMPRISING PROTEASE VARIANTS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims priority to and benefit of U.S. Provisional Patent Application No. 61/285,127, filed on December 9, 2009, and U.S. Provisional Patent Application No. 61/392,373, filed on October 12, 2010, the disclosure of each of which is incorporated herein by reference in its entirety for all purposes.

FIELD OF THE INVENTION

The present invention provides protease variants, compositions comprising protease variants, and methods of using such protease variants and compositions thereof.

BACKGROUND OF THE INVENTION

Although proteases have long been known in the art of industrial enzymes, there remains a need for engineered proteases that are suitable for particular conditions and uses. The present invention fills these and other needs.

SUMMARY OF THE INVENTION

In a first aspect, the invention provides an isolated protease variant of a parent protease enzyme, the protease variant having proteolytic activity and comprising an amino acid sequence which comprises an alteration at one or more amino acid positions corresponding to amino acid positions of SEQ ID NO:2 selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217, wherein the at least one alteration is independently (i) an insertion of one or more amino acid residues upstream or downstream of the amino acid residue which occupies the position, (ii) a deletion of the amino acid residue which occupies the position, or (iii) a substitution of the amino acid residue which occupies the position with a different amino acid residue, wherein each amino acid position is numbered by correspondence with an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin protease BPN' set forth in SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2.

In a second aspect, the invention provides an isolated protease variant of a parent protease, the variant comprising an amino acid sequence comprising three amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2.

In a third aspect, the invention provides an isolated polypeptide having protease activity, said polypeptide comprising an amino acid sequence having at least 85% sequence identity to a polypeptide sequence selected from the group consisting of:

a) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+G131H+N243V+L257G;

b) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S033T+N076D;

c) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S009T+N109G+K141R+N243V;

d) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S162G+K256R;

e) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+A116T;

f) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+L257G;

g) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S162G+L257G;

h) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N061G+N109G+N243V;

i) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+N109G+N243V+S248A;

j) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S033T+N076D+N109G+N218S+N243V+S248N+K256R;

k) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+A116T+N243V+K256R;

l) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+G131H+N243V;

m) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G;

n) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+N243V;

o) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+T158S+L257G;

p) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N061S+N109G+N243V;

q) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+P040A+N109G+N243V+S248N+K256R;

r) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S009T+S018T+Y021N+N109G+K141R;

s) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+T158S+N243V+K256R;

t) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+T158S+N218S+L257G;

u) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+K256R;

v) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+N109G+N243V+K256R;

w) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+K256R;

x) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+N109G;

y) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S063G;

z) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+N076D;

aa) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S033T+N076D+N218S;

bb) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N076D+N218S; and

cc) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q, wherein each amino acid position of the variant is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2.

In a fourth aspect, the invention provides an isolated polypeptide having protease activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 95%, 96%, 97%, 98%, or 99% sequence identity to the polypeptide sequence of SEQ ID NO:6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least high stringency conditions with (i) the polynucleotide sequence of SEQ ID NO:5 or (ii) a complementary polynucleotide sequence of (i); and (c) a polypeptide encoded by a polynucleotide comprising a polynucleotide sequence having at least 95% sequence identity to the polynucleotide sequence of SEQ ID NO:5.

In a fifth aspect, the invention provides an isolated protease variant of a parent protease, wherein: (a) the variant comprises an amino acid sequence having no more than 20, 15, or 10 alterations relative to the parent protease, wherein (i) the alterations are independently selected from an insertion, a deletion, or a substitution, and (ii) the alterations include a substitution of glycine at positions 24 and 53, a substitution of asparagine at positions 78 and 101, a substitution of alanine or serine at position 128, and a substitution of glutamine at position 217, (b) the parent protease has at least 90% sequence identity to SEQ ID NO:2, (c) the amino acid sequence of SEQ ID NO:2 is used for determining position numbering; and (d) the variant has increased proteolytic activity relative to the parent protease, wherein each amino acid position is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2.

In a sixth aspect, the invention provides an isolated protease variant of a parent protease, wherein (a) the variant comprises an amino acid sequence (i) having at least 85% identity to the sequence of SEQ IDNO:2 and (ii) comprising a substitution of glycine at positions 24 and 53, a substitution of asparagine at positions 78 and 101, a substitution of alanine or serine at position 128, and a substitution of glutamine at position 217; (b) the parent protease has at least 85% sequence identity to SEQ ID NO:2; (c) each amino acid position of the variant is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2; and (d) the variant has increased proteolytic activity relative to the parent protease.

In another aspect, the invention provides an isolated or recombinant nucleic acid comprising a polynucleotide sequence encoding at least one polypeptide variant (e.g., protease variant) of the invention, or a complementary polynucleotide sequence thereof.

In another aspect, the invention provides an isolated or recombinant nucleic acid comprising a polynucleotide sequence having at least 80% sequence identity to the polynucleotide sequence set forth in SEQ ID NO:3 or SEQ ID NO:5, or a complementary polynucleotide sequence thereof.

In another aspect, the invention provides an expression vector comprising at least one nucleic acid of the invention. Also provided is a recombinant host cell or cell culture comprising at least one nucleic acid or an expression vector of the invention.

In another aspect, the invention provides a method of producing at least one polypeptide (e.g., protease variant) of the invention, the method comprising: (a) introducing a recombinant expression vector of the invention which encodes a polypeptide (e.g., protease variant) of the invention into a population of cells; (b) culturing the cells in a culture medium under conditions conducive to produce the polypeptide (e.g., protease variant) encoded by the expression vector; and optionally (c) isolating or recovering the variant from the cells or from the culture medium.

In another aspect, the invention provides a composition comprising at least one protease variant or polypeptide of the invention, optionally in combination with another enzyme. Such composition may comprise an adjunct ingredient, such as a surfactant and/or builder, or a carrier. Such composition may be a cleaning composition or a detergent composition and may be useful in cleaning methods described elsewhere herein. Such composition may be a fabric and home care product or such composition may not be a fabric and home care product.

In another aspect, the invention provides a method for cleaning an item, object, or surface in need of cleaning, the method comprising contacting the item, object, or surface with a polypeptide or protease variant of the invention or a composition of the invention, and optionally rinsing the item, object, or surface with water.

In another aspect, the invention provides a method for cleaning an item or surface (e.g., hard surface), the method comprising contacting at least a portion of the item or surface (e.g., hard surface) to be cleaned with a polypeptide or protease variant of the invention or a composition of the invention for a sufficient time and/or under conditions sufficient or effective to clean or wash the item or surface (e.g., hard surface) to a desired degree, and optionally comprising rinsing the item or surface (e.g., hard surface) with water.

Other aspects of the invention are described below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a plasmid map of pHPLT-BPN'-v3.

Figure 2 provides a plasmid map of pHPLT-BPN'-v3+S78N.

Figure 3 provides a plasmid map of pHPLT-BPN' partial opt.

Figure 4 provides a plasmid map of pHPLT-BPN'-v36.

Figure 5 provides an alignment of the mature reference subtilisin proteases including: BPN' (SEQ ID NO:2) and GG36 (SEQ ID NO:755). Each amino acid position of each protease variant described herein, including each cold water protease variant, is numbered according to the numbering of the corresponding amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin protease BPN' (SEQ ID NO:2), as shown in Figure 5, as determined by alignment of the

protease variant amino acid sequence with the *Bacillus amyloliquefaciens* subtilisin protease BPN' amino acid sequence. Thus, unless otherwise specified herein, substitution positions are given in relationship to BPN'.

Figure 6 provides map of pHPLT-GG36.

Figure 7 provides a map of pRA68.

Figure 8 provides a map of pRA96.

DESCRIPTION OF THE INVENTION

Definitions

Unless otherwise indicated, the practice of the present invention involves conventional techniques commonly used in molecular biology, protein engineering, microbiology, and recombinant DNA, which are within the skill of the art. Such techniques are known to those of skill in the art and are described in numerous texts and reference works well known to those of skill in the art. All patents, patent applications, articles and publications mentioned herein, both *supra* and *infra*, are hereby expressly incorporated herein by reference.

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Many technical dictionaries are known to those of skill in the art. Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, some suitable methods and materials are described herein. Accordingly, the terms defined immediately below are more fully described by reference to the specification as a whole. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary, depending upon the context they are used by those of skill in the art.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of protein purification, molecular biology, microbiology, recombinant DNA techniques and protein sequencing, all of which are within the skill of those in the art.

Furthermore, the headings provided herein are not limitations of the various aspects of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole. Nonetheless, in order to facilitate understanding of the invention, a number of terms are defined below.

As used herein, the terms "protease" and "proteinase" refer to an enzyme protein that has the ability to break down other proteins. A protease has the ability to conduct "proteolysis," which begins protein catabolism by hydrolysis of peptide bonds that link amino acids together in a peptide or polypeptide chain forming the protein. This activity of a protease as a protein-digesting enzyme is

referred to as "proteolytic activity." Many well known procedures exist for measuring proteolytic activity (see, e.g., Kalisz, "Microbial Proteinases," *In*: Fiechter (ed.), Advances in Biochemical Engineering/Biotechnology (1988)). For example, proteolytic activity may be ascertained by comparative assays which analyze the respective protease's ability to hydrolyze a commercial substrate. Exemplary substrates useful in the analysis of protease or proteolytic activity, include, but are not limited to, di-methyl casein (Sigma C-9801), bovine collagen (Sigma C-9879), bovine elastin (Sigma E-1625), and bovine keratin (ICN Biomedical 902111). Colorimetric assays utilizing these substrates are well known in the art (see, e.g., WO 99/34011 and U.S. Pat. No. 6,376,450, both of which are incorporated herein by reference). The pNA assay (see, e.g., Del Mar et al., Anal. Biochem. 99:316-320 [1979]) also finds use in determining the active enzyme concentration for fractions collected during gradient elution. This assay measures the rate at which *p*-nitroaniline is released as the enzyme hydrolyzes the soluble synthetic substrate, succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (suc-AAPF-pNA). The rate of production of yellow color from the hydrolysis reaction is measured at 410 nm on a spectrophotometer and is proportional to the active enzyme concentration. In addition, absorbance measurements at 280 nanometers (nm) can be used to determine the total protein concentration. The active enzyme/total protein ratio gives the enzyme purity.

As used herein, the term "subtilisin" refers any member of the S8 serine protease family as described in MEROPS - The Peptidase Data base (see Rawlings et al., MEROPS: the peptidase database, Nucl. Acids Res., 34 Database issue, D270-272 [2006]). As described therein, the peptidase family S8 contains the serine endopeptidase subtilisin and its homologues (Rawlings and Barrett, Biochem. J. 290:205-218, [1993]). Family S8, also known as the subtilase family, is the second largest family of serine peptidases. The tertiary structures for several members of family S8 have now been determined. A typical S8 protein structure consists of three layers with a seven-stranded β sheet sandwiched between two layers of helices. Subtilisin (S08.001) is the type structure for clan SB (SB). Despite the different structure, the active sites of subtilisin and chymotrypsin (S01.001) can be superimposed, which suggests the similarity is the result of convergent rather than divergent evolution.

A "protease variant" (or "variant protease") may refer to a protease that differs in its amino acid sequence from the amino acid sequence of a reference protease or parent protease by at least one amino acid residue. A parent protease or reference protease need not be a wild-type protease, but may itself be a variant of a wild-type protease. It is not intended that the reference or parent protease be limited to any particular amino acid sequence. A protease variant of a reference or parent protease may comprise an amino acid sequence comprising at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence of the parent protease or reference protease and at least one amino acid substitution, insertion, or deletion relative to the amino acid sequence of the parent protease or reference protease. In one aspect, the invention includes a variant of a serine protease, wherein the variant has at least one mutation relative to the serine protease. In one aspect, the present

invention includes a "BPN' variant" (or "BPN' subtilisin variant") comprising an amino acid sequence comprising one or more mutations relative to the mature BPN' sequence of SEQ ID NO:2.

A parent protease or reference protease can be, but is not limited to, e.g., a known protease (including, but not limited to, e.g., BPN') or a commercially available protease or a variant of the commercially available protease. A parent protease or reference protease may itself be a variant of a known or commercially available protease. A protease variant can be derived from a parent protease that is commercially available or a variant of such commercially available parent protease. Commercially available proteases, include, but are not limited to, e.g., proteases sold under the tradenames SAVINASE®, POLARZYME®, KANNASE®, LIQUANASE®, LIQUANASE ULTRA®, SAVINASE ULTRA®, OVOZYME®, (by Novozymes A/S); MAXACAL®, PROPERASE®, PURAFECT®, FN3®, FN4® and PURAFECT OXP®, PURAFAST™, PURAFECT® PRIME, PURAMAX® (by Danisco US Inc., formerly Genencor International, Inc.); and those available from Henkel/Kemira, namely BLAP (amino acid sequence shown in Figure 29 of US 5,352,604 with the following mutations S99D+S101R+S103A+ V104I+G159S, hereinafter referred to as BLAP) and BLAP X (BLAP with S3T+V4I+V205I).

As used herein, a "cold water protease" is an enzyme that exhibits one or more of the following four criteria: (a) a performance index of at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from 1.1 to about 10, from 1.1 to about 8, or even from 1.1 to about 5 on BMI at pH 8 and 16°C (60°F) when compared to PURAFECT® Prime (SEQ ID NO:2 with the amino acid substitution Y217L), as defined in the "Test Method" set forth herein in Part I Example 1; (b) a performance index of at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from 1.3 to about 10, from 1.3 to about 8, or even from 1.3 to about 5 on BMI at pH 8 and 16°C (60°F) when compared to BPN' (SEQ ID NO:2), as defined in the "Test Method" set forth herein in Part I Example 1; (c) a performance index of at least 0.9, at least 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from 0.9 to about 10, from 0.9 to about 8, or even from 0.9 to about 5 on BMI at pH 8 and 16°C (60°F) when compared to BPN'-v3 (SEQ ID NO:4), as defined in the "Test Method" set forth herein in Part I Example 1; and/or (d) a performance index of at least 0.9, at least 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from 0.9 to about 10, from 0.9 to about 8, from 0.9 to about 5, from 1.0 to about 10, from 1.0 to about 8, or even from 1.0 to about 5 on BMI at pH 8 and 16°C (60°F) when compared to BPN'-v36 (SEQ ID NO:6), as defined in the "Test Method" set forth herein in Part I Example 1.

Some suitable cold water proteases are derived from subtilisins, particularly those derived from subtilisin BPN' (SEQ ID NO:2). A cold water protease can be a variant of BPN' having the amino acid sequence of SEQ ID NO:2 (e.g., "BPN' variant" or "BPN' subtilisin variant"). Some such cold water proteases comprise one or more of the amino acid substitutions set forth herein.

As used herein, the genus *Bacillus* includes all species within the genus *Bacillus*, as known to those of skill in the art, including but not limited to *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. clausii*, *B. halodurans*, *B. megaterium*, *B. coagulans*, *B. circulans*, *B. lautus*, and *B. thuringiensis*. It is recognized that the genus *Bacillus* continues to undergo taxonomical reorganization. Thus, it is intended that the genus include species that have been reclassified, including but not limited to such organisms as *B. stearothermophilus*, which is now named "*Geobacillus stearothermophilus*." The production of resistant endospores in the presence of oxygen is considered the defining feature of the genus *Bacillus*, although this characteristic also applies to the recently named *Alicyclobacillus*, *Amphibacillus*, *Aneurinibacillus*, *Anoxybacillus*, *Brevibacillus*, *Filobacillus*, *Gracilibacillus*, *Halobacillus*, *Paenibacillus*, *Salibacillus*, *Thermobacillus*, *Ureibacillus*, and *Virgibacillus*.

The terms "polynucleotide" and "nucleic acid," which are used interchangeably herein, refer to a polymer of any length of nucleotide monomers covalently bonded in a chain. DNA (deoxyribonucleic acid), a polynucleotide comprising deoxyribonucleotides, and RNA (ribonucleic acid), a polymer of ribonucleotides, are examples of polynucleotides or nucleic acids having distinct biological function. Polynucleotides or nucleic acids include, but are not limited to, a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The following are non-limiting examples of polynucleotides: genes, gene fragments, chromosomal fragments, expressed sequence tag(s) (EST(s)), exons, introns, messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), ribozymes, complementary DNA (cDNA), recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. Some polynucleotides comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. A sequence of nucleotides may be interrupted by non-nucleotide components.

As used herein, the term "vector" refers to a nucleic acid construct or polynucleotide construct used to introduce or transfer nucleic acid(s) or polynucleotide(s) into a target cell or tissue. A vector is typically used to introduce foreign DNA into another cell or tissue. A vector generally comprises a DNA sequence that is a transgene and a larger polynucleotide sequence that serves as the "backbone" of the vector. The vector typically serves to transfers genetic information, such as the inserted transgene, to a target cell or tissue so as to isolate, multiply, or express the insert in the target cell or tissue. Vectors include plasmids, cloning vectors, bacteriophages, viruses (e.g., viral vector), cosmids, expression vectors, shuttle vectors, cassettes, and the like. A vector typically includes an origin of replication, a multicloning site, and a selectable marker. The process of inserting a vector into a target cell is typically referred to as transformation in bacterial and yeast cells and as transfection in mammalian cells. The present invention includes a vector that comprises a DNA sequence encoding a protease variant (e.g.,

precursor or mature protease variant) that is operably linked to a suitable prosequence (e.g., secretory, signal peptide sequence, etc.) capable of effecting the expression of the DNA sequence in a suitable host.

As used herein, the term "expression cassette" or "expression vector" refers to a nucleic acid construct or vector generated recombinantly or synthetically for the expression of a nucleic acid of interest (e.g., a foreign nucleic acid or transgene) in a target cell. The nucleic acid of interest typically expresses a protein of interest. An expression vector or expression cassette typically comprises a promoter nucleotide sequence that drives or promotes expression of the foreign nucleic acid. The expression vector or cassette also typically includes any other specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. A recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Some expression vectors have the ability to incorporate and express heterologous DNA fragments in a host cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those of skill in the art. Selection of appropriate expression vectors for expression of a protein from a nucleic acid sequence incorporated into the expression vector is within the knowledge of those of skill in the art.

A DNA construct is an artificially constructed segment of nucleic acid that may be introduced into a target cell or tissue. A DNA construct typically comprises a DNA insert comprising a nucleotide sequence encoding a protein of interest that has been subcloned into a vector. The vector may contain bacterial resistance genes for growth in bacteria and a promoter for expression of the protein of interest in an organism. The DNA may be generated *in vitro* by PCR or any other suitable technique(s) known to those in the art. The DNA construct may comprise a nucleic acid sequence of interest. In one aspect, the sequence is operably linked to additional elements such as control elements (e.g., promoters, etc.). The DNA construct may further comprise a selectable marker and may further comprise an incoming sequence flanked by homology boxes. The construct may comprise other non-homologous sequences, added to the ends (e.g., stuffer sequences or flanks). The ends of the sequence may be closed such that the DNA construct forms a closed circle. The nucleic acid sequence of interest, which is incorporated into the DNA construct, using techniques well known in the art, may be a wild-type, mutant, or modified nucleic acid. The DNA construct may comprise one or more nucleic acid sequences homologous to the host cell chromosome. The DNA construct may comprise one or more non-homologous nucleotide sequences. Once the DNA construct is assembled *in vitro,* it may be used, e.g., to: 1) insert heterologous sequences into a desired target sequence of a host cell; and/or 2) mutagenize a region of the host cell chromosome (i.e., replace an endogenous sequence with a heterologous sequence); 3) delete target genes; and/or 4) introduce a replicating plasmid into the host. "DNA construct" is used interchangeably herein with "expression cassette."

As used herein, a "plasmid" refers to an extrachromosomal DNA molecule which is capable of replicating independently from the chromosomal DNA. A plasmid is double stranded (ds) and may be circular and is typically used as a cloning vector.

As used herein in the context of introducing a nucleic acid sequence into a cell, the term "introduced" refers to any method suitable for transferring the nucleic acid sequence into the cell. Such methods for introduction include but are not limited to protoplast fusion, transfection, transformation, electroporation, conjugation, and transduction (see, e.g., Ferrari et al., "Genetics," in Hardwood et al. (eds.), Bacillus, Plenum Publishing Corp., pp. 57-72 [1989]).

Transformation refers to the genetic alteration of a cell which results from the uptake, genomic incorporation, and expression of genetic material (e.g., DNA).

As used herein, a nucleic acid is "operably linked" with another nucleic acid sequence when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably linked to a nucleotide coding sequence if the promoter affects the transcription of the coding sequence. A ribosome binding site may be operably linked to a coding sequence if it is positioned so as to facilitate translation of the coding sequence. Typically, "operably linked" DNA sequences are contiguous. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, synthetic oligonucleotide adaptors or linkers may be used in accordance with conventional practice.

As used herein, "recombinant" when used with reference to a cell typically indicates that the cell has been modified by the introduction of a heterologous nucleic acid sequence or that the cell is derived from a cell so modified. For example, a recombinant cell may comprise a gene not found in identical form within the native (non-recombinant) form of the cell, or a recombinant cell may comprise a native gene (found in the native form of the cell) but which has been modified and re-introduced into the cell. A recombinant cell may comprise a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques known to those of ordinary skill in the art. Recombinant DNA (rDNA) is a form of artificial DNA that is created by combining two or more nucleotide sequences that would not normally occur together through the process of gene splicing. Recombinant DNA technology includes techniques for the production of recombinant DNA *in vitro* and transfer of the recombinant DNA into cells where it may be expressed or propagated, thereby producing a recombinant polypeptide.

As used herein, the term nucleic acid or gene "amplification" refers to a process by which specific DNA sequences are disproportionately replicated such that the amplified nucleic acid or gene becomes present in a higher copy number than was initially present in the genome. Selection of cells by growth in the presence of a drug (e.g., an inhibitor of an inhibitable enzyme) may result in the amplification of either the endogenous gene encoding the gene product required for growth in the presence of the drug or by amplification of exogenous (i.e., input) sequences encoding this nucleic acid or gene product or both.

As used herein, the term "primer" refers to an oligonucleotide (a polymer of nucleotide residues), whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable

of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced (i.e., in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). A primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer may comprise an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact length of a primer depends on a variety of factors, including temperature, source of primer, and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, which is typically capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labeled with any "reporter molecule," so that it is detectable in any detection system, including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the invention be limited to any particular detection system or label.

As used herein, the term "polymerase chain reaction" (PCR) refers to the methods of U.S. Patent Nos. 4,683,195 4,683,202, and 4,965,188, hereby incorporated by reference, which include methods for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence is well known in the art.

As used herein, the term "amplification reagents" refers to those reagents (e.g., deoxyribonucleotide triphosphates, buffer, etc.) needed for amplification except for primers, nucleic acid template, and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

As used herein, the term "restriction endonuclease" or "restriction enzyme" refers to an enzyme (e.g., bacterial enzyme) that is capable of cutting double-stranded or single-stranded DNA at or near a specific sequence of nucleotides known as a restriction site. The nucleotide sequence comprising the restriction site is recognized and cleaved by a given restriction endonuclease or restriction enzyme and is frequently the site for insertion of DNA fragments. A restriction site can be engineered into an expression vector or DNA construct.

As is known in the art, a DNA sequence can be transcribed by an RNA polymerase to produce an RNA sequence, but an RNA sequence can be reverse transcribed by reverse transcriptase to produce a DNA sequence.

"Host strain" or "host cell" refers to a suitable host for an expression vector comprising a DNA sequence of interest. A DNA sequence of interest may express a protein of interest in the host strain or host cell.

A "protein" or "polypeptide" or "peptide" is a polymeric sequence of amino acid residues. A carboxyl group of one amino acid is linked to the amino group of another. The terms "protein" and "polypeptide" and "peptide" may be used interchangeably herein. A peptide comprises two or more amino acids. Peptides typically contain fewer amino acids than do polypeptides or proteins. The single and 3-letter code for amino acids as defined in conformity with the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) is used through out this disclosure. The single letter X refers to any of the twenty amino acids. It is also understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code.

In describing enzyme variants, the following nomenclature is used typically for ease of reference: Original amino acid(s):position(s):substituted amino acid(s). The accepted IUPAC single letter or triple letter amino acid abbreviation is employed. The single letter "X" refers to any amino acid residue However, when in the context of an amino acid substitution (e.g. "X003C"), it is to be understood that "X" refers to an amino acid residue other than the amino acid residue resulting from the substitution (e.g., X is an amino acid residue other than C). Mutations are typically named by the one letter code for the parent amino acid, followed by a three or two or one digit amino acid position number in an amino acid sequence and then the one letter code for the substituted amino acid. For example, mutating the amino acid glycine (G) at amino acid position 87 in an amino acid sequence by substituting to the amino acid serine (S) for glycine (G) is represented as "G087S" or "G87S". Typically, the substitution of a glycine at position 2 with a threonine is represented as G002T; however, such substitution may also be represented as G02T or G2T. One or two leading zeroes ("0") may be included simply to provide a convenient three number designation for each amino acid position. The amino acid position "001" is the same as "1" and thus "A001C" is the same as "A1C". "X001G" refers to the substitution of glycine (G) at amino acid position 1 in an amino acid sequence, wherein the amino acid that is to be replaced by glycine is any amino acid. Multiple mutations are indicated by inserting a "-" between the mutations or by using a plus (+) sign between the mutations. For example, amino acid substitutions at amino acid residue positions 87 and 90 in an amino acid sequence are represented as either "G087S-A090Y" or "G87S-A90Y" or "G87S + A90Y" or "G087S + A090Y". For deletions, the one letter code "Z" is used. For an insertion relative to the parent sequence, the one letter code "Z" is on the left side of the position number. For a deletion, the one letter code "Z" is on the right side of the position number. For insertions, the position number is the position number before the inserted amino acid(s), plus 0.01 for each amino acid. For example, an insertion of three amino acids alanine (A), serine (S) and tyrosine (Y) between position 87 and 88 is shown as "Z087.01A-Z087.02S-Z087.03Y." Thus, combining all the mutations above plus a deletion at position 100 is: "G087S- Z087.01A-Z087.02S-Z087.03Y-A090Y-A100Z."

A "prosequence" or "propetide sequence" refers to an amino acid sequence between the signal peptide sequence and mature protease sequence that is necessary for the secretion of the protease. Cleavage of the prosequence or propeptide sequence results in a mature active protease.

The term "signal sequence" or "signal peptide" refers to a sequence of amino acid residues that may participate in the secretion or direct transport of the mature or precursor form of a protein. The signal sequence is typically located N-terminal to the precursor or mature protein sequence. The signal sequence may also be referred to as a leader sequence. The signal sequence may be endogenous or exogenous. One exemplary exogenous signal sequence comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536). A signal sequence is normally absent from the mature protein. A signal sequence is typically cleaved from the protein by a signal peptidase after the protein is transported.

The term "hybrid signal sequence" refers to signal sequences in which part of sequence is obtained from the expression host fused to the signal sequence of the gene to be expressed. Synthetic sequences can be utilized.

The term "mature" form of a protein, polypeptide, or peptide refers to the functional form of the protein, polypeptide, or peptide without the signal peptide sequence and propeptide sequence.

The term "precursor" form of a protein or peptide refers to a mature form of the protein having a prosequence operably linked to the amino or carbonyl terminus of the protein. The precursor may also have a "signal" sequence operably linked to the amino terminus of the prosequence. The precursor may also have additional polynucleotides that are involved in post-translational activity (e.g., polynucleotides cleaved therefrom to leave the mature form of a protein, polypeptide, or peptide).

The term "wild-type" in reference to an amino acid sequence or nucleic acid sequence indicates that the amino acid sequence or nucleic acid sequence is native or naturally occurring sequence. As used herein, the term "naturally-occurring" refers to anything (e.g., proteins, amino acids, or nucleic acid sequences) that is found in nature (e.g., has not been manipulated by means of recombinant or chemical methods). As used herein, the term "non-naturally occurring" refers to anything that is not found in nature (e.g., recombinant or chemically synthesized nucleic acids produced in the laboratory).

An amino acid residue in a particular amino acid sequence may be numbered by correspondence with an amino acid residue in a position of a reference amino acid sequence. An amino acid residue of an amino acid sequence of interest which is in a position that "corresponds to" or is "corresponding to" or in "correspondence with" the position of an amino acid residue of a reference amino acid sequence indicates that the amino acid residue of the sequence of interest is located at a position that is equivalent or homologous to the position of an amino acid residue in the reference amino acid sequence. One skilled in the art can determine whether a particular residue position in a polypeptide corresponds to a position of a homologous reference sequence. For example, a protease variant may be aligned with that of a reference sequence (e.g., BPN' sequence of SEQ ID NO:2) using known techniques. The positions of the amino acid residues in the reference sequence are used for numbering of the amino acid residues in the sequence of interest. Accordingly, the amino acid residues of the protease variant may be numbered according to the corresponding amino acid residue position numbering of the reference sequence. For example, the

amino acid residues in the reference sequence of SEQ ID NO: 2 may be used for determining amino acid residue position numbering of each amino acid residue of a protease variant of interest.

As used herein, "homology" refers to sequence similarity or identity, with identity being preferred. Homology may be determined using standard techniques known in the art (see, e.g., Smith and Waterman, Adv. Appl. Math. 2:482 [1981]; Needleman and Wunsch, J. Mol. Biol. 48:443 [1970; Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 [1988]; software programs such as GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, Madison, WI); and Devereux et al., Nucl. Acid Res. 12:387-395 [1984]). One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pair-wise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (see Feng and Doolittle, J. Mol. Evol. 35:351-360 [1987]). The method is similar to that described by Higgins and Sharp (see Higgins and Sharp, CABIOS 5:151-153 [1989]). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps. Another example of a useful algorithm is the BLAST algorithm, described by Altschul et al., (see Altschul et al., J. Mol. Biol. 215:403-410 [1990]; and Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 [1993]). A particularly useful BLAST program is the WU-BLAST-2 program (see Altschul et al., Meth. Enzymol. 266:460-480 [1996]). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. However, the values may be adjusted to increase sensitivity.

The percent sequence identity (% sequence identity or simply % identity) between a subject polypeptide sequence and a reference polypeptide sequence means that the subject amino acid sequence is identical on an amino acid residue-by-amino acid residue basis by a specified percentage to the reference polypeptide sequence over a comparison length when the sequences are optimally aligned, as determined, for example, by an amino acid sequence comparison algorithm or visual inspection. The percent sequence identity between a subject nucleic acid sequence and a reference nucleic acid sequence similarly means the subject nucleotide sequence is identical on a nucleic acid residue-by-nucleic acid residue basis by a specified percentage to the reference nucleotide sequence over a comparison length when the sequences are optimally aligned.

The percent sequence identity (percent identity or % sequence identity or % identity) between a reference sequence and a subject sequence of interest may be readily determined by one skilled in the art. The percent identity shared by two polypeptide sequences can be determined, for example, by direct comparison of the amino acid residues in each sequence by aligning the residues of the respective sequences for maximum similarity and determining the number of identical amino acid residues between

the sequences by using a sequence comparison algorithm known in the art or by visual inspection. The two optimally aligned polypeptide sequences can be compared over the comparison length and the number of positions in the optimal alignment at which identical amino acid residues occur in both polypeptide sequences can be determined, thereby providing the number of matched positions, and the number of matched positions is then divided by the total number of positions over the comparison length. The resulting number is multiplied by 100 to yield the percent identity of the subject polypeptide sequence to the reference (or query) polypeptide sequence. The percent identity shared by two nucleic acid sequences can be similarly determined by direct comparison of the nucleotide residues in each sequence by aligning the residues of the respective sequences for maximum similarity and determining the number of identical nucleic acid residues between the nucleic acid sequences by using a sequence comparison algorithm or by visual inspection. The percent identity between two or more sequences may also be described as the sequences being a particular percent identical.

An example of an algorithm that is suitable for determining sequence identity is the BLAST algorithm, (see Altschul, et al., J. Mol. Biol., 215:403-410 [1990]). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 [1992]) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin and Altschul, *supra*). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a protease-encoding nucleic acid of this invention if the smallest sum probability in a comparison of the test nucleic acid to a protease-encoding nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. Where the test nucleic acid encodes a protease polypeptide, it is considered similar to a specified protease-encoding nucleic acid if the comparison results in a smallest sum probability of less than about 0.5, and more preferably less than about 0.2.

"Optimal alignment" or "optimally aligned" refers to the alignment of two (or more) sequences giving the highest percent identity score. For example, optimal alignment of two polypeptide sequences can be achieved by manually aligning the sequences such that the maximum number of identical amino acid residues in each sequence are aligned together or by using software programs or procedures described herein or known in the art. Optimal alignment of two nucleic acid sequences can be achieved by manually aligning the sequences such that the maximum number of identical nucleotide residues in each sequence are aligned together or by using software programs or procedures described herein or known in the art.

Two sequences (e.g., polypeptide sequences) may be deemed "optimally aligned" when they are aligned using defined parameters, such as a defined amino acid substitution matrix, gap existence penalty (also termed gap open penalty), and gap extension penalty, so as to achieve the highest identity score possible for that pair of sequences. The BLOSUM62 scoring matrix (see Henikoff and Henikoff, *supra*) is often used as a default scoring substitution matrix in polypeptide sequence alignment algorithms (e.g., BLASTP). The gap existence penalty is imposed for the introduction of a single amino acid gap in one of the aligned sequences, and the gap extension penalty is imposed for each residue position in the gap. Exemplary alignment parameters employed are: BLOSUM62 scoring matrix, gap existence penalty=11, and gap extension penalty=1. The alignment score is defined by the amino acid positions of each sequence at which the alignment begins and ends (e.g., the alignment window), and optionally by the insertion of a gap or multiple gaps into one or both sequences, so as to achieve the highest possible similarity score.

Optimal alignment between two or more sequences can be determined manually by visual inspection or by using a computer, such as, but not limited to e.g., the BLASTP program for amino acid sequences and the BLASTN program for nucleic acid sequences (see, e.g., Altschul et al., Nucleic Acids Res. 25(17):3389-3402 (1997); see also the National Center for Biotechnology Information (NCBI) website) or CLUSTALW program.

A polypeptide of interest may be said to be "substantially identical" to a reference polypeptide if the polypeptide of interest comprises an amino acid sequence having at least about 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% sequence identity to the amino acid sequence of the reference polypeptide. The percent identity between two such polypeptides can be determined manually by inspection of the two optimally aligned polypeptide sequences or by using software programs or algorithms (e.g., BLAST, ALIGN, CLUSTAL) using standard parameters. One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, e.g., where the two peptides differ only by a conservative amino acid substitution or one or more conservative amino acid substitutions.

A nucleic acid of interest may be said to be "substantially identical" to a reference nucleic acid if the nucleic acid of interest comprises a nucleotide sequence having at least about 60%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% sequence identity to the nucleotide sequence of the reference nucleic acid. The percent identity between two such nucleic acids can be determined manually by inspection of the two optimally aligned nucleic acid sequences or by using software programs or algorithms (e.g., BLAST, ALIGN, CLUSTAL) using standard parameters. One indication that two nucleic acid sequences are substantially identical is that the two nucleic acid molecules hybridize to each other under stringent conditions (e.g., within a range of medium to high stringency).

As used herein, "isolated" in reference to a particular component of interest means that component is essentially or substantially free of other components. For example, an "isolated" polypeptide means the polypeptide is essentially or substantially free of other components, including, but not limited to, e.g., other polypeptides and cellular components. An "isolated" nucleic acid means the nucleic acid is essentially or substantially free of other components, including, but not limited to, e.g., other nucleic acids and cellular components. For purposes of this application, "isolated" refers to nucleic acids or polypeptides that are not part of a library (e.g., screening library).

Purity and homogeneity are typically determined using analytical chemistry techniques, such as polyacrylamide gel electrophoresis or high-performance liquid chromatography. A protein or nucleic acid that is the predominant species present in a preparation is substantially purified. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel, chromatographic eluate, and/or a media subjected to density gradient centrifugation. Particularly, "purified" means that when isolated, the isolate contains at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 99.5% or more of nucleic acid or protein by weight of the isolate. Purified polypeptides may be obtained by a number of methods including, but not limited to, e.g., laboratory synthesis, chromatography (e.g., high-performance liquid chromatography) preparative electrophoresis, polyacrylamide gel electrophoresis followed by visualization upon staining, centrifugation, precipitation, affinity purification, etc. (see, generally, R Scopes, Protein Purification, Springer-Verlag, N.Y. (1982), Deutscher, Methods in Enzymol., Vol. 182: Guide to Protein Purification, Academic Press, Inc. N.Y. (1990)). The invention includes an isolated or purified polypeptides (e.g., isolated protease variants or subtilisin variants of the invention) and isolated or purified nucleic acids (e.g., nucleic acids encoding protease variants or subtilisin variants of the invention).

In a related sense, the invention provides methods of enriching compositions for one or more molecules of the invention, such as one or more polypeptides of the invention (e.g., one or more protease variants of the invention) or one or more nucleic acids of the invention (e.g., one or more nucleic acids encoding one or more protease variants of the invention). A composition is enriched for a molecule when there is a substantial increase in the concentration of the molecule after application of a purification or enrichment technique. A substantially pure polypeptide or nucleic acid will typically comprise at least

about 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98, 99%, 99.5% or more by weight (on a molar basis) of all macromolecular species in a particular composition.

As used herein, the term "combinatorial mutagenesis" refers to methods in which libraries of nucleic acid variants of a reference nucleic acid sequence are generated. In these libraries, the variants contain one or several mutations chosen from a predefined set of mutations. The methods also provide means to introduce random mutations which were not members of the predefined set of mutations. Some such methods include those set forth in U.S. Patent No. 6,582,914, hereby incorporated by reference. Some such combinatorial mutagenesis methods include and/or encompass methods embodied in commercially available kits (e.g., QUIKCHANGE® Multi Site-Directed Mutagenesis Kit (Stratagene)).

As used herein, having "improved properties" used in connection with a protease variant refers to a protease variant having improved properties compared to a reference or parent protease. Protease variants of the invention may exhibit one of more of the following properties: enhanced or improved proteolytic activity, enhanced or improved stability, enhanced or improved ability to clean a surface or item, enhanced or improved cleaning performance, enhanced or improved fabric or laundry cleaning performance or wash performance, enhanced or improved hand wash performance, enhanced or improved hand or manual dishwashing performance, enhanced or improved automatic dishwashing performance, enhanced or improved laundry performance compared to a reference protease or parent protease of interest.

As used herein, the term "functional assay" refers to an assay that provides an indication of a protein's activity. The term typically refers to assay systems in which a protein is analyzed for its ability to function in its usual capacity. For example, in the case of enzymes, a functional assay involves determining the effectiveness of the enzyme in catalyzing a reaction.

The term "property" or grammatical equivalents thereof in the context of a molecule may refer to any characteristic or attribute of the molecule that can be selected or detected. For example, in the context of a polypeptide, a property may be enzymatic activity (e.g., proteolytic activity), stability, or other property.

A "mutant" nucleic acid sequence typically refers to a nucleic acid sequence that has an alteration in at least one codon occurring in a host cell's wild-type sequence such that the expression product of the mutant nucleic acid sequence is a protein with an altered amino acid sequence relative to the wild-type protein. The expression product may have an altered functional capacity (e.g., enhanced enzymatic activity).

As used herein, the term "net charge" is defined as the sum of all charges present in a molecule. "Net charge changes" can be made to a parent protein molecule to provide a protein variant that has a net charge that differs from that of the parent protein molecule (i.e., the variant has a net charge that is not the same as that of the parent molecule). For example, substitution of a neutral amino acid of a protein with a negatively charged amino acid or substitution of a positively charged amino acid of a protein with a neutral amino acid results in net charge of -1 with respect to the unmodified protein. Substitution of a

positively charged amino acid of a protein with a negatively charged amino acid results in a net charge of -2 with respect to the unmodified protein. Substitution of a neutral amino acid of a protein with a positively charged amino acid or substitution of a negatively charged amino acid of a protein with a neutral amino acid results in net charge of +1 with respect to the parent. Substitution of a negatively charged amino acid of a protein with a positively charged amino acid results in a net charge of +2 with respect to the unmodified protein. The net charge of a parent protein can also be altered by deletion and/or insertion of one or more charged amino acids.

The terms "thermally stable" and "thermostable" and "thermostability" in reference to a polypeptide indicates that the polypeptide is resistant to a permanent change in its activity caused solely by heat, such as, e.g., via exposure to higher temperature. For example, a thermally stable enzyme means that the enzyme is resistant to a permanent change in its enzymatic activity caused solely by heat, such as, e.g., via exposure to higher temperature. Typically, a protease that is thermally stable is able to retain at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% of its proteolytic activity after exposure to increased temperatures over a given time period, e.g., at least about 60 minutes, 120 minutes, 180 minutes, 240 minutes, 300 minutes, etc.

Cleaning activity of a polypeptide or protease may refer to a cleaning performance achieved by a protease. Cleaning activity may be determined by using various assays for cleaning one or more of various enzyme-sensitive stains on an object, item, or surface (e.g., a stain resulting from food, grass, blood, ink, blood/milk/ink, milk/oil/pigment, egg yolk, milk, oil, and/or egg protein). Cleaning performance of a polypeptide (e.g., a polypeptide of the invention (such as a protease variant) or a reference polypeptide (e.g., reference protease) may be determined by subjecting the stain on the object, item, or surface to standard wash condition(s) and assessing the degree to which the stain is removed by using various chromatographic, spectrophotometric, or other quantitative methodologies. Exemplary cleaning assays and methods are known in the art and include, but are not limited to those described in WO 99/34011 and U.S. Pat. 6,605,458, both of which are herein incorporated by reference, as well as those cleaning assays and methods included in the Part I Examples and Part II Examples provided below.

The term "cleaning effective amount" of a protease variant or reference protease refers to the amount of protease that achieves a desired level of enzymatic activity in a specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and are based on many factors, such as the particular protease used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, tablet, bar) composition is required, etc.

The term "cleaning adjunct material" refers to any liquid, solid, or gaseous material included in cleaning composition other than a protease variant of the invention. The cleaning compositions of the present invention may include one of more cleaning adjunct materials. Each cleaning adjunct material is typically selected depending on the particular type and form of cleaning composition (e.g., liquid, granule, powder, bar, paste, spray, tablet, gel, foam, or other composition). Preferably, each cleaning adjunct material is compatible with the protease enzyme used in the composition.

The term "enhanced performance" in the context of cleaning activity refers to an increased or greater cleaning activity by an enzyme on certain enzyme sensitive stains such as egg, milk, grass, ink, oil, and/or blood, as determined by usual evaluation after a standard wash cycle and/or multiple wash cycles.

The term "diminished performance" in the context of cleaning activity refers to a decreased or lesser cleaning activity by an enzyme on certain enzyme sensitive stains such as egg, milk, grass or blood, as determined by usual evaluation after a standard wash cycle.

As used herein, the term "institutional cleaning composition" refers to products suitable for use in institutions including but not limited to schools, hospitals, factories, stores, corporations, buildings, restaurants, office complexes and buildings, processing and/or manufacturing plants, veterinary hospitals, factory farms, factory ranches, etc.

As used herein, "fabric and home care product" refers to a product or device generally intended to be used or consumed in the form in which it is sold and that is for treating fabric, hard surface and any other surface in the area of fabric and home care, including: air care including air freshener and scent delivery systems, car care, dishwashing, fabric conditioning (including softening and/or freshening), laundry detergency, laundry and rinse additive and/or care, hard surface cleaning and/or treatment including floor and toilet bowl cleaners, and other cleaning products for consumer and institutional use.

As used herein, the terms "cleaning composition" and "cleaning formulation" refer to compositions that find use in the removal of undesired compound(s) from an item(s) to be cleaned, such as, but not limited to, e.g., fabric, laundry, dishes, dishware, contact lenses, other solid substrates, hair (including human or animal hair) (shampoos), skin (soaps, cosmetics, and creams), teeth (mouthwashes, toothpastes), non-fabric and home care objects, filters, membranes (e.g., filtration membrane, including, but not limited to, ultrafiltration membranes), hard surfaces and other surfaces, including, but not limited to, e.g., the hard surface of a table (table top or legs), wall, another furniture item or object, floor, ceiling, etc. A cleaning composition or cleaning formulation may be useful in a personal care application and/or in personal care item, including, e.g., but not limited to, shampoo (for cleaning human or animal hair); soap, cream or cosmetics (for skin cleaning and/or skin care); mouthwash (for oral care); toothpaste (for cleaning teeth and/or oral care). The terms encompass any material/compound selected for the particular type of cleaning composition or formulation desired and the form of the product (e.g., liquid, gel, granule, or spray composition), as long as the composition or formulation is compatible with the protease and/or other enzyme(s) used in the composition or formulation. The specific selection of cleaning composition or formulation materials are readily made by considering the surface, object, or item (e.g., fabric) to be cleaned, and the desired form of the composition or formulation for the cleaning conditions during use. In one aspect, a cleaning composition or formulation may be a fabric and home care product (e.g., a cleaning composition for cleaning laundry). In another aspect, a cleaning composition or formulation is not a fabric and home care product (e.g., a cleaning composition for cleaning contact lens, hair, teeth, or skin, or useful in personal care applications and/or personal care items).

Cleaning compositions and cleaning formulations include any composition that is suited for cleaning, bleaching, disinfecting, and/or sterilizing any object, item, and/or surface. Such compositions and formulations include, but are not limited to, for example, liquid and/or solid compositions, including cleaning or detergent compositions (e.g., liquid, tablet, gel, bar, granule, and/or solid laundry cleaning or detergent compositions and fine fabric detergent compositions; hard surface cleaning compositions and formulations, such as for glass, wood, ceramic and metal counter tops and windows; carpet cleaners; oven cleaners; fabric fresheners; fabric softeners; and textile, laundry booster cleaning or detergent compositions, laundry additive cleaning compositions, and laundry pre-spotter cleaning compositions; dishwashing compositions, including hand or manual dishwash compositions (e.g., "hand" or "manual" dishwashing detergents) and automatic dishwashing compositions (e.g., "automatic dishwashing detergents").

Cleaning compositions or cleaning formulations, as used herein, include, unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, granular, gel, solid, tablet, or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) detergent or heavy-duty powder detergent (HDD) types; liquid fine-fabric detergents; hand or manual dishwashing agents, including those of the high-foaming type; hand or manual dishwashing, automatic dishwashing, or dishware or tableware washing agents, including the various tablet, powder, solid, granular, liquid, gel, and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, mouthwashes, denture cleaners, car shampoos, carpet shampoos, bathroom cleaners; personal care items, such as, but not limited to, e.g., hair shampoos and/or hair-rinses for humans (and other animals), shower gels and foam baths, skin care items, cosmetics, creams, bath and personal human soaps; metal cleaners; as well as cleaning auxiliaries, such as bleach additives and "stain-stick" or pre-treat types. Some granular compositions are in "compact" form; some liquid compositions are in a "concentrated" form.

In one aspect, the invention provides a cleaning composition or detergent composition comprising at least one protease variant or polypeptide of the invention, wherein the cleaning composition or detergent composition is useful for cleaning contact lens(es). In another aspect, the invention provides a cleaning composition or detergent composition comprising at least one protease variant or polypeptide of the invention, wherein the cleaning composition or detergent composition is useful in a personal care application. In another aspect, the invention provides a cleaning composition or detergent composition comprising at least one protease variant or polypeptide of the invention, wherein the cleaning composition or detergent composition is useful for cleaning or rinsing hair, including human hair and/or animal hair (e.g., hair shampoo and/or hair-rinse). In another aspect, the invention provides a cleaning composition or detergent composition comprising at least one protease variant or polypeptide of the invention, wherein the cleaning composition or detergent composition is useful for cleaning or treating skin (e.g., human and/or animal skin) (e.g., shower gel, foam bath, skin care cleaner, cosmetic, cream, and/or bath soap). In another aspect, the invention provides a cleaning composition or detergent

composition comprising at least one protease variant or polypeptide of the invention, wherein the cleaning composition or detergent composition is useful for cleaning teeth and/or dentures and/or for oral care. Such cleaning compositions or detergent compositions may comprise at least one adjunct ingredient or carrier, at least one additional enzyme, at least one builder, and/or at least one surfactant and may be formulated or in a form appropriate to their use. Such cleaning or detergent compositions may contain phosphate or may be phosphate-free. Additional details regarding compositions of the invention are provided elsewhere herein.

As used herein, "fabric cleaning compositions" include hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the soaking and/or pretreatment of stained fabrics (e.g., clothes, linens, and other textile materials).

As used herein, "non-fabric cleaning compositions" include non-textile (i.e., non-fabric) surface cleaning compositions, including, but not limited to, for example, hand or manual or automatic dishwashing detergent compositions, oral cleaning compositions, denture cleaning compositions, and personal cleansing compositions.

As used herein, the term "detergent composition" or "detergent formulation" is used in reference to a composition intended for use in a wash medium for the cleaning of soiled or dirty objects, including particular fabric and/or non-fabric objects or items. Such compositions of the present invention are not limited to any particular detergent composition or formulation. Indeed, the detergents of the invention may comprise at least one protease variant of the invention and, in addition, one or more surfactants, transferase(s), hydrolytic enzymes, perhydrolases, oxido reductases, builders (e.g., a builder salt), bleaching agents, bleach activators, bluing agents, fluorescent dyes, caking inhibitors, masking agents, enzyme activators, antioxidants, and/or solubilizers. In some instances, a builder salt is a mixture of a silicate salt and a phosphate salt, preferably with more silicate (e.g., sodium metasilicate) than phosphate (e.g., sodium tripolyphosphate). Some compositions of the invention, such as, but not limited to, cleaning compositions or detergent compositions, do not contain any phosphate (e.g., phosphate salt or phosphate builder).

As used herein, "dishwashing composition" refers to all forms of compositions for cleaning dishware, including cutlery, including, but not limited to, granular and liquid forms. In some aspects, the dishwashing composition is an "automatic dishwashing" composition that finds use in automatic dishwashing machines. It is not intended that the present invention be limited to any particular type of dishware composition. Indeed, the present invention finds use in cleaning dishware (e.g., dishes, including, but not limited to plates, cups, glasses, bowls, etc.) and cutlery (e.g., utensils, including, but not limited to ,spoons, knives, forks, serving utensils, etc.) of any material, including, but not limited to, ceramics, plastics, metals, china, glass, acrylics, etc. The term "dishware" is used herein in reference to both dishes and cutlery.

As used herein, the term "bleaching" refers to the treatment of a material (e.g., fabric, laundry, pulp, etc.) or surface for a sufficient length of time and/or under appropriate pH and/or temperature

conditions to effect a brightening (*i.e.*, whitening) and/or cleaning of the material. Examples of chemicals suitable for bleaching include, but are not limited to, for example, ClO_2, H_2O_2, peracids, NO_2, etc.

As used herein, "wash performance" of a protease (e.g., a protease variant of the invention) refers to the contribution of a protease variant to washing that provides additional cleaning performance to the detergent as compared to the detergent without the addition of the protease variant to the composition. Wash performance is compared under relevant washing conditions. In some test systems, other relevant factors, such as detergent composition, sud concentration, water hardness, washing mechanics, time, pH, and/or temperature, can be controlled in such a way that condition(s) typical for household application in a certain market segment (e.g., hand or manual dishwashing, automatic dishwashing, dishware cleaning, tableware cleaning, fabric cleaning, etc.) are imitated.

The term "relevant washing conditions" is used herein to indicate the conditions, particularly washing temperature, time, washing mechanics, sud concentration, type of detergent and water hardness, actually used in households in a hand dishwashing, automatic dishwashing, or laundry detergent market segment.

The term "improved wash performance" is used to indicate that a better end result is obtained in stain removal under relevant washing conditions, or that less protease variant, on weight basis, is needed to obtain the same end result relative to the corresponding wild-type or starting parent protease.

As used herein, the term "disinfecting" refers to the removal of contaminants from the surfaces, as well as the inhibition or killing of microbes on the surfaces of items. It is not intended that the present invention be limited to any particular surface, item, or contaminant(s) or microbes to be removed.

The "compact" form of the cleaning compositions herein is best reflected by density and, in terms of composition, by the amount of inorganic filler salt. Inorganic filler salts are conventional ingredients of detergent compositions in powder form. In conventional detergent compositions, the filler salts are present in substantial amounts, typically about 17 to about 35% by weight of the total composition. In contrast, in compact compositions, the filler salt is present in amounts not exceeding about 15% of the total composition. The filler salt can be present in amounts that do not exceed about 10%, or more preferably, about 5%, by weight of the composition. The inorganic filler salts can be selected from the alkali and alkaline-earth-metal salts of sulfates and chlorides. The filler salt may be sodium sulfate.

The position of an amino acid residue in a given amino acid sequence is typically numbered herein using the numbering of the position of the corresponding amino acid residue of the *B. amyloliquefaciens* subtilisin BPN' amino acid sequence shown in SEQ ID NO:2. The *B. amyloliquefaciens* subtilisin BPN' amino acid sequence of SEQ ID NO:2 thus serves as a reference sequence. A given amino acid sequence, such as a protease variant amino acid sequence described herein, can be aligned with the BPN' sequence (SEQ ID NO:2) using an alignment algorithm as described herein, and an amino acid residue in the given amino acid sequence that aligns (preferably optimally

aligns) with an amino acid residue in the BPN' sequence can be conveniently numbered by reference to the corresponding amino acid residue in the subtilisin BPN' sequence.

Generally, the nomenclature used herein and many of the laboratory procedures in cell culture, molecular genetics, molecular biology, nucleic acid chemistry, and protein chemistry described below are well known and commonly employed by those of ordinary skill in the art. Methods for production and manipulation of recombinant nucleic acid methods, nucleic acid synthesis, cell culture methods, and transgene incorporation (e.g., transfection, electroporation) are known to those skilled in the art and are described in numerous standard texts. Oligonucleotide synthesis and purification steps are typically performed according to specifications. Techniques and procedures are generally performed according to conventional methods well known in the art and various general references that are provided throughout this document. Procedures therein are believed to be well known to those of ordinary skill in the art and are provided for the convenience of the reader.

A fabric and home care product may comprise a protease (including a protease variant), including one or more protease variants of the invention and a material selected from the group consisting of an encapsulate comprising a perfume, a hueing agent, an amphiphilic cleaning polymer and mixtures thereof, with the balance of any aspects of the aforementioned composition is made up of one or more adjunct materials, is disclosed. In one aspect of the aforementioned fabric and home care product, said fabric and home care product may comprise, based on total fabric and home care product weight, from about 0.005 weight percent (0.0005 wt%) to about 0.1 wt%, from about 0.001 wt% to about 0.05 wt%, or even from about 0.002 wt% to about 0.03 wt% of said protease. In one aspect of the aforementioned fabric and home care product, said fabric and home care product may comprise, based on total fabric and home care product weight, about 0.00003 wt% to about 0.1 wt%, from about 0.00008 wt% to about 0.05 wt%, or even from about 0.0001 wt% to about 0.04 wt% fabric hueing agent. In one aspect of the aforementioned fabric and home care product, said fabric and home care product may comprise, based on total fabric and home care product weight, from about 0.001 wt% to about 5 wt%, from about 0.01 wt% to about 2 wt%, or even from about 0.03 wt% to about 0.5 wt%, perfume capsules. In one aspect of the aforementioned fabric and home care product, said fabric and home care product may comprise, based on total fabric and home care product weight, from about 0.1 wt% to about 5 wt%, from about 0.25 wt% to about 2.5 wt%, or even from about 0.3 wt% to about 1.5 wt% amphiphilic cleaning polymer.

Polypeptides of the Invention

The present invention provides novel polypeptides, which may be collectively referred to as "polypeptides of the invention". Polypeptides of the invention include isolated, recombinant, substantially pure, or non-naturally occurring protease variants, including, for example, subtilisin protease variant polypeptides, which have enzymatic activity (e.g., proteolytic activity) and/or additional properties discussed in greater detail elsewhere herein (e.g., cleaning activity, stability, etc.).

Polypeptides of the invention include isolated, recombinant, substantially pure, or non-naturally occurring cold water proteases having proteolytic activities, cleaning activities, stability, and other properties discussed elsewhere herein. Such polypeptides, including cold water proteases, of the invention may have enhanced performance relative to known proteases (e.g., enhanced proteolytic activity, enhanced cleaning performance or activity, enhanced stability, etc). Polypeptides of the invention are useful in cleaning applications and may be incorporated into cleaning compositions that are useful in methods of cleaning an item or a surface (e.g., of surface of an item) in need of cleaning. Polypeptides of the invention having proteolytic activity are useful in fabric and home care products, including, e.g., fabric and home care cleaning compositions. Some polypeptides of the invention having proteolytic activity are useful in personal care compositions. Polypeptides of the invention having proteolytic activity are also useful in non-fabric and home care products (i.e., those products that are not fabric and home care products are described herein). A protease variant of the invention may be a subtilisin protease variant. The invention includes *Bacillus species* protease variants and *Bacillus species* subtilisin protease variants.

Polypeptides of the invention are disclosed throughout this specification, including, but not limited to, Part I Examples and Part II Examples provided below. The invention includes an isolated, recombinant, substantially pure, or non-naturally occurring protease variant (e.g., a subtilisin variant) having proteolytic activity, which polypeptide comprises an amino acid sequence having at least about 60%, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% sequence identity to a specific protease variant amino acid sequence set forth in any of the Part I and Part II Examples below. In one aspect, the invention includes an isolated, recombinant, substantially pure, or non-naturally occurring subtilisin polypeptide having proteolytic activity, wherein said polypeptide is a protease variant of the BPN' sequence of SEQ ID NO:2 and said polypeptide comprises an amino acid sequence having at least about 60%, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to SEQ ID NO:2 and a set of amino acid substitutions set forth in any of Part I Examples. In one aspect, the invention includes an isolated, recombinant, substantially pure, or non-naturally occurring subtilisin polypeptide having proteolytic activity, wherein said polypeptide is a protease variant of the GG36 sequence of SEQ ID NO:755 and said polypeptide comprises an amino acid sequence having at least about 60%, 70%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to SEQ ID NO:755 and a set of amino acid substitutions set forth in any of Part II Examples.

In a first aspect, the invention provides an isolated or non-naturally occurring polypeptide protease variant of a parent protease enzyme, the variant having proteolytic activity and comprising an amino acid sequence which comprises an alteration at one or more amino acid positions corresponding to amino acid positions of SEQ ID NO:2 selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217, wherein the at least one alteration is independently (i) an insertion of one or more amino acid residues upstream or downstream of the amino acid residue which occupies the position, (ii) a

deletion of the amino acid residue which occupies the position, or (iii) a substitution of the amino acid residue which occupies the position with a different amino acid residue, wherein each amino acid position is numbered by correspondence with an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin protease BPN' set forth in SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2. A variant of a protease enzyme according to the first aspect of the invention may be termed a protease variant. A variant according to the first aspect of the invention may be a non-naturally occurring protease. A variant according to the first aspect of the invention may be isolated or purified. A variant according to the first aspect of the invention may be a subtilisin variant. A variant according to the first aspect of the invention may have proteolytic activity. A variant according to the first aspect of the invention may have enhanced proteolytic activity compared to the proteolytic activity of the BPN' amino acid sequence set forth in SEQ ID NO:2.

A variant according to the first aspect of the invention may be a variant of a parent protease that is a subtilisin protease, and the subtilisin protease may be a *Bacillus species* protease. A parent *Bacillus* protease according to the first aspect of the invention may be selected from the group consisting of *B. amyloliquefaciens* subtilisin protease BPN' (SEQ ID NO:2), *B. stearothermophilus*, *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. clausii*, *B. halodurans*, *B. megaterium*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. species* TS-23, *B. thuringiensis*, BPN'-v3 (SEQ ID NO4:), and BPN'-v36 (SEQ ID NO:6). A parent protease according to the first aspect of the invention may comprise an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

A variant according to the first aspect of the invention may be a protease variant having a mature form. A variant according to the first aspect of the invention may be a protease variant having a mature form.

A variant according to the first aspect of the invention may comprise an amino acid sequence comprising an alteration at two amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising an alteration at three amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising an alteration at four amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising an alteration at five amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising an alteration at six amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising an alteration at each of the amino acid positions corresponding to positions of SEQ

ID NO:2 selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at a position selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

A variant according to the first aspect of the invention may comprise an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of two or three positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of four positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of five positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprises an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of six or seven positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of positions 24, 53, 78, 101, 128, and 217, wherein each position is numbered by correspondence with a position in SEQ ID NO:2.

A variant according to the first aspect of the invention may comprise an amino acid sequence comprising at least one amino acid substitution selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising at least two amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising at least three amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising at least four amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising at least five amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising at least six amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising a set of substitutions selected from the group consisting of: (a) X128A/S and/or X217L/Q, (b) G128A/S and/or Y217L/Q, and (c) G097A, G128A/S, and Y217L/Q. A variant according to the first aspect of the invention may comprise

an amino acid sequence comprising amino acid substitutions X024G/R+X053G+X078N+X101N+X128A/S+X217Q/L, wherein the variant has proteolytic activity; and wherein optionally the variant has enhanced proteolytic activity or enhanced cleaning activity compared to the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6 or a performance index in a proteolytic assay (e.g., AAPF assay) or cleaning assay (e.g., BMI, grass, or egg microswatch assay) that is greater than that of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

A variant according to the first aspect of the invention may comprise at least one amino acid substitution selected from the group of S024G, S053G, S078N, S101N, G128A/S, and Y217Q. A variant according to the first aspect of the invention may comprise an amino acid sequence comprising amino acid substitutions S024G+S053G+S078N+S101N+G128A+Y217Q.

A variant according to the first aspect of the invention may comprise an amino acid sequence having at least 60%, 65%, 70%, 80%, or 85% sequence identity to the amino acid sequence of SEQ ID NO:2. A variant according to the first aspect of the invention may comprise an amino acid sequence having at least 90% sequence identity to SEQ ID NO:2. A variant according to the first aspect of the invention may comprise an amino acid sequence having at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:2. A variant according to the first aspect of the invention may comprise an amino acid sequence having at least 95% sequence identity to SEQ ID NO:2.

A variant according to the first aspect of the invention may have enhanced proteolytic activity compared to the proteolytic activity of the protease set forth in SEQ ID NO:2. A variant according to the first aspect of the invention may have enhanced proteolytic activity compared to the proteolytic activity of the protease set forth in SEQ ID NO:4. A variant according to the first aspect of the invention may have enhanced proteolytic activity compared to the proteolytic activity of the protease set forth in SEQ ID NO:6. A variant according to the first aspect of the invention may have enhanced cleaning activity compared to the cleaning activity of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A variant according to the first aspect of the invention may a performance index in a proteolytic assay (e.g., AAPF assay) or cleaning assay (e.g., BMI, grass, or egg microswatch assay) that is greater than that of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

In a second aspect, the invention provides an isolated or non-naturally occurring polypeptide protease variant of a parent protease, the variant comprising an amino acid sequence comprising three amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2. A variant according to the first aspect of the invention may have enhanced proteolytic activity or enhanced cleaning activity compared to the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A variant according to the first aspect of the invention may have a performance index in a proteolytic assay

(e.g., AAPF assay) or cleaning assay (e.g., BMI, grass, or egg microswatch assay) that is greater than that of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

A variant according to the second aspect of the invention may comprise an amino acid sequence comprising three amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2. A variant according to the second aspect of the invention may comprise an amino acid sequence comprising four amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q. A variant according to the second aspect of the invention may comprise an amino acid sequence comprising five amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q. A variant according to the second aspect of the invention may comprise an amino acid sequence comprising six amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q. A variant according to the second aspect of the invention may comprise an amino acid sequence further comprising amino acid substitution X097A. A variant according to the second aspect of the invention may comprise an amino acid sequence comprising amino acid substitutions X024G/R+X053G+X078N+X101N+X128A/S+X217L/Q or X097A+X128A/S+X217L/Q.

A variant according to the second aspect of the invention may comprise an amino acid sequence comprising amino acid substitutions S024G/R+S053G+S078N+S101N+G128A/S+Y217L/Q or G097A+G128A/S+Y217L/Q. A variant according to the second aspect of the invention may comprise an amino acid sequence comprising amino acid substitutions S024G+S053G+S078N+S101N+G128S+Y217Q or S024G+S053G+S078N+S101N+G128A+Y217Q.

The amino acid sequence of a variant according to the second aspect of the invention may further comprise the substitution N109G. The amino acid sequence of a variant according to the second aspect of the invention may further comprise the substitution N076D. The amino acid sequence of a variant according to the second aspect of the invention may further comprise the substitution S033T. The amino acid sequence of a variant according to the second aspect of the invention may further comprise the substitution N243V. The amino acid sequence of a variant according to the second aspect of the invention may further comprise the substitution S248A. The amino acid sequence of a variant according to the second aspect of the invention may further comprise the substitution A088T. The amino acid sequence of a variant according to the second aspect of the invention may further comprise the substitution S063G. The amino acid sequence of the variant according to the second aspect of the invention may further comprise two or more substitutions selected from the group consisting of N109G, N076D, S033T, N243V, S248A, A088T, and S063G.

The amino acid sequence of a variant according to the second aspect of the invention may may comprise an amino acid sequence comprising amino acid substitutions

S024G+S053G+S078N+S101N+G128S+Y217Q or S024G+S053G+S078N+S101N+G128A+Y217Q and may further comprise a set of amino acid substitutions selected from the group consisting of: A088T+N109G+A116T+G131H+N243V+L257G, S033T+N076D, S009T+N109G+K141R+N243V, S162G+K256R, N109G+A116T, N109G+L257G, S162G+L257G, N061G+N109G+N243V, N109G+N243V+S248A, S033T+N076D+N109G+N218S+N243V+S248N+K256R, N109G+A116T+N243V+K256R, A088T+N109G+A116T+G131H+N243V, A088T+N109G, N109G+N243V, T158S+L257G, N061S+N109G+N243V, P040A+N109G+N243V+S248N+K256R, S009T+S018T+Y021N+N109G+K141R, A088T+N109G+A116T+T158S+N243V+K256R, A088T+N109G+A116T+T158S+N218S+L257G, N109G+K256R, N109G+N243V+K256R, S063G+K256R, S063G+N109G, S063G, S063G+N076D, S033T+N076D+N218S, and N076D+N218S.

A variant according to the second aspect of the invention may comprise an amino acid sequence comprising amino acid substitutions S024G+S053G+S078N+S101N+G128A+Y217Q and may further comprise a set of amino acid substitutions selected from the group consisting of: A088T+N109G+A116T+G131H+N243V+L257G, S033T+N076D, S009T+N109G+A128S+K141R+N243V, S162G+K256R, N109G+A116T, N109G+L257G, S162G+L257G, N061G+N109G+N243V, N109G+A128S+N243V+S248A, S033T+N076D+N109G+A128S+N218S+N243V+S248N+K256R, N109G+A116T+N243V+K256R, A088T+N109G+A116T+G131H+N243V, A088T+N109G, N109G+N243V, T158S+L257G, N061S+N109G+N243V, P040A+N109G+A128S+N243V+S248N+K256R, S009T+S018T+Y021N+N109G+A128S+K141R, A088T+N109G+A116T+T158S+N243V+K256R, A088T+N109G+A116T+T158S+N218S+L257G, N109G+K256R, N109G+A128S+N243V+K256R, S063G+K256R, S063G+N109G, S063G+A128S, S063G+N076D, S033T+N076D+A128S+N218S, and N076D+N218S, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2. Notably, for example, a variant according to the second aspect of the invention which comprises a sequence comprising substitutions S024G+S053G+S078N+S101N+**G128A**+Y217Q, and which further comprises the set of substitutions S009T+N109G+**A128S**+K141R+N243V, has a serine (S) at position 128 because the alanine in position 128 (i.e., G128A substitution) is substituted with S (A128S substitution).

The amino acid sequence of a variant according to the second aspect of the invention may have at least 60%, 65%, or 70% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. The amino acid sequence of a variant according to the second aspect of the invention may have at least 80% or 85% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. The amino acid sequence of a variant according to the second aspect of the invention may have at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

The patent protease according to the second aspect of the invention may be a subtilisin protease. The parent protease according to the second aspect of the invention may comprise an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A variant according to the second aspect of the invention may be a protease variant having a mature form. A variant according to the first aspect of the invention may be a protease variant having a mature form.

The invention also provides an isolated polypeptide (e.g., protease variant) according to the second aspect of the invention comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:6 and comprising a set of amino acid substitutions selected from the group consisting of:

A088T+N109G+A116T+G131H+N243V+L257G,
S033T+N076D,
S009T+N109G+A128S+K141R+N243V,
S162G+K256R,
N109G+A116T,
N109G+L257G,
S162G+L257G,
N061G+N109G+N243V,
N109G+A128S+N243V+S248A,
S033T+N076D+N109G+A128S+N218S+N243V+S248N+K256R,
N109G+A116T+N243V+K256R,
A088T+N109G+A116T+G131H+N243V,
A088T+N109G,
N109G+N243V,
T158S+L257G,
N061S+N109G+N243V,
P040A+N109G+A128S+N243V+S248N+K256R,
S009T+S018T+Y021N+N109G+A128S+K141R,
A088T+N109G+A116T+T158S+N243V+K256R,
A088T+N109G+A116T+T158S+N218S+L257G,
N109G+K256R,
N109G+A128S+N243V+K256R,
S063G+K256R,
S063G+N109G,
S063G+A128S,
S063G+N076D,

S033T+N076D+A128S+N218S, and

N076D+N218S,

wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2. Such polypeptide may have enhanced proteolytic activity or enhanced cleaning activity compared to the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. Such polypeptide may have a performance index in a proteolytic assay (e.g., AAPF assay) or cleaning assay (e.g., BMI, grass, or egg microswatch assay) that is greater than that of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A variant according to the second aspect of the invention may have enhanced proteolytic activity compared to the proteolytic activity of the BPN' protease having the sequence of SEQ ID NO:2. A variant according to the second aspect of the invention may have enhanced proteolytic activity compared to the proteolytic activity of the protease having the sequence of SEQ ID NO:4 or SEQ ID NO:6. The parent protease of a variant according to the second aspect of the invention may be a subtilisin protease, and optionally the subtilisin protease may be a *Bacillus species*. The parent subtilisin protease of a variant according to the second aspect of the invention may be selected from the group consisting of *B. amyloliquefaciens* subtilisin protease BPN' (SEQ ID NO:2), *B. stearothermophilus, B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. clausii, B. halodurans, B. megaterium, B. coagulans, B. circulans, B. lautus, B. species* TS-23, *B. thuringiensis,* BPN'-v3 (SEQ ID NO4:), and BPN'-v36 (SEQ ID NO:6). A parent protease according to the second aspect of the invention may comprise an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

A variant according to the second aspect of the invention may be a protease variant having a mature form. A parent protease according to the second aspect of the invention may be protease having a mature form.

A variant according to the second aspect of the invention may comprise an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO:2, SEQ ID NO:6, or SEQ ID NO:4.

In a third aspect, the invention provides an isolated or non-naturally occurring polypeptide having protease activity, said polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a polypeptide sequence selected from the group consisting of:

a) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+ G131H+N243V+L257G;

b) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S033T+N076D;

c) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S009T+N109G+

K141R+N243V;

d) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S162G+K256R;

e) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+A116T;

f) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+L257G;

g) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S162G+L257G;

h) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N061G+N109G+N243V;

i) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+N109G+N243V+S248A;

j) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S033T+N076D+N109G+ N218S+N243V+S248N+K256R;

k) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+A116T+N243V+ K256R;

l) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+ G131H+N243V;

m) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G;

n) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+N243V;

o) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+T158S+L257G;

p) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N061S+N109G+N243V;

q) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+P040A+N109G+N243V+ S248N+K256R;

r) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S009T+S018T+Y021N+ N109G+K141R;

s) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+ T158S+N243V+K256R;

t) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+ T158S+N218S+L257G;

u) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+K256R;

v) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+N109G+N243V+K256R;

w) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+K256R;

x) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+N109G;

y) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S063G;

z) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+N076D;

aa) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S033T+N076D+N218S;

bb) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N076D+N218S; and

cc) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q, wherein each amino acid position of the variant is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2. A variant according to the third aspect of the invention may have enhanced proteolytic activity or enhanced cleaning activity compared to the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A

variant according to the third aspect of the invention may have a performance index in a proteolytic assay (e.g., AAPF assay) or cleaning assay (e.g., BMI, grass, or egg microswatch assay) that is greater than that of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

A polypeptide according to the third aspect of the invention may comprise or consist of the amino acid sequence set forth in any of a) through cc) above or a fragment of any thereof having proteolytic activity. A variant or parent protease according to the third aspect of the invention may be in a mature form.

In a fourth aspect, the invention provides an isolated or non-naturally occurring polypeptide having protease activity selected from the group consisting of: (a) a polypeptide comprising an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the polypeptide sequence of SEQ ID NO:6; (b) a polypeptide encoded by a polynucleotide that hybridizes under at least high stringency conditions with (i) the polynucleotide sequence of SEQ ID NO:5 or (ii) a complementary polynucleotide sequence of (i); and (c) a polypeptide encoded by a polynucleotide comprising a nucleotide sequence having at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to the polynucleotide sequence of SEQ ID NO:5. A polypeptide according to the fourth aspect of the invention may comprise or consist of the amino acid sequence of SEQ ID NO:6, or a fragment thereof having proteolytic activity. The variant according to the fourth aspect of the invention may have increased proteolytic activity and/or cleaning performance or activity compared to that of mature BPN' (SEQ ID NO:2). The variant according to the fourth aspect of the invention may have increased proteolytic activity and/or cleaning performance or activity compared to that of SEQ ID NO:4 or SEQ ID NO:6. A variant or parent protease according to the fourth aspect of the invention may be in a mature form.

In a fifth aspect, the invention provides an isolated or non-naturally occurring protease variant of a parent protease, wherein: (a) the variant comprises an amino acid sequence having no more than 25, 20, 15, or 10 alterations relative to the parent protease, wherein (i) the alterations are independently selected from an insertion, a deletion, or a substitution, and (ii) the alterations include a substitution of glycine at positions 24 and 53, a substitution of asparagine at positions 78 and 101, a substitution of alanine or serine at position 128, and a substitution of glutamine at position 217, (b) the parent protease has at least 90% sequence identity to SEQ ID NO:2, (c) the amino acid sequence of SEQ ID NO:2 is used for determining position numbering; and (d) the variant has increased proteolytic activity relative to the parent protease, wherein each amino acid position is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2. The amino acid sequence of a variant according to the fifth aspect of the invention may have at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6.

The parent protease according to the fifth aspect of the invention may have at least 80%, 85%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. The parent protease according to the fifth aspect of the invention may be a protease having the

amino acid sequence of SEQ ID NO:2. A variant or parent protease according to the fifth aspect of the invention may be in a mature form.

The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise the substitution N109G. The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise the substitution N076D. The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise the substitution S033T. The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise the substitution N243V. The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise the substitution S248A. The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise the substitution A088T. The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise the substitution S063G.

The amino acid sequence of a variant according to the fifth aspect of the invention may further comprise a set of amino acid substitutions selected from the group consisting of: A088T+N109G+A116T+G131H+N243V+L257G, S033T+N076D, S009T+N109G+K141R+N243V, S162G+K256R, N109G+A116T, N109G+L257G, S162G+L257G, N061G+N109G+N243V, N109G+N243V+S248A, S033T+N076D+N109G+N218S+N243V+S248N+K256R, N109G+A116T+N243V+K256R, A088T+N109G+A116T+G131H+N243V, A088T+N109G, N109G+N243V, T158S+L257G, N061S+N109G+N243V, P040A+N109G+N243V+S248N+K256R, S009T+S018T+Y021N+N109G+K141R, A088T+N109G+A116T+T158S+N243V+K256R, A088T+N109G+A116T+T158S+N218S+L257G, N109G+K256R, N109G+N243V+K256R, S063G+K256R, S063G+N109G, S063G, S063G+N076D, S033T+N076D+N218S, and N076D+N218S.

The parent protease according to the fifth aspect of the invention may be a subtilisin protease. The parent protease according to the fifth aspect of the invention may be BPN' set forth in SEQ ID NO:2. The variant according to the fifth aspect of the invention may have increased proteolytic activity and/or cleaning performance or activity compared to that of SEQ ID NO:4 or SEQ ID NO:6.

In a sixth aspect, the invention provides an isolated or non-naturally occurring protease variant of a parent protease, wherein (a) the variant comprises an amino acid sequence (i) having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, or 97% identity to the sequence of SEQ ID NO:2 and (ii) comprising a substitution of glycine at positions 24 and 53, a substitution of asparagine at positions 78 and 101, a substitution of alanine or serine at position 128, and a substitution of glutamine at position 217; (b) the parent protease has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO:2; (c) each amino acid position of the variant is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2; and (d) the variant has increased proteolytic activity relative to the parent protease. The parent protase according to the sixth aspect of the invention may be a subtilisin protease. The parent protease according to the sixth aspect of the invention may be a protease having the amino acid sequence of SEQ ID NO:2. The variant

according to the sixth aspect of the invention may have increased proteolytic activity and/or cleaning activity compared to the proteolytic activity and/or cleaning activity, respectively, of the parent protease.

The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise the substitution N109G. The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise the substitution N076D. The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise the substitution S033T. The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise the substitution N243V. The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise the substitution S248A. The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise the substitution A088T. The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise the substitution S063G.

The amino acid sequence of a variant according to the sixth aspect of the invention may further comprise a set of amino acid substitutions selected from the group consisting of: A088T+N109G+A116T+G131H+N243V+L257G, S033T+N076D, S009T+N109G+K141R+N243V, S162G+K256R, N109G+A116T, N109G+L257G, S162G+L257G, N061G+N109G+N243V, N109G+N243V+S248A, S033T+N076D+N109G+N218S+N243V+S248N+K256R, N109G+A116T+N243V+K256R, A088T+N109G+A116T+G131H+N243V, A088T+N109G, N109G+N243V, T158S+L257G, N061S+N109G+N243V, P040A+N109G+N243V+S248N+K256R, S009T+S018T+Y021N+N109G+K141R, A088T+N109G+A116T+T158S+N243V+K256R, A088T+N109G+A116T+T158S+N218S+L257G, N109G+K256R, N109G+N243V+K256R, S063G+K256R, S063G+N109G, S063G, S063G+N076D, S033T+N076D+N218S, and N076D+N218S.

The variant according to the sixth aspect of the invention may have increased proteolytic activity and/or cleaning performance or activity compared to that of SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A variant according to the sixth aspect of the invention may have a performance index in a proteolytic assay (e.g., AAPF assay) or cleaning assay (e.g., BMI, grass, or egg microswatch assay) that is greater than that of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A variant or parent protease according to the sixth aspect of the invention may be in a mature form.

In a seventh aspect, the invention provides an isolated or non-naturally occurring protease variant of BPN' subtilisin protease having the amino acid sequence shown in SEQ ID NO:2, said protease variant selected from the group consisting of:

(a) a protease variant comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence of SEQ ID NO:2, said variant having increased proteolytic activity and/or increased cleaning activity compared to the protease of SEQ ID NO:2 and/or SEQ ID NO:4, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2 and wherein said variant comprises at least one set of amino acid substitution(s) relative to SEQ ID NO:2 selected from groups (i) through (x):

(i) G097A-G128A-P210S-Y217Q-N218A and G097A-G128A-P210S-Y217Q;

(ii) S063T-S078N-G097A-S101A-G128A-S183T-Y217Q-T244N, N061A-S078N-G097A-G128A-Y217Q-S224A, S053G-S078N-G097A-G128A-P129T-Q185T-Y217Q, S063T-S078N-G097A-S101A-G128A-S183T-Y217Q, S063T-S078N-G097A-S101A-G128A-Y217Q, S063T-S078N-G097A-S101A-G128A-Y217Q-T244I, and S078N-G097A-G128A-P129T-Y217Q;

(iii) S063T-S078N-G097A-S101A-G128A-S183T-Y217Q, S063T-S078N-G097A-S101A-G128A-S183T-Y217Q-T244N, S063T-S078N-G097A-S101A-G128A-Y217Q, and S063T-S078N-G097A-S101A-G128A-Y217Q-T244I;

(iv) G097A-I111V-M124V-Y217Q, G097A-I111V-Y167A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128A-V203Y-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128S-V203Y-Y217Q, and V068A-A092G-Y217Q;

(v) N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128S-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-I111V-G128S-Y217Q;

(vi) N061P-G097A-G128S-Y217Q, N061P-G097A-S101N-G128A-P210S-Y217Q, N061P-N062Q-G097A-S101N-I111V-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128S-Y217Q, and S024G-N025G-S053G-N061P-S078N-G097A-S101N-I111V-G128S-Y217Q;

(vii) N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, N025G-G097A-S101N-G128A-Y217Q, N025G-S038G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-G128A-Y217Q, N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, N025G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, N025G-S078N-G097A-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-S101N-G128A-Y217Q, N061P-S101N-G128A-Y217Q, S024G-N025G-N061P-G097A-G128A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G128A-Y217Q, S024G-N025G-T055P-G097A-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-N061P-G097A-G128A-Y217Q, S024G-S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-T055P-N061P-G097A-G128A-Y217Q, S053G-G097A-S101N-G128A-Y217Q, S053G-N061P-G097A-S101N-G128A-Y217Q-S249N, S053G-N061P-S078N-G097A-G128A-Y217Q, S053G-S078N-G097A-S101N-G128A-Y217Q, S053G-T055P-G097A-S101N-G128A-Y217Q, S053G-

T055P-N061P-S101N-G128A-Y217Q, S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, T055P-G097A-S101N-G128A-Y217Q, and T055P-N061P-S078N-G097A-S101N-G128A-Y217Q;

(viii) S024G-N025G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-T055P-N061P-S078N-S101N-G128A-Y217Q, S053G-G097A-S101N-G128A-Y217Q, and T055P-N061P-S078N-G128A-Y217Q;

(ix) N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N061P-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-G097A-S101N-G128A-Y217Q, S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-N025G-T055P-G097A-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-G128A-Y217Q; and

(x) S024G-G097A-S101N-G128A-Y217Q, S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-S101N-G128A-Y217Q, S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-T055P-N061P-G097A-S101N-G128A, S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A, N061P-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-T055P-N061P-S078N-S101N-G128A-Y217Q, N025G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G128A-Y217Q, S024G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, T055P-N061P-S078N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S038G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-G128A-Y217Q, N025G-S053G-N061P-S078N-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S078N-S101N-G128A-Y217Q, T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G128A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-Y217Q, S053G-N061P-G097A-S101N-G128A-Y217Q-S249N, N025G-S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, S024G-T055P-G097A-G128A-Y217Q, T055P-N061P-G097A-A116S-G128A, S024G-N025G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A-Y217Q, S053G-T055P-G097A-S101N-G128A-Y217Q, T055P-G097A-S101N-G128A-Y217Q, S024G-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-G097A-S101N-G128A-Y217Q,

G097A-G128S-Y217Q, S024G-S053G-N061P-G097A-G128A-Y217Q, S024G-N025G-N061P-G097A-G128A-Y217Q, S024G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-T055P-N061P-G097A-G128A-Y217Q, S024G-S038G-S053G-S078N-S101N-G128A-Y217Q, S053G-G097A-S101N-G128A-Y217Q, N025G-T055P-G097A-G128A-Y217Q, S024G-T055P-S078N-G097A-S101N-G128A-Y217Q, S053G-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-G097A-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-G128A-Y217Q, N025G-S078N-G097A-S101N-G128A-Y217Q, N025G-G097A-S101N-G128A-Y217Q, S024G-S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-S078N-G097A-S101N-G128A-Y217Q, N025G-S078N-G097A-G128A-Y217Q, and S024G-N025G-S053G-N061P-G097A-G128A-S130G-Y217Q; and

(b) a protease variant comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence of SEQ ID NO:6, said variant having increased proteolytic activity and/or increased cleaning activity compared to SEQ ID NO:2, SEQ ID NO:4 and/or SEQ ID NO:6, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2 and wherein said variant comprises at least one set of amino acid substitution(s) relative to SEQ ID NO:6 selected from groups (i) through (xv):

(i) at least one substitution relative to SEQ ID NO:6 selected from the group consisting of A116V, G160S, I111L, I115V, N109S, N117M, P005G, Q059V, T164S, Y262M, A015Q, A015S, A098E, A098N, A098S, A098T, A098V, A098Y, A114S, A114T, A116G, A116L, A116S, A116T, A116W, A133G, A133H, A133T, A133V, A137G, A137I, A137L, A137S, A137T, A138S, A216E, A216F, A216V, D099S, D181E, F261A, F261Q, G024F, G024I, G024Q, G024Y, G097S, G160T, G211L, G211V, H017F, H017W, H039V, H226A, I031V, I111V, I268V, K170R, K265R, L016Q, L016T, L135M, L209T, L209V, L233M, L257T, L257V, L267A, L267V, N025A, N025I, N025Q, N025R, N025T, N025V, N101I, N101Q, N101S, N109A, N109G, N109H, N109L, N109M, N109Q, N109T, N117Q, N184A, N184L, N184T, N184W, N212G, N212L, N212V, N243P, N252G, N252M, P005T, P014S, P040G, P040L, P040Q, P129A, P129S, P172G, P172S, P194Q, P210A, P210S, Q185F, Q185G, Q185I, Q185M, Q185N, Q185S, Q275H, R186K, S009A, S009G, S009H, S009M, S018T, S130T, S132N, S145K, S159T, S161I, S161K, S161N, S161T, S162I, S162M, S162Y, S163G, S182F, S182G, S182V, S182W, S183F, S183L, S183M, S183T, S183V, S183W, S224A, S236T, S249V, T022A, T022G, T022Q, T022V, T208V, T242S, T253N, T253S, T254A, T254S, T255L, T255S, T255V, V004A, V004P, V004W, V084C, V139C, V165M, V203F, Y021K, Y021N, Y021T, Y021V, Y167F, Y171F, Y214F, Y262F, and Y262T;

(ii) at least one substitution relative to SEQ ID NO:6 selected from the group consisting of A216E, L090I, A098R, A098W, A098Y, A116G, A116R, A116S, A133M, I107L, I115V, M124L, N101I, N109H, N109S, N109T, N117R, P005G, Q185L, S089V, V095A, A015Y, A029G, A098D, A098E, A098G, A098N, A098S, A098T, A098V, A114S, A114T, A116E, A116L, A116T, A116V,

A133H, A133L, A133S, A137G, A137I, A137L, A137S, A137V, A138S, A144S, A144V, A176S, A176T, A187T, A216F, A216P, A216Q, A216R, A216S, A216T, A216V, A216Y, D041E, D120A, D120E, D120Q, D120R, D120S, D181S, G020A, G020S, G024A, G097A, G097D, G097S, G131Q, G160S, G166I, G211L, G215N, H039N, H238N, I111L, I111V, I122A, L075I, L075Q, L135M, L209T, L209V, L233V, L235M, L235R, L257A, M119I, N025A, N025G, N025T, N061K, N101F, N101H, N101L, N101Q, N101R, N101S, N101T, N109A, N109G, N109K, N109L, N117E, N117H, N117K, N117S, N212G, N212S, N218F, N218G, N218H, N218L, N218S, N218W, N240Q, N252M, N252R, N252S, P005T, P040A, P040G, P040T, P129D, P129S, P194S, P210R, Q019R, Q019W, Q103L, Q103W, Q185A, Q185G, Q185M, Q185R, Q185T, Q206G, Q206Y, Q217A, Q217E, Q217R, Q217S, Q217T, S003Q, S009H, S018M, S033T, S130A, S130F, S130G, S130T, S130V, S145T, S159A, S161N, S161T, S162V, S162Y, S182L, S182W, S183F, S183L, S183V, S183W, S188K, S188W, S236Q, S236T, S248L, T022H, T022K, T208C, T253H, T255V, V044I, V121I, V139C, V143H, V143Q, V143T, V143W, V143Y, Y006K, Y021A, Y104W, A001F, A001G, A001H, A001K, A001L, A001Q, A001S, A001Y, A013V, A015G, A015K, A015R, A015S, A015T, A015W, A048S, A073N, A073S, A092S, A098K, A098P, A116D, A116W, A128S, A133P, A133T, A133V, A134G, A134S, A137H, A137N, A137T, A144D, A144K, A144L, A144M, A144N, A144R, A179G, A179S, A187V, A216G, A216L, A216W, A223S, A230C, A272K, A272L, A272P, A272S, A272T, A272W, A273G, A273S, A274G, A274M, A274T, D120K, D140E, D181A, D181E, D181G, D181H, D181T, D259E, D259N, D259Q, E054D, E156D, E156T, E251L, E251T, E251V, F058Y, F189W, F261K, F261Q, F261R, G007A, G007S, G020F, G020H, G020N, G020Q, G020T, G020Y, G024F, G024Q, G024R, G024T, G024V, G024W, G024Y, G053T, G097K, G097M, G097R, G097T, G131A, G131H, G131P, G131R, G131T, G131V, G160H, G160T, G166C, G166Q, G166S, G166T, G211A, G211D, G211K, G211M, G211N, G211Q, G211R, G211V, G211W, G215S, G215T, G215W, H017T, H017W, H017Y, H039V, H226A, H226F, H226I, H226L, H226M, H226V, I035V, I079A, I079S, I108V, I205V, I234L, I234V, I268V, K012S, K043P, K136H, K136R, K141A, K141F, K141T, K141W, K170A, K170G, K170R, K213A, K213R, K213S, K237A, K237H, K237L, K237S, K237V, K256A, K256G, K256H, K256M, K256P, K256Q, K256R, L016A, L016Q, L016T, L016V, L042V, L075M, L075T, L082M, L082V, L135F, L196I, L209H, L209Q, L209R, L209S, L209W, L233A, L233M, L233Q, L235I, L235K, L250I, L257S, L257T, L257V, L267A, L267Q, L267T, L267V, M119C, M199V, N025C, N025E, N025F, N025I, N025K, N025L, N025M, N025Q, N025V, N025Y, N061F, N061P, N061S, N061T, N076G, N078S, N101A, N109M, N109Q, N109R, N117A, N117M, N117Q, N118D, N118G, N118H, N118Q, N118R, N118S, N184A, N184C, N184G, N184L, N184R, N184S, N184T, N184V, N184W, N212C, N212F, N212I, N212K, N212L, N212P, N212Q, N212R, N212V, N212W, N212Y, N218A, N218P, N218T, N240A, N240E, N240G, N240H, N240L, N240R, N240S, N240T, N243C, N243Q, N243T, N243V, N252A, N252G, N252K, N252Q, P014G, P014Q, P014R, P014S, P014T, P040F, P040L, P040Q, P040S, P040V, P086C, P086H, P086S, P129A, P129E, P129G, P129K, P129R, P172A, P172K, P172Q, P172S, P194A, P194G, P194H, P194L, P194M, P194Q, P194R, P194V, P194W, P194Y,

P210A, P210G, P210L, P210S, P239K, P239R, Q002A, Q002S, Q010A, Q010N, Q010R, Q010T, Q019A, Q019C, Q019D, Q019G, Q019S, Q019T, Q019V, Q059I, Q059V, Q103S, Q185F, Q185H, Q185I, Q185K, Q185N, Q185S, Q185Y, Q206H, Q206L, Q206P, Q206W, Q217F, Q217H, Q217I, Q217K, Q217L, Q217N, Q217V, Q271G, Q271R, Q271T, Q275F, Q275P, Q275R, R186A, R186I, R186K, S003A, S003F, S003G, S003H, S003K, S003R, S003T, S009T, S018N, S018T, S037G, S037T, S037V, S038G, S038Q, S063N, S063Q, S063T, S089M, S089N, S130K, S130L, S130R, S130W, S132N, S145G, S145K, S145M, S145R, S145V, S159C, S159H, S159L, S159Q, S159R, S159T, S159W, S161A, S161C, S161G, S161H, S161I, S161K, S161P, S161Q, S161R, S162F, S162G, S162I, S162L, S162M, S162N, S162P, S162R, S163G, S173A, S173G, S182F, S182G, S182K, S182N, S182Q, S182V, S183A, S183M, S183Q, S183R, S183T, S188A, S188F, S188G, S188P, S188R, S188T, S188V, S190C, S204A, S204G, S204I, S204L, S204Q, S204R, S204V, S207G, S224A, S224T, S236C, S236D, S236E, S236G, S236N, S248A, S248F, S248K, S248M, S248T, S249A, S249R, S249T, S249V, S249W, S249Y, S260G, S260H, S260K, S260N, T022A, T022G, T022Q, T022S, T022V, T022Y, T055A, T055K, T158A, T158S, T208L, T208S, T208V, T220S, T242D, T242N, T242S, T244E, T244G, T244I, T244R, T244V, T244W, T253A, T253G, T253N, T253S, T254S, T254V, T255H, T255I, T255K, T255L, T255Q, T255R, T255S, T255Y, V004A, V004N, V004P, V004W, V008A, V008M, V026I, V045S, V045W, V051I, V081Q, V081T, V084C, V093I, V095C, V143A, V143E, V143F, V143N, V143S, V147I, V147L, V147S, V147T, V148I, V149C, V149I, V165M, V180A, V180C, V180I, V180L, V180T, V192A, V192C, V192I, V192S, V192T, V192Y, V198L, V203A, V203F, V203K, V203L, V203M, V203N, V203Y, W241Y, Y006A, Y006G, Y006H, Y006L, Y006N, Y006P, Y006Q, Y006T, Y021E, Y021K, Y021L, Y021N, Y021Q, Y021R, Y021S, Y021T, Y104F, Y104I, Y104V, Y171F, Y214F, Y214L, Y214W, Y262F, and Y262S;

(iii) at least one set of amino acid substitutions relative to SEQ ID NO:6 selected from the group consisting of A088T-L257G, A116T-A128S, N061S-N109G-A128S-N243V-S260P, S009T-N109G-A128S-K141R-N243V, S009T-S018T-Y021N-N109G-A128S-K141R, and S162G-K256R;

(iv) at least one set of amino acid substitution(s) relative to SEQ ID NO:6 selected from the group consisting of A116T, A088T-N243V, G024E-A116T, K043Y, N076D-A116T, N218S-S248N, S033T-N243V, S033T-S063G, S248N-L257G, A001E-S249A, A088T-A116T, A088T-A128S, A088T-G131H, A088T-L257G, A088T-N109G, A088T-S248N, A088T-S249A, A116T-N243V, A116T-T158S, A128S, A128S-K256R, A128S-L257G, A128S-N243V, A128S-S248N, A128S-T158S, G024E-A088T, G024E-A128S, G024E-G131H, G024E-K256R, G024E-L257G, G024E-N218S, G024E-N243V, G024E-S162G, G024E-S249A, G024E-T158S, G131H, G131H-K256R, G131H-S249A, K043Y-A088T, K043Y-A116T, K256R, N076D-K256R, N109G, N109G-A116T, N109G-A128S, N109G-A128S-N243V-K256R, N109G-A128S-N243V-S248A, N109G-G131H, N109G-K256R, N109G-L257G, N109G-N218S, N109G-N243V, N109G-S248N, N218S-L257G, N243V, N243V-K256R, N243V-L257G, N243V-S248N, N243V-S249A, Q103H-A128S, Q103H-G131H, Q103H-K256R, Q103H-L257G, Q103H-N243V, Q103H-S248N, Q103H-S249A, Q103H-T158S, Q206D-N243V, S033T-

A128S, S033T-K256R, S033T-N076D, S033T-N218S, S033T-S248N, S033T-T158S, S063G-A128S, S063G-K256R, S063G-N243V, S063G-S162G, S063G-T158S, S162G-K256R, S248N-K256R, S249A, T158S-N243V, and T158S-S249A;

(v) at least one set of amino acid substitution(s) relative to SEQ ID NO:6 selected from the group consisting of A088T-L257G, G024E-K256R, G024E-L257G, N109G-A116T, N109G-L257G, N243V-K256R, S033T-N109G, S033T-T158S, S063G-L257G, A001E-L257G, A088T-A128S, A088T-G169A, A088T-K256R, A088T-N109G, A088T-N218S, A088T-N243V, A088T-S248N, A088T-T158S, A116T, A116T-A128S, A116T-G131H, A116T-K256R, A116T-L257G, A116T-N218S, A116T-S162G, A116T-T158S, A128S, A128S-G169A, A128S-K256R, A128S-L257G, A128S-N218S, G024E, G024E-A128S, G024E-G131H, G024E-N109G, G024E-N243V, G024E-S033T, G024E-S063G, G024E-S248N, G024E-S249A, G024E-T158S, G131H, G131H-G169A, G131H-K256R, G131H-N218S, G131H-S249A, G169A, G169A-L257G, G169A-N243V, K043Y-A088T, K043Y-N109G, K256R, K256R-L257G, N061G-N109G-N243V, N076D-N109G, N109G, N109G-A128S, N109G-G131H, N109G-K256R, N109G-N218S, N109G-S162G, N109G-S248N, N109G-S249A, N109G-T158S, N218S, N218S-K256R, N218S-L257G, N218S-S248N, N243V, N243V-L257G, N243V-S248N, N243V-S249A, P040A-N109G-A128S-N243V-S248N-K256R, Q103H-K256R, Q103H-L257G, Q103H-N109G, S009T-S018T-Y021N-N109G-A128S-K141R, S033T-A088T, S033T-A116T, S033T-A128S, S033T-G131H, S033T-K043Y, S033T-K256R, S033T-L257G, S033T-N076D, S033T-N218S, S033T-N243V, S033T-Q103H, S033T-S063G, S033T-S162G, S033T-S248N, S033T-S249A, S063G, S063G-A088T, S063G-A116T, S063G-A128S, S063G-G131H, S063G-K256R, S063G-N109G, S063G-N218S, S063G-N243V, S063G-S248N, S063G-S249A, S063G-T158S, S162G-K256R, S162G-N218S, S162G-N243V, S162G-S248N, S162G-S249A, S248N, S249A, S249A-L257G, T158S, T158S-L257G, and T158S-N243V;

(vi) at least one set of amino acid substitution(s) relative to SEQ ID NO:6 selected from the group consisting of T158S-L257G, K256R, L257G, S033T-N109G, S162G-K256R, S162G-L257G, G024E-K256R, G024E-L257G, G024E-S033T, N109G-A116T, N218S-L257G, S033T-A088T, S033T-A116T, S033T-N243V, S033T-Q103H, S162G-N218S, S162G-N243V, T158S, T158S-N218S, T158S-N243V, A088T, A088T-G169A, A088T-K256R, A088T-L257G, A088T-S162G, A088T-T158S, A116T-K256R, A116T-L257G, A116T-N243V, A128S-L257G, A128S-N218S, A128S-N243V, A128S-S248N, G024E-A116T, G024E-A128S, G024E-G131H, G024E-N243V, G024E-S248N, G024E-S249A, G024E-T158S, G131H-N243V, G131H-T158S, G169A-N218S, G169A-N243V, G169A-S248N, K256R-L257G, N109G-A128S, N109G-G131H, N109G-N218S, N109G-N243V, N109G-S249A, N218S, N218S-K256R, N218S-N243V, N218S-S249A, N243V, N243V-K256R, N243V-L257G, N243V-S248N, Q103H-N109G, Q103H-N218S, S033T-A128S, S033T-L257G, S033T-N218S, S033T-S162G, S033T-S248N, S033T-T158S, S063G-K256R, S063G-L257G, S162G, S162G-G169A, S162G-S248N, S248N, S248N-K256R, S248N-L257G, S249A, T158S-S162G, and T158S-S248N;

(vii) at least one set of amino acid substitutions relative to SEQ ID NO:6 selected from the group consisting of S033T-N076D-A128S-N218S, A001E-S033T-N109G-N218S, S033T-N218S,

S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243V, A128S-G169A, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S018T-Y021N-S033T-N109G-A128S-N243V-S248N-K256R, S033T-A128S-G131H-N243P, P040E-N109G-A128S-G131H, S033T-A128S, S033T-N109G-A128S-N243V-S248N-K256R, N109G-G169A, S063G-N109G-A128S-G131H, G169A, N109G-A128S-G131H-N243V-S248N-K256R, S033T-A128S-G131H-N243V, A128S-N218S, A001E-G169A, A088T-G169A, G169A-L257G, N109G-N218S, S033T-N109G-A128S-N243P-S248N-K256R, G169A-K256R, N076D-G169A, A001E-G131H-G169A-N243V, G169A-S249A, S033T-N109G, G169A-S248N, K043Y-G169A, K043Y-N218S, N218S-L257G, N218S-N243V, S063G-G169A, A001E-A128S-G131H-N243V, A001E-S033T-N109G-N243V, A088T-N218S, G024E-N218S, G024E-S033T, G169A-Q206D, N076D-N218S, S033T-L257G, S162G-G169A, A001E-N218S, A116T-N218S, G169A-N243V, N218S, P040A-N109G-A128S-N243V-S248N-K256R, S033T-N076D, A001E-S033T, A128S-G131H, N218S-S248N, S018T-Y021N-N109G-A128S, S033T-K043Y, S033T-N243V, S033T-Q206D, S063G-N218S, S162G-N218S, T158S-G169A, A116T-G169A, G131H-G169A, N061S-N109G-A128S-S260P, N109G-A128S-N243V-K256R, N109G-A128S-N243V-S248A, N109G-A128S-N243V-S248A-K256R, N109G-A128S-N243V-S248N-K256R-L257G, N218S-K256R, S009T-N109G-A128S-K141R, S009T-S018T-Y021N-N109G-A128S-K141R, S033T-A088T, S033T-S063G, S033T-S162G, T158S-N218S, A001E-N076D-N109G-A128S, N109G-A128S-N243V-S248N-K256R, N109G-A128S-S248N-K256R, S009T-N109G-A128S-K141R-N243V, S018T-Y021N-N061S-N109G-A128S-S260P, S033T-A116T, S033T-S248N, S033T-S249A, S033T-T158S, G131H-N218S, N109A-A128S-N243V-K256R, N109G-A128S, N109G-A128S-S162G-N243V-S248N-K256R, N109G-A128S-T158S-N243V-S248N-K256R, N218S-S249A, Q206D-N218S, S018T-Y021N-N109G-A128S-N243V, S018T-Y021N-N109G-A128S-N243V-S248N-K256R, S033T-K256R, A116T-A128S, N061S-N109G-A128S-N243V-S260P, N109G-A128S-N243V-S248N, S009T-N109G-A128S-K141R-N243V-S248N-K256R, G024E-A128S, N061S-N109G-A128S-N243V-S248N-K256R-S260P, N109S-A128S-N243V-K256R, S033T, S033T-G131H, A001E-A128S, A128S, A128S-L257G, A128S-Q206D, N109Q-A128S-N243V-K256R, S009T-A128S-K141R-N243V, S009T-S018T-Y021N-A128S-K141R-N243V, A088T-A128S, A128S-K256R, A128S-N243V, N061P-N109G-N243V, N061S-A128S-N243V-S260P, S018T-Y021N-A128S-N243V, A128S-N243V-S248N-K256R, A128S-S248N, A128S-S249A, N076D-A128S, S063G-A128S, A128S-S162G, A128S-T158S, S018T-Y021N-N061S-A128S-N243V-S260P, S033T-Q103H-A128S-G131H, N061S-N109G-N243V, K043Y-A128S, N061P-N109G-G131H-N243V, N109G-L257G, A001E-G024E-S204E-Q206D, A001E-L257G, A088T-N109G, G024E-N109G, K043Y-N109G, N061G-N109G-N243V, N076D-N109G, N109G, N109G-A116T, N109G-K256R, N109G-N243V-K256R, N109G-N243V-S248A-K256R, N109G-Q206D, S063G-N109G, A001E-A116T, A001E-N109G, A001E-Q206D, A088T-A116T, A088T-N243V, A116T-L257G, G024E-A116T, G024E-L257G, G024E-N243V, G024E-Q206D, N109G-G131H, N109G-N243V, N109G-S162G, N109G-S248N, N109G-S248N-K256R, N109G-S249A, N109G-T158S, N243V-L257G, A001E-A088T, A001E-G024E, A001E-K256R, A001E-N076D, A001E-N243V, A088T, A088T-L257G, A088T-Q206D, A116T, A116T-K256R,

A116T-N243V, G024E-A088T, G024E-K043Y, G024E-K256R, G024E-N076D, G024E-S162G, G024E-S248N, K043Y-A088T, K043Y-A116T, K043Y-L257G, K043Y-N243V, K043Y-Q206D, K256R-L257G, N076D-A116T, N076D-L257G, N076D-N243V, N076D-Q206D, N109G-N243V-S248N, N109G-N243V-S248N-K256R, N243V-K256R, Q206D, Q206D-L257G, Q206D-N243V, Q206D-S248N, S063G-K256R, S063G-L257G, T158S-L257G, A001E, A001E-K043Y, A001E-S162G, A001E-S248N, A001E-S249A, A001E-T158S, A088T-K256R, A088T-S162G, A088T-S248N, A088T-S249A, A116T-Q206D, A116T-S248N, A116T-S249A, G024E, G024E-G131H, G024E-S249A, G024E-T158S, G131H, G131H-K256R, G131H-L257G, K043Y-K256R, K043Y-N076D, K256R, L257G, N076D-A088T, N076D-K256R, N076D-S162G, N076D-S248N, N076D-S249A, N109G-N243P-S248A-K256R, N109G-N243P-S248N-K256R, N243V, Q206D-K256R, S033T-P040E-Q103H-N109G, S063G, S063G-A116T, S063G-Q206D, S162G-K256R, S162G-L257G, S162G-N243V, S162G-Q206D, S162G-S248N, S248N, S248N-L257G, S249A, S249A-L257G, T158S, T158S-N243V, and T158S-Q206D;

(viii) at least one set of amino acid substitution(s) relative to SEQ ID NO:6 selected from the group consisting of A088T-A116T-N243V-K256R-L257G, A088T-A116T-N243V-L257G, A088T-T158S-N218S-K256R, A088T-T158S-N218S-N243V-L257G, A088T-A116T-T158S-N218S-N243V-K256R-L257G, A088T-N109G-A116T-G131H-A153S-N218S-S248N-L257G, A088T-N109G-A116T-T158S-S248N-K256R-L257G, A088T-N109G-T158S-L257G, A114S-A116T-N218S-N243V-S248N-K256R-L257G, A116T-T158S-K256R, A088T-A116T-G131H-T158S-S248N-L257G, A088T-A116T-T158S, A088T-N109G-A116T-G131H-L257G, A088T-N109G-A116T-T158S-N243V-S248N-L257G, A088T-N109G-N243V-L257G, A088T-N109G-N243V-S248N, A088T-N109G-T158S-N243V-L257G, A088T-N109G-T158S-N243V-S248N-L257G, A116T-T158S-S248N-L257G, Y006H-A116T-G131H-S248N, A088T-A116T-G131H-T158S-N218S-N243V, A088T-A116T-G131H-T158S-N243V, A088T-A116T-G131H-T158S-N243V-K256R-L257G, A088T-A116T-N218S-N243V-K256R-L257G, A088T-A116T-S248N-K256R-L257G, A088T-A116T-T158S-N218S-N243V, A088T-A116T-T158S-N243V-K256R-L257G, A088T-A116T-T158S-N243V-S248N-L257G, A088T-G131H-N243V-S248N-K256R-L257G, A088T-N109G-A116T-T158S-L257G, A088T-N109G-A116T-T158S-N212D-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-S248N-K256R, A088T-N109G-A116T-T158S-S248N-L257G, A088T-N109G-G131H-V148A-N218S-N243V-K256R-L257G, A088T-N109G-K256R, A088T-N109G-N243V-S248N-L257G, A088T-N109G-T158S-K256R, A088T-N109G-T158S-N243V, A088T-T158S-N243V-K256R-L257G, A116T, A116T-N218S-N243V-L257G-N269S, A116T-T158S-K256R-L257G, N109G-A116T-K256R-L257G, N109G-A116T-N243V, N109G-A116T-T158S-N243V-K256R-L257G, N109G-G131H-L257G, N109G-G131H-S248N-K256R-L257G, N109G-G131H-T158S-K256R-L257G, S003P-A116T-T158S-S248N-K256R, T158S-S248N-K256R, A088T-A116T-G131H-N243V-K256R, A088T-A116T-G131H-S248N-K256R-L257G, A088T-A116T-G131H-V147A-T158S-N218S-N243V-S248N-L257G, A088T-A116T-S248N-L257G, A088T-A116T-T158S-N218S, A088T-A116T-T158S-N218S-K256R-L257G, A088T-A116T-T158S-N218S-L257G, A088T-G131H-N243V-

L257G, A088T-G131H-T158S-S248N-L257G, A088T-L257G, A088T-N109G-A116T, A088T-N109G-A116T-G131H-N218S, A088T-N109G-A116T-G131H-N218S-S248N-L257G, A088T-N109G-A116T-G131H-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-S248N-K256R-L257G, A088T-N109G-A116T-N218S-N243V-K256R, A088T-N109G-A116T-N218S-N243V-L257G, A088T-N109G-A116T-N243V-S248N-K256R, A088T-N109G-A116T-N243V-S248N-K256R-L257G, A088T-N109G-A116T-T158S-L257G, A088T-N109G-A116T-T158S-N243V-L257G, A088T-N109G-G131H-T158S-N243V-S248N-K256R, A088T-N109G-G131H-T158S-W241R-S248N-K256R, A088T-N109G-K256R-L257G, A088T-N109G-L257G, A088T-N109G-N243V, A088T-N109G-N243V-K256R, A088T-N109G-N243V-K256R-L257G, A088T-N109G-S248N-K256R, A088T-N109G-T158S-N218S-K256R-L257G, A088T-N109G-T158S-N218S-N243V-S248N-K256R, A088T-N109G-T158S-N243V-K256R, A088T-N109G-T158S-N243V-K256R-L257G, A088T-N109G-T158S-N243V-S248N-A274D, A088T-N109G-T158S-S248N-L257G, A088T-T158S-K256R, A088T-T158S-N218S-N243V-K256R-L257G, A088T-T158S-N243V-L257G, A116T-G131H-N218S-N243V-S248N, A116T-G131H-S248N-L257G, A116T-S248N-K256R-L257G, A116T-T158S-N218S-N243V-K256R, A116T-T158S-N218S-S248N-L257G-Q271R, A116T-T158S-N243V-K256R-L257G, A116T-T158S-N243V-S248N-L257G, G131H-S248N, G131H-T158S-I234T-N243V-K256R, G131H-W241L-N243V-S248N-K256R, N109G-A116T-G131H-A137V-T158S-S248N-K256R-L257G, N109G-A116T-G131H-A151S-N218S-K256R-L257G, N109G-A116T-G131H-T158S-N218S-N243V-K256R, N109G-A116T-G131H-T158S-N218S-S248N, N109G-A116T-G131H-T158S-N243V-S248N, N109G-A116T-S248N, N109G-A116T-T158S-L257G, N109G-A116T-T158S-N218S-W241R-N243V, N109G-A116T-T158S-N243V-S248N-L257G, N109G-A116T-T158S-S248N-K256R-L257G, N109G-A116T-T158S-S248N-L257G, N109G-G131H-N218S-L257G, N109G-G131H-N218S-S248N-K256R-L257G, N109G-G131H-T158S-N218S-S248N-K256R-L257G-A274T, N109G-K256R, N109G-N243V-L257G, N109G-T158S-N218S-K256R-L257G, N109G-T158S-N218S-L257G, N109G-T158S-S248N-K256R, P014L-A015L-L016C-H017T-S018L-Q019K-G020A-Y021T-T022L-G023E, S003F-A088T-N109G-A116T-T158S-N243V-K256R-L257G, V004A-A088T-A116T-T158S-N218S, V004A-N109G-A116T-G131H-S248N-K256R-L257G, V004L-A116T-N218S-N243V-S248N-L257G, Y006H-N109G-N218S-N243V-S248N, A001T-A116T-T158S-N243V-L257G, A088T-A116T, A088T-A116T-G131H-L257G, A088T-A116T-G131H-N218S-L257G, A088T-A116T-G131H-N218S-S248N-K256R-L257G, A088T-A116T-G131H-N218S-S248N-L257G, A088T-A116T-G131H-N243V-K256R-L257G, A088T-A116T-G131H-N243V-L257G, A088T-A116T-G131H-N243V-S248N, A088T-A116T-G131H-T158S-K256R-L257G, A088T-A116T-G131H-T158S-L257G, A088T-A116T-G131H-T158S-N218S, A088T-A116T-G131H-T158S-N218S-N243V-K256R-A273T, A088T-A116T-G131H-T158S-N218S-S248N-K256R, A088T-A116T-G131H-T158S-N218S-S248N-L257G, A088T-A116T-G131H-T158S-N243V-S248N-K256R, A088T-A116T-G131H-T158S-S248N, A088T-A116T-G131H-T158S-S248N-L257G, A088T-A116T-K256R, A088T-A116T-K256R-L257G, A088T-A116T-N218S-N243V-L257G, A088T-A116T-N243V-K256R, A088T-A116T-N243V-L257G, A088T-A116T-N243V-S248N-K256R-L257G, A088T-A116T-S248N-K256R, A088T-A116T-

T158S-K256R, A088T-A116T-T158S-N218S, A088T-A116T-T158S-N218S-N243V-K256R, A088T-A116T-T158S-N218S-N243V-K256R-N269S, A088T-A116T-T158S-N218S-N243V-S248N, A088T-A116T-T158S-N218S-N243V-S248N, A088T-A116T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-A116T-T158S-N243V-K256R, A088T-A116T-T158S-N243V-L257G, A088T-A116T-T158S-N243V-S248N-K256R, A088T-A116T-T158S-N243V-S248N-K256R-L257G, A088T-A116T-T158S-S248N-K256R, A088T-A116T-V143A-N218S-S248N-K256R, A088T-A116T-V147I-T158S-N218S-N243V-L257G, A088T-G131H-K256R-L257G, A088T-G131H-N218S-N243V-S248N, A088T-G131H-N218S-S248N-L257G, A088T-G131H-S248N-K256R-L257G, A088T-G131H-T158S-L257G, A088T-G131H-T158S-N218S-K256R, A088T-G131H-T158S-N218S-N243V-K256R-L257G, A088T-G131H-T158S-N218S-N243V-L257G, A088T-G131H-T158S-N218S-S248N, A088T-G131H-T158S-N243V, A088T-G131H-T158S-N243V, A088T-G131H-T158S-N243V-S248N, A088T-G131H-T158S-N243V-S248N-K256R, A088T-G131H-T158S-N243V-S248N-L257G, A088T-I107T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-G131H-L257G, A088T-N109G-A116T-G131H-N218S, A088T-N109G-A116T-G131H-N218S-L257G, A088T-N109G-A116T-G131H-N218S-N243V, A088T-N109G-A116T-G131H-N218S-N243V-K256R-L257G, A088T-N109G-A116T-G131H-N218S-N243V-L257G, A088T-N109G-A116T-G131H-N218S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N243V, A088T-N109G-A116T-G131H-N243V-L257G, A088T-N109G-A116T-G131H-N243V-S248N-L257G, A088T-N109G-A116T-G131H-S248N, A088T-N109G-A116T-G131H-S248N-K256R, A088T-N109G-A116T-G131H-S248N-L257G, A088T-N109G-A116T-G131H-T158S-L257G, A088T-N109G-A116T-G131H-T158S-N218S, A088T-N109G-A116T-G131H-T158S-N218S-S248N-K256R, A088T-N109G-A116T-G131H-T158S-N218T-N243V, A088T-N109G-A116T-G131H-T158S-N243V-K256R, A088T-N109G-A116T-G131H-T158S-N243V-K256R-L257G, A088T-N109G-A116T-G131H-T158S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N243V-S248N-K256R, A088T-N109G-A116T-G131H-T158S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-S248N-L257G, A088T-N109G-A116T-N218S, A088T-N109G-A116T-N218S-L257G, A088T-N109G-A116T-N218S-N243V, A088T-N109G-A116T-N218S-N243V-S248N-L257G, A088T-N109G-A116T-N218S-S248N-K256R, A088T-N109G-A116T-N218T-K256R, A088T-N109G-A116T-N218T-K256R-L257G, A088T-N109G-A116T-N243V, A088T-N109G-A116T-N243V-K256R-L257G, A088T-N109G-A116T-N243V-K256R-L257G-N269D, A088T-N109G-A116T-S248N-K256R, A088T-N109G-A116T-T158S, A088T-N109G-A116T-T158S-N218S-L257G, A088T-N109G-A116T-T158S-N218S-N243V, A088T-N109G-A116T-T158S-N218S-N243V-K256R, A088T-N109G-A116T-T158S-N218S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-L257G, A088T-N109G-A116T-T158S-N218S-S248N, A088T-N109G-A116T-T158S-N243V, A088T-N109G-A116T-T158S-N243V-K256R, A088T-N109G-A116T-T158S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-S248N-L257G, A088T-N109G-G131H-L257G, A088T-N109G-G131H-N218S-K256R-L257G, A088T-N109G-G131H-N218S-N243V-K256R, A088T-N109G-G131H-N218S-N243V-L257G, A088T-N109G-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-

G131H-N243V, A088T-N109G-G131H-N243V-L257G, A088T-N109G-G131H-N243V-S248N-K256R, A088T-N109G-G131H-N243V-S248N-L257G, A088T-N109G-G131H-S248N-L257G, A088T-N109G-G131H-T158S-L257G, A088T-N109G-G131H-T158S-N218S-N243V-S248N-K256R, A088T-N109G-G131H-T158S-N243V, A088T-N109G-G131H-T158S-N243V-K256R, A088T-N109G-G131H-T158S-N243V-K256R-L257G, A088T-N109G-G131H-T158S-N243V-L257G, A088T-N109G-L257G, A088T-N109G-N218S-K256R, A088T-N109G-N218S-N243V-S248N-L257G, A088T-N109G-N218S-S248N-K256R-L257G, A088T-N109G-N243V-K256R-L257G, A088T-N109G-N243V-S248N-K256R-L257G, A088T-N109G-N243V-S248N-L257G-I268V, A088T-N109G-S248N-K256R-L257G, A088T-N109G-T158S-N218S-K256R, A088T-N109G-T158S-N218S-N243V-L257G, A088T-N109G-T158S-N218S-N243V-L257G, A088T-N109G-T158S-N243V-K256R-I268V, A088T-N109G-T158S-N243V-S248N-Q275R, A088T-N218S-N243V, A088T-N218S-N243V-S248N-K256R-L257G, A088T-N218S-S248N, A088T-N218S-S248N-L257G, A088T-N243V, A088T-N243V, A088T-N243V-K256R, A088T-N243V-L257G, A088T-S145T-T158S-S248N, A088T-T158S-L257G, A088T-T158S-N218S-S248N-L257G, A088T-T158S-N243V-K256R-L257G-Q271H, A088T-T158S-S248N, A088T-V143A-T158S-K256R, A116T-G131H-K256R, A116T-G131H-N218S, A116T-G131H-N243V, A116T-G131H-N243V-K256R, A116T-G131H-N243V-L257G, A116T-G131H-S248N-K256R, A116T-G131H-T158S-N218S-I234T-N243V-S248N-K256R, A116T-G131H-T158S-N243V-L257G, A116T-G131H-T158S-N243V-S248N-K256R, A116T-G131H-V143F-T158S-N218S, A116T-L257G, A116T-N218S, A116T-N218S-L257G, A116T-N218S-N243V-L257G, A116T-N243V, A116T-N243V-K256R, A116T-N243V-S248N, A116T-N243V-S248N-K256R-L257G, A116T-S248N, A116T-T158S, A116T-T158S-N218S-N243V, A116T-T158S-N218S-S248N, A116T-T158S-N243V, A116T-T158S-N243V-K256R, A116T-T158S-N243V-L257G, A116T-T158S-N243V-S248N, A116T-T158S-S248N-K256R-L257G, A116T-V149I-T158S-N243V-S248N-K256R-Q271H, G131H-N218S-N243V-L257G, G131H-N243V, G131H-N243V-S248N-K256R, G131H-T158S, G131H-T158S-N218S-N243V-K256R, G131H-T158S-N243V-K256R-L257G, G131H-T158S-N243V-S248N-L257G, N109G-A116T-G131H-N218S-K256R-L257G, N109G-A116T-G131H-N218S-L257G, N109G-A116T-G131H-N218S-N243V-K256R-L257G, N109G-A116T-G131H-N218S-S248N-K256R, N109G-A116T-G131H-N243V-K256R, N109G-A116T-G131H-N243V-L257G, N109G-A116T-G131H-N243V-S248N-K256R-L257G, N109G-A116T-G131H-S248N, N109G-A116T-G131H-S248N-I268V, N109G-A116T-G131H-T158S-N218S-N243V-S248N-K256R, N109G-A116T-G131H-T158S-N218S-S248N-L257G, N109G-A116T-G131H-T158S-S248N, N109G-A116T-G131H-T158S-S248N-K256R, N109G-A116T-N218S, N109G-A116T-N218S-N243V-K256R, N109G-A116T-N218S-N243V-K256R-L257G, N109G-A116T-N218S-S248N-L257G, N109G-A116T-N243V-K256R, N109G-A116T-N243V-S248N-K256R-L257G, N109G-A116T-S248N-L257G, N109G-A116T-T158S-G211V-N243V-S248N-K256R, N109G-A116T-T158S-K256R-L257G, N109G-A116T-T158S-N218S, N109G-A116T-T158S-N218S-N243V-K256R-L257G, N109G-A116T-T158S-N218S-N243V-L257G, N109G-A116T-T158S-N218S-N243V-S248N-L257G, N109G-A116T-T158S-N218S-S248N-K256R-L257G, N109G-A116T-T158S-N243V, N109G-A116T-T158S-Q275R, N109G-G131H-A137V-

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(ix) at least one set of amino acid substitution(s) relative to SEQ ID NO:6 selected from the group consisting of A088T-N109G-A116T-T158S-N243V-L257G, A116T-N218S-N243V-L257G-N269S, A088T-A116T-K256R, A088T-G131H-K256R, A088T-N109G-A116T-T158S-S248N-K256R-L257G, A088T-N109G-T158S-L257G, A088T-A116T-G131H-T158S-N218S-N243V-K256R-A273T, A088T-A116T-N243V-L257G, A088T-A116T-S248N-K256R-L257G, A088T-A116T-T158S-N243V-L257G, A088T-A116T-T158S-N243V-S248N-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N243V-L257G, A088T-N109G-A116T-G131H-T158S-L257G, A088T-N109G-A116T-T158S-N218S-L257G, A088T-N109G-A116T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-G131H-N218S-K256R-L257G, A088T-N109G-N218S-S248N-L257G, A088T-T158S-N218S-N243V-K256R-I268V, A088T-T158S-N218S-S248N-L257G, A116T-N218S-K256R-L257G, N109G-A116T, N109G-A116T-G131H-T158S-L257G, N109G-A116T-N243V, N109G-A116T-N243V-K256R, N109G-A116T-T158S-L257G, N109G-K256R, N109G-N243V-K256R-L257G, S003P-N109G-G131H-T158S-K256R, A088T-A116T, A088T-A116T-G131H-N218S-K256R-L257G, A088T-A116T-G131H-N218S-L257G, A088T-A116T-G131H-N218S-N243V-S248N-L257G, A088T-A116T-G131H-N243V-K256R-L257G, A088T-A116T-G131H-N243V-S248N-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V, A088T-A116T-G131H-T158S-N218S-N243V-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N, A088T-A116T-G131H-T158S-S248N-K256R-L257G, A088T-A116T-G131H-T158S-S248N-L257G, A088T-A116T-N218S-N243V-L257G, A088T-A116T-N218S-N243V-S248N-K256R-L257G, A088T-A116T-N218S-N243V-S248N-K256R-Q275R, A088T-A116T-T158S-A216S-N218S-N243V-K256R-L257G, A088T-A116T-T158S-K256R, A088T-A116T-T158S-N218S-L257G, A088T-A116T-T158S-N218S-N243V, A088T-A116T-T158S-N218S-N243V-K256R, A088T-A116T-T158S-N218S-N243V-K256R-L257G, A088T-A116T-T158S-N218S-N243V-K256R-N269S, A088T-A116T-T158S-N243V, A088T-A116T-T158S-N243V-K256R, A088T-A116T-V147I-T158S-N218S-N243V-L257G, A088T-G131H-K256R-L257G, A088T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-G131H-S248N-K256R-L257G, A088T-

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G131H-S248N-K256R, N109G-A116T-G131H-T158S-K256R, N109G-A116T-G131H-T158S-K256R-L257G, N109G-A116T-G131H-T158S-N218S-K256R, N109G-A116T-G131H-T158S-N218S-K256R-L257G, N109G-A116T-G131H-T158S-N218S-L257G, N109G-A116T-G131H-T158S-N218S-N243V-K256R, N109G-A116T-G131H-T158S-N218S-N243V-S248N-L257G, N109G-A116T-G131H-T158S-N218S-S248N, N109G-A116T-G131H-T158S-N243V-L257G, N109G-A116T-G131H-T158S-N243V-S248N, N109G-A116T-G131H-T158S-S248N, N109G-A116T-G131H-T158S-S248N-K256R, N109G-A116T-G131H-T158S-S248N-K256R-L257G, N109G-A116T-G131H-V149A-T158S-N218S-N243V-S248N-L257G, N109G-A116T-N218S, N109G-A116T-N218S-K256R, N109G-A116T-N218S-N243V-K256R-L257G, N109G-A116T-N218S-N243V-S248N-I268V, N109G-A116T-N243V-K256R-L257G, N109G-A116T-N243V-S248N, N109G-A116T-N243V-S248N-L257G, N109G-A116T-T158S, N109G-A116T-T158S-N218S-N243V-K256R-L257G, N109G-A116T-T158S-N218S-N243V-S248N-K256R-L257G, N109G-A116T-T158S-N218S-N243V-S248N-L257G, N109G-A116T-T158S-N218S-S248N-K256R-L257G, N109G-A116T-T158S-N243V-K256R-L257G, N109G-A116T-T158S-N243V-L257G, N109G-A116T-T158S-N243V-S248N-L257G, N109G-A116T-T158S-Q275R, N109G-A116T-T158S-S248N-K256R-L257G, N109G-G131H-L257G, N109G-G131H-N218S-N243V-S248N-K256R-L257G, N109G-G131H-N218S-N243V-S248N-L257G, N109G-G131H-N218S-S248N-K256R-L257G, N109G-G131H-N243V-K256R, N109G-G131H-S145F-N218S-N243V-K256R-L257G, N109G-G131H-S248N-K256R-L257G, N109G-G131H-S248N-L257G, N109G-G131H-T158S-K256R, N109G-G131H-T158S-N218S-L257G, N109G-G131H-T158S-N218S-N243V, N109G-G131H-T158S-N218S-N243V-K256R, N109G-G131H-T158S-N218S-N243V-K256R-L257G, N109G-G131H-T158S-N218S-N243V-S248N-L257G, N109G-G131H-T158S-N218S-S248N-K256R, N109G-G131H-T158S-N218S-S248N-K256R-L257G-A274T, N109G-G131H-T158S-N218S-S248N-L257G, N109G-G131H-T158S-N243V-S248N, N109G-G131H-T158S-N243V-S248N-K256R-L257G, N109G-G131H-T158S-S248N-K256R-L257G, N109G-G131H-T158S-S248N-L257G, N109G-N218S-K256R-L257G, N109G-N218S-L257G, N109G-N218S-N243V-K256R, N109G-N218S-N243V-S248N-S260F, N109G-N218S-S248N, N109G-N243V-K256R, N109G-N243V-L257G, N109G-N243V-S248N, N109G-N243V-S248N-K256R-L257G, N109G-S182F-S204F-S207L-N218S-S236F-S248N-L257G, N109G-S248N-K256R, N109G-T158S-K256R, N109G-T158S-N218S-N243V-K256R-L257G, N109G-T158S-N218S-S248N-L257G, N109G-T158S-N243V, N109G-T158S-N243V-K256R, N109G-T158S-N243V-S248N, N109G-T158S-N243V-S248N-K256R, N109G-T158S-S248N-K256R, N109G-T158S-S248N-L257G, N218S, N218S-N243V-S248N-K256R, N218S-S248N-L257G, N243V-K256R, N243V-S248N-K256R, N243V-S248N-K256R-L257G, N243V-S248N-L257G-Q271R, S003P-A116T-T158S-S248N-K256R, S248N, T158S-N218S, T158S-N218S-A272V, T158S-N218S-L257G, T158S-N218S-N243V-K256R-L257G, T158S-N218S-N243V-L257G, T158S-N218S-S248N-K256R-L257G, T158S-N243V-K256R, T158S-N243V-K256R-L257G, T158S-N243V-S248N-K256R, V004A-N109G-A116T-G131H-S248N-K256R-L257G, and Y006H-A116T-G131H-S248N;

(x) at least one set of amino acid substitutions relative to SEQ ID NO:6 selected from the group consisting of S018F-S162L, S018P-D120N, P014T-S037T, S009T-K141R, and S161P-S162L;

(xi) at least one set of amino acid substitutions selected relative to SEQ ID NO:6 from the group consisting of I031V-S038W, P014T-S037T, S018F-S162L, S018P-D120N, and S162L-D181H;

(xii) at least one set of amino acid substitutions relative to SEQ ID NO:6 selected from the group consisting of Y21H-D259G, S183T-S249R, N61D-Q206R, Y262N-Q275R, K043R-N076S, A133V-D259N, and I079V-Q217H;

(xiii) at least one set of amino acid substitutions relative to SEQ ID NO:6 selected from the group consisting of N61P-S63G-N109Q-A128S-S224A-N243V, A88T-N109G-A114S-A116T-A128S-N243V, A88T-N109G-A114S-A116T-A128S-S183L-S224A-N243V, N109G-A128S-S183V, N109G-A128S-N243V-K256R, N109M-A128S-S224A, A88T-N109S-A116T-A128S-S224A-N243V, N109Q-A128S-S224A-N243V, A88T-N109M-A116T-A128S-S224A-N243V, N109S-A128S-S224A-N243V, A88T-N109G-A116T-N243V, N101Q-N109Q-A128S-S224A-N243V, N109G-A116T-N243V-K256R, N109G-A128S-P129S-S130T-S224A-N243V, and A88T-N109Q-A116T-A128S-S224A-N243V;

(xiv) at least one set of amino acid substitutions relative to SEQ ID NO:6 selected from the group consisting of S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V, S33T-N61G-S63G-N109G-A128S-N218S-N243V, S33T-S63G-N109G-A128S-N218S-N243V, N61P-S63G-N109Q-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-A88T-N109G-A116T-A128S-N218S-N243V, S33T-N109G-A128S-N218S-N243V, S33T-A128S-N218S, A1G-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-S224A-N243V-S249Q, S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-S224A-N243V, A88T-N109G-A114S-A116T-A128S-N243V, A88T-N109G-A114S-A116T-A128S-S183L-S224A-N243V, N109G-A114S-A128S, N109G-A114S-A128S-S183L-S224A, N109G-A114S-A128S-S224A, N109G-A114S-A128S-S224A-N243V, A88T-N109G-A116T-A128S-S224A-N243V, N61G-A88T-N109G-A116T-A128S-S224A-N243V, N109G-A128S-S183V, N109G-A114S-A128S-N243V, N109G-A128S-N243V-S248A, N109G-A128S-S224A-N243V, N109G-A128S-N243V-K256R, N109G-A128S-S224A, N109G-A128S-S183L-S224A, N61G-N109G-A128S-S224A, N109M-A128S-S224A, A88T-N109S-A116T-A128S-S224A-N243V, N109M-A128S-S224A-N243V, S63G-A128S, A88T-N109G-A116T-N243V, N101Q-N109Q-A128S-S224A-N243V, N109G-A116T-N243V-K256R, N109G-A116T, S63G-N109G, A88T-N109G, N109G-K256R, N61G-N109G-N243V, S33T-N109G-A128S-G169A-N218S-N243V, S33T-N109G-A128S-N218S-S224A-N243V, N109G-A128S-P129S-S130T-S224A-N243V, and A88T-N109Q-A116T-A128S-S224A-N243V; and

(xv) at least one set of amino acid substitutions relative to SEQ ID NO:6 selected from the group consisting of G24S-G53S-N78S-G97A-N101S-A128S, I31L-S33T-S63G-N109G-A128S-G169A-N218S-N243V, A1G-I31L-S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-S63G-N109G-A128S-G131H-G169A-N218S-N243V, S33T-S63G-N109G-A128S-G169A-N218S-N243V, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-

S224A-N243V, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V-S249Q, S33T-N61G-S63G-N109G-A128S-N218S-N243V, S33T-S63G-N109G-A128S-N218S-N243V, S33T-T55P-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-A88T-N109G-A116T-A128S-N218S-N243V, S33T-N109G-A128S-N218S-N243V, S33T-N76D-N109G-A128S-N218S-N243V, S33T-N76D-N109G-A128S-N218S-N243V-S248N-K256R, S33T-N61G-N109G-A128S-N218S-N243V, S33T-N76D-A128S-N218S, S33T-A128S-N218S, A1G-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-S224A-N243V-S249Q, N61P-S63G-N109Q-A128S-G131H-S224A-N243V, S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-S224A-N243V, A88T-N109G-A114S-A116T-A128S-N243V, A88T-N109G-A114S-A116T-A128S-S183L-S224A-N243V, N109G-A114S-A128S, N109G-A114S-A128S-S183L-S224A, N109G-A114S-A128S-S224A, N109G-A114S-A128S-S224A-N243V, A88T-N109G-A116T-A128S-S224A-N243V, N61G-A88T-N109G-A116T-A128S-S224A-N243V, N109G-A128S-S183V, N109G-A114S-A128S-N243V, N109G-A128S-N243V-S248A, N109G-A128S-S224A-N243V, N109G-A128S-N243V-K256R, N109G-A128S-S224A, N109G-A128S-S183L-S224A, N61G-N109G-A128S-S224A, N76D-N109G-A128S-S224A, N109M-A128S-S224A, N109G-A128S-S183L, S33T-N76D, A88T-N109S-A116T-A128S-S224A-N243V, N109Q-A128S-S224A-N243V, N109S-A128S-S224A, A88T-N109M-A116T-A128S-S224A-N243V, N101Q-N109Q-A128S-P129S-S130T-S224A-N243V, S63G-N109Q-A128S-S224A-N243V, N109M-A128S-S224A-N243V, S63G-A128S, N109S-A128S-S224A-N243V, A88T-N109G-A116T-N243V, N61S-N109G-N243V, N101Q-N109Q-A128S-S224A-N243V, N109G-A116T-N243V-K256R, A88T-N109G-A116T-T158S-N243V-K256R, N109G-A116T, S63G-N109G, A88T-N109G, N109G-K256R, N61G-N109G-N243V, S33T-N61P-S63G-N109G-A128S-G131H-G169A-N218S-N243V, S33T-N109G-A128S-G169A-N218S-N243V, S33T-N109G-A128S-N218S-S224A-N243V, N109G-A128S-P129S-S130T-S224A-N243V, and A88T-N109Q-A116T-A128S-S224A-N243V.

In an eighth aspect, the invention provides an isolated or non-naturally occurring protease variant of a BPN' subtilisin protease having the amino acid sequence of SEQ ID NO:2, said variant having proteolytic activity and comprising at least one set of amino acid substitution(s) selected from the group consisting of: S182E, N109I, N109D-Y217L-S248R, N109D-S188R-Y217L, S87D-Y217L-S248R, S87R-N109D-Y217L-S248R, S87R-N109D-S188D-Y217L-S248R, G128A-Y217Q, I111V-M124V, M124V-Y217Q, N62Q-G97A, S89Y-M124V, V68A, V68A-A92G, V68A-G97A, V68A-I111V, V68A-S89Y, V68A-V227T, V68A-Y217Q, W106F-Y217Q, G97A-G128A-Y217Q, G97A-L126A-Y217Q, G97A-M124V-L126A-Y217Q, G97A-N123G-Y217Q, L96T-G97A-Y217Q, M124V-L126A-Y217Q, N62Q-G128A-Y217Q, N62Q-G97A-Y217Q, G97N-G128A-Y217M, G97G-G128S-Y217E, G97A-G128A-Y217Q, G97M-G128S-Y217E, G97A-G128S-Y217Q, G97D-G128S-Y217Q, G97M-G128G-Y217M, G97G-G128S-Y217Q, G97S-G128S-Y217Q, G97G-G128A-Y217Q, G97S-G128A-Y217E, G97A-G128S-Y217L, G97A-G128A-Y217N, G97Q-G128S-Y217L, G97A-G128A-Y217M, G97A-G128A-Y217S, G97D-G128A-Y217Q, G97M-G128S-Y217Q, G97Q-G128G-Y217D-S87Y, G97S-

G128A-Y217N, G97A-G128S-Y217T, G97D-G128S-Y217E, G97D-G128A-Y217L, G97G-G128S-Y217E-S78P-A272T, G97T-G128S-Y217D, G97D-G128A-Y217I, G97Q-G128S-Y217Q, G97G-G128A-Y217D, G97Q-G128A-Y217N, G97S-G128A-Y217M, G97S-G128S-Y217N, G97S-G128S-Y217M, G97E-G128S-Y217M, G97S-G128P-Y217Q, G97T-G128S-Y217Q, G97D-G128S-Y217Q-A73T, G97E-G128S-Y217N, G97G-G128A-Y217I, G97Q-G128A-Y217D, G97Q-G128S-Y217M, G97R-G128T-Y217Q-S162P, G97S-G128S-Y217D, G97T-G128P-Y217I, G97Q-G128G-Y217E, G97C-G128G-Y217N, G97D-G128S-Y217H, G97M-G128S-Y217L, G97M-G128S-Y217N, G97S-G128S-Y217E, G97M-G128S-Y217I, G97A-G128P-Y217A, G97R-G128S-Y217D, G97A-G128A-Y217Q-S145D, G97A-G128A-Y217Q-P239R, G97A-G128A-Y217Q-N61E-P129E-S162K-K213L-N240K, G97A-G128A-Y217Q-N61E, G97A-G128A-Y217Q-P40E-A144K-K213L, G97A-G128A-Y217Q-P129E, G97A-G128A-Y217Q-N61E-P129E-S159K, G97A-G128A-Y217Q-K213L, G97A-G128A-Y217Q-S87D, G97A-G128A-Y217Q-Q206E, G97A-G128A-Y217Q-S24R-P40E-S145D-S159K-K213L, G97A-G128A-Y217Q-K265N, G97A-G128A-Y217Q-S24R, G97A-G128A-Y217Q-P40E, G97A-G128A-Y217Q-Q275E, G97A-G128A-Y217Q-P129E-S145D-N240K, G97A-G128A-Y217Q-A144K, G97A-G128A-Y217Q-S159K, G97A-G128A-Y217Q-S162K, G97A-G128A-Y217Q-N240K, G97A-G128A-Y217Q-S53G, G97A-G128A-Y217Q-S78N, G97A-G128A-Y217Q-S53G-S78N, G97A-G128A-Y217Q-S53G-I111V, G97A-G128A-Y217Q-S53G-N117S, G97A-G128A-Y217Q-S53G-S132N, G97A-G128A-Y217Q-Y104N-S132N, G97A-G128A-Y217Q-S53G-S78N-I111V, G97A-G128A-Y217Q-S53G-S78N-N117S, G97A-G128A-Y217Q-S53G-S78N-S132N, G97A-G128A-Y217Q-S53G-Y104N-S132N, G97A-G128A-Y217Q-S78N-Y104N-S132N, Y217L-V068C-A069G, Y217L-I079F-A098G, Y217L-P086T-S101D-Q103S-V147I, Y217L-A088T-P129S-G146D, Y217L-V093I-G128D-P129R, Y217L-Z096.01D-A098R, Y217L-Z096.01H-A098G, Y217L-G097S-Z097.01S-A098G-A273T, Y217L-A098S-D099G-G100D, Y217L-Z098.01N, Y217L-D099G-Z099.01N, Y217L-D099G-Z099.01S, Y217L-D099V-S101D, Y217L-Z099.01S, Y217L-G100D, Y217L-S101D-Q103H, Y217L-S101G-A151V, Y217L-S101H-G102S, Y217L-S101H-Q103D, Y217L-G102R-Q103C-Y104C-V192I, Y217L-Q103D, Y217L-V121I-I122S-N123C, Y217L-V121L-N123C, Y217L-I122S-N123S, Y217L-M124I, Y217L-M124V, Y217L-L126F-P129Z-S182N, Y217L-L126Y, Y217L-G127S-P129D, Y217L-Z127.01N-G128S-P129S, Y217L-G128H-P129Y, Y217L-G128S-P129D, Y217L-G128S-P129D-S248R, Y217L-G128S-P129G, Y217L-P129G-G131Z, Y217L-P129G-S130H-S132Z, Y217L-P129H-G131Z, Y217L-P129L, Y217L-P129S-S130H-S132Z, Y217L-P129Z, Y217L-P129Z-S130G, Y217L-P129Z-S130G-G131H-S132H, Y217L-P129Z-S130H, Y217L-S130V-G131D-S132I, S87T-A88L-S89G-G97A-G128A-Y217Q, N61P-S63H-G97A-G128A-Y217Q, S87G-A88V-S89A-G97A-G128A-Y217Q, P86S-S87G-A88V-G97A-G128A-Y217Q, Q59S-N61P-G97A-G128A-Y217Q, S24G-N25G-G97A-G128A-Y217Q, N61P-N62S-G97A-G128A-Y217Q, G97A-G128A-P129Q-S130G-G131S-Y217Q, L75S-N76Y-G97A-G128A-Y217Q, G97A-G128A-V203Y-Y217Q, T55P-G97A-G128A-Y217Q, A88V-L90I-G97A-G128A-Y217Q, G97A-G128A-G211R-N212S-K213V-Y217Q, G23A-S24G-N25G-G97A-G128A-Y217Q, T22N-S24A-G97A-G128A-Y217Q, S24R-G97A-G128A-Y217Q, G97A-A98S-G128A-Y217Q,

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A variant according to the eighth aspect of the invention may have increased proteolytic activity and/or increased cleaning activity compared to the protease having the sequence of SEQ ID NO:2 and/or SEQ ID NO:4 and may be in a mature form. A variant according to the eighth aspect of the invention

may comprise an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:4, wherein the variant has proteolytic activity and optionally has enhanced proteolytic activity and/or enhanced cleaning activity compared to the protease having the sequence of SEQ ID NO:2 or SEQ ID NO:4.

A variant according to the eighth aspect of the invention may comprise three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14 or 15 amino acid substitutions at positions selected from the group of consisting of positions 24, 25, 40, 52, 53, 55, 58, 59, 61, 62, 63, 68, 78, 86, 87, 88, 89, 92, 96, 97, 100, 101, 103, 104, 106, 111, 114, 115, 116, 117, 118, 123, 124, 125, 126, 128, 129, 130, 131, 132, 133, 134, 144, 145, 159, 161, 162, 167, 194, 203, 206, 213, 217, 227, 232, 239, 240, 242, 265, 267, and 275. A variant according to the eighth aspect may comprise a total of three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14 or 15 amino acid substitutions selected from groups (a) and (b): (a) charged amino acid substitutions selected from the group consisting of N61E, A144K, P129E , P239R, P40E, Q103E, Q206E, Q7275E, S145D, S159K, S162K, S24R, S63H, S87D and T242R; and (b) neutral amino acid substitutions selected from the group consisting of A114G, A116N, A133V, A134T, A232T, A88V, A92G, F58G, G100T, G128A, G131S, G97A, I111V, I115V, K213L, K265N, L126A, L267V, L96T, M124V, N117S, N118G, N123G, N240K, N25G, N61P, N62Q, N62R, N62S, P129Q, P129V, P194L, P239V, P52L, P86S, Q59S, S101N, S125A, S130G, S132N, S161P, S24G, S53G, S78N, S87G, S89Y, T55P, V203Y, V227T, V68A, W106F, Y104N, Y167A, and Y217Q. A variant according to the eighth aspect may comprise an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:2.

In a ninth aspect, the invention provides an isolated or non-naturally occurring protease variant selected from the group of:

(a) a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, or 98% identity to SEQ ID NO:2, and comprising at least one set of amino acid substitution(s) relative to SEQ ID NO:2 selected from groups (i) through (vii):

(i) Q059V-S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q, S078N-G097A-I108V-G128A-V147Q/-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-Y217Q-N252Q, and S078N-S087E-G097A-M124I-G128A-Y217Q-S224A;

(ii) Q059V-S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q, S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-Y217Q-N252Q, and S078N-S087E-G097A-M124I-G128A-Y217Q-S224A;

(iii) G097A-M124V-Y167A-Y217Q, V068A-Y167A-Y217Q, G097A-I111V-M124V-Y167A, I111V-M124V-Y167A-Y217Q, V068A-I111V-Y167A-Y217Q, G097A-I111V-M124V-Y167A-Y217Q, and P052L-V068A-I111V;

(iv) G097A-N123A-Y217Q, G097A-N123V-Y217Q, N061P-G102A-G128S-Y217Q, N061P-S101N-G102A-G128S-Y217Q, Y217Q, S078N-G097A-I111V-N123Q-Y217Q, and G102A-N123Q-Y217Q;

(v) G097A-N123A-Y217Q, G097A-N123V-Y217Q, N061P-N062Q-G097A-G100D-Y217Q, N061P-S101N-G102A-G128S-Y217Q, Y217Q, N061P-G102A-G128S-Y217Q, S078N-G097A-I111V-N123Q-Y217Q, and G102A-N123Q-Y217Q;

(vi) N061P-N062Q-G097A-G100N-Y217Q, N061P-G097A-M124I-Y217Q, G102A-N123Q-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-N123Q-Y217Q, S053G-N061P-G097A-S101N-N123Q-Y217Q, N061P-G097A-S101N-N123Q-Y217Q, N061P-N062Q-G097A-G100Q-P210S-Y217Q, S053G-N061P-G102A-P129S-P210S-Y217Q, G097A-I111V-M124V-P210S-Y217Q, G097A-N123Q-P210S-Y217Q, N061P-G097A-N123Q-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-M124I-Y217Q, S053G-N061P-G097A-S101N-M124I-Y217Q, S053G-N061P-G097A-M124I-Y217Q, N061P-S078N-G097A-I111V-M124I-Y217Q, N061P-G097A-M124V-Y217Q, N061P-N062Q-G100N-G102A-Y217Q, N061P-N062Q-G097A-G100Q-S101N-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, S078N-G097A-I111V-N123Q-Y217Q, S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, N061P-N062Q-G097A-G100N-S101N-Y217Q, N061P-N062Q-S078N-G097A-G100N-I111V-Y217Q, N061P-N062Q-G097A-G100D-Y217Q, N061P-N062Q-G097A-G100Q-Y217Q, N061P-S101N-G102A-G128S-Y217Q, N061P-G102A-G128S-Y217Q, S024G-N025G-S053G-N061P-S101N-G102A-P129S-Y217Q, S053G-N061P-S101N-G102A-P129S-Y217Q, N061P-S078N-G102A-I111V-P129S-Y217Q, G097A-N123V-Y217Q, S053G-N061P-G102A-P129S-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, N061P-N062Q-G097A-S101N-I111V-Y217Q, N061P-N062Q-G097A-I111V-Y217Q, N062Q-S078N-G097A-I111V-Y217Q, N062Q-G097A-I111V-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, N061P-G097A-S101N-I111V-M124V-Y217Q, G097A-N123Q-Y217Q, N061P-G097A-I111V-M124V-Y217Q, S078N-G097A-I111V-M124V-Y217Q, G097A-I111V-M124I-Y217Q, S053G-S078N-G097A-I111V-G128S-Y217Q, S078N-G097A-G128S-Y217Q, S053G-N061P-G097A-G128S-Y217Q, G097A-N123A-Y217Q. and Y217Q; and

(vii) S101N-G128A-Y217Q, S024G-T055P-N061P-G097A-S101N-G128A, S024G-N025G-N061P-S078N-G097A-S101N-G128A, S024G-T055P-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S053G-N061P-G097A-S101N-G128A-Y217Q-S249N, N025G-S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-G097A-S101N-G128A-Y217Q, S024G-S053G-N061P-G097A-G128A-Y217Q, S024G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S024G-T055P-N061P-G097A-G128A-Y217Q, S024G-S038G-S053G-S078N-S101N-G128A-Y217Q, S053G-S078N-G097A-S101N-

G128A-Y217Q, N025G-S078N-G097A-G128A-Y217Q, S024G-N025G-S053G-N061P-G097A-G128A-S130G-Y217Q, and S024G-S053G-S078N-G097A-S101N-G128A-Y217Q; and

(b) a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the amino acid sequence of SEQ ID NO:6 and comprising at least one set of amino acid substitution(s) relative to SEQ ID NO:6 selected from groups (i) through (xii):

(i) A001C, A001D, A001E, A001F, A001H, A001K, A001L, A001M, A001N, A001P, A001Q, A001R, A001S, A001T, A001V, A013C, A013S, A015C, A015D, A015E, A015L, A015R, A048C, A048E, A048S, A073N, A073T, A074G, A074S, A085C, A085G, A085S, A085T, A085V, A088M, A088S, A092S, A098G, A114G, A133E, A133P, A133R, A137E, A137H, A137R, A142C, A144D, A144G, A144H, A144K, A144L, A144N, A144R, A152S, A153G, A153S, A153V, A176C, A179G, A187C, A187F, A187G, A187L, A187N, A187P, A187Q, A187S, A187T, A187V, A187W, A200G, A216C, A216H, A216R, A216W, A223S, A228S, A230G, A230S, A230T, A230V, A231V, A232C, A232V, A272E, A272G, A272K, A272P, A272R, A273D, A273G, A273H, A273L, A273P, A273Q, A273R, A273T, A273V, A274H, A274M, A274R, D036E, D036N, D036Q, D036S, D041C, D060G, D099A, D099H, D099Q, D120E, D181A, D181G, D181H, D181T, D197T, D259A, D259G, D259H, D259N, D259P, D259Q, D259S, D259T, E156A, E156C, E156G, E156H, E156L, E156Q, E156S, E156V, E195G, E251C, E251I, E251L, E251Q, E251T, F058Y, F189A, F189G, F189H, F189L, F189R, F189S, F189T, F189W, F189Y, F261C, F261D, F261E, F261K, F261P, G020C, G020E, G020F, G020H, G020L, G020N, G020Q, G020R, G020T, G020Y, G024A, G024D, G024P, G046D, G053A, G053D, G053E, G053F, G053L, G053M, G053Q, G053R, G053S, G053Y, G097K, G097M, G097R, G131C, G131D, G131R, G146A, G157A, G157N, G157P, G157S, G157T, G160A, G160L, G160R, G160V, G166C, G166I, G166L, G166Q, G166V, G166W, G169A, G178A, G211E, G211K, G215C, G215D, G215E, G215H, G215L, G215S, G215T, G215W, G258A, G258D, G258P, G258S, H017I, H039S, H226L, H238N, H238Y, I011L, I011T, I011V, I031C, I031F, I031L, I079E, I079F, I079K, I079L, I079M, I079Q, I079R, I107L, I111M, I175L, I205A, I205C, I205V, I268L, I268M, K012A, K012C, K012E, K012F, K012G, K012H, K012L, K012N, K012S, K012T, K012W, K027A, K027N, K027S, K027T, K043A, K043C, K043D, K043E, K043F, K043G, K043H, K043I, K043L, K043M, K043N, K043P, K043Q, K043R, K043T, K043V, K043W, K043Y, K136G, K136H, K141A, K141G, K141H, K141L, K141M, K141N, K141Q, K141R, K141T, K141V, K141W, K170A, K170C, K170G, K170Q, K170S, K213A, K213G, K213H, K213I, K213L, K213N, K213Q, K213R, K213S, K213T, K213V, K237A, K237H, K237I, K237L, K237N, K237S, K237T, K237V, K256A, K256C, K256D, K256E, K256G, K256H, K256M, K256P, K256Q, K256S, K256T, K256V, K256W, K265G, K265H, K265N, K265S, K265Y, L016E, L042C, L042F, L042M, L042V, L075G, L075H, L075I, L075T, L082A, L082E, L082F, L082H, L082R, L082S, L082T, L090M, L135F, L196M, L209A, L209C, L209E, L209G, L209H, L209R, L209S, L233E, L233G, L233S, L235M, L235R, L235V, L235W, L257C, L257D, L257E, L257G, L257P, L257R, L257W, L267E, L267F, M050L, M050Y, M119C,

M119I, M124L, M222A, M222F, M222L, M222S, M222T, N025C, N025E, N025P, N056D, N056S, N061A, N061C, N061D, N061G, N061I, N061K, N061L, N061Q, N061R, N062A, N062C, N062H, N062L, N062Q, N062R, N062S, N062T, N062V, N062Y, N076A, N076D, N076E, N076L, N076M, N076P, N076Q, N076S, N076T, N076V, N078D, N078E, N078F, N078G, N078H, N078K, N078P, N078Q, N078R, N101D, N101F, N101R, N117G, N117R, N117S, N118A, N118D, N118H, N118Q, N118R, N118S, N118T, N184C, N184E, N184P, N184R, N212C, N212D, N212E, N212R, N212W, N218C, N218D, N218E, N218F, N218G, N218M, N218P, N218R, N218T, N218V, N218W, N240A, N240G, N240Q, N240S, N240W, N243C, N243G, N243S, N252D, N252E, N252V, N269C, N269H, P005A, P005D, P005M, P005Q, P005V, P005W, P014A, P014D, P014F, P014K, P014M, P014R, P014V, P040F, P040R, P040W, P057A, P057W, P129E, P129R, P129V, P172E, P172K, P172R, P194E, P194H, P194R, P194W, P201A, P201G, P201T, P210E, P210L, P239A, P239G, P239H, P239K, P239N, P239R, P239T, Q002D, Q002E, Q002G, Q002I, Q002K, Q002L, Q002P, Q002R, Q002V, Q010D, Q010R, Q010W, Q019C, Q019D, Q019E, Q019H, Q019L, Q019P, Q019R, Q059A, Q059C, Q059D, Q059E, Q059L, Q059R, Q059S, Q059T, Q059W, Q103W, Q185D, Q185E, Q185K, Q185R, Q185W, Q206D, Q206G, Q206H, Q206L, Q206V, Q206W, Q217A, Q217C, Q217E, Q217F, Q217G, Q217H, Q217K, Q217L, Q217R, Q217V, Q245A, Q245D, Q245E, Q245H, Q245M, Q245R, Q271A, Q271C, Q271D, Q271E, Q271F, Q271G, Q271L, Q271P, Q271R, Q271T, Q271W, Q271Y, Q275A, Q275F, Q275G, Q275I, Q275L, Q275P, Q275R, R186A, R186H, R186I, R186L, R186M, R186V, R186W, S003D, S003E, S003F, S003K, S003R, S009C, S009E, S009K, S018C, S018D, S018R, S037A, S037D, S037E, S037G, S037H, S037K, S037L, S037P, S037R, S037Y, S038D, S038M, S038P, S038R, S038Y, S049C, S049T, S063A, S063C, S063D, S063F, S063L, S063M, S063R, S063Y, S087C, S087D, S087K, S087L, S087M, S087N, S087R, S087Y, S089A, S089C, S089D, S089E, S089F, S089G, S089H, S089I, S089K, S089P, S089R, S089V, S089Y, S105T, S125A, S130C, S130D, S130E, S130K, S130R, S130W, S145D, S145G, S145L, S145R, S145T, S159C, S159D, S159L, S159P, S159W, S161C, S161E, S161R, S162C, S162E, S162W, S163A, S173T, S173V, S182C, S182E, S182R, S183C, S183D, S183P, S183R, S188C, S188D, S188E, S188F, S188K, S188L, S188P, S188R, S188W, S190A, S190C, S190G, S190T, S191G, S204E, S204G, S204R, S204Y, S207G, S224G, S224T, S236C, S236D, S236E, S236G, S248C, S248D, S248E, S248H, S248R, S249E, S249L, S249R, S260A, S260C, S260E, S260G, S260K, S260Q, S260R, S260V, S260Y, T022L, T022P, T055C, T055D, T055E, T055I, T055K, T055M, T055R, T055S, T055V, T055W, T055Y, T071A, T158A, T158D, T158E, T158G, T158L, T158P, T158Q, T158R, T158V, T158Y, T164A, T164G, T164K, T164Q, T164R, T208S, T220A, T242D, T242G, T244D, T244E, T244R, T253E, T253R, T253Y, T254G, T255C, T255D, T255E, T255K, T255R, V004D, V004E, V004T, V026A, V028I, V028L, V030I, V044A, V044C, V044P, V044T, V045C, V045D, V045E, V045G, V045I, V045N, V045R, V045T, V051H, V072L, V081A, V081G, V081H, V081R, V081S, V084I, V084M, V095A, V095C, V143A, V143C, V143E, V143F, V143G, V143H, V143Q, V143T, V143W, V147A, V147Q, V147S, V148I, V148L, V149C, V149I, V149L, V165C, V165L, V180A, V180C, V180M, V180S, V192C, V192F, V192G, V192I, V192Q, V192Y, V203A, V203C,

V203D, V203E, V203G, V203K, V203M, V203R, V203S, V270C, V270G, V270L, V270P, W241F, W241L, Y006A, Y006C, Y006D, Y006E, Y006M, Y006N, Y006R, Y006S, Y021C, Y091W, Y104T, Y104V, Y104W, Y214H, Y214Q, Y262C, Y262D, Y262E, Y262H, Y262I, Y262R, and Y262V;

(ii) A001C, A001E, A001P, A015C, A015E, A048C, A048E, A073T, A085C, A085G, A088I, A088M, A114G, A128H, A137R, A142C, A187C, A187F, A187L, A187N, A187P, A187W, A216C, A216H, A230G, A230S, A273D, A273H, A273P, A273Q, A273R, A273T, A274H, D036N, D036Q, D036S, D041C, D041N, D099A, D099H, D099N, D099Q, D099S, D197T, D259H, E156C, E156G, E156H, E156L, E156Q, F058G, F189A, F189G, F189H, F189L, F189R, F189S, F189T, F261C, F261D, F261E, G046D, G053E, G053L, G053M, G053Q, G053R, G131C, G131D, G146A, G157N, G157P, G157T, G160V, G178A, G215C, G215L, G258A, G258P, H039A, H039S, H067T, H238Y, I011L, I031F, I079E, I111M, I175L, I268M, K012A, K012C, K012E, K012F, K012G, K012H, K012L, K012N, K012W, K027N, K027S, K027T, K043C, K043D, K043G, K043L, K043R, K043W, K136E, K136G, K141G, K141L, K141M, K141N, K141R, K170C, K170Q, K213V, K256D, K265G, K265N, K265Q, K265S, K265Y, L042C, L042F, L075G, L075V, L082A, L082E, L082F, L082H, L082R, L082S, L090M, L090T, L126W, L233E, L233G, L233S, L235V, L257D, L257E, L257G, L257P, L257R, L257W, M050L, M222A, M222F, M222L, M222S, M222T, N025R, N056S, N062C, N062H, N062L, N062Q, N062R, N062T, N062Y, N076D, N076P, N078D, N078E, N078F, N078R, N078V, N117G, N118L, N184P, N269C, N269H, N269Q, P005V, P005W, P014K, P057A, P057W, P086F, P129V, P201T, P225G, P225S, P239A, P239G, P239H, P239N, P239T, Q002D, Q002I, Q002K, Q002L, Q002P, Q002R, Q002V, Q010W, Q019L, Q019P, Q059C, Q059D, Q059E, Q059W, Q185W, Q217C, Q245A, Q245H, Q271C, Q271D, Q271E, Q271L, Q271W, Q275A, Q275G, R186M, S009C, S009L, S018C, S018D, S037E, S037H, S037K, S037L, S037P, S038P, S038Y, S049C, S049N, S063C, S063D, S063F, S063L, S063Y, S087C, S087K, S087L, S087N, S087R, S087Y, S089D, S089E, S089F, S089G, S089P, S089W, S105T, S125A, S130C, S145D, S159D, S159P, S163A, S173V, S182P, S183P, S190A, S190G, S191G, S204E, S224G, S248E, S248H, S249E, S260V, S260Y, T022P, T055E, T055M, T055R, T055W, T158D, T158E, T164A, T164G, T164K, T164Q, T164R, T220A, T242G, T242P, T253E, T255C, T255G, V004D, V004T, V044A, V044L, V044P, V044T, V045C, V045G, V045I, V045K, V045L, V045N, V045R, V045T, V045V, V051H, V081A, V081G, V081H, V081R, V084S, V143G, V147A, V148L, V165C, V180S, V203D, V203G, V203S, V270C, V270G, V270P, V270S, W241L, Y104T, Y214H, Y214Q, Y262D, Y262E, Y262G, Y262H, Y262L, Y262N, Y263G, and Y263W;

(iii) A001E, A001E-A088T, A001E-A116T, A001E-A128S, A001E-A128S-G131H-N243V, A001E-G024E, A001E-G131H, A001E-G131H-G169A-N243V, A001E-G169A, A001E-K043Y, A001E-K256R, A001E-L257G, A001E-N076D, A001E-N076D-N109G-A128S, A001E-N109G, A001E-N218S, A001E-N243V, A001E-Q103H, A001E-Q206D, A001E-S033T, A001E-S033T-N109G-N218S, A001E-S033T-N109G-N243V, A001E-S063G, A001E-S162G, A001E-S248N, A001E-S249A, A001E-T158S, A088T-A128S, A088T-G169A, A088T-N218S, A088T-Q206D, A116T-G169A, A116T-N218S, A116T-Q206D, A128S, A128S-G131H, A128S-G169A, A128S-N218S, A128S-N243V, A128S-

Q206D, G024E, G024E-A088T, G024E-A128S, G024E-G131H, G024E-K043Y, G024E-N076D, G024E-N218S, G024E-Q103H, G024E-Q206D, G024E-S033T, G024E-S063G, G024E-S162G, G024E-S248N, G024E-S249A, G131H-G169A, G131H-N218S, G131H-N243V, G131H-Q206D, G169A, G169A-K256R, G169A-L257G, G169A-N218S, G169A-N243V, G169A-Q206D, G169A-S248N, G169A-S249A, K043Y, K043Y-A116T, K043Y-A128S, K043Y-G131H, K043Y-G169A, K043Y-L257G, K043Y-N076D, K043Y-N109G, K043Y-N218S, K043Y-Q103H, K043Y-Q206D, K043Y-S063G, K043Y-S162G, K043Y-S248N, K043Y-S249A, K043Y-T158S, N076D, N076D-A088T, N076D-A116T, N076D-A128S, N076D-G131H, N076D-G169A, N076D-N218S, N076D-N243V, N076D-Q103H, N076D-Q206D, N076D-S162G, N076D-S248N, N076D-S249A, N109G-G169A, N109G-Q206D, N109G-S248N, N218S, N218S-K256R, N218S-L257G, N218S-S248N, N218S-S249A, P040E-N109G-A128S-G131H, Q103H, Q103H-G169A, Q103H-Q206D, Q206D, Q206D-K256R, Q206D-L257G, Q206D-N218S, Q206D-N243V, Q206D-S248N, Q206D-S249A, S018T-Y021N-S033T-N109G-A128S-N243V-S248N-K256R, S033T, S033T-A088T, S033T-A116T, S033T-A128S, S033T-A128S-G131H-N243P, S033T-G131H, S033T-G169A, S033T-K043Y, S033T-K256R, S033T-L257G, S033T-N076D, S033T-N076D-A128S-N218S, S033T-N076D-N109G-A128S-N218S-N243V-S248N-K256R, S033T-N109G, S033T-N109G-A128S-N243V-S248N-K256R, S033T-N218S, S033T-P040E-Q103H-N109G, S033T-Q103H-A128S-G131H, S033T-Q206D, S033T-S063G, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243V, S033T-S162G, S033T-S248N, S033T-S249A, S063G-A116T, S063G-G131H, S063G-G169A, S063G-N109G-A128S-G131H, S063G-N218S, S063G-N243V, S063G-Q206D, S063G-S249A, S162G-G169A, S162G-N218S, S162G-Q206D, S162G-S249A, S248N-K256R, S248N-S249A, S249A-K256R, S249A-L257G, T158S-G169A, T158S-K256R, T158S-Q206D, and T158S-S162G;

(iv) A001E, A001E-A088T, A001E-A116T, A001E-A128S-G131H-N243V, A001E-G024E, A001E-G131H-G169A-N243V, A001E-G169A, A001E-K043Y, A001E-L257G, A001E-N076D, A001E-N076D-N109G-A128S, A001E-N109G, A001E-Q103H, A001E-Q206D, A001E-S033T-N109G-N218S, A001E-S033T-N109G-N243V, A001E-S248N, A001E-T158S, A088T-G169A, A088T-Q206D, A116T-G169A, A116T-N218S, A116T-Q206D, A128S-G131H, A128S-N243V-S248N-K256R, A128S-Q206D, G024E-K043Y, G024E-N076D, G024E-Q206D, G131H-G169A, G131H-N218S, G131H-N243V-K256R, G131H-Q206D, G131H-S248N, G169A-K256R, G169A-N218S, G169A-N243V, G169A-Q206D, G169A-S249A, K043Y-G169A, K043Y-N076D, K043Y-N218S, K043Y-Q206D, N061P-N109G-G131H-N243V, N061P-N109G-N243V, N061S-N109G-A128S-N243V-S248N-K256R-S260P, N061S-N109G-N243V, N076D, N076D-G131H, N076D-L257G, N076D-N109G, N076D-Q103H, N076D-Q206D, N076D-S249A, N109G-A128S-N243V-S248N, N109G-A128S-N243V-S248N-K256R, N109G-A128S-S248N-K256R, N109G-G169A, N109G-N243P-S248A-K256R, N109G-N243P-S248N-K256R, N109G-N243V-K256R, N109G-N243V-S248A-K256R, N109G-N243V-S248N, N109G-N243V-S248N-K256R, N109G-S248N-K256R, N218S-S249A, N243V-S248N-K256R, P040E-N109G-A128S-G131H, Q103H-Q206D, Q206D, Q206D-K256R, Q206D-L257G, Q206D-N218S,

Q206D-S248N, Q206D-S249A, S009T-N109G-A128S-K141R-N243V-S248N-K256R, S009T-S018T-Y021N-A128S-K141R-N243V, S018T-Y021N-A128S-N243V, S018T-Y021N-N061S-A128S-N243V-S260P, S018T-Y021N-N061S-N109G-A128S-S260P, S018T-Y021N-S033T-N109G-A128S-N243V-S248N-K256R, S033T, S033T-A128S-G131H-N243P, S033T-A128S-G131H-N243V, S033T-G169A, S033T-N076D-A128S-N218S, S033T-N076D-N109G-A128S-N218S-N243V-S248N-K256R, S033T-N109G-A128S-N243P-S248N-K256R, S033T-N109G-A128S-N243V-S248N-K256R, S033T-P040E-Q103H-N109G, S033T-Q103H-A128S-G131H, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243V, S063G-G131H, S063G-G169A, S063G-N109G-A128S-G131H, S063G-Q206D, S162G-Q206D, T158S-Q206D, and T158S-S162G;

(v) A001E-A088T, A001E-A128S, A001E-A128S-G131H-N243V, A001E-G024E, A001E-G024E-S204E-Q206D, A001E-G131H-G169A-N243V, A001E-K043Y, A001E-N076D, A001E-N076D-N109G-A128S, A001E-N218S, A001E-N243V, A001E-Q103H, A001E-Q206D, A001E-S033T, A001E-S033T-N109G-N218S, A001E-S162G, A116T-Q206D, A128S-N243V-S248N-K256R, A128S-Q206D, G024E-Q206D, G131H-Q206D, K043Y-G131H, K043Y-Q103H, K043Y-Q206D, K043Y-S162G, N061S-N109G-A128S-N243V-S260P, N076D-A128S, N076D-Q206D, N076D-S162G, N076D-S248N, N109G-A128S-S248N-K256R, N109G-A128S-T158S-N243V-S248N-K256R, N109G-N243P-S248N-K256R, N109G-Q206D, N243V-S248N-K256R, P040E-N109G-A128S-G131H, Q103H-Q206D, Q206D-K256R, Q206D-N243V, Q206D-S248N, Q206D-S249A, S018T-Y021N-N061S-A128S-N243V-S260P, S018T-Y021N-N061S-N109G-A128S-S260P, S033T-A128S-G131H-N243P, S033T-N076D-A128S-N218S, S033T-N076D-N109G-A128S-N218S-N243V-S248N-K256R, S033T-P040E-Q103H-N109G, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S063G-Q206D, S162G-Q206D, and T158S-Q206D;

(vi) A001E, A001E-A128S, A001E-G024E, A001E-G131H, A001E-G169A, A001E-K043Y, A001E-K256R, A001E-L257G, A001E-N076D, A001E-N109G, A001E-N218S, A001E-N243V, A001E-Q103H, A001E-Q206D, A001E-S033T, A001E-S063G, A001E-S162G, A001E-S248N, A001E-S249A, A001E-T158S, A088T-A116T, A088T-G131H, A088T-N109G, A088T-N218S, A088T-Q206D, A116T-A128S, A116T-G131H, A116T-Q206D, A116T-S248N, A128S, A128S-G131H, A128S-Q206D, G024E, G024E-N076D, G024E-N109G, G024E-Q206D, G131H, G131H-L257G, G131H-Q206D, G169A-K256R, G169A-Q206D, K043Y, K043Y-A088T, K043Y-A116T, K043Y-A128S, K043Y-G131H, K043Y-G169A, K043Y-K256R, K043Y-L257G, K043Y-N076D, K043Y-N109G, K043Y-N218S, K043Y-N243V, K043Y-Q103H, K043Y-S063G, K043Y-S162G, K043Y-S248N, K043Y-S249A, K043Y-T158S, N076D-A088T, N076D-A116T, N076D-A128S, N076D-G131H, N076D-G169A, N076D-N109G, N076D-N218S, N076D-N243V, N076D-Q103H, N076D-Q206D, N076D-S248N, N076D-T158S, N109G, N109G-G169A, N109G-Q206D, N109G-S162G, N109G-T158S, N218S-S248N, Q103H, Q103H-A128S, Q103H-G131H, Q103H-Q206D, Q103H-S248N, Q103H-S249A, Q103H-T158S, Q206D, Q206D-K256R, Q206D-L257G, Q206D-N218S, Q206D-N243V, Q206D-S248N, Q206D-S249A, S033T, S033T-K256R, S033T-S063G, S033T-S249A,

S063G-A088T, S063G-G131H, S063G-G169A, S063G-N076D, S063G-N109G, S063G-N218S, S063G-Q103II, S063G-Q206D, S162G-Q206D, S162G-S249A, S248N-S249A, S249A-K256R, T158S-Q206D, and T158S-S249A;

(vii) G131H-S162G, G131H-S248N, G131H-T158S, K043Y-G131H, K043Y-S162G, Q103H-A128S, Q103H-N218S, S033T-Q103H, S063G-A088T, S063G-G131H, S063G-S248N, and T158S-S162G;

(viii) A015S-A088T-N109G-G131II-T158S-N218S-S248N, A088T-A098S-G131II-S248N-K256R-L257G, A088T-A098S-N218S-K256R, A088T-A116T-G131H-G146C, A088T-A116T-G131H-K256R, A088T-A116T-G131H-K256R-L257G-L267M, A088T-A116T-G131H-N218S-N243V-K256R, A088T-A116T-G131H-N218S-N243V-K256R-L257G, A088T-A116T-G131H-N218S-N243V-K256R-L257G, A088T-A116T-G131H-N218S-N243V-S248N, A088T-A116T-G131H-N218S-S248N, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-N243V, A088T-A116T-G131H-S248N, A088T-A116T-G131H-S248N-L257G, A088T-A116T-G131H-S248N-L257G, A088T-A116T-G131H-T158S-L257G, A088T-A116T-G131H-T158S-N218S, A088T-A116T-G131H-T158S-N218S-K256R-L257G, A088T-A116T-G131H-T158S-N218S-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-A116T-G131H-T158S-N218S-S248N, A088T-A116T-G131H-T158S-N218S-S248N-K256R, A088T-A116T-K256R-L257G, A088T-A116T-N218S, A088T-A116T-N218S-I268V, A088T-A116T-N218S-K256R, A088T-A116T-N218S-L257G, A088T-A116T-N218S-N243V-Q271R, A088T-A116T-N218S-N243V-S248N-K256R, A088T-A116T-N218S-N243V-S248N-K256R-Q275R, A088T-A116T-N218S-S248N, A088T-A116T-N218S-S248N-K256R, A088T-A116T-N243V-S248N-K256R, A088T-A116T-T158S, A088T-A116T-T158S-K256R, A088T-A116T-T158S-K256R-L257G, A088T-A116T-T158S-N218S-K256R, A088T-A116T-T158S-N218S-N243V-L257G, A088T-A116T-T158S-N218S-N243V-S248N-E251K-K256R-L257G, A088T-A116T-T158S-N218S-N243V-S248N-K256R, A088T-A116T-T158S-N218S-N243V-S248N-L257G, A088T-A116T-T158S-N218S-S248N, A088T-A116T-T158S-N218S-S248N-K256R, A088T-A116T-T158S-N218S-S248N-K256R-L257G, A088T-A116T-T158S-N218S-S248N-L257G, A088T-A116T-T158S-N243V, A088T-A116T-T158S-N243V-K256R-L257G, A088T-A116T-T158S-S248N-K256R-L257G, A088T-A116T-T158S-S248N-L257G, A088T-A138E-N218S-N243V-K256R, A088T-G131H, A088T-G131H, A088T-G131H-K141E-N218S-N243V-S248N-L257G, A088T-G131H-K256R, A088T-G131II-N218S-K237R-K256R-L257G, A088T-G131II-N218S-K256R, A088T-G131II-N218S-K256R, A088T-G131H-N218S-K256R-L257G, A088T-G131H-N218S-N243V-K256R-L257G, A088T-G131H-N218S-N243V-L257G, A088T-G131H-N218S-N243V-L257G, A088T-G131H-N218S-N243V-S248N, A088T-G131H-N218S-N243V-S248N-K256R, A088T-G131H-N218S-N243V-S248N-K256R, A088T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-G131H-N218S-N243V-S248N-L257G, A088T-G131H-N218S-S248N, A088T-G131H-N218S-

S248N-K256R, A088T-G131H-N218S-S248N-K256R, A088T-G131H-N243V, A088T-G131H-N243V-K256R, A088T-G131H-N243V-K256R-L257G, A088T-G131H-N243V-S248N, A088T-G131H-N243V-S248N, A088T-G131H-S248N, A088T-G131H-S248N-K256R, A088T-G131H-T158S-N218S-I234T-S248N-L257G, A088T-G131H-T158S-N218S-K256R-L257G, A088T-G131H-T158S-N218S-L257G, A088T-G131H-T158S-N218S-N243V, A088T-G131H-T158S-N218S-N243V-K256R-L257G, A088T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-G131H-T158S-N218S-S248N-K256R, A088T-G131H-T158S-N218S-S248N-K256R-L257G, A088T-G131H-T158S-S248N-K256R, A088T-G131H-T158S-S248N-K256R-L257G, A088T-G131H-T158S-S248N-K256R-L257G, A088T-G131H-V149L-T158S-N243V-S248N-K256R-L257G, A088T-I107T-N109G-G131H-N218S-A223G-S248N-K256R, A088T-I107T-N109G-G131H-N218S-S248N-K256R, A088T-I108T-N109G-G131H-T158S-N218S-S248N-K256R-L257G, A088T-K213N-N243V-S248N-K256R, A088T-K256R-L257G, A088T-L257G, A088T-N109G-A116T-G131H-A232S-N243V-K256R, A088T-N109G-A116T-G131H-D140G-S248N-L257G, A088T-N109G-A116T-G131H-D140G-T158S-N218S-N243V-K256R, A088T-N109G-A116T-G131H-K141E-N218S, A088T-N109G-A116T-G131H-N218S-L257G, A088T-N109G-A116T-G131H-N218S-N243V-K256R, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-N269D, A088T-N109G-A116T-G131H-N218S-N243V-S248N-Q275R, A088T-N109G-A116T-G131H-N218S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N243V-S248N, A088T-N109G-A116T-G131H-T158S, A088T-N109G-A116T-G131H-T158S-N218S-L257G-I268V, A088T-N109G-A116T-G131H-T158S-N218S-N243F-S248N, A088T-N109G-A116T-G131H-T158S-N218S-N243V-K256R, A088T-N109G-A116T-G131H-T158S-N218S-N243V-K256R-L257G, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-N109G-A116T-G131H-T158S-N218S-S248N-L257G, A088T-N109G-A116T-G131H-T158S-N218S-S248N-L257G, A088T-N109G-A116T-G131H-T158S-N243V-S248N-K256R-I268V, A088T-N109G-A116T-G131H-T158S-N243V-S248N-L257G, A088T-N109G-A116T-G131H-T158S-S248N, A088T-N109G-A116T-G131H-T158S-S248N, A088T-N109G-A116T-G131H-V149A-N218S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-W241L-S248N-K256R-L257G, A088T-N109G-A116T-K256R, A088T-N109G-A116T-M124I-G131H-T158S-N218S-S248N-L257G, A088T-N109G-A116T-N218S-K256R-L257G, A088T-N109G-A116T-N218S-N243V-S248N-K256R, A088T-N109G-A116T-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-N218S-S248N, A088T-N109G-A116T-T158S-K256R-L257G, A088T-N109G-A116T-T158S-N218S, A088T-N109G-A116T-T158S-N218S-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-L257G, A088T-N109G-A116T-T158S-N218S-N243V-S248N-

L257G, A088T-N109G-A116T-T158S-N243V-S248N, A088T-N109G-A116T-T158S-N243V-S248N-K256R, A088T-N109G-A116T-V148A-N218S-N243V, A088T-N109G-D140G-N243V, A088T-N109G-G131H-A138V-T158S-N218S-N243V-S248N-L257G, A088T-N109G-G131H-D140G-T158S-N243V-S248N-K256R, A088T-N109G-G131H-K141E-T158S-N218S-K256R, A088T-N109G-G131H-K256R-L257G, A088T-N109G-G131H-N218S-N243V, A088T-N109G-G131H-N218S-N243V-S248N, A088T-N109G-G131H-N218S-N243V-S248N-L257G, A088T-N109G-G131H-N218S-S248N, A088T-N109G-G131H-N218S-S248N-K256R-L257G, A088T-N109G-G131H-N218S-S248N-K256R-L257G-Q275R, A088T-N109G-G131H-N218S-S248N-K256R-Q271R, A088T-N109G-G131H-N218S-S248N-L257G, A088T-N109G-G131H-N243V-S248N, A088T-N109G-G131H-N243V-S248N-K256R-L257G, A088T-N109G-G131H-T158S, A088T-N109G-G131H-T158S-K256R, A088T-N109G-G131H-T158S-L233S-N243V-S248N, A088T-N109G-G131H-T158S-N218S-N243V-K256R, A088T-N109G-G131H-T158S-N218S-N243V-K256R-L257G, A088T-N109G-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-G131H-T158S-N218S-N243V-S248N-L257G, A088T-N109G-G131H-T158S-N218S-S248N-K256R, A088T-N109G-G131H-T158S-N218S-S248N-K256R, A088T-N109G-G131H-T158S-N243V-S248N-K256R, A088T-N109G-G131H-T158S-S248N-K256R, A088T-N109G-G131H-T158S-S248N-K256R-L257G, A088T-N109G-G131H-V149A-K256R-L257G, A088T-N109G-G131H-V149L-T158S-K256R-L257G, A088T-N109G-N218S-K256R-L257G, A088T-N109G-N218S-N243V-K256R, A088T-N109G-N218S-N243V-L257G, A088T-N109G-N218S-N243V-S248N, A088T-N109G-N218S-N243V-S248N-K256R-L257G, A088T-N109G-N218S-S248N-K256R, A088T-N109G-N218S-S248N-T255K-K256R-L257G, A088T-N109G-N243V-K256R, A088T-N109G-N243V-S248N-K256R, A088T-N109G-S248N-K256R-L257G, A088T-N109G-T158S, A088T-N109G-T158S-K256R-L257G, A088T-N109G-T158S-N218S, A088T-N109G-T158S-N218S-K256R-L257G-Q271K, A088T-N109G-T158S-N218S-L257G, A088T-N109G-T158S-N218S-N243V-K256R, A088T-N109G-T158S-N218S-N243V-K256R-L257G, A088T-N109G-T158S-N218S-N243V-S248N-K256R, A088T-N109G-T158S-N218S-N243V-S248N-L257G, A088T-N109G-T158S-N218S-S248N, A088T-N109G-T158S-S248N, A088T-N109G-T158S-S248N, A088T-N109G-T158S-S248N-K256R, A088T-N109G-W241R-S248N-K256R, A088T-N218S-N243V-K256R, A088T-N218S-N243V-L257G, A088T-N218S-N243V-S248N, A088T-N218S-N243V-S248N-K256R, A088T-N218S-N243V-S248N-K256R, A088T-N218S-S248N, A088T-N218S-S248N-L257G, A088T-N218S-S248N-L257G-Q271R, A088T-S248N-K256R-L257G, A088T-T158S-K256R, A088T-T158S-N218S-K256R-L257G, A088T-T158S-N218S-L257G, A088T-T158S-N218S-N243V-K256R, A088T-T158S-N218S-N243V-K256R, A088T-T158S-N218S-N243V-L257G, A088T-T158S-N218S-N243V-S248N, A088T-T158S-N218S-N243V-S248N-K256R, A088T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-T158S-N218S-N243V-S248N-L257G, A088T-T158S-N218S-Q245K-S248N-K256R, A088T-T158S-N218S-S248N, A088T-T158S-N218S-S248N-K256R, A088T-T158S-N218S-S248N-K256R, A088T-T158S-N218S-S248N-L257G, A088T-T158S-N218S-S248N-L257G-Q275K, A088T-T158S-N243V, A088T-T158S-N243V-

K256R, A088T-T158S-N243V-S248N, A088T-T158S-S248N-K256R-L257G, A088T-T158S-S248N-L257G, A088T-T158S-V203I-N218S-K256R-L257G, A088T-V147I-N218S-N243V-K256R-L257G, A088T-Y104H-A116T-G131H-N218S-N243V, A116T-D140G-T158S-N218S-N243V-S248N, A116T-G131H-K141E-N218S-N243V-S248N-L257G, A116T-G131H-L257G, A116T-G131H-N218S-N243V, A116T-G131H-N218S-N243V-K256R, A116T-G131H-N218S-N243V-K256R-L257G, A116T-G131H-N218S-N243V-S248N-K256R, A116T-G131H-N218S-N243V-S248N-K256R-L257G, A116T-G131H-N218S-S248N, A116T-G131H-N218S-S248N-K256R, A116T-G131H-N218S-W241R-N243V-S248N-K256R-L257G, A116T-G131H-N243V-S248N-K256R, A116T-G131H-T158S-N218S-L257G, A116T-G131H-T158S-N218S-N243V-S248N, A116T-G131H-T158S-N218S-S248N, A116T-G131H-T158S-N218S-S248N-L257G-N269D, A116T-G131H-T158S-N218S-S248N-Q271R, A116T-G131H-T158S-N243V-K256R, A116T-G131H-T158S-N243V-S248N, A116T-G131H-V139I-N218S-N243V-S248N, A116T-G131H-V150A-T158S-N243V-S248N-K256R-L257G, A116T-G157E-T158S-N243V-S248N-K256R, A116T-K141E-N218S-N243V-S248N-K256R-L257G, A116T-K256R, A116T-N218S-N243V-S248N, A116T-N218T-N243V-S248N, A116T-N243V-K256R-L257G, A116T-S248N-K256R, A116T-T158S-N218S, A116T-T158S-N218S-K256R, A116T-T158S-N218S-K256R-L257G, A116T-T158S-N218S-N243V-L257G, A116T-T158S-N218S-N243V-S248N, A116T-T158S-N218S-S248N-L257G, G024S-G053S-N078S-G097A-N101S, G053S-A088T-N109G-A116T-G131H-T158S-G169S-N218S-S248N-K256R-L257G, G065D-A088T-G131H-N243V-S248N, G131H-K141R-T158S-N218S-K256R, G131H-K256R, G131H-N218S-K256R-L257G, G131H-N218S-L257G, G131H-N218S-N243V-S248N-L257G, G131H-N218S-S248N, G131H-N218S-S248N-K256R, G131H-N243V-S248N, G131H-N243V-S248N-L257G, G131H-T158S-N218S, G131H-T158S-N218S-N240H-N243V-S248N-K256R-L257G, G131H-T158S-N218S-N243V, G131H-T158S-N218S-N243V-S248N-K256R-L257G, G131H-T158S-N218S-N243V-S248N-L257G, G131H-T158S-N218S-S248N-I268V, G131H-T158S-N218S-S248N-K256R-L257G-N269S, G131H-T158S-N243V-L257G, G131H-T158S-N243V-S248N, G131H-T158S-S248N, I107T-G131H-T158S-N243V-S248N-K256R-L257G, I107T-N109G-G131H-N218S-L257G, K256R, K256R-L257G, L090I-N109G-T158S-N243V, L257G, N109G-A116T-G131H-N218S-N243V, N109G-A116T-G131H-N218S-N243V-L257G, N109G-A116T-G131H-N218S-S248N-L257G, N109G-A116T-G131H-N218S-W241R-N243V-K256R, N109G-A116T-G131H-S248N-L257G, N109G-A116T-G131H-T158S-N218S-K256R, N109G-A116T-G131H-T158S-N218S-K256R-L257G-Q271R, N109G-A116T-G131H-T158S-N218S-N243V-K256R-L257G, N109G-A116T-G131H-T158S-N218S-N243V-S248N, N109G-A116T-G131H-T158S-N218S-N243V-S248N-L257G, N109G-A116T-G131H-T158S-N243V-K256R-L257G, N109G-A116T-G131H-T158S-N243V-S248N-K256R, N109G-A116T-G131H-T158S-S248N-K256R-L257G, N109G-A116T-I234T-N243V-S248N-K256R-L257G, N109G-A116T-K141E-T158S-N218S-N243V-L257G, N109G-A116T-N218S-N243V-S248N, N109G-A116T-N218S-W241R-N243V-S248N-K256R-L257G, N109G-A116T-N243V-K256R-L257G, N109G-A116T-T158S-N218S-K237R-N243V-S248N, N109G-A116T-T158S-N218S-N243V-S248N, N109G-G131H-K141E-L257G, N109G-G131H-N218S-N243V, N109G-G131H-N218S-N243V-S248N, N109G-G131H-S248N,

N109G-G131H-T158S, N109G-G131H-T158S-L257G, N109G-G131H-T158S-N218S-N243V-S248N-K256R, N109G-G131H-T158S-N218S-S248N-K256R, N109G-G131H-T158S-N218S-S248N-L257G, N109G-G131H-T158S-N243V-S248N-K256R-L257G, N109G-G131H-T158S-S248N-K256R, N109G-N218S-K256R-L257G, N109G-N218S-N243V-S248N-K256R, N109G-N218S-S248N-L257G, N109G-S248N, N109G-T158S-N218S, N109G-T158S-N218S-N243V, N109G-T158S-N218S-N243V-L257G, N109G-T158S-N218S-S248N-K256R, N109G-T158S-N243V-L257G, N109G-T158S-N243V-S248N-K256R-L257G, N218S-K256R, N218S-N243V-K256R, N218S-N243V-S248N, N218S-S248N, N218S-S248N-K256R, N218S-S248N-K256R-L257G, N243V-L257G, S003P-N109G-A116T-G131H-N218S-N243V-S248N, S003P-N109G-A116T-G131H-T158S-N218S-K256R, S003P-N109G-G131H-T158S-L257G, S003P-S248N-L257G, S105H-W106G-I107L-I108S-N109A-G110A-I111S-E112N-W113G-A114P, S248N-L257G, T158S, T158S-K256R, T158S-N218S, T158S-N218S-K256R, T158S-N218S-L233S-S248N, T158S-N218S-L257G, T158S-N218S-N243V-K256R, T158S-N218S-N243V-S248N, T158S-N218S-N243V-S248N-L257G, T158S-N218S-S248N-K256R-L257G, T158S-N218S-S248N-L257G, T158S-N243V-S248N-K256R, T158S-N243V-S248N-K256R-L257G, T158S-N243V-S248N-L257G, T158S-S248N, T158S-S248N-K256R-L257G, V004A-A088T-G131H-N218S-N243V-S248N-L257G, V004L-A088T-G131H-T158S-N218S-S248N-L257G, Y006H-N218S-N243V-S248N, and Y104H-N109G-G131H-N243V-S248N;

(ix) A088T-A098S-G131H-S248N-K256R-L257G, A088T-A116T-G131H-K256R, A088T-A116T-G131H-L257G, A088T-A116T-G131H-N218S-N243V, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-N218S-S248N-L257G, A088T-A116T-G131H-N243V-S248N-L257G, A088T-A116T-G131H-S248N, A088T-A116T-G131H-S248N-K256R-L257G, A088T-A116T-G131H-T158S-K256R, A088T-A116T-G131H-T158S-L257G, A088T-A116T-G131H-T158S-N218S-L257G, A088T-A116T-G131H-T158S-N218S-N243V-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-A116T-G131H-T158S-N243V-K256R-L257G, A088T-A116T-G131H-T158S-N243V-S248N-L257G, A088T-A116T-G131H-V150A-N218S-S248N-L257G, A088T-A116T-K256R-L257G, A088T-A116T-K256R-L257G, A088T-A116T-L257G, A088T-A116T-N218S, A088T-A116T-N218S, A088T-A116T-N218S-N243V-Q271R, A088T-A116T-N218S-N243V-S248N, A088T-A116T-N218S-S248N-K256R, A088T-A116T-T158S, A088T-A116T-T158S, A088T-A116T-T158S-K256R, A088T-A116T-T158S-K256R-L257G, A088T-A116T-T158S-N218S-N243V-L257G, A088T-A116T-T158S-N218S-S248N, A088T-A116T-T158S-N218S-S248N-K256R, A088T-A116T-T158S-N243V-K256R-L257G, A088T-A116T-T158S-N243V-S248N-K256R, A088T-A138E-N218S-N243V-K256R, A088T-G131D-T158S-N243V-S248N, A088T-G131H, A088T-G131H-L257G, A088T-G131H-N218S-K237R-K256R-L257G, A088T-G131H-N218S-K256R, A088T-G131H-N218S-N243V-S248N-K256R, A088T-G131H-N218S-N243V-S248N-L257G, A088T-G131H-N243V-K256R, A088T-G131H-N243V-L257G, A088T-G131H-N243V-S248N, A088T-G131H-N243V-S248N-K256R, A088T-G131H-T158S-N218S-I234T-S248N-L257G, A088T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-G131H-T158S-N243V-K256R-L257G, A088T-

G131H-T158S-S248N, A088T-G131H-T158S-S248N-K256R, A088T-G131H-T158S-S248N-K256R, A088T-G131H-T158S-S248N-K256R-L257G, A088T-G131H-V149L-T158S-N243V-S248N-K256R-L257G, A088T-I108T-N109G-G131H-T158S-N218S-S248N-K256R-L257G, A088T-K213N-N243V-S248N-K256R, A088T-K256R-L257G, A088T-N109G-A116T-G131H-D140G-S248N-L257G, A088T-N109G-A116T-G131H-K141E-N218S, A088T-N109G-A116T-G131H-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-Q275R, A088T-N109G-A116T-G131H-N218S-S248N, A088T-N109G-A116T-G131H-N218S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N243V-S248N-K256R, A088T-N109G-A116T-G131H-N243V-S248N-K256R, A088T-N109G-A116T-G131H-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-K256R, A088T-N109G-A116T-G131H-T158S-N218S-L257G-I268V, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-S248N, A088T-N109G-A116T-G131H-V149A-T158S-N218S-K256R, A088T-N109G-A116T-G131H-W241L-S248N-K256R-L257G, A088T-N109G-A116T-M124I-G131H-T158S-N218S-S248N-L257G, A088T-N109G-A116T-N218S-N243V-S248N-K256R, A088T-N109G-A116T-N218S-S248N-K256R-L257G, A088T-N109G-A116T-N243V-K256R, A088T-N109G-A116T-N243V-S248N-L257G, A088T-N109G-A116T-S248N-L257G, A088T-N109G-A116T-T158S-K256R-L257G, A088T-N109G-A116T-T158S-N212D-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-K256R, A088T-N109G-A116T-T158S-N218S-N243V-L257G, A088T-N109G-A116T-T158S-N218S-S248N, A088T-N109G-A116T-T158S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N243V-S248N, A088T-N109G-A116T-T158S-S248N-L257G-Q271P, A088T-N109G-A116T-V148A-N218S-N243V, A088T-N109G-A137E-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-D140G-N243V, A088T-N109G-G131H-A152S-T158S-N218S-S248N-K256R, A088T-N109G-G131H-D140G-T158S-N243V-S248N-K256R, A088T-N109G-G131H-K256R-L257G, A088T-N109G-G131H-K256R-L257G, A088T-N109G-G131H-N218S, A088T-N109G-G131H-N218S-L257G, A088T-N109G-G131H-N218S-N243V, A088T-N109G-G131H-N218S-N243V-S248N-L257G, A088T-N109G-G131H-N218S-S248N, A088T-N109G-G131H-N243V-S248N, A088T-N109G-G131H-T158S-L233S-N243V-S248N, A088T-N109G-G131H-T158S-L257G, A088T-N109G-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-G131H-T158S-N218S-S248N-K256R, A088T-N109G-G131H-T158S-N218S-W241R-S248N-L257G, A088T-N109G-G131H-T158S-N243V-S248N-L257G, A088T-N109G-G131H-T158S-S248N-K256R, A088T-N109G-L257G, A088T-N109G-N218S-K256R-L257G, A088T-N109G-N218S-S248N-K256R, A088T-N109G-N218S-S248N-T255K-K256R-L257G, A088T-N109G-N243V-K256R-L257G, A088T-N109G-N243V-S248N-K256R, A088T-N109G-S248N-K256R-L257G, A088T-N109G-T158S, A088T-N109G-T158S-N218S-N243V, A088T-N109G-T158S-N218S-N243V-L257G, A088T-N109G-T158S-N218S-N243V-S248N-L257G, A088T-N109G-T158S-N218S-S248N, A088T-N109G-T158S-N218S-S248N, A088T-N109G-T158S-N243V-S248N-K256R, A088T-N109G-T158S-N243V-S248N-Q275R, A088T-N109G-T158S-S248N, A088T-N109G-T158S-S248N-K256R, A088T-

N109G-W241R-S248N-K256R, A088T-N218S-N243V-S248N-K256R-L257G, A088T-N218S-S248N-L257G, A088T-N243V-L257G, A088T-S248N, A088T-S248N-K256R-L257G, A088T-S248N-L257G, A088T-S248N-L257G-I268V, A088T-T158S-K256R, A088T-T158S-N218S-K256R, A088T-T158S-N218S-L257G, A088T-T158S-N218S-L257G, A088T-T158S-N218S-N243V-S248N, A088T-T158S-N218S-N243V-S248N-L257G, A088T-T158S-N218S-Q245K-S248N-K256R, A088T-T158S-N218S-S248N-K256R, A088T-T158S-N218S-S248N-L257G, A088T-T158S-N218S-S248N-L257G-Q275K, A088T-T158S-N243V-K256R, A088T-T158S-N243V-K256R-L257G, A088T-T158S-N243V-L257G, A088T-T158S-N243V-S248N-K256R, A088T-T158S-S248N, A088T-T158S-S248N-K256R-L257G, A088T-T158S-S248N-L257G, A088T-T158S-S248N-L257G, A088T-T158S-V203I-N218S-K256R-L257G, A088T-Y104H-A116T-G131H-N218S-N243V, A088T-Y104H-N109G-A116T-A153S-N218S-N243V-S248N-L257G-N269D, A088T-Y104H-N109G-G131H-A137E-T158S-N218S-N243V-S248N-K256R, A098S-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A116T-D140G-T158S-N218S-N243V-S248N, A116T-G131H-K141E-N218S-N243V-S248N-L257G, A116T-G131H-N218S-W241R-N243V-S248N-K256R-L257G, A116T-G131H-N243V, A116T-G131H-T158S-K256R-L257G, A116T-G131H-T158S-N218S-N243V, A116T-G131H-T158S-N218S-S248N-K256R, A116T-G131H-T158S-N243V-S248N, A116T-G131H-V139I-N218S-N243V-S248N, A116T-G157E-T158S-N243V-S248N-K256R, A116T-N218S-S248N, A116T-N218S-S248N-K256R, A116T-N243V-K256R, A116T-S248N-K256R, A116T-T158S, A116T-T158S-L257G-Q271R, A116T-T158S-N218S-N243V-K256R-L257G, G053S-A088T-N109G-A116T-G131H-T158S-G169S-N218S-S248N-K256R-L257G, G065D-A088T-G131H-N243V-S248N, G131H-N218S-L257G, G131H-N218S-S248N, G131H-N243V-K256R, G131H-S248N-K256R, G131H-T158S, G131H-T158S-K256R, G131H-T158S-N243V-L257G, K256R, K256R-L257G, L090I-N109G-T158S-N243V, L257G, N109G-A116T-G131H, N109G-A116T-G131H-N243V, N109G-A116T-G131H-N243V-K256R-L257G, N109G-A116T-G131H-T158S-N218S-N243V-S248N, N109G-A116T-G131H-T158S-N218S-N243V-S248N-K256R, N109G-A116T-I234T-N243V-S248N-K256R-L257G, N109G-A116T-N218S-N243V-L257G, N109G-A116T-N218S-W241R-N243V-S248N-K256R-L257G, N109G-A116T-S248N-K256R, N109G-A116T-T158S-K256R-L257G, N109G-A116T-T158S-N218S-K237R-N243V-S248N, N109G-A116T-T158S-N218S-S248N-K256R, N109G-A116T-T158S-N243V, N109G-A116T-T158S-S248N, N109G-A116T-T158S-S248N-K256R, N109G-G131H-A137V-T158S-N218S-S248N, N109G-G131H-K141E-L257G, N109G-G131H-T158S, N109G-G131H-T158S-N243V-K256R-L257G, N109G-G131H-T158S-N243V-S248N-L257G, N109G-G131H-T158S-S248N-Q271R, N109G-N218S-S248N-K256R, N109G-N243V-S248N-L257G, N109G-S248N, N109G-T158S-K256R-L257G, N109G-T158S-N218S-N243V-L257G, N109G-T158S-N243V-K256R-L257G, N109G-T158S-N243V-S248N-K256R-L257G, N218S-N243V-S248N-K256R-L257G, N243V, P014L-A015L-L016C-H017T-S018L-Q019K-G020A-Y021T-T022L-G023E, S003P-N109G-A116T-G131H-T158S-N218S-K256R, S003P-N109G-G131H-T158S-L257G, S003P-S248N-L257G, S105H-W106G-I107L-I108S-N109A-G110A-I111S-E112N-W113G-A114P, T158S-N218S-N243V-K256R, T158S-N218S-N243V-S248N, T158S-N218S-S248N-K256R, T158S-N243V-S248N, T158S-S248N,

T158S-S248N-K256R, T158S-S248N-K256R-L257G, V004A-A088T-A116T-T158S-N218S, V004A-A088T-G131H-N218S-N243V-S248N-L257G, V004M-A116T-V148A-T158S-N243V-S248N-K256R, Y006H-N109G-N218S-N243V-S248N, Y104H-A116T-T158S-S248N, Y104H-N109G-G131H-N243V-S248N, and Y104H-N218S-L257G;

(x) A045S-S236G, A045S-S236Y, A134T-S260G, G024A-S037W, I031V-S038W, I115T-S183T, I115V-N184Y, N025K-P129K, N025K-P129R, N025K-S037P, N061D-S260I, Q010L-S037P, Q010R-S037T, Q019L-S260N, Q019L-S260P, S037P-T254S, S161P-T253A, and S162L-D181H;

(xi) A045S-S236G, A045S-S236Y, A133V-S260N, A134T-S260G, G024A-S037W, I031V-S038W, I115T-S183T, I115V-N184Y, N025K-P129R, N025K-S037P, N061D-S260I, Q010R-S037T, Q019L-S260N, Q019L-S260P, and S162L-D181H; and

(xii) A045S-S236G, A045S-S236Y, I115T-S183T, N025K-S037P, N061S-S260P, Q010L-S037P, Q010R-S037T, Q019L-S260N, S037P-T254S, and S161P-S260P; wherein each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2.

A variant according to the ninth aspect of the invention may have enhanced proteolytic activity and/or enhanced cleaning activity compared to the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6, respectively. A variant according to the ninth aspect of the invention may have a performance index in a proteolytic assay (e.g., AAPF assay) or cleaning assay (e.g., BMI, grass, or egg microswatch assay) that is greater than that of the protease set forth in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6. A variant according to the ninth aspect of the invention may be a protease variant of BPN' subtilisin protease (SEQ ID NO:2).

In a twenty-first aspect, the invention provides an isolated or non-naturally occurring cold water protease that is a variant of a parent protease, said cold water protease comprising a total of three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14 or 15 mutations selected from groups (a) and (b) below, wherein at least one mutation is selected from group (a):

(a) 1, 9, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 33, 43, 76, 102, 109, 137, 141, 158, 169, 204, 210, 218, 243, 248, 249, 256, 257, 260, and 269; and

(b) 24, 25, 40, 52. 53, 55, 58, 59, 61, 62, 63, 68, 78, 86, 87, 88, 89, 92, 96, 97, 100, 101, 103, 104, 106, 111, 114, 115, 116, 117, 118, 123, 124, 125, 126, 128, 129, 130, 131, 132, 133, 134, 144, 145, 159, 161, 162, 167, 194, 203, 206, 213, 217, 227, 232, 239, 240, 242, 265, 267, and 275, wherein amino acid positions are numbered by correspondence with SEQ ID NO:2. The parent protease according to the twenty-first aspect of the invention may comprise an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence of SEQ ID NO:2 and the variant according to the twenty-first aspect of the invention, may comprise a total of three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14 or 15 mutations selected from groups (a) and (b) below, wherein at least one mutation is selected from group (a):

(a) A1E/T, S9/T, P14L, A15L, L16C, H17T, S18L/T, Q19K, G20A, Y21N/T, T22L, G23E, S33T, K43Y, N76D, G102A, N109A/S/G, A137V, K141R, T158S, G169A, S204E, P210S, N218S, N243P/V, S248N/A, S249A, K256R, L257G, S260P, and N269D; and

(b) S24G/R/E, N25G, P40A/E, P52L, S53G, T55P, F58G, Q59S, N61E/P/G/S, N62Q/R/S, S63G/H, V68A, S78N, P86S, S87D/G, A88T/V, S89Y, A92G, L96T, G97A, G100N/Q/T, S101N, Q103E/H, Y104N, W106F, I111V, A114G, I115V, A116N/T, N117S, N118G, N123A/G/Q/V, M124I/V, S125A, L126A, G128A/S, P129E/Q/S/V, S130G, G131S/H, S132N, A133V, A134T, A144K, S145D, S159K, S161P, S162G/K, Y167A, P194L, V203Y, Q206D/E, K213L, Y217Q/L/D, V227T, A232T, P239R/V, N240K, T242R, K265N, L267V, and Q275E. A variant and parent protease according to the second aspect of the invention may be in a mature form.

A variant according to the twenty-first aspect of the invention may have a total net charge of -1, 0 or +1 relative to the BPN' wild-type.

In the first, second, fourth, fifth, sixth, seventh, eighth, ninth, or twenty-first aspect of the invention, the variant may be a subtilisin protease variant having improved wash performance or cleaning performance in a detergent as compared to that of the parent subtilisin protease, which may be a subtilisin protease. In the third aspect of the invention, the polypeptide may have improved wash performance or cleaning performance in a detergent as compared to that of the protease of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. Exemplary detergents for such aspects are shown in the Part I Examples and Part II Examples.

A protease variant according to the first, second, third, fourth, fifth, sixth, seventh, eighth, ninth, or twenty-first aspect of the invention may be a cold water protease. In one aspect, a protease variant according to the first, second, third, fourth, fifth, sixth, seventh, eighth, ninth, or twenty-first aspect of the invention may be a cold water protease having: (i) a performance index (PI) greater than 1, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from at least 1 to about 10, from at least 1 to about 8, or from at least 1 to about 5 on BMI at pH 8 and 60°F when compared to an enzyme having the amino acid sequence of SEQ ID NO:4, as defined in the Test Method set forth in Part I Example 1; or (ii) a performance index of at least 1, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from at least 1 to about 10, from at least 1 to about 8, or from at least 1 to about 5 on BMI at pH 8 and 60°F when compared to an enzyme having the amino acid sequence of SEQ ID NO:6, as defined in the Test Method set forth in Part I Example 1.

As noted above, the protease variant polypeptides of the invention have enzymatic activities (e.g., proteolytic activities) and thus are useful in cleaning applications, including but not limited to, methods for cleaning dishware items, tableware items, fabrics, and items having hard surfaces (e.g., the hard surface of a table, table top, wall, furniture item, floor, ceiling, etc.). Exemplary cleaning compositions comprising one or more protease variant polypeptides of the invention are described *infra*. The enzymatic activity (e.g., protease activity) of a protease variant polypeptide of the invention can be

determined readily using procedures well known to those of ordinary skill in the art. The Examples presented *infra* describe methods for evaluating the enzymatic activity, cleaning performance, and/or washing performance. The performance of protease variants of the invention in removing stains (e.g., a proteinaceous stain), cleaning hard surfaces, or cleaning laundry, dishware or tableware item(s) can be readily determined using procedures well known in the art and/or by using procedures set forth in the Part I Examples and/or Part II Examples.

A polypeptide of the invention can be subject to various changes, such as one or more amino acid insertions, deletions, and/or substitutions, either conservative or non-conservative, including where such changes do not substantially alter the enzymatic activity of the polypeptide. Similarly, a nucleic acid of the invention can also be subject to various changes, such as one or more substitutions of one or more nucleic acids in one or more codons such that a particular codon encodes the same or a different amino acid, resulting in either a silent variation (e.g., mutation in a nucleotide sequence results in a silent mutation in the amino acid sequence, for example when the encoded amino acid is not altered by the nucleic acid mutation) or non-silent variation, one or more deletions of one or more nucleic acids (or codons) in the sequence, one or more additions or insertions of one or more nucleic acids (or codons) in the sequence, and/or cleavage of or one or more truncations of one or more nucleic acids (or codons) in the sequence. Many such changes in the nucleic acid sequence may not substantially alter the enzymatic activity of the resulting encoded protease variant compared to the protease variant encoded by the original nucleic acid sequence. A nucleic acid of the invention can also be modified to include one or more codons that provide for optimum expression in an expression system (e.g., bacterial expression system), while, if desired, said one or more codons still encode the same amino acid(s).

The present invention includes a genus of polypeptides comprising protease variant polypeptides having the desired enzymatic activity (e.g., protease activity or cleaning performance activity) which comprise sequences having the amino acid substitutions described herein and also which comprise one or more additional amino acid substitutions, such as conservative and non-conservative substitutions, wherein the polypeptide exhibits, maintains, or approximately maintains the desired enzymatic activity (e.g., protease activity or subtilisin activity, as reflected in the cleaning activity or performance of the protease variant). Amino acid substitutions in accordance with the invention may include, but are not limited to, one or more non-conservative substitutions and/or one or more conservative amino acid substitutions. A conservative amino acid residue substitution typically involves exchanging a member within one functional class of amino acid residues for a residue that belongs to the same functional class (identical amino acid residues are considered functionally homologous or conserved in calculating percent functional homology). A conservative amino acid substitution typically involves the substitution of an amino acid in an amino acid sequence with a functionally similar amino acid. For example, alanine, glycine, serine, and threonine are functionally similar and thus may serve as conservative amino acid substitutions for one another. Aspartic acid and glutamic acid may serve as conservative substitutions for one another. Asparagine and glutamine may serve as conservative substitutions for one another.

Arginine, lysine, and histidine may serve as conservative substitutions for one another. Isoleucine, leucine, methionine, and valine may serve as conservative substitutions for one another. Phenylalanine, tyrosine, and tryptophan may serve as conservative substitutions for one another.

Other conservative amino acid substitution groups can be envisioned. For example, amino acids can be grouped by similar function or chemical structure or composition (e.g., acidic, basic, aliphatic, aromatic, sulfur-containing). For instance, an aliphatic grouping may comprise: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I). Other groups containing amino acids that are considered conservative substitutions for one another include: aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); sulfur-containing: Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H); Acidic: Aspartic acid (D), Glutamic acid (E); non-polar uncharged residues, Cysteine (C), Methionine (M), and Proline (P); hydrophilic uncharged residues: Serine (S), Threonine (T), Asparagine (N), and Glutamine (Q). Additional groupings of amino acids are well-known to those of skill in the art and described in various standard textbooks. Listing of a polypeptide sequence herein, in conjunction with the above substitution groups, provides an express listing of all conservatively substituted polypeptide sequences.

More conservative substitutions exist within the amino acid residue classes described above, which also or alternatively can be suitable. Conservation groups for substitutions that are more conservative include: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Thus, for example, the invention includes an isolated or recombinant protease variant polypeptide (e.g., subtilisin variant) having proteolytic activity, said protease variant polypeptide comprising an amino acid sequence having at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 99.5% sequence identity to the amino acid sequence of SEQ ID NO:2. A conservative substitution of one amino acid for another in a protease variant of the invention is not expected to alter significantly the enzymatic activity or cleaning performance activity of the protease variant. Enzymatic activity or cleaning performance activity of the resultant protease can be readily determined using the standard assays and the assays described herein.

Conservatively substituted variations of a polypeptide sequence of the invention (e.g., protease variants of the invention) include substitutions of a small percentage, sometimes less than about 25%, 20%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, or 6% of the amino acids of the polypeptide sequence, or less than about 5%, 4%, 3%, 2%, or 1%, of the amino acids of the polypeptide sequence, with a conservative amino acid.

As described elsewhere herein in greater detail and in the Examples provided herein, polypeptides of the invention may have cleaning abilities that may be compared to known proteases, including known subtilisins. Exemplary known subtilisin proteases include, but are not limited to, for example, *B. lentus* subtilisin GG36, *B. amyloliquefaciens* subtilisin BPN', *B. amyloliquefaciens* subtilisin BPN'-Y217L, and *B. clausii* PB92.

Numerous polypeptides of the invention, including protease variants, such as, e.g., subtilisin protease variants described and set forth throughout the specification, including, but not limited to, in the Examples. Part I Examples describe protease variants of the invention, including, e.g., but not limited to, BPN' protease variants. Part II Examples describe additional protease variants of the invention, including, e.g., but not limited to, GG36 protease variants.

The present invention provides protease variants, including serine protease variants, having one or more substitutions as compared to a reference serine protease. In one aspect, the present invention provides cold water proteases. In addition, the present invention provides compositions comprising one or more such protease variants, e.g., serine protease variants. A composition may comprise at least one such protease variant of the invention and an adjunct ingredient, as described elsewhere herein. In one aspect, the present invention provides cleaning compositions comprising at least one of these protease variants, e.g., serine protease variants. A cleaning composition may be a detergent composition. The trend in cleaning is to use lower wash temperatures to save energy. Enzymes have lower activity at lower temperatures resulting in reduced cleaning performance. There is a need in the art for enzymes with enhanced performance at coldwater washing conditions over those currently known in the art. The present invention addresses this need.

The present invention provides cleaning compositions comprising at least one serine protease variant described herein, such as a subtilisin protease variant. The subtilisin protease variant may be a BPN' protease variant. As discussed in further detail *supra*, the invention includes a composition comprising a BPN' protease variant and a GG36 protease variant. Some protease variants, including, but not limited to, e.g., subtilisin variants of the invention, are cold water proteases. The cleaning composition may be a laundry detergent. The laundry detergent may be a detergent having a pH between 3 and 11 (e.g., between pH 4, pH 5, pH 6, pH 7, pH 7.5, pH 8, pH 9, pH 10, pH 10.5, etc.) cold water detergent, a low pH detergent (e.g., pH 3-6), neutral pH detergent (e.g., pH 6.5-7.5), alkaline pH detergent (e.g., pH 9-11) or a compact detergent. A detergent may include phosphate or be without phosphate. The cleaning composition may be a dishwashing detergent. In one aspect, the dishwashing detergent is a phosphate-free detergent, while in another aspect, the dishwashing detergent is a phosphate-containing detergent. In one aspect, the cleaning composition of the invention further comprises at least one additional enzyme, which may optionally be selected from the group of a neutral metalloprotease, lipase, cutinase, amylase, carbohydrase, cellulase, pectinase, mannanase, arabinase, galactanase, xylanase, perhydrolase, oxidase, and peroxidase. Also provided are isolated nucleic acids encoding a serine protease variant of the invention, expression vectors comprising one or more such nucleic acids of the invention, and host cells comprising at least one such expression vector of the invention.

Throughout the specification, for ease of reference, a "set of amino acid substitutions" or "set of substitutions" may refer to a set of multiple amino acid substitutions (i.e., G097A+G128A+Y217Q) or set of a single amino acid substitution (i.e., Y217Q). Thus, a BPN' variant comprising the BPN'

sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q and Y217Q indicates the BPN' variant comprises an amino acid sequence comprising BPN'-G097A-G128A-Y217Q or BPN'-Y217Q.

The amino acid sequence of the mature BPN'-v3 subtilisin protease variant, which is set forth in SEQ ID NO:4, can be represented as BPN'-G097A-G128A-Y217Q, which means the BPN' amino acid sequence of SEQ ID NO:2 with the three substitutions G097A,G128A, and Y217Q. In this format, each dash (-) is equivalent to using a plus sign (+). Thus, BPN'-G097A-G128A-Y217Q can be written alternatively as BPN'+G097A+G128A+Y217Q. The amino acid sequence of the mature BPN'-v36 subtilisin protease variant, which is set forth in SEQ ID NO:6, can be written as BPN'-S24G-S53G-S78N-S101N-G128A-Y217Q or BPN'+S24G+S53G+S78N+S101N+G128A+Y217Q. BPN' variants of the invention may be depicted using these formats.

In addition, the present invention provides subtilisin variants, wherein each such variant is a mature form having proteolytic activity and comprises the BPN'-v3 amino acid sequence (SEQ ID NO:4) comprising at least one set of amino acid substitutions selected from the group consisting of S87T-A88L-S89G, N61P-S63H, S87G-A88V-S89A, P86S-S87G-A88V, Q59S-N61P, S24G-N25G, N61P-N62S, P129Q-S130G-G131S, L75S-N76Y, V203Y, T55P, A88V-L90I, G211R-N212S-K213V, G23A-S24G-N25G, T22N-S24A, S24R, A98S, T158G-S159G, Q59E-N61P, and A98E, and wherein the amino acid positions are numbered by correspondence with the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' set forth as SEQ ID NO:2.

Note that the BPN'-v3 amino acid sequence, which is set forth as SEQ ID NO:4, may be written as BPN'-G97A-G128A-Y217Q. The invention includes a protease variant having proteolytic activity, wherein said variant comprises the amino acid sequence of SEQ ID NO:4 having an amino acid substitution A128S in SEQ ID NO:4. Thus, the invention includes the amino acid sequence BPN'-G97A-G128A-Y217Q. Throughout this specification, polypeptide variants of the invention may be described as a variant of a reference polypeptide comprising one or more particular amino acid substitutions at one or more specified positions in the reference polypeptide sequence, respectively.

The present invention also provides subtilisin variants, wherein each such variant is a mature form having proteolytic activity and comprises the BPN'-v3 amino acid sequence (SEQ ID NO:4) comprising at least one set of amino acid substitutions selected from the group consisting of P86S-S87G-A88V-A116N-N117S-N118G, S24G-N25G-N61P-S101N, S24G-N25G-S53G-T55P-S87T-A88L-S89G-S101N-V203Y, N61P-S78N-S101N-V203Y, T55P-N61P-S78N-S101N-V203Y, S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N, V203Y-L267V, S24G-N25G-T55P-S101N, A134T-L267V, S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G, S24G-N25G-S53G-N61P-S101N-V203Y, N25Y-Q59S-N61P, I111V-S161P, I115V-L267V, T55P-S78N-S87T-A88L-S89G-S101N-V203Y, N25Y-P129Q-S130G-G131S-A137T, N61P-S63H-A128S-P129Q, S53G-N61P-S101N-V203Y, S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N, N61P-S78N-S87T-A88L-S89G-S101N, N25Y-N61P-S63H, Q59S-N61P-V203Y, V8L-N25Y-P129Q-S130G-G131S, P86S-S87G-A88V-P239R, S24G-N25G-S53G-

T55P-N61P-S101N-V203Y, S24G-N25G-P129Q-S130G-G131S, N240K, G23A-S24G-N25G-G211R-N212S-K213V, N61P-S63H-S78N-I111V-A134T, S63T-P86S-S87G-A88V, G23A-S24G-N25G-A116N-N117S-N118G, S78N-S87T-A88L-S89G-S101N, S24G-N25G-A116N-N117S-N118G, T55P-N240K, T55P-P129V-P194S, N25Y-S87G-A88V-S89A, S24G-N25G-S87T-A88L-S89G-S101N, P129Q-S130G-G131S-V203Y, Q59S-N61P-N240K, S24R-P40E-P129E-S159K-K265R, P52S-T55P-V203Y, S24R-P129E, S24G-N25G-S53G-N61P-S78N, S24G-N25G-T55P-S78N-S101N, P86S-S87G-A88V-A116S-N117G-N118R, N61P-S87T-A88L-S89G, S24G-N25G-S53G-T55P-S78N-S87T-A88L-S89G, G23A-S24G-N25G-N61P-S63H, S24R-Q59S-N61P, N61P-P129Q-S130G-G131S, S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N, S24G-N25G-S53G-T55P-S101N-V203Y, N61P-S78N-S87T-A88L-S89G-S101N-V203Y, S24G-N25G-S53G-T55P-S78N-S101N, S24G-N25G-S53G-S101N-V203Y, S24G-N25G-S78N-S101N-V203Y, P129Q-S130G-G131S-A133V-L267V, S87T-A88L-S89G-S101N, G23A-S24G-N25G-P239R, S87G-A88V-S89A-A116N-N117S-N118G, Q59S-N61P-A116S-N117G-N118R, Q59S-N61P-S87T-A88L-S89G, S24G-N25G-S53G-S87T-A88L-S89G-V203Y, A134T-G211T, T55P-A128S-P129Q, T55P-S78N-S87T-A88L-S89G-S101N, P86S-S87G-A88V-T242R, S161P-V203Y, S24G-N25G-T55P-N61P-S78N-S101N-V203Y, G211T-L267V, P40E-T55P-N269K, S24R-A128S-P129G, S24G-N25G-N61P-N62S-P194L-A232T, T55P-A116S-N117G-N118R, S24G-N25G-S53G-S78N-S101N-V203Y, P129Q-S130G-G131S-N240K, S53G-T55P-N61P-S78N-S87T-A88L-S89G, N25Y-P129Q-S130G-G131S, T55P-I115V, N25Y-T55P, G23A-S24G-N25G-A128S-P129D, S53G-S78N-S87T-A88L-S89G-S101N-P129S-V203Y, T55P-A134T, N61P-S63H-S78N-I111V, N61P-A97G-G102A-A128G-P129S, S53G-N61P-S101N, Q59S-N61P-S87G-A88V-S89A, S53G-S87T-A88L-S89G-S101N-V203Y, S87T-A88L-S89G-P129S, S53G-T55P-S78N-S101N-V203Y, T55P-P129Q-S130G-G131S, Q59S-N61P-P129Q-S130G-G131S, A134T-P239R, T55P-V203Y, T55P-S78N-S89Y, T22N-S24A-N61P-S63H, S161P-L267V, T55P-L75H-N76G, A134T-S161P, S87T-A88L-S89G-A134T, T55P-A116N-N117S-N118G, A128S, T55P-S78N-I115V, Y6Q-P129Q-S130G-G131S, S24R-P129Q-S130G-G131S, S24G-N25G-S53G-S78N-S101N, T55P-P129V, N61P-N62Q-G100N-A128G, T55P-P129Q, S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y, S87T-A88L-S89G-N240K, A134T-N240K, S87T-A88L-S89G-P239R, P129Q-S130G-G131S-L267V, P129Q-N240K, S78N-S87T-A88L-S89G-V203Y, I111V-A273S, S24G-N25G-T55P-S78N-A88V-S101N, S24G-N25G-T55P-S78N, S24G-N25G-S53G-S78N-S87T-A88L-S101N-V203Y, S24G-N25G-S53G-S78N-S87T-A88L-S89G-V203Y, S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N-V203Y, S87G-A88V-S89A-P129Q-S130G-G131S, N61P-S63H-S78N-S161P, T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y, I111V-P129Q-S130G-G131S, T22N-S24A-T55P, I115V-N240K, S87G-A88V-S89A-A116N-N117S-N118G-P172H, S24G-N25G-S78N-S87T-A88L-S89G-S101N, S24G-N25G-I115V-A134T, T55P-A128S-P129D, I111V-S159K, N240K-A273S, S159K-L267V, I111V-P129Q-G211T, I115V-A273S, S89Y, S24R-A116N-N117S-N118G, N61E-A144K, P129Q-S130G-G131S-P239R, S87T-A88L-S89G-I115V, T55P-A92G, S145D-S159K-N240K-Q275E, S89Y-P129Q-S130G-G131S, P129Q-S130G-G131S-S162K, I111V-A134T, P40E-S53Y-S78Y-P86S-S87G-A88V, S24G-

N25G-L75H-N76G, N61P-A128G-P129S-S130P, S24R-S145D, S24R-S145D-P239R-Q275E, S24R-S78N-S182P-L267V, S53G-N61P-S87T-A88L-S89G-S101N-V203Y, P5S-S87G-A88V-S89A-A116G-N117R, S53G-N61P-S78N-S87T-A88L-S89G-S101N-V203Y, Q59S-N61P-A116N-N117S-N118G, P239R-A273S, S53G-S78N-S87T-A88L-S89G-S101N-V203Y, S24R-P129V, I111V-P239R, S87T-A88L-S89G-S101N-V203Y, T55P-P129L, S87T-A88L-S89G-I111V, S145D-A273S, P129Q-S130G-G131S-T242R, S3F-S87T-A88L-S89G-G211T, S87G-A88V-S89A-S162K, S89Y-G211T, S87T-A88L-S89G-A144K, P129Q-S130G-G131S-S159K, A116N-N117S-N118G-P129Q-S130G-G131S, S24G-N25G-P129V, S24G-N25G-S78N-S87T-A88L-S89G-S101N-V203Y, N123G-A128G, N61P-N62Q-G100N-G102A-M124I, S24G-N25G-K141E-T242R, S87G-A88V-S89A-A116N-N117S-N118G-A144T, T55P-N61P-S87T-A88L-S89G-G110C-S130P, L75S-N76Y-A116S-N117G-N118R, S145D-S159K-K213L-P239R-N240K, S24R-S87T-A88L-S89G, G23A-S24G-N25G-P129V, A134T-K213L, S89Y-A273S, S24R-P239R, N123G-A128G-P129S, S89Y-P239R, S24G-N25G-A92G, N61P-S63H-I115V-A228V, A97G-A128G-Q217L, L75S-N76Y-P129V, S24R-P129L, S87G-A88V-S89A-P129Q-S182Y-S204Y-P239Q, S24R-A92G, S24R-A116S-N117G-N118R, G23A-S24G-N25G-A116G-N117R, S24G-N25G-P129L, S87T-A88L-S89G-S101G, G23A-S24G-N25G-P129L, S53G-N61P-G102A-V203Y, T55P-V147P, Y6Q-L75S-N76Y, N61P-S63H-V147P, S24R-V147P, S24G-N25G-V68C-A69G, S24G-N25G-N61P-L75S-N76Y-S101N-V203Y, L75S-N76Y-S78N-S101N-V203Y, L75S-N76Y-S78N-S87T-A88L-S89G-S101N-S130P, S24G-N25G-S53G-S101N-S130P-V203Y, G47E-M50I-L75S-N76Y-S162K, S53G-T55P-S87T-A88L-S89G-S101N-S130P-V203Y, S24G-N25G-L75S-N76Y-A128T-P129T-S130G-G131Q-S132C-A133G-A134T, P129Q-S130G-G131S-V147P, S53G-T55P-N61P-L75S-N76Y-S87T-A88L-S89G-G102A-S130P-V203Y, and S53G-T55P-N61P-L75S-N76Y-S101N-S130P-V203Y, and wherein the amino acid positions are numbered by correspondence with the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' set forth as SEQ ID NO:2.

The present invention also provides subtilisin variants, wherein the variant is a mature form having proteolytic activity and comprises the BPN'-v3 amino acid sequence (SEQ ID NO:4) comprising at least one set of amino acid substitutions selected from the group consisting of P40E-S78N-S87D-Y217L, P40E-Y217L, T22V-S78N-Q206E-K213N-Y217L, T22V-S78N-K213N-Y217L, S87D-Y217L, S78N-Y217L, K213N-Y217L, Q206E-Y217L, and T22V-Y217L, and wherein the amino acid positions are numbered by correspondence with the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' of SEQ ID NO:2.

The present invention also provides subtilisin variants, wherein each such variant is a mature form having proteolytic activity and the variant comprises the BPN'-v3 amino acid sequence (SEQ ID NO:4) comprising at least one set of amino acid substitutions selected from the group consisting of S87D-N76D-S78N, P40E-S78N-S87D, P40E-S87D, S78N-P40E, S87D-N76D, P40E, S78N-S87D, S87D, and S78N, and wherein the amino acid positions are numbered by correspondence with the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' of SEQ ID NO:2. In one aspect, the substitutions confer improved enzyme stability.

The present invention also provides subtilisin variants, wherein each such variant is a mature form having proteolytic activity and comprises the BPN'-v3 amino acid sequence (SEQ ID NO:4) comprising at least one set of amino acid substitutions selected from the group consisting of S101N, A137V, N61P, S130P, Q103N, S63T, G102A, N109D-S248R, S87R, S188D, S87D-S248R, S188D-S248R, S248D, N109D-S188D-S248R, N109D, S87R-S248R, N109D-S188R, N76D, S87D-N109D-S188D-S248R, S87R-N109D-S188D-S248R, S87R-S188R-S248R, A187D, N109D-S248D, S87R-N109R-S188R-S248R, F189D, G100N, S87R-N109D-S188D, S87D-N109D-S188D, S87R-S188D-S248D, N62D, and S87D-N109D-S188D-S248D, and wherein the amino acid positions are numbered by correspondence the positions of with the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' of SEQ ID NO:2.

The present invention also provides subtilisin variants, wherein each such variant is a mature form having proteolytic activity and comprises the BPN'-v3 amino acid sequence (SEQ ID NO:4) comprising at least one set of amino acid substitutions selected from S78N-L267V, S78N-S161P, S78N-I115V, S78N-A273S, S78N-G211T , S78N, S78N-I111V, S78N-V147L, S78N-I108V, S78N-S89Y, S78N-A138T, S78N-P172V, S78N-Q59G, P129T-V147Q-S159D-S161P-S183T-Q185T-G211A-S224A, Q059V-I108V-V147Q-G211A-N252Q, S78N-Y167A, S78N-A92G, S78N-P129L, N061A-S087E-M124I-S161P-S224A, S78N-N62Q, S78N-V68A, S063T-S101A-L126V-S183T-T244N, S78N-M124T, P040L-S053G-Q059V-N061A-N062Q-S063T-S087E-G100N, N062Q-G100N-S125A-S159D-N240S, V68A-G102A-G211A-S125A, and S053G-V068A-G102A-P129T-Q185T, and wherein the amino acid positions are numbered by correspondence with the amino acid residue positions of the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' set forth as SEQ ID NO:2.

The present invention also provides subtilisin variants, wherein each such variant is a mature form having proteolytic activity and comprises an amino acid sequence comprising at least one set of amino acid substitutions selected from the group consisting of G97A-G128S-Y217Q-S24G-N25G-N61P-S101N, G97A-G128S-Y217Q-S53G-N61P-S101N-V203Y, G97A-G128S-Y217Q-S24G-N25G-S53G-T55P-N61P-S101N-V203Y, G97A-G128A-Y217Q-S24G-N25G-S53G-N61P-S101N-V203Y, P52L-V68A-G97A-I111V, I111V-M124V-Y167A-Y217Q, Y104N-G128A-Y217Q, M124V-Y167A-Y217Q, I111V-M124V-Y217Q, P52L-V68A-G97A, G97A-I111V-M124V, V68A-A92G-G97A, G97A-I111V-M124V-Y167A-Y217Q, P52L-V68A-I111V-Y217Q, P52L-V68A-I111V, V68A-A92G-I111V, P52L-V68A-G97A-I111V-Y217Q, V68A-G97A-I111V, G97A-I111V-Y217Q, G97A-I111V-M124V-Y167A, S89Y-I111V-M124V, V68A-S89Y-I111V, V68A-A92G-Y217Q, I111V-Y167A-Y217Q, G97A-I111V-Y167A-Y217Q, G97A-I111V-M124V-Y217Q, V68A-I111V-Y167A-Y217Q, I111V-G128A-Y217Q, G97A-M124V-Y217Q, V68A-Y167A-Y217Q, I111V-M124V-Y167A, N62Q-G97A-I111V, G97A-M124V-Y167A-Y217Q, G97A-L126A-Y217Q, V68A-I111V-Y217Q, S89Y-M124V-Y217Q, and L96T-G97A-Y217Q, and wherein the positions are numbered by correspondence with the amino acid residue positions of the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' set forth as SEQ ID

NO:2. Some such variants comprise the BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from said group.

The present invention also provides subtilisin variants, wherein each such variant is a mature form having proteolytic activity and comprises a BPN' amino acid sequence comprising at least one, two, three, four, five, six, seven, eight, nine, ten, eleven or more substitutions selected from S182E, N109I, N117H, K237D, L257Q, P225N, S105H, S236I, L235H, S249E, N76E, S145N, N243D, R247N, E195N, A98K, S182N, S161II, G83II, G131D, T71C, K136Q, P40D, A187II, L250K, S9I, N76M, S132D, Q19F, E112H, S249P, S53D, V68E, D41I, K43H, V4H, A13Y, N62P, L196E, and V44D, and wherein the positions are numbered by correspondence with the amino acid residue positions of amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' set forth as SEQ ID NO:2.

The present invention also provides dishwashing compositions comprising the subtilisin variant(s), and fabric cleaning compositions comprising the subtilisin variant(s). In some preferred aspects, the dishwashing and fabric cleaning compositions further comprise at least one additional enzyme. In some preferred aspects, the additional enzyme is selected from: a protease (e.g., a neutral metalloprotease, a wild type serine protease, or a second variant serine protease) a lipase, a cutinase, an amylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, a perhydrolase, an oxidase, and a peroxidase. Moreover, the present invention provides dishwashing methods, comprising the steps of: providing i) the dishwashing composition comprising the subtilisin variant, and ii) dishware in need of cleaning; and contacting the dishware with the dishwashing composition under conditions effective to provide cleaning of the dishware. Similarly, the present invention provides fabric cleaning methods, comprising the steps of: providing i) the fabric cleaning composition comprising the subtilisin variant, and ii) laundry in need of cleaning; and contacting the laundry with the fabric cleaning composition under conditions effective to provide cleaning of the laundry. In still further aspects, the present invention provides an isolated nucleic acid encoding the variant, an expression vector comprising the isolated nucleic acid in operable combination with a promoter, and/or host cells comprising the expression vector are provided.

The present invention also provides cleaning compositions comprising the subtilisin variants provided herein. In one aspect, the cleaning compositions comprise a liquid, gel, tablet, powder and/or granule detergent. In some further aspects, the cleaning compositions are selected from laundry detergents and dish detergents. In some preferred aspects, the cleaning compositions comprise laundry detergents. In some particularly preferred aspects, the cleaning compositions are heavy duty detergents. In some additional aspects, the cleaning compositions comprise dish detergents selected from hand dishwashing and automatic dishwashing detergents. In some further preferred aspects, the cleaning compositions provided herein further comprise at least one bleaching agent. In some additional aspects, the cleaning compositions provided herein are phosphate-free, while in some alternative aspects, the cleaning compositions provided herein are phosphate-containing detergents. In some still further aspects, the cleaning compositions provided herein are cold water detergents. In yet some additional aspects, the

cleaning compositions provided herein further comprise at least one additional enzyme. In one aspect, the cleaning compositions comprise at least one additional enzyme selected from the group consisting of a hemicellulase, cellulase, peroxidase, protease, metalloprotease, xylanase, lipase, phospholipase, esterase, perhydrolase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, ß-glucanase, arabinosidase, hyaluronidase, chondroitinase, laccase, and amylase; or mixtures of any thereof.

The present invention also provides methods for cleaning, comprising providing an item to be cleaned and a composition comprising at least one cleaning composition provided herein, and contacting them with the composition, under conditions effective to provide cleaning of the item. In one aspect, the methods further comprise the step of rinsing the item after contacting the item with the cleaning composition. In some preferred aspects, the item to be cleaned comprises dishware. In some alternative aspects, the item to be cleaned comprises fabric.

In some further aspects, any of the above mutations, deletions, insertions or combinations thereof can be applied to either the enzyme of SEQ ID NO:2 containing the mutation Y217L or the enzyme of SEQ ID NO:2 containing the mutations G97A-G128A-Y217Q.

In one aspect, the present invention provides a suitable cold water protease derived from a subtilisin, particularly subtilisin BPN' (SEQ ID NO:2) (i.e., a BPN' subtilisin variant). In one aspect, a cold water protease one, two, three, four, five, six, seven, eight, nine, ten, eleven or more of the following amino acid substitutions relative to SEQ ID NO:2: Q002W, P005K, P005L, P005Y, G007T, V008G, V008K, V008P, I011G, I011H, I011S, K012L, A013M, L016W, G023S, V026H, V026W, V026Y, K027P, V028Q, V028S, V028T, A029C, A029D, A029S, A029T, A029V, V030D, V030E, V030G, V030T, I031E, I031G, I031H, I031K, I031N, I031Q, I031S, I031Y, S033F, S033H, D036L, S037P, D041A, D041C, D041M, D041N, D041S, L042S, L042Y, V044H, V044Q, V044T, G046F, G046L, G046M, G046V, G047T, G047W, S049I, S049V, M050D, M050I, M050R, V051A, V051G, V051S, P052C, P052I, P052L, P052M, P052V, P052W, P052Y, E054R, N056G, N056I, N056K, N056M, N056Q, N056R, N056V, N056Y, P057I, P057K, P057L, P057R, P057T, P057V, F058T, Q059P, Q059W, N061P, N062D, N062M, N062Q, S063C, S063Q, S063T, G065Q, V068A, V068G, V068M, V068S, A069C, A069D, A069F, A069H, A069M, A069N, A069P, A069Q, A069R, A069T, T071D, T071E, T071G, T071K, T071M, V072D, V072G, V072K, V072Q, V072S, V072T, A073E, A073I, A073K, A073M, A073Q, A073S, A073V, A074E, A074F, A074H, A074I, A074L, A074M, A074Q, A074R, A074V, A074W, A074Y, L075A, N076A, N077G, N077L, N077P, N077Q, N077R, N077S, N077T, S078W, G080H, V081D, V081F, V081H, V081N, V081Q, V081R, V081W, L082G, L082N, L082W, A085I, A085T, A085V, P086A, P086G, P086M, P086Q, P086R, P086T, P086W, P086Y, S087F, S087I, S087L, S087M, S087Q, S087V, S087W, A088D, A088E, A088K, A088P, A088Q, S089D, S089Q, S089V, L090D, L090E, L090F, L090H, L090P, L090S, L090T, Y091L, Y091Q, Y091T, A092C, A092I, A092M, A092N, A092P, A092V, V093D, V093F, V093L, V093T, K094C, K094R, K094S, V095I, L096F, L096H, L096I, L096M, L096N, L096Q, L096S, L096T, L096V, L096W, L096Y,

G097A, G097C, G097D, G097E, G097F, G097L, G097M, G097P, G097Q, G097S, G097V, G097W, G097Y, D099C, D099E, D099I, D099M, D099P, D099V, D099Y, G100D, G100E, G100H, G100I, G100K, G100M, G100Q, G100T, G100V, G100Y, S101A, S101E, S101G, S101N, S101P, S101Q, S101T, S101V, G102A, G102S, Q103E, Q103G, Q103H, Q103K, Q103N, Y104L, Y104M, Y104N, Y104T, Y104V, S105D, S105I, S105R, S105V, W106A, W106C, W106E, W106F, W106G, W106I, W106L, W106M, W106S, W106T, W106V, I107R, I107S, I107T, I108S, I108T, G110S, G110T, I111A, I111C, I111F, I111L, I111M, I111T, I111V, E112I, E112L, E112T, W113H, A114I, A114V, I115A, I115E, I115F, I115H, I115M, I115N, I115P, I115Q, I115R, I115S, I115T, I115V, I115Y, N117K, N117V, N117W, N118I, N118L, N118V, M119F, M119N, D120Y, I122R, I122S, I122T, N123A, N123C, N123G, N123S, M124A, M124H, M124N, M124Q, M124S, M124T, M124V, S125A, L126A, L126Q, L126S, L126T, L126Y, G128E, G128N, G128T, P129V, S130P, S132I, S132N, S132P, S132Q, S132V, A134I, A134L, A134M, L135I, L135T, L135V, L135W, L135Y, K136D, K136F, K136I, K136V, K136Y, A137P, A138D, A138E, A138F, A138H, A138Q, A138Y, A142G, A142I, A142T, A142V, V143W, A144P, G146L, G146T, G146Y, V147D, V147P, V147W, V147Y, V148N, V148Y, V149E, A151T, A153T, A153V, S159K, T164W, V165T, Y167A, Y167D, Y167E, Y167M, Y167P, Y167S, Y167T, P168L, P168T, G169C, K170E, K170N, K170P, K170Q, K170S, K170T, K170Y, Y171C, Y171D, Y171L, Y171N, Y171W, P172E, P172G, P172I, P172L, P172V, P172Y, S173H, S173W, S173Y, V174A, V174I, V174L, V174S, I175A, I175F, I175R, I175T, I175V, A176V, V177W, V180F, V180H, V180Q, S182W, N184A, N184L, N184V, Q185D, Q185E, Q185I, Q185S, Q185T, R186C, R186D, R186E, R186F, R186G, R186I, R186L, R186N, R186P, R186Q, R186S, R186T, R186V, R186W, R186Y, A187D, A187E, A187Q, S190G, S190N, Y192D, Y192E, Y192L, Y192Q, G193H, G193Q, G193T, G193V, P194E, P194I, P194L, P194M, P194N, P194T, P194V, E195C, E195K, E195W, L196I, L196M, L196T, L196V, D197I, D197M, M199F, M199Q, A200C, A200H, A200N, A200T, A200V, A200Y, P201L, P201T, P201V, V203G, I205L, I205T, T208C, T208L, T208M, T208P, T208V, L209C, L209W, P210C, P210D, P210E, P210F, P210G, P210Q, P210R, P210S, P210V, G211A, G211D, G211E, G211P, G211T, G211V, G211W, N212E, N212T, K213Q, K213T, Y214A, Y214D, Y214N, Y214P, Y214S, G215D, G215Q, G215V, A216E, Y217E, Y217L, Y217M, N218P, A223W, S224D, S224N, S224Q, H226E, H226T, V227G, V227L, V227S, A230H, A230N, A231W, A231Y, A232N, I234W, L235N, S236W, H238A, H238G, H238I, P239H, P239S, W241G, W241Q, R247H, R247L, R247W, R247Y, L250E, L250T, N252Q, T253Y, T254D, T254Q, T254R, T255L, T255P, K256G, K256R, G258P, Y263D, Y263K, Y263R, K265P, I268S, I268T, I268W, V270F, A273K, A273P, A273R, A273V, A273W, A274W, S182E, and N109I, wherein positions of the variant sequence are numbered by correspondence to positions of SEQ ID NO:2.

In another aspect, the invention provides suitable cold water proteases, including subtilisin variants, particularly variant of mature BPN' (SEQ ID NO:2), comprising one, two, three, four, five, six, seven, eight, nine, ten, eleven or more of the following sets of mutations relative to SEQ ID NO:2: N109D-Y217L-S248R, N109D-S188R-Y217L, S87D-Y217L-S248R, S87R-N109D-Y217L-S248R,

S87R-N109D-S188D-Y217L-S248R, G128A-Y217Q, I111V-M124V, M124V-Y217Q, N62Q-G97A, S89Y-M124V, V68A, V68A-A92G, V68A-G97A, V68A-I111V, V68A-S89Y, V68A-V227T, V68A-Y217Q, W106F-Y217Q, G97A-G128A-Y217Q, G97A-L126A-Y217Q, G97A-M124V-L126A-Y217Q, G97A-N123G-Y217Q, L96T-G97A-Y217Q, M124V-L126A-Y217Q, N62Q-G128A-Y217Q, N62Q-G97A-Y217Q, G97N-G128A-Y217M, G97G-G128S-Y217E, G97A-G128A-Y217Q, G97M-G128S-Y217E, G97A-G128S-Y217Q, G97D-G128S-Y217Q, G97M-G128G-Y217M, G97G-G128S-Y217Q, G97S-G128S-Y217Q, G97G-G128A-Y217Q, G97S-G128A-Y217E, G97A-G128S-Y217L, G97A-G128A-Y217N, G97Q-G128S-Y217L, G97A-G128A-Y217M, G97A-G128A-Y217S, G97D-G128A-Y217Q, G97M-G128S-Y217Q, G97Q-G128G-Y217D-S87Y, G97S-G128A-Y217N, G97A-G128S-Y217T, G97D-G128S-Y217E, G97D-G128A-Y217L, G97G-G128S-Y217E-S78P-A272T, G97T-G128S-Y217D, G97D-G128A-Y217I, G97Q-G128S-Y217Q, G97G-G128A-Y217D, G97Q-G128A-Y217N, G97S-G128A-Y217M, G97S-G128S-Y217N, G97S-G128S-Y217M, G97E-G128S-Y217M, G97S-G128P-Y217Q, G97T-G128S-Y217Q, G97D-G128S-Y217Q-A73T, G97E-G128S-Y217N, G97G-G128A-Y217I, G97Q-G128A-Y217D, G97Q-G128S-Y217M, G97R-G128T-Y217Q-S162P, G97S-G128S-Y217D, G97T-G128P-Y217I, G97Q-G128G-Y217E, G97C-G128G-Y217N, G97D-G128S-Y217H, G97M-G128S-Y217L, G97M-G128S-Y217N, G97S-G128S-Y217E, G97M-G128S-Y217I, G97A-G128P-Y217A, G97R-G128S-Y217D, G97A-G128A-Y217Q-S145D, G97A-G128A-Y217Q-P239R, G97A-G128A-Y217Q-N61E-P129E-S162K-K213L-N240K, G97A-G128A-Y217Q-N61E, G97A-G128A-Y217Q-P40E-A144K-K213L, G97A-G128A-Y217Q-P129E, G97A-G128A-Y217Q-N61E-P129E-S159K, G97A-G128A-Y217Q-K213L, G97A-G128A-Y217Q-S87D, G97A-G128A-Y217Q-Q206E, G97A-G128A-Y217Q-S24R-P40E-S145D-S159K-K213L, G97A-G128A-Y217Q-K265N, G97A-G128A-Y217Q-S24R, G97A-G128A-Y217Q-P40E, G97A-G128A-Y217Q-Q275E, G97A-G128A-Y217Q-P129E-S145D-N240K, G97A-G128A-Y217Q-A144K, G97A-G128A-Y217Q-S159K, G97A-G128A-Y217Q-S162K, G97A-G128A-Y217Q-N240K, G97A-G128A-Y217Q-S53G, G97A-G128A-Y217Q-S78N, G97A-G128A-Y217Q-S53G-S78N, G97A-G128A-Y217Q-S53G-I111V, G97A-G128A-Y217Q-S53G-N117S, G97A-G128A-Y217Q-S53G-S132N, G97A-G128A-Y217Q-Y104N-S132N, G97A-G128A-Y217Q-S53G-S78N-I111V, G97A-G128A-Y217Q-S53G-S78N-N117S, G97A-G128A-Y217Q-S53G-S78N-S132N, G97A-G128A-Y217Q-S53G-Y104N-S132N, G97A-G128A-Y217Q-S78N-Y104N-S132N, Y217L-V068C-A069G, Y217L-I079F-A098G, Y217L-P086T-S101D-Q103S-V147I, Y217L-A088T-P129S-G146D, Y217L-V093I-G128D-P129R, Y217L-Z096.01D-A098R, Y217L-Z096.01H-A098G, Y217L-G097S-Z097.01S-A098G-A273T, Y217L-A098S-D099G-G100D, Y217L-Z098.01N, Y217L-D099G-Z099.01N, Y217L-D099G-Z099.01S, Y217L-D099V-S101D, Y217L-Z099.01S, Y217L-G100D, Y217L-S101D-Q103H, Y217L-S101G-A151V, Y217L-S101H-G102S, Y217L-S101H-Q103D, Y217L-G102R-Q103C-Y104C-V192I, Y217L-Q103D, Y217L-V121I-I122S-N123C, Y217L-V121L-N123C, Y217L-I122S-N123S, Y217L-M124I, Y217L-M124V, Y217L-L126F-P129Z-S182N, Y217L-L126Y, Y217L-G127S-P129D, Y217L-Z127.01N-G128S-P129S, Y217L-G128H-P129Y, Y217L-G128S-P129D, Y217L-G128S-P129D-S248R, Y217L-

G128S-P129G, Y217L-P129G-G131Z, Y217L-P129G-S130H-S132Z, Y217L-P129H-G131Z, Y217L-P129L, Y217L-P129S-S130H-S132Z, Y217L-P129Z, Y217L-P129Z-S130G, Y217L-P129Z-S130G-G131H-S132H, Y217L-P129Z-S130H, Y217L-S130V-G131D-S132I, G97A-G128A-Y217Q-A134T-K213L, G97A-G128A-Y217Q-G23A-S24G-N25G-P129V, G97A-G128A-Y217Q-S24R-P239R, and G97A-G128A-Y217Q-S24R-S87T-A88L-S89G, wherein positions of the variant sequence are numbered by correspondence to positions of SEQ ID NO:2.

In another aspect, the invention provides suitable cold water proteases, including subtilisin variants, particularly variants of mature BPN' (SEQ ID NO:2) comprise one, two, three, four, five, six, seven, eight, nine, ten, eleven or more of the following sets of mutations relative to SEQ ID NO:2: S87T-A88L-S89G-G97A-G128A-Y217Q, N61P-S63H-G97A-G128A-Y217Q, S87G-A88V-S89A-G97A-G128A-Y217Q, P86S-S87G-A88V-G97A-G128A-Y217Q, Q59S-N61P-G97A-G128A-Y217Q, S24G-N25G-G97A-G128A-Y217Q, N61P-N62S-G97A-G128A-Y217Q, G97A-G128A-P129Q-S130G-G131S-Y217Q, L75S-N76Y-G97A-G128A-Y217Q, G97A-G128A-V203Y-Y217Q, T55P-G97A-G128A-Y217Q, A88V-L90I-G97A-G128A-Y217Q, G97A-G128A-G211R-N212S-K213V-Y217Q, G23A-S24G-N25G-G97A-G128A-Y217Q, T22N-S24A-G97A-G128A-Y217Q, S24R-G97A-G128A-Y217Q, G97A-A98S-G128A-Y217Q, G97A-G128A-T158G-S159G-Y217Q, Q59E-N61P-G97A-G128A-Y217Q, G97A-A98E-G128A-Y217Q, G97A-G128A-Y217Q-P86S-S87G-A88V-A116N-N117S-N118G, G97A-G128A-Y217Q-S63T-P86S-S87G-A88V, G97A-G128A-Y217Q-P86S-S87G-A88V-P239R, G97A-G128A-Y217Q-S24G-N25G-N61P-N62S-P194L-A232T, G97A-G128A-Y217Q-P129Q-S130G-G131S-A133V-L267V, G97A-G128A-Y217Q-A134T-L267V, G97A-G128A-Y217Q-S24R-P40E-P129E-S159K-K265R, G97A-G128A-Y217Q-A134T-G211T, G97A-G128A-Y217Q-S24R-P129E, G97A-G128A-Y217Q-I111V-S161P, G97A-G128A-Y217Q-T55P-P129Q, G97A-G128A-Y217Q-I115V-L267V, G97A-G128A-Y217Q-P86S-S87G-A88V-A116S-N117G-N118R, G97A-G128A-Y217Q-V203Y-L267V, G97A-G128A-Y217Q-S24G-N25G-S78N-S101N-V203Y, G97A-G128A-Y217Q-P52S-T55P-V203Y, G97A-G128A-Y217Q-Q59S-N61P-A116S-N117G-N118R, G97A-G128A-Y217Q-S24G-N25G-P129Q-S130G-G131S, G97A-G128A-Y217Q-P86S-S87G-A88V-T242R, G97A-G128A-Y217Q-P40E-T55P-N269K, G97A-G128A-Y217Q-G23A-S24G-N25G-A116N-N117S-N118G, G97A-G128A-Y217Q-V8L-N25Y-P129Q-S130G-G131S, G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-G211T-L267V, G97A-G128A-Y217Q-S24R-A116N-N117S-N118G, G97A-G128A-Y217Q-S24R-A128S-P129G, G97A-G128A-Y217Q-P129Q-S130G-G131S-N240K, G97A-G128A-Y217Q-N25Y-P129Q-S130G-G131S, G97A-G128A-Y217Q-S87T-A88L-S89G-A134T, G97A-G128A-Y217Q-P129Q-S130G-G131S-L267V, G97A-G128A-Y217Q-S87G-A88V-S89A-A116N-N117S-N118G, G97A-G128A-Y217Q-N61P-P129Q-S130G-G131S, G97A-G128A-Y217Q-N61P-S78N-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-T55P-P129V-P194S, G97A-G128A-Y217Q-T55P-P129V, G97A-G128A-Y217Q-S24G-N25G-T55P-S78N-S101N, G97A-G128A-Y217Q-T55P-S78N-I115V, G97A-G128A-Y217Q-N25Y-S87G-A88V-S89A, G97A-G128A-Y217Q-A134T-N240K, G97A-G128A-Y217Q-S24R-Q59S-N61P, G97A-G128A-

Y217Q-G23A-S24G-N25G-P239R, G97A-G128A-Y217Q-T55P-A116S-N117G-N118R, G97A-G128A-Y217Q-A134T-S161P, G97A-G128A-Y217Q-S24G-N25G-S53G-N61P-S101N-V203Y, G97A-G128A-Y217Q-N25Y-Q59S-N61P, G97A-G128A-Y217Q-N25Y-P129Q-S130G-G131S-A137T, G97A-G128A-Y217Q-G23A-S24G-N25G-N61P-S63H, G97A-G128A-Y217Q-T55P-N61P-S78N-S101N-V203Y, G97A-G128A-Y217Q-P129Q-N240K, G97A-G128A-Y217Q-T55P-A134T, G97A-G128A-Y217Q-N25Y-N61P-S63H, G97A-G128A-Y217Q-S87T-A88L-S89G-P129S, G97A-G128A-Y217Q-T55P-L75II-N76G, G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-T55P-I115V, G97A-G128A-Y217Q-T55P-A116N-N117S-N118G, G97A-G128A-Y217Q-S24G-N25G-A116N-N117S-N118G, G97A-G128A-Y217Q-S24R-P129Q-S130G-G131S, G97A-G128A-Y217Q-G23A-S24G-N25G-G211R-N212S-K213V, G97A-G128A-Y217Q-S24G-N25G-T55P-N61P-S78N-S101N-V203Y, G97A-G128A-Y217Q-T55P-S78N-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-I115V-A273S, G97A-G128A-Y217Q-N25Y-T55P, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-Q59S-N61P-N240K, G97A-G128A-Y217Q-S161P-L267V, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-S24G-N25G-N61P-S101N, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S101N-V203Y, G97A-G128A-Y217Q-N240K, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-S24G-N25G-T55P-S101N, G97A-G128A-Y217Q-N61P-S63H-A128S-P129Q, G97A-G128A-Y217Q-S89Y-P129Q-S130G-G131S, G97A-G128A-Y217Q-P129Q-S130G-G131S-V203Y, G97A-G128A-Y217Q-I115V-N240K, G97A-G128A-Y217Q-S53G-N61P-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-S161P-V203Y, G97A-G128A-Y217Q-S87T-A88L-S89G-N240K, G97A-G128A-Y217Q-S87T-A88L-S89G-P239R, G97A-G128A-Y217Q-T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-S24G-N25G-I115V-A134T, G97A-G128A-Y217Q-Y6Q-P129Q-S130G-G131S, G97A-G128A-Y217Q-T55P-S78N-S89Y, G97A-G128A-Y217Q-S24G-N25G-T55P-S78N-A88V-S101N, G97A-G128A-Y217Q-N61P-S63H-S78N-I111V-A134T, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S78N-S101N, G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S101N-V203Y, G97A-G128A-Y217Q-S53G-N61P-S101N-V203Y, G97A-G128A-Y217Q-S53G-T55P-S78N-S101N-V203Y, G97A-G128A-Y217Q-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-N240K-A273S, G97A-G128A-Y217Q-S78N-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-Q59S-N61P-S87T-A88L-S89G, G97A-G128A-Y217Q-N61P-S63H-S78N-S161P, G97A-G128A-Y217Q-N61P-S63H-S78N-I111V, G97A-G128A-Y217Q-T55P-A128S-P129Q, G97A-G128A-Y217Q-N61P-S78N-S101N-V203Y, G97A-G128A-Y217Q-N61E-A144K, G97A-G128A-Y217Q-A134T-P239R, G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S101N-V203Y, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S101N-V203Y, G97A-G128A-Y217Q-N61P-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-T55P-N240K, G97A-G128A-Y217Q-S24G-N25G-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-P129Q-S130G-G131S-P239R, G97A-G128S-Y217Q, G97A-G128A-Y217Q-S53G-N61P-

S101N, G97A-G128A-Y217Q-I111V-P129Q-G211T, G97A-G128A-Y217Q-S24G-N25G-S53G-S101N-V203Y, G97A-G128A-Y217Q-Q59S-N61P-S87G-A88V-S89A, G97A-G128A-Y217Q-S24G-N25G-S78N-S87T-A88L-S89G-S101N, G97A-G128A-Y217Q-P129Q-S130G-G131S-S162K, G97A-G128A-Y217Q-T55P-P129Q-S130G-G131S, G97A-G128A-Y217Q-T55P-V203Y, G97A-G128A-Y217Q-S87G-A88V-S89A-P129Q-S130G-G131S, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G, G97A-G128A-Y217Q-I111V-P129Q-S130G-G131S, G97A-G128A-Y217Q-I111V-A273S, G97A-G128A-Y217Q-N61P-S87T-A88L-S89G, G97A-G128A-Y217Q-T22N-S24A-N61P-S63H, G97A-G128A-Y217Q, G97A-G128A-Y217Q-S53G-S78N-S87T-A88L-S89G-S101N-P129S-V203Y, G97A-G128A-Y217Q-S159K-L267V, G97A-G128A-Y217Q-P40E-S53Y-S78Y-P86S-S87G-A88V, G97A-G128A-Y217Q-S24R-S145D, G97A-G128A-Y217Q-I111V-S159K, G97A-G128A-Y217Q-T55P-P129L, G97A-G128A-Y217Q-Q59S-N61P-V203Y, G97A-G128A-Y217Q-T55P-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S101N, G97A-G128A-Y217Q-S53G-N61P-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-S89Y, G97A-G128A-Y217Q-S24R-P129V, G97A-G128A-Y217Q-S87G-A88V-S89A-A116N-N117S-N118G-P172H, G97A-G128A-Y217Q-I111V-A134T, G97A-G128A-Y217Q-Q59S-N61P-P129Q-S130G-G131S, G97A-G128A-Y217Q-P5S-S87G-A88V-S89A-A116G-N117R, Y217Q-N61P-A97G-G102A-A128G-P129S, G97A-G128A-Y217Q-S24G-N25G-S53G-N61P-S78N, G97A-G128A-Y217Q-S145D-S159K-N240K-Q275E, G97A-G128A-Y217Q-T55P-A128S-P129D, G97A-G128A-Y217Q-G23A-S24G-N25G-A128S-P129D, G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S89G-V203Y, G97A-G128A-Y217Q-I111V-P239R, G97A-G128A-Y217Q-S87G-A88V-S89A-S162K, G97A-G128A-Y217Q-S87T-A88L-S89G-I115V, G97A-G128A-Y217Q-S24G-N25G-T55P-S78N, G97A-G128A-Y217Q-T55P-A92G, G97A-G128A-Y217Q-S24G-N25G-S53G-S87T-A88L-S89G-V203Y, G97A-G128A-Y217Q-T22N-S24A-T55P, G97A-G128A-Y217Q-S53G-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S78N-S87T-A88L-S89G, G97A-G128A-Y217Q-P129Q-S130G-G131S-S159K, G97A-Y217Q-N61P-N62Q-G100N-A128G, G97A-G128A-Y217Q-S24R-S78N-S182P-L267V, G97A-G128A-Y217Q-P239R-A273S, G97A-G128A-Y217Q-S53G-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-P129Q-S130G-G131S-T242R, G97A-G128A-Y217Q-S3F-S87T-A88L-S89G-G211T, G97A-G128A-Y217Q-S24G-N25G-L75H-N76G, G97A-G128A-Y217Q-S53G-T55P-N61P-S78N-S87T-A88L-S89G, G97A-G128A-Y217Q-S87T-A88L-S89G-A144K, G97A-G128A-Y217Q-S78N-S87T-A88L-S89G-V203Y, G97A-G128A-Y217Q-Q59S-N61P-A116N-N117S-N118G, G97A-G128A-Y217Q-S87T-A88L-S89G-I111V, G97A-G128A-Y217Q-S24R-S145D-P239R-Q275E, G97A-G128A-Y217Q-S145D-A273S, G97A-G128A-Y217Q-S24G-N25G-K141E-T242R, G97A-G128A-Y217Q-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-A116N-N117S-N118G-P129Q-S130G-G131S, G97A-G128A-Y217Q-S89Y-G211T, G97A-G128A-Y217Q-S87G-A88V-S89A-A116N-N117S-N118G-A144T, G97A-G128A-Y217Q-S24G-N25G-S78N-S87T-A88L-S89G-S101N-V203Y, G97A-G128A-Y217Q-S24G-N25G-P129V, G97A-Y217Q-N61P-A128G-P129S-S130P, G97A-G128A-Y217Q-T55P-N61P-S87T-A88L-S89G-G110C-S130P, G97A-

Y217Q-N123G-A128G, G97A-G128A-Y217Q-N61P-N62Q-G100N-G102A-M124I, S78N-G97A-G128A-Y217Q, G97A-S101N-G128A-Y217Q, G97A-G128A-A137V-Y217Q, N61P-G97A-G128A-Y217Q, G97A-G128A-S130P-Y217Q, G97A-Q103N-G128A-Y217Q, S63T-G97A-G128A-Y217Q, G97A-G102A-G128A-Y217Q, G97A-N109D-G128A-Y217Q-S248R, S87R-G97A-G128A-Y217Q, G97A-G128A-S188D-Y217Q, S87D-G97A-G128A-Y217Q-S248R, G97A-G128A-S188D-S248R-Y217Q, G97A-G128A-S248D-Y217Q, S78N-G97A-G128A-Y217Q-L267V, S78N-G97A-G128A-Y217Q-S161P, S78N-G97A-G128A-Y217Q-I115V, S78N-G97A-G128A-Y217Q-A273S, S78N-G97A-G128A-Y217Q-G211T, S78N-G97A-G128A-Y217Q, S78N-G97A-G128A-Y217Q-I111V, S78N-G97A-G128A-Y217Q-V147L, S78N-G97A-G128A-Y217Q-I108V, S78N-G97A-G128A-Y217Q-S89Y, and S78N-G97A-G128A-Y217Q-A138T, wherein positions of the variant sequence are numbered by correspondence to positions of SEQ ID NO:2.

In another aspect, the cold water protease is variant of subtilisin BPN' having SEQ ID NO:2, wherein the variant comprises three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, or more mutations selected from amino acid positions 24, 25, 40, 52, 53, 55, 58, 59, 61, 62, 63, 68, 78, 86, 87, 88, 89, 92, 96, 97, 100, 101, 103, 104, 106, 111, 114, 115, 116, 117, 118, 123, 124, 125, 126, 128, 129, 130, 131, 132, 133, 134, 144, 145, 159, 161, 162, 167, 194, 203, 206, 213, 217, 227, 232, 239, 240, 242, 265, 267, and 275, wherein the positions of the variant sequence are numbered by correspondence to positions in the amino acid sequence of SEQ ID NO:2.

In another aspect, the cold water protease is variant of subtilisin BPN' comprising SEQ ID NO:2 (i.e., a BPN' subtilisin variant), wherein the variant comprises a total of three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen or more mutations selected from groups (a) and (b): (a) charged mutations selected from the group consisting of N61E, A144K, P129E, P239R, P40E, Q103E, Q206E, Q7275E, S145D, S159K, S162K, S24R, S63H, S87D and T242R; and (b) neutral mutations selected from the group consisting of A114G, A116N, A133V, A134T, A232T, A88V, A92G, F58G, G100T, G128A, G131S, G97A, I111V, I115V, K213L, K265N, L126A, L267V, L96T, M124V, N117S, N118G, N123G, N240K, N25G, N61P, N62Q, N62R, N62S, P129Q, P129V, P194L, P239V, P52L, P86S, Q59S, S101N, S125A, S130G, S132N, S161P, S24G, S53G, S78N, S87G, S89Y, T55P, V203Y, V227T, V68A, W106F, Y104N, Y167A, and Y217Q, wherein the positions of the variant sequence are numbered by correspondence to positions in the amino acid sequence of SEQ ID NO:2.

Nucleic Acids of the Invention

The invention provides isolated, non-naturally occurring, or recombinant nucleic acids (also referred to herein as "polynucleotides"), which may be collectively referred to as "nucleic acids of the invention" or "polynucleotides of the invention", which encode polypeptides (e.g., protease variants) of the invention. Nucleic acids of the invention, including all described below, are useful in recombinant production (e.g., expression) of polypeptides of the invention, typically through expression of a plasmid expression vector comprising a sequence encoding the polypeptide of interest or fragment thereof. As

discussed above, polypeptides include protease variant polypeptides, including subtilisin variant polypeptides having enzymatic activity (e.g., proteolytic activity) which are useful in cleaning applications and cleaning compositions for cleaning an item or a surface (e.g., surface of an item) in need of cleaning.

The invention includes an isolated, recombinant, substantially pure, or non-naturally occurring nucleic acid comprising a polynucleotide sequence encoding any protein, polypeptide, or protease variant (including any fusion protein, etc.) of the invention described above in the section entitled "Polypeptides of the Invention" and elsewhere herein, including, but not limited, to, the Examples, including Part I Examples and Part II Examples, or a complementary polynucleotide sequence thereof. The invention also provides an isolated, recombinant, substantially pure, or non-naturally-occurring nucleic acid comprising a polynucleotide sequence encoding a combination of two or more of any polypeptides, proteins, or protease variants (including any fusion protein) of the invention described above and elsewhere herein, including, but not limited, to, the Examples, including Part I Examples and Part II Examples.

In a tenth aspect, the invention provides an isolated, non-naturally occurring, or recombinant nucleic acid comprising a polynucleotide sequence encoding a variant (or polypeptide as in the third aspect) of the first, second, third, fourth, fifth, sixth, seventh, eighth, or ninth aspect of the invention, or a complementary polynucleotide sequence thereof.

In an eleventh aspect, the invention provides an isolated, non-naturally-occurring, or recombinant nucleic acid comprising a polynucleotide sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the polynucleotide sequence set forth in SEQ ID NO:3 or SEQ ID NO:5, or a complementary polynucleotide sequence thereof.

The present invention further provides nucleic acid encoding a protease variant comprising an amino acid sequence which differs from the amino acid sequence of SEQ ID NO:2, and wherein the total net charge of the protease variant is +1, +2, +3, +4, +5, 0, -1, -2, -3, -4, or -5 relative to the total net charge of the *B. amyloliquefaciens* BPN' subtilisin protease, and wherein amino acid positions of the protease variant are numbered according to the numbering of corresponding amino acid positions in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' shown in SEQ ID NO:2 as determined by alignment of the protease variant amino acid sequence with the *Bacillus amyloliquefaciens* subtilisin BPN' amino acid sequence.

The present invention provides nucleic acids encoding a subtilisin variant of *B. amyloliquefaciens* BPN' subtilisin protease, wherein the BPN' subtilisin protease comprises the amino acid sequence shown in SEQ ID NO:2, and wherein the protease variant comprises an amino acid sequence which differs from the amino acid sequence of SEQ ID NO:2 in no more than two, three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 mutations at amino acid positions selected from amino acid positions 1-275, wherein the amino acid positions of the subtilisin variant are numbered by correspondence with the amino acid sequence of *B. amyloliquefaciens* subtilisin BPN' set forth as SEQ ID NO:2, wherein amino acid positions of the protease variant are

numbered according to the numbering of corresponding amino acid positions in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' shown in SEQ ID NO:2 as determined by alignment with the protease variant.

The present invention provides nucleic acids encoding a subtilisin variant comprising an amino acid sequence which differs from the amino acid sequence of SEQ ID NO:2, and wherein the total net charge of the protease variant is +1, +2, +3, +4, +5, 0, -1, -2, -3, -4, or -5 relative to the total net charge of the *Bacillus lentus* subtilisin BPN' protease, and wherein amino acid positions of the protease variant are numbered according to the numbering of corresponding amino acid positions in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' shown in SEQ ID NO:2 as determined by alignment of the protease variant amino acid sequence with the *Bacillus amyloliquefaciens* subtilisin BPN' amino acid sequence.

The above low ionic strength protease variants may form part of a detergent composition that is diluted in water, typically within a laundry washing machine, to form a laundry detergent wash liquor, whose conductivity is from about 0.1 mS/cm to about 3 mS/cm, from about 0.3 mS/cm to about 2.5 mS/cm, or even from about 0.5 mS/cm to about 2 mS/cm. The protease variants may be high ionic strength protease variants. Such high ionic strength protease variants comprise two or more mutations, and have a total net charge of +5, +4, +3, +2, +1 or 0 relative to wild-type BPN' subtilisin protease wild-type shown in SEQ ID NO:2.

The above high ionic strength protease variants may form part of a detergent composition that is diluted in water, typically within a laundry washing machine, to form a laundry detergent wash liquor, whose conductivity is from about 3 mS/cm to about 30 mS/cm, from about 3.5 mS/cm to about 20 mS/cm, or even from about 4mS/cm to about 10 mS/cm.

The charge of the protease variants is expressed relative to BPN' having the amino acid sequence of SEQ ID NO:2. The amino acids that impart a single negative charge are D and E and those that impart a single positive charge are R, H and K. Any amino acid change versus SEQ ID NO:2 that changes a charge is used to calculate the charge of the protease variant. For example, introducing a negative charge mutation from a wild-type neutral position will add a net charge of -1 to the protease variant, whereas introducing a negative charge mutation (D or E) from a wild-type positive amino acid residue (R, H or K) will add a net charge of -2. Summing the charge changes from all the amino acid residues that are different for the protease variant versus BPN' having the amino acid sequence of SEQ ID NO:2 gives the charge change of the protease variant. Without wishing to be bound by theory, it is believed that: the preferred charge range for cold water proteases to be used in low conductivity laundry detergent solutions is -5, -4, -3, -2, -1, 0, particularly -2, -1; the preferred charge range for cold water proteases to be used in high conductivity laundry detergent solutions is +5, +4, +3, +2, +1, 0, particularly +2, +1. By correctly selecting the charge unexpectedly improved levels of cold water cleaning performance can be obtained. "Low conductivity laundry detergent solutions" are defined as having a conductivity of from about 0.1 mS/cm to about 3 mS/cm, from about 0.3 mS/cm to about 2.5 mS/cm, or even from about 0.5 mS/cm to

about 2 mS/cm. "High conductivity laundry detergent solutions" are defined as having a conductivity of from about 3 mS/cm to about 30 mS/cm, from about 3.5 mS/cm to about 20 mS/cm, or even from about 4 mS/cm to about 10 mS/cm. It is intended that the above examples be non-limiting. Once mutations are combined to optimize cold water performance, the enzyme charge can also be balanced by mutations in further positions.

The invention includes cold water proteases and products comprising at least one cold water protease. In one aspect, the cold water protease is a variant of subtilisin BPN' having SEQ ID NO:2, said variant comprising an amino acid sequence having one or more mutations, and having a total net charge of -1, 0 or +1 relative to wild-type BPN' (SEQ ID NO:2). The amino acids that impart negative charge are typically D and E and those that impart positive charge are typically R, H and K. Without wishing to be bound by theory, it is believed that this charge range (-1, 0, +1) is the optimal charge to deliver cold water performance. However, these examples should be viewed as non-limiting. In one aspect, once mutations are combined to optimize cold water performance, the enzyme charge is balanced by mutations in further positions.

The invention provides an isolated, recombinant, substantially pure, or non-naturally occurring protease variant (e.g., subtilisin variant) having proteolytic activity, said protease variant comprising an amino acid sequence which differs from the amino acid sequence shown in SEQ ID NO:2 by 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, or 8 amino acid residues, wherein amino acid positions are numbered according to the numbering of corresponding amino acid positions in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' shown in SEQ ID NO:2, as determined by alignment of the protease variant amino acid sequence with the *Bacillus amyloliquefaciens* subtilisin BPN' amino acid sequence, wherein the subtilisin variant includes the amino acid sequence of BPN'-v3 (SEQ ID NO:4) or BPN'-v36 (SEQ ID NO:6).

In one aspect, the invention provides an isolated, recombinant, substantially pure, or non-naturally occurring protease variant (e.g., subtilisin variant) having proteolytic activity, said protease variant comprising an amino acid sequence which differs from the amino acid sequence shown in SEQ ID NO:2 by no more than 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 6, 5, 4, 3, 2 amino acid residues, wherein amino acid positions are numbered according to the numbering of corresponding amino acid positions in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' shown in SEQ ID NO:2, as determined by alignment of the protease variant amino acid sequence with the *Bacillus amyloliquefaciens* subtilisin BPN' amino acid sequence, as set forth herein.

The invention includes an isolated, recombinant, or non-naturally occurring nucleic acid comprising a polynucleotide sequence encoding: (a) at least one protease variant of the invention, including any protease variant described herein, including, but not limited to, those protease variants set forth in the Part I Examples, (b) at least one cold water protease of the invention, or (c) at least one protease variant of the invention, including any protease variant described herein, including, but not limited to, a Series I GG36 variant set forth in the Part II Examples, or a complementary polynucleotide

sequence of any thereof. In another aspect, the invention provides an expression vector comprising at least one nucleic acid of the invention. The expression vector may be operably linked to a promoter.

In another aspect, the invention provides a nucleic acid comprising a polynucleotide sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to the polynucleotide sequence set forth in SEQ ID NO:3 or SEQ ID NO:5, or a complementary polynucleotide sequence thereof.

Nucleic acids of the invention can be generated by using any suitable synthesis, manipulation, and/or isolation techniques, or combinations thereof. For example, a polynucleotide of the invention may be produced using standard nucleic acid synthesis techniques, such as solid-phase synthesis techniques that are well-known to those skilled in the art. In such techniques, fragments of up to 50 or more nucleotide bases are typically synthesized, then joined (e.g., by enzymatic or chemical ligation methods, or polymerase mediated recombination methods) to form essentially any desired continuous nucleic acid sequence. The synthesis of the nucleic acids of the invention can be also facilitated (or alternatively accomplished) by any suitable method known in the art, including but not limited to chemical synthesis using the classical phosphoramidite method (see, e.g., Beaucage et al. Tetrahedron Letters 22:1859-69 (1981)); or the method described by Matthes et al., EMBO J. 3:801-805 (1984), as is typically practiced in automated synthetic methods. Nucleic acids of the invention also can be produced by using an automatic DNA synthesizer. Customized nucleic acids can be ordered from a variety of commercial sources (e.g., The Midland Certified Reagent Company, the Great American Gene Company, Operon Technologies Inc., and DNA2.0). Other techniques for synthesizing nucleic acids and related principles are known in the art (see, e.g., Itakura et al., Ann. Rev. Biochem. 53:323 (1984); and Itakura et al., Science 198:1056 (1984)).

As indicated above, recombinant DNA techniques useful in modification of nucleic acids are well known in the art. For example, techniques such as restriction endonuclease digestion, ligation, reverse transcription and cDNA production, and polymerase chain reaction (e.g., PCR) are known and readily employed by those of skill in the art. Nucleotides of the invention may also be obtained by screening cDNA libraries (e.g., cDNA libraries generated using mutagenesis techniques commonly used in the art, including those described herein) using one or more oligonucleotide probes that can hybridize to or PCR-amplify polynucleotides which encode a protease variant polypeptide(s) of the invention. Procedures for screening and isolating cDNA clones and PCR amplification procedures are well known to those of skill in the art and described in standard references known to those skilled in the art. Some nucleic acids of the invention can be obtained by altering a naturally occurring polynucleotide backbone (e.g., that encodes an enzyme or parent protease) by, for example, a known mutagenesis procedure (e.g., site-directed mutagenesis, site saturation mutagenesis, and *in vitro* recombination).

The invention includes nucleic acids that hybridize to a target nucleic acid of the invention (e.g., SEQ ID NO:3 or 5), or a complementary polynucleotide sequence thereof, wherein hybridization is over substantially the entire length of the target nucleic acid. The hybridizing nucleic acid may hybridize to a

nucleotide sequence of the invention under at least stringent conditions or under at least high stringency conditions. Moderately stringent, stringent, and highly stringent hybridization conditions for nucleic acid hybridization experiments are known. Examples of factors that can be combined to achieve such levels of stringency are briefly discussed herein.

Nucleic acids hybridize due to a variety of well-characterized physico-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. An extensive guide to the hybridization of nucleic acids is found in P. Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology – Hybridization with Nucleic Acid Probes, vol. 24, part I, chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays," (Elsevier, N.Y.) (hereinafter "Tijssen"). See also Hames and Higgins (1995) Gene Probes 1 and 2. An indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under at least stringent conditions. Stringent hybridization conditions in the context of nucleic acid hybridization experiments, such as Southern and northern hybridizations, are sequence dependent, and are different under different environmental parameters. High stringency conditions are typically selected such that hybridization occurs at about 5°C or less than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the test sequence hybridizes to a perfectly matched probe. The Tm indicates the temperature at which the nucleic acid duplex is 50% denatured under the given conditions and its represents a direct measure of the stability of the nucleic acid hybrid. Thus, the Tm corresponds to the temperature corresponding to the midpoint in transition from helix to random coil; it depends on length, nucleotide composition, and ionic strength for long stretches of nucleotides. Under stringent conditions, a probe will typically hybridize to its target subsequence, but to no other sequences. Very stringent condition" are selected to be equal to the Tm for a particular probe.

The Tm of a DNA-DNA duplex can be estimated using equation (1): Tm (°C) = 81.5°C+16.6 ($\log_{10}$M)+0.41 (% G+C)-0.72 (% f)-500/n, where M is the molarity of the monovalent cations (usually Na+), (% G+C) is the percentage of guanosine (G) and cytosine (C) nucleotides, (% f) is the percentage of formalize and n is the number of nucleotide bases (i.e., length) of the hybrid. The Tm of an RNA-DNA duplex can be estimated using equation (2): Tm (°C) = 79.8°C+18.5 ($\log_{10}$M)+0.58 (% G+C)-11.8(% G+C)^2-0.56 (% f)-820/n, where M is the molarity of the monovalent cations (usually Na+), (% G+C) is the percentage of guanosine (G) and cytosine (C) nucleotides, (% f) is the percentage of formamide and n is the number of nucleotide bases (i.e., length) of the hybrid. Equations 1 and 2 above are typically accurate only for hybrid duplexes longer than about 100-200 nucleotides. The Tm of nucleic acid sequences shorter than 50 nucleotides can be calculated as follows: Tm (°C) = 4G+C)+2(A+T), where A (adenine), C, T (thymine), and G are the numbers of the corresponding nucleotides.

Non-hybridized nucleic acid material is typically removed by a series of washes, the stringency of which can be adjusted depending upon the desired results, in conducting hybridization analysis. Low stringency washing conditions (e.g., using higher salt and lower temperature) increase sensitivity, but can

product nonspecific hybridization signals and high background signals. Higher stringency conditions (e.g., using lower salt and higher temperature that is closer to the hybridization temperature) lower the background signal, typically with only the specific signal remaining. For additional guidance, see Hames and Higgins, *supra.*

Exemplary stringent conditions for analysis of at least two nucleic acids comprising at least 100 nucleotides include incubation in a solution or on a filter in a Southern or northern blot comprises 50% formalin (or formamide) with 1 milligram (mg) of heparin at 42°C, with the hybridization being carried out overnight. A regular stringency wash can be carried out using a solution comprising 0.2x SSC buffer wash at about 65°C for about 15 minutes (see Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, and the third edition thereof (2001) for a description of SSC buffer). The regular stringency wash can be preceded by a low stringency wash to remove background probe signal. A low stringency wash can be carried out in, for example, a solution comprising 2x SSC buffer at about 40°C for about 15 minutes. A highly stringent wash can be carried out using a solution comprising 0.15 M NaCl at about 72°C for about 15 minutes. Exemplary moderate stringency conditions include overnight incubation at 37°C in a solution comprising 20% formalin (or formamide), 0.5x SSC, 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1x SSC at about 37-50°C. High stringency conditions are conditions that (a) use low ionic strength and high temperature for washing, such as 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate (SDS) at 50°C, (b) employ a denaturing agent during hybridization, such as formamide, e.g., 50% (v/v) formamide with 0.1% bovine serum albumin (BSA)/0.1% Ficoll/0.1% polyvinylpyrrolidone (PVP)/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C, or (c) employ 50% formamide, 5x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5x Denhardt's solution, sonicated salmon sperm DNA (50µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C with washes at (1) 42°C in 0.2x SSC, (2) 55°C in 50% formamide, and (3) 55°C in 0.1x SSC (preferably with EDTA). A signal to noise ratio of 2x or 2.5x-5x that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Detection of at least stringent hybridization between two sequences in the context of the present invention indicates relatively strong structural similarity or homology to a nucleic acid of the invention.

Vectors, Cells, and Methods for Making Protease Variant Polypeptides of the Invention

A variety of methods are known in the art that are suitable for generating modified polynucleotides of the invention that encode protease variants of the invention (such as cold water proteases of the invention), including, but not limited to, e.g., site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, deletion mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches. Methods for making modified polynucleotides and proteins (e.g., protease variants) include DNA shuffling

methodologies (see, e.g., Stemmer WP, Proc. Natl. Acad. Sci. USA 91(22):10747-51 (1994)); methods based on non-homologous recombination of genes, e.g., ITCHY (Ostermeier et al., Bioorg. Med. Chem.7:2139-44 [1999]); SCRATCHY (Lutz et al., Proc. Natl. Acad. Sci. USA 98:11248-53 [2001]); SHIPREC (Sieber et al., Nat. Biotechnol. 19:456-60 [2001]); NRR (Bittker et al., Nat. Biotechnol. 20:1024-9 [2001]; Bittker et al., Proc Natl. Acad. Sci. USA 101:7011-6 [2004]); methods that rely on the use of oligonucleotides to insert random and targeted mutations, deletions and/or insertions (Ness et al., Nat. Biotechnol. 20:1251-5 [2002]; Coco et al., Nat. Biotechnol. 20:1246-50 [2002]; Zha et al., Chembiochem. 4:34-9 [2003]; Glaser et al., J. Immunol. 149:3903-13 [1992]); see also Arkin and Youvan, Biotechnology 10:297-300 (1992); Reidhaar-Olson et al., Methods Enzymol. 208:564-86 (1991).

In one aspect, a full-length parent polynucleotide is ligated into an appropriate expression plasmid, and the following mutagenesis method is used to facilitate the construction of the modified protease of the present invention, although other methods may be used. The method is based on that described by Pisarchik et al. (Pisarchik et al., Prot. Eng. Des. Select. 20:257-265 [2007]). In one aspect, an added advantage is provided in that the restriction enzyme cuts outside its recognition sequence, which allows digestion of practically any nucleotide sequence and precludes formation of a restriction site scar.

In one approach, a naturally-occurring gene encoding a full-length protease is obtained and sequenced and scanned for one or more points at which it is desired to make a mutation (e.g., deletion, insertion, substitution) at one or more amino acids. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by primer extension in accord with generally known methods. Fragments to the left and to the right of the desired point(s) of mutation are amplified by PCR and to include the *Eam*1104I restriction site. The left and right fragments are digested with *Eam*1104I to generate a plurality of fragments having complementary three base overhangs, which are then pooled and ligated to generate a library of modified sequences containing one or more mutations. This method avoids the occurrence of frame-shift mutations. This method also simplifies the mutagenesis process because all of the oligonucleotides can be synthesized so as to have the same restriction site, and no synthetic linkers are necessary to create the restriction sites as is required by some other methods.

In one aspect, the invention includes a method of making a cold water protease, the method comprising: (a) providing a library of nucleic acid variants of a DNA substrate molecule that encodes a parent protease; (b) transforming the library of nucleic acid variants into host cells; (c) expressing the library to provide polypeptide expression products; (d) screening the polypeptide expression products to identify a mutant protease having at least one property selected from the group consisting of: (i) having a performance index of from 1.1 to 10 on a blood/milk/ink (BMI) stain at pH 8 and 16°C compared to PURAFECT® Prime (SEQ ID NO:2 with the amino acid substitution Y217L) as defined in the "Test Method" set forth in Part I Example 1; (ii) a performance index of from 1.3 to 10 on BMI stain at pH 8 and 16°C compared to BPN' (SEQ ID NO:2), as defined in the "Test Method" set forth herein in Part I

Example 1; (iii) a performance index of from 0.9 to about 10 on BMI stain at pH 8 and 16°C compared to BPN'-v3 (SEQ ID NO:4), as defined in the "Test Method" set forth herein in Part I Example 1; and (d) a performance index of from 1.0 to about 10 on BMI at pH 8 and 16°C compared to BPN'-v36 (SEQ ID NO:6), as defined in the "Test Method" set forth herein in Part I Example 1, wherein a cold water protease is identified.

In another aspect, the invention provides an expression vector comprising at least one nucleic acid of the invention. Such nucleic acid may comprise a polynucleotide sequence encoding: (a) at least one protease variant of the invention, including any protease variant described herein, including, but not limited to, those protease variants set forth in the Part I Examples, (b) at least one cold water protease of the invention, or (c) at least one protease variant of the invention, including any protease variant described herein, including, but not limited to, a Series I GG36 variant set forth in the Part II Examples, or a complementary polynucleotide sequence of any thereof. Such expression vector may be operably linked to a promoter.

In another aspect, the invention provides a recombinant host cell comprising: (a) a nucleic acid of the invention, (b) an expression vector comprising a nucleic acid of the invention, (c) a protease variant of the invention, including, but not limited to, e.g., a cold water protease of the invention. The recombinant host cell may be a bacterial cell, such as, but not limited to, e.g., a *Bacillus* cell. An exemplary *Bacillus* cell is a *Bacillus* subtilis cell.

In another aspect, the invention provides a cell culture comprising: (a) a nucleic acid of the invention, (b) an expression vector comprising a nucleic acid of the invention, (c) a protease variant of the invention, including, but not limited to, e.g., a cold water protease of the invention variant.

In another aspect, the invention provides isolated or recombinant vectors comprising at least one polynucleotide of the invention described herein (e.g., a polynucleotide encoding a protease variant of the invention described herein), isolated or recombinant expression vectors or expression cassettes comprising at least one nucleic acid or polynucleotide of the invention, isolated, substantially pure, or recombinant DNA constructs comprising at least one nucleic acid or polynucleotide of the invention, isolated or recombinant cells comprising at least one polynucleotide of the invention, cell cultures comprising cells comprising at least one polynucleotide of the invention, cell cultures comprising at least one nucleic acid or polynucleotide of the invention, and compositions comprising one or more such vectors, nucleic acids, expression vectors, expression cassettes, DNA constructs, cells, cell cultures, or any combination or mixtures thereof.

In another aspect, the invention provides recombinant cells comprising at least one vector (e.g., expression vector or DNA construct) of the invention which comprises at least one nucleic acid or polynucleotide of the invention. Some such recombinant cells are transformed or transfected with such at least one vector. Such cells are typically referred to as host cells. Some such cells comprise bacterial cells, including, but are not limited to, e.g., *Bacillus sp.* cells, such as, e.g., *Bacillus subtilis* cells. The invention also provides recombinant cells (e.g., recombinant host cells) comprising at least one protease

variant of the invention.

In another aspect, the invention provides a vector comprising a nucleic acid or polynucleotide of the invention. The vector may be an expression vector or expression cassette in which a polynucleotide sequence of the invention which encodes a protease variant of the invention is operably linked to one or additional nucleic acid segments required for efficient gene expression (e.g., a promoter operably linked to the polynucleotide of the invention which encodes a protease variant of the invention). A vector may include a transcription terminator and/or a selection gene, such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antimicrobial-containing media.

An expression vector may be derived from plasmid or viral DNA, or in alternative aspects, contains elements of both. Exemplary vectors include, but are not limited to pXX, pC194, pJH101, pE194, pHP13 (Harwood and Cutting (eds.)), Molecular Biological Methods for *Bacillus*, John Wiley & Sons (1990), see, e.g., chapter 3; suitable replicating plasmids for *B. subtilis* include those listed on p. 92; Perego, M. (1993) Integrational Vectors for Genetic Manipulations in *Bacillus subtilis*, pp. 615-624; A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology and molecular genetics, American Society for Microbiology, Washington, D.C.

For expression and production of a protein of interest (e.g., protease variant) in a cell, at least one expression vector comprising at least one copy of a polynucleotide encoding the modified protease, and preferably comprising multiple copies, is transformed into the cell under conditions suitable for expression of the protease. In one aspect, a polynucleotide sequence encoding the protease variant (as well as other sequences included in the vector) is integrated into the genome of the host cell, while in another aspect, a plasmid vector comprising a polynucleotide sequence encoding the protease variant remains as autonomous extra-chromosomal element within the cell. The invention provides both extrachromosomal nucleic acid elements as well as incoming nucleotide sequences that are integrated into the host cell genome. The vectors described herein are useful for production of the protease variants of the invention. In one aspect, a polynucleotide construct encoding the protease variant is present on an integrating vector that enables the integration and optionally the amplification of the polynucleotide encoding the protease variant into the bacterial chromosome. Examples of sites for integration include are well known to those skilled in the art. In one aspect, transcription of a polynucleotide encoding a protease variant of the invention is effectuated by a promoter that is the wild-type promoter for the selected precursor protease. In some other aspects, the promoter is heterologous to the precursor protease, but is functional in the host cell. Specifically, examples of suitable promoters for use in bacterial host cells include, but are not limited to, e.g., the amyE, amyQ, amyL, pstS, sacB, pSPAC, pAprE, pVeg, pHpaII promoters, the promoter of the *B. stearothermophilus* maltogenic amylase gene, the *B. amyloliquefaciens* (BAN) amylase gene, the *B. subtilis* alkaline protease gene, the *B. clausii* alkaline protease gene the *B. pumilis* xylosidase gene, the *B. thuringiensis* cryIIIA, and the *B. licheniformis* alpha-amylase gene.

Additional promoters include, but are not limited to the A4 promoter, as well as phage Lambda P_R or P_L promoters, and the *E. coli* lac, trp or tac promoters.

Protease variants of the invention can be produced in host cells of any suitable Gram-positive microorganism, including bacteria and fungi. For example, in one aspect, the protease variant is produced in host cells of fungal and/or bacterial origin. In one aspect, the host cells are *Bacillus sp., Streptomyces sp., Escherichia sp. or Aspergillus sp.* In one aspect, the protease variants are produced by *Bacillus sp.* host cells. Examples of *Bacillus* sp. host cells that find use in the production of the protease variants of the invention include, but are not limited to *B. licheniformis, B. lentus, B. subtilis, B. amyloliquefaciens, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. coagulans, B. circulans, B. pumilis, B. thuringiensis, B. clausii,* and *B. megaterium,* as well as other organisms within the genus *Bacillus.* In one aspect, *B. subtilis* host cells are used for production of protease variants. U.S. Patents 5,264,366 and 4,760,025 (RE 34,606) describe various *Bacillus* host strains that can be used for producing protease variants of the invention, although other suitable strains can be used.

Several industrial bacterial strains that can be used to produce protease variants of the invention include non-recombinant (i.e., wild-type) *Bacillus sp.* strains, as well as variants of naturally-occurring strains and/or recombinant strains. In one aspect, the host strain is a recombinant strain, wherein a polynucleotide encoding a polypeptide of interest has been introduced into the host. In one aspect, the host strain is a *B. subtilis* host strain and particularly a recombinant *Bacillus subtilis* host strain. Numerous *B. subtilis* strains are known, including, but not limited to, e.g., 1A6 (ATCC 39085), 168 (1A01), SB19, W23, Ts85, B637, PB1753 through PB1758, PB3360, JH642, 1A243 (ATCC 39,087), ATCC 21332, ATCC 6051, MI113, DE100 (ATCC 39,094), GX4931, PBT 110, and PEP 211strain (see, e.g., Hoch et al., Genetics 73:215–228 (1973)) (see also U.S. Patent Nos. 4,450,235 and 4,302,544, and EP 0134048, each of which is incorporated by reference in its entirety). The use of *B. subtilis* as an expression host cells is well known in the art (see, e.g., Palva et al., Gene 19:81-87 (1982); Fahnestock and Fischer, J. Bacteriol. 165:796–804 (1986); and Wang et al., Gene 69:39–47 (1988)).

In one aspect, the *Bacillus* host cell is a *Bacillus* sp. that includes a mutation or deletion in at least one of the following genes, *degU, degS, degR* and *degQ.* The mutation may be in a *degU* gene, and in some instances the mutation may be *degU(Hy)32.* See, e.g., Msadek et al., J. Bacteriol. 172:824-834 (1990) and Olmos et al., Mol. Gen. Genet. 253:562–567 (1997)). A typical host strain is a *Bacillus subtilis* carrying a *degU32(Hy)* mutation. The *Bacillus* host may comprise an amino acid mutation (e.g., substitution) or deletion in *scoC4* (see, e.g., Caldwell et al., J. Bacteriol. 183:7329-7340 (2001)); *spoIIE* (see, e.g., Arigoni et al., Mol. Microbiol. 31:1407-1415 (1999)); and/or *oppA* or other genes of the *opp* operon (see, e.g., Perego et al., Mol. Microbiol. 5:173-185 (1991)). Indeed, it is contemplated that any mutation in the *opp* operon that causes the same phenotype as a mutation in the *oppA* gene will find use in one aspect of the altered *Bacillus* strain of the invention. Such mutations may occur alone or combinations of mutations may be present. In one aspect, an altered *Bacillus* host cell strain that can be used to produce a protease variant of the invention is a *Bacillus* host strain that already includes a

mutation in one or more of the above-mentioned genes. In addition, *Bacillus* sp. host cells that comprise mutation(s) and/or deletions of endogenous protease genes find use. The *Bacillus* host cell may comprise a deletion of the *aprE* and the *nprE* genes. The *Bacillus* sp. host cell may comprise a deletion of 5 protease genes, or the *Bacillus sp.* host cell may comprise a deletion of 9 protease genes (see, e.g., U.S. Pat. Appn. Pub. No. 2005/0202535).

Host cells are transformed with at least one nucleic acid encoding at least one protease variant of the invention using any suitable method known in the art. Whether the nucleic acid is incorporated into a vector or is used without the presence of plasmid DNA, it is typically introduced into a microorganism, in one aspect, preferably an *E. coli* cell or a competent *Bacillus* cell. Methods for introducing a nucleic acid (e.g., DNA) into *Bacillus* cells or *E. coli* cells utilizing plasmid DNA constructs or vectors and transforming such plasmid DNA constructs or vectors into such cells are well known. In one aspect, the plasmids are subsequently isolated from *E. coli* cells and transformed into *Bacillus* cells. However, it is not essential to use intervening microorganisms such as *E. coli*, and in one aspect, a DNA construct or vector is directly introduced into a *Bacillus* host.

Those of skill in the art are well aware of suitable methods for introducing nucleic acid or polynucleotide sequences of the invention into *Bacillus* cells (see, e.g., Ferrari et al., "Genetics," in Harwood et al. (ed.), Bacillus, Plenum Publishing Corp. (1989), pp. 57-72; Saunders et al., J. Bacteriol. 157:718-726 (1984); Hoch et al., J. Bacteriol. 93:1925 -1937 (1967); Mann et al., Current Microbiol. 13:131-135 (1986); and Holubova, Folia Microbiol. 30:97 (1985); Chang et al., Mol. Gen. Genet. 168:11-115 (1979); Vorobjeva et al., FEMS Microbiol. Lett. 7:261-263 (1980); Smith et al., Appl. Env. Microbiol. 51:634 (1986); Fisher et al., Arch. Microbiol. 139:213-217 (1981); and McDonald, J. Gen. Microbiol. 130:203 (1984)). Indeed, such methods as transformation, including protoplast transformation and congression, transduction, and protoplast fusion are well known and suited for use in the present invention. Methods of transformation are used to introduce a DNA construct or vector comprising a nucleic acid encoding a protease variant of the present invention into a host cell. Methods known in the art to transform *Bacillus* cells include such methods as plasmid marker rescue transformation, which involves the uptake of a donor plasmid by competent cells carrying a partially homologous resident plasmid (Contente et al., Plasmid 2:555-571 (1979); Haima et al., Mol. Gen. Genet. 223:185-191 (1990); Weinrauch et al., J. Bacteriol. 154:1077-1087 (1983); and Weinrauch et al., J. Bacteriol. 169:1205-1211 (1987)). In this method, the incoming donor plasmid recombines with the homologous region of the resident "helper" plasmid in a process that mimics chromosomal transformation.

In addition to commonly used methods, in one aspect, host cells are directly transformed with a DNA construct or vector comprising a nucleic acid encoding a protease variant of the invention (i.e., an intermediate cell is not used to amplify, or otherwise process, the DNA construct or vector prior to introduction into the host cell). Introduction of the DNA construct or vector of the invention into the host cell includes those physical and chemical methods known in the art to introduce a nucleic acid sequence (e.g., DNA sequence) into a host cell without insertion into a plasmid or vector. Such methods include,

but are not limited to calcium chloride precipitation, electroporation, naked DNA, liposomes and the like. In additional aspects, DNA constructs or vector are co-transformed with a plasmid, without being inserted into the plasmid. In further aspects, a selective marker is deleted from the altered *Bacillus* strain by methods known in the art (see Stahl et al., J. Bacteriol. 158:411-418 (1984); and Palmeros et al., Gene 247:255 -264 (2000)).

In one aspect, the transformed cells of the present invention are cultured in conventional nutrient media. The suitable specific culture conditions, such as temperature, pII and the like are known to those skilled in the art. In addition, some culture conditions may be found in the scientific literature such as Hopwood (2000) Practical *Streptomyces* Genetics, John Innes Foundation, Norwich UK; Hardwood et al., (1990) Molecular Biological Methods for *Bacillus*, John Wiley and from the American Type Culture Collection (ATCC). In one aspect, the invention provides a culture (e.g., cell culture) comprising at least one protease variant or at least one nucleic acid of the invention. Also provided is a composition comprising at least one nucleic acid, vector, or DNA construct of the invention.

Host cells transformed with at least one polynucleotide sequence encoding at least one protease variant of the invention may be cultured in a suitable nutrient medium under conditions permitting the expression of the present protease, after which the resulting protease is recovered from the culture. The medium used to culture the cells comprises any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., in catalogues of the American Type Culture Collection). The protease produced by the cells may be recovered from the culture medium by conventional procedures, including, but not limited to, e.g., separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt (e.g., ammonium sulfate), chromatographic purification (e.g., ion exchange, gel filtration, affinity, etc.). Any method suitable for recovering or purifying a protease variant of the invention can be used.

In one aspect, a protease variant produced by a recombinant host cell is secreted into the culture medium. A nucleic acid sequence that encodes a purification facilitating domain may be used to facilitate purification of soluble proteins. A vector or DNA construct comprising a polynucleotide sequence encoding a protease variant may further comprise a nucleic acid sequence encoding a purification facilitating domain to facilitate purification of the protease variant (see, e.g., Kroll, D.J. et al., DNA Cell Biol. 12:441-53 (1993)). Such purification facilitating domains include, but are not limited to, e.g., metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J., Protein Expr. Purif. 3:263-281 (1992)), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and the heterologous protein also find use to facilitate purification.

Assays for detecting and measuring the enzymatic activity of an enzyme, such as a protease variant of the invention, are well known. Various assays for detecting and measuring activity of proteases, such as, e.g., protease variants of the invention, are also known to those of ordinary skill in the art. In particular, assays are available for measuring protease activity that are based on the release of acid-soluble peptides from casein or hemoglobin, measured as absorbance at 280 nm or colorimetrically using the Folin method (see, e.g., Bergmeyer et al., "Methods of Enzymatic Analysis" vol. 5, Peptidases, Proteinases and their Inhibitors, Verlag Chemie, Weinheim (1984)). Other exemplary assays involve the solubilization of chromogenic substrates (see, e.g., Ward, "Proteinases," in Fogarty (ed.). Microbial Enzymes and Biotechnology, Applied Science, London, (1983), pp. 251-317). Other exemplary assays include, but are not limited to succinyl-Ala-Ala-Pro-Phe-*para* nitroanilide assay (SAAPFpNA) and the 2,4,6-trinitrobenzene sulfonate sodium salt assay (TNBS assay). Numerous additional references known to those in the art provide suitable methods (see, e.g., Wells et al., Nucleic Acids Res. 11:7911-7925 (1983); Christianson et al., Anal. Biochem. 223:119 -129 (1994); and Hsia et al., Anal Biochem. 242:221-227 (1999)).

A variety of methods can be used to determine the level of production of a mature protease (e.g., mature protease variant of the invention) in a host cell. Such methods include, but are not limited to, e.g., methods that utilize either polyclonal or monoclonal antibodies specific for the protease. Exemplary methods include, but are not limited, e.g., to enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), fluorescent immunoassays (FIA), and fluorescent activated cell sorting (FACS). These and other assays are well known in the art (see, e.g., Maddox et al., J. Exp. Med. 158:1211 (1983)).

In another aspect, the invention provides methods for making or producing a mature protease variant of the invention. A mature protease variant does not include a signal peptide or a propeptide sequence. Some such methods comprising making or producing a protease variant of the invention in a recombinant bacterial host cell, such as, e.g., a *Bacillus sp.* cell, including, e.g., *Bacillus subtilis* cell. In one aspect, the invention provides a method of producing a protease variant of the invention, the method comprising cultivating a recombinant host cell comprising a recombinant expression vector comprising a nucleic acid encoding a protease variant of the invention under conditions conducive to the production of the protease variant. Some such methods further comprise recovering the protease variant from the culture.

In one aspect, the invention provides a method of producing a protease variant of the invention, the method comprising: (a) introducing a recombinant expression vector comprising a nucleic acid encoding a protease variant of the invention into a population of cells (e.g., bacterial cells, such as *Bacillus subtilis* cells); and (b) culturing the cells in a culture medium under conditions conducive to produce the protease variant encoded by the expression vector. Some such methods further comprise: (c) isolating the protease variant from the cells or from the culture medium.

In addition to recombinant production, the protease variant polypeptides of the invention may be produced by direct peptide synthesis using solid-phase techniques (see, e.g., Stewart et al. (1969) Solid-Phase Peptide Synthesis, W.H. Freeman Co, San Francisco; Merrifield (1963) J. Am. Chem. Soc 85:2149-2154). Peptide synthesis may be performed using manual or automated techniques. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City, Calif.) in accordance with the instructions provided by the manufacturer. For example, subsequences may be chemically synthesized separately and combined using chemical methods to provide protease variants or functional fragments thereof. Alternatively, such variant polypeptide sequences may be ordered from any number of companies that specialize in production of polypeptides. Most commonly, polypeptides of the invention are produced by expressing coding nucleic acids and recovering polypeptides, e.g., as described above and in the Examples.

In another aspect, the invention provides a method of producing a protease variant, the method comprising cultivating a recombinant host cell of the invention, said host cell comprising an expression vector which comprising at least one nucleic acid of the invention, under conditions conducive to produce the variant. The method may further comprise recovering the variant from the culture.

In another aspect, the invention includes a method of producing a protease variant, the method comprising: (a) introducing the recombinant expression vector of the invention into a population of cells; and (b) culturing the cells in a culture medium under conditions conducive to produce the subtilisin variant encoded by the expression vector. The method may further comprise (c) isolating the variant from the cells or from the culture medium.

In another aspect, the invention provides methods for producing a serine protease variant of a *Bacillus* serine protease, comprising: transforming a host cell with an expression vector comprising a nucleic acid encoding the serine protease variant; and cultivating the transformed host cell under conditions suitable for the production of the serine protease variant. In one aspect, the methods further comprise the step of harvesting the produced serine protease variant. In one aspect, the host cell is a *Bacillus* cell, and in a subset of these aspects, the *Bacillus* cell is a *B. subtilis* cell. Furthermore, the present invention provides methods of cleaning, comprising the step of contacting a surface and/or an article comprising a fabric with a cleaning composition comprising a serine protease variant. In some alternative methods, the present invention provides methods of cleaning, comprising the step of contacting a surface and/or an article comprising dishware with a cleaning composition comprising a serine protease variant.

In another aspect, the invention provides a method for identifying a wild-type subtilisin protease, said subtilisin protease comprising an amino acid sequence which comprises the following amino acids: a glycine or arginine at amino acid position 24, a glycine at amino acid position 53, an asparagine at amino acid position 78, an alanine at amino acid position 97, an asparagine at amino acid position 101, an alanine or serine at amino acid position 128, and/or a leucine or glutamine at amino acid position 217, wherein said amino acid positions are numbered by correspondence with amino acid positions in the

amino acid sequence of BPN' set forth in SEQ ID NO:2, said method comprising: a) providing a sample comprising at least one wild-type subtilisin protease or a library of wild-type subtilisin proteases, or providing a DNA sample comprising one or more DNA sequences encoding wild-type subtilisin proteases; and b) identifying a wild-type subtilisin protease or DNA sequence that encodes a wild-type protease.

In a twelfth aspect, the invention provides an expression vector comprising at least one nucleic acid of the tenth or eleventh aspect of the invention. An expression vector according to the twelfth aspect of the invention may be operably linked to a promoter. Such expression vector may be in isolated or purified form.

In a thirteenth aspect, the invention provides a recombinant host cell comprising: (a) a nucleic acid of the tenth or eleventh aspect of the invention or (b) an expression vector of the twelfth aspect of the invention. A recombinant host cell of the thirteenth aspect of the invention may be a bacterial cell, which may optionally be a *Bacillus* cell. A recombinant host cell of the thirteenth aspect of the invention may be a *Bacillus* subtilis cell.

In a fourteenth aspect, the invention provides a cell culture comprising: (a) a nucleic acid of the tenth or eleventh aspect of the invention or (b) an expression vector of the twelfth aspect of the invention.

In a fifteenth aspect, the invention provides a method of producing a protease variant, the method comprising cultivating a recombinant host cell of the thirteenth aspect of the invention under conditions conducive to produce the variant. A method according to the fifteenth aspect of the invention may further comprise isolating or recovering the variant from the culture.

In a sixteenth aspect, the invention provides a method of producing a protease variant, the method comprising: (a) introducing the recombinant expression vector of the twelfth aspect of the invention into a population of cells; and (b) culturing the cells in a culture medium under conditions conducive to produce the protease variant encoded by the expression vector. The method according to the sixteenth aspect of the invention may further comprise (c) isolating or recovering the variant from the cells or from the culture medium.

Compositions of the Invention

The invention includes a composition comprising at least one polypeptide (e.g., at least one protease variant) of the invention. Polypeptides of the invention are described *infra* and *supra*, including in the Part I Examples and Part II Examples. Such compositions may comprise at least one excipient, carrier, adjunct ingredient, or other substituent, component, or material.

For example, in one aspect, the invention includes a composition comprising at least one protease variant and at least one excipient, carrier, adjunct ingredient, or other substituent, component, or material. Such at least one protease variant may be any one of those set forth herein, including, but not limited to, in the Part I Examples. Such at least one protease variant may be a BPN' protease variant. For example, a composition of the invention may be a BPN' protease variant, such as BPN'-v36 (SEQ ID NO:6) or

BPN'-v3 (SEQ ID NO:4) or any protease variant set forth herein or in Part I Examples 2-23. Such composition may be a fabric and home care product or fabric and home care composition. Alternatively, such composition may be a fabric and home care product or fabric and home care composition.

Such composition may further comprise (in addition to a BPN' protease variant) at least one GG36 protease variant, such as at least one Series I GG36 protease variant described in the Part II Examples. Such compositions are useful in a variety of applications as described elsewhere herein.

In a seventeenth aspect, the invention provides a composition comprising a variant (or polypeptide as in the third aspect) of the first, second, third, fourth, fifth, sixth, seventh, eighth, or ninth aspect of the invention. A composition according to the seventeenth aspect of the invention may comprise an adjunct ingredient or carrier as described in greater detail elsewhere herein. A composition according to the seventeenth aspect of the invention may comprise at least one builder and/or at least one surfactant. A composition according to the seventeenth aspect of the invention may comprise phosphate or may not comprise phosphate.

A composition according to the seventeenth aspect of the invention may be a cleaning composition or a detergent composition. A composition according to the seventeenth aspect of the invention may be a fabric or home care product. A composition according to the seventeenth aspect of the invention may not be a fabric or home care product. A composition according to the seventeenth aspect of the invention may be a cleaning composition or detergent composition that is a fabric or home care product. A composition according to the seventeenth aspect of the invention may be a cleaning composition or detergent composition that is not a fabric or home care product..

A composition according to the seventeenth aspect of the invention may comprise may further comprise one, two, three, four, five or more additional enzymes. Such additional enzyme(s) may be selected from the group consisting of additional enzymes selected from the group consisting of hemicellulase, cellulase, amylase, peroxidase, protease, xylanase, lipase, phospholipase, esterase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, ß-glucanase, arabinosidase, hyaluronidase, chondroitinase, and laccase.

The additional enzyme according to the seventeenth aspect of the invention may be a GG36 protease variant having proteolytic activity, such as, e.g., a Series I GG36 protease variant, which Series I GG36 protease variant may comprise an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to the subtilisin *Bacillus lentus* GG36 of SEQ ID NO:755 and at least one amino acid substitution selected from the group consisting of A1R, Q2S, V4R, V4S, S9A, R10S, P14K, A16S, H17R, N18R, G20R, T22A, T22R, S24R, S24W, G25R, G25V, V26F, L42I, N43R, N43A, G46R, P52F, P52E, P52N, T57R, Q59A, N62E, N62Q, V68A, V68C, T71G, I72C, A74C, L75A, L75F, L75R, N76D, S78R, L82R, P86W, E89P, E89T, E89G, E89H, E89I, E89V, E89W, Y91N, K94N, G100S, S101A, S101N, S101G, S101D, S103G, S103N, V104L,

V104I, S106V, S106G, A108I, L111V, E112V, G115K, G115R, N117F, G118I, V121F, S128D, S128F, S128L, S128N, P129E, S144R, L148I, A158E. G159E, S160D, S166D, N185E, N185I, R186II, S188E, S188D, D197F, V203E, Y209S, Y209N, Y209F, Y209T, Y209E, Y209H, Y209G, P210R, S212I, S212F, Y214F, A215N, A215D, A215E, L217E, L217N, T224A, A230E, A231I, Q236F, N238R, N238K, P239K, P239G, P239R, P239S, W241R, S242R, S242L, N243R, V244R, N248I, N248V, H249R, L250I, N252R, T253R, L262D, Y263F, S265F, L267V, L267N. N269I, N269R, E271F, E271I, E271II, E271P, E271T, E271V, E271L, and A272F, wherein each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' protease set forth in SEQ ID NO:2. A composition according to the seventeenth aspect of the invention that comprises an additional enzyme (such as a protease, such as, e.g., a Series I G36 protease variant as described herein) may be a fabric and home care product. Alternatively, such composition may not be a fabric and home care product.

A composition according to the seventeenth aspect of the invention may comprise a Series I GG36 protease variant which comprises an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the subtilisin *Bacillus lentus* GG36 of SEQ ID NO:755 and at least one amino acid substitution selected from the group consisting of T022R-S024R, S009A-E271L, N018R-W241R, N018R-G115R, N043R-H249R, G020R-H249R, V004R-H249R, G020R-S024R, N018R-H249R, S009A-G020R, G020R-W241R, S009A-S078R, G020R-G115R, N018R-S024R, S024R-S242R, T022R-G115R, N018R-N043R, G020R-N043R, N018R-S242R, S242R-N269R, N018R-V244R, S024R-N269R, G020R-E271L, S024R-E271L, V004R-S009A, G020R-N269R, A001R-S024R, V244R-E271L, S009A-N018R, W241R-E271L, V004R-S024R, S009A-II249R, S009A-T022R, N062E-P129E, N062E-G159E, A016S-L148I, A158E-II249R, A016S-N062E, L111V-S188D, T022A-N062E, N062E-L148I, T022A-P129E, N062E-E271F, N062E-A158E, A016S-G159E, N062E-R186H, S128N-G159E, N062E-S188D, N062E-S128N, L148I-G159E, S103G-A158E, L111V-G159E, A158E-E271F, A016S-S188D, T022A-L111V, S128N-A158E, A016S-A158E, V104L-A158E, S128N-R186H, G159E-Y209E, N062E-S101A, L111V-Y209E, L148I-S188D, S101A-Y209E, T022A-S188D, A016S-T022A, S128N-P129E, A016S-Y209E, A016S-S128N, T022A-E089P, S128N-Y209E, E089P-A158E, N062E-S103G, R186H-E271F, A016S-P129E, E089P-G159E, L111V-H249R, S101A-P129E, L148I-Y209E, T022A-G159E, P129E-H249R, P129E-Y209E, V104L-P129E, S128N-S188D, L111V-A158E, T022A-A158E, N062E-Y209E, N062E-H249R, S101A-R186H, E089P-P129E, P129E-E271, T22A-L111V-G159E, S101A-S103G-V104L-Y209E, S101A-S103G-V104L-G159E, S101A-S103G-V104L-S188D, S101G-S103A-V104I-G159D, T22A-S103G-G159E, T22A-S128N-E271F-Y209E, T22A-Y209E-E271F, T22A-S101A-Y209E, S101A-Y209E-E271F, T22A-L111V-S128N, T22A-S101A-G159E, S101A-S103G-V104L, T22A-S101A-S103G-V104L, S101A-S103G-V104L, S101G-S103A-V104I, S101A-S103G-V104L-S128N, S103A-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-A232V-Q236H-Q245R-

N248D-N252K, S101G-S103A-V104L-G159D-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236II-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N248D, N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-S24R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-T253R, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159E-A232V-Q245R-N248D-H249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N238R, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N76D, and S101G-S103A-V104I-G159E-A232V-Q245R-N248D-E271F, , wherein each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' protease set forth in SEQ ID NO:2. Such composition may be a fabric and home care product or fabric and home care composition. In another aspect, such composition may not be a fabric and home care product or fabric and home care composition.

A composition according to the seventeenth aspect of the invention may comprise an Series I GG36 protease variant which comprises an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to the subtilisin *Bacillus lentus* GG36 of SEQ ID NO:755 and comprises three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or even 25 amino acid substitutions selected from the group consisting of: A1R, Q2S, V4R, V4S, S9A, R10S, P14K, A16S, H17R, N18R, G20R, T22A, T22R, S24R, S24W, G25R, G25V, V26F, I42I, N43R, N43A, G46R, P52F, P52E, P52N, T57R, Q59A, N62E, N62Q, V68A, V68C, T71G, I72C, A74C. L75A, L75F, L75R, N76D, S78R, L82R, P86W, E89P, E89T, E89G, E89H, E89I, E89V, E89W, Y91N, K94N, G100S, S101A, S101N, S101G, S101D, S103G, S103N, V104L, V104I, S106V, S106G, A108I, L111V, E112V, G115K, G115R, N117F, G118I, V121F, S128D, S128F, S128L, S128N, P129E, S144R, L148I, A158E. G159E, S160D, S166D, N185E, N185I, R186H, S188E, S188D, D197F, V203E, Y209S, Y209N, Y209F, Y209T, Y209E, Y209H, Y209G, P210R, S212I, S212F, Y214F, A215N, A215D, A215E, L217E, L217N, T224A, A230E, A231I, Q236F, N238R, N238K, P239K, P239G, P239R, P239S, W241R, S242R, S242L, N243R, V244R, N248I, N248V, II249R, L250I, N252R, T253R, L262D, Y263F, S265F, L267V, L267N, N269I, N269R, E271F, E271I, E271H, E271P, E271T, E271V, E271L, and A272F; and optionally at least one amino acid substitution selected from the group consisting of: S103A, G159D, Q236H, Q245R, N248D, and N252K, , wherein each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' protease set forth in SEQ ID NO:2. Such composition may be a fabric and home care product or fabric and home care composition. In

another aspect, such composition may not be a fabric and home care product or fabric and home care composition.

A composition according to the seventeenth aspect of the invention may comprise a Series I GG36 protease variant which comprises an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to the subtilisin *Bacillus lentus* GG36 of SEQ ID NO:755 and (a) two or more substitutions selected from the group consisting of A1R, Q2S, V4R, V4S, S9A, R10S, P14K, A16S, T22A, T22R, S24R, G25V, V26F, L42I, P52F, P52E, P52N, N62E, N62Q, V68A, V68C, T71G, I72C, A74C. L75A, L75F, S78R, E89P, E89T, E89G, E89H, E89W, Y91N, K94N, G100S, S101A, S101N, S101G, S101D, S103G, S103N, V104L, V104I, A108I, L111V, E112V, G115K, N117F, V121F, S128D, S128F, S128L, S128N, P129E, L148I, A158E. G159E, S160D, S166D, N185E, R186H, S188E, S188D, V203E, Y209S, Y209N, Y209F, Y209T, Y209E, Y209H, Y209G, P210R, S212I, S212F, Y214F, A215N, A215D, A215E, L217E, L217N, T224A, A230E, A231I, Q236F, N238R, N238K, P239K, P239G, P239R, N248V, H249R, L250I, L262D, Y263F, S265F, L267V, L267N. N269I, N269R, E271F, E271I, E271H and A272F, and/or (b) one or more sets of substitutions selected from the group consisting of N062E-P129E, N062E-G159E, A016S-L148I, A158E-H249R, A016S-N062E, L111V-S188D, T022A-N062E, N062E-L148I, T022A-P129E, N062E-E271F, N062E-A158E, A016S-G159E, N062E-R186H, S128N-G159E, N062E-S188D, N062E-S128N, L148I-G159E, S103G-A158E, L111V-G159E, A158E-E271F, A016S-S188D, T022A-L111V, S128N-A158E, A016S-A158E, V104L-A158E, S128N-R186H, G159E-Y209E, N062E-S101A, L111V-Y209E, L148I-S188D, S101A-Y209E, T022A-S188D, A016S-T022A, S128N-P129E, A016S-Y209E, A016S-S128N, T022A-E089P, S128N-Y209E, E089P-A158E, N062E-S103G, R186H-E271F, A016S-P129E, E089P-G159E, L111V-H249R, S101A-P129E, L148I-Y209E, T022A-G159E, P129E-H249R, P129E-Y209E, V104L-P129E, S128N-S188D, L111V-A158E, T022A-A158E, N062E-Y209E, N062E-H249R, S101A-R186H, E089P-P129E, P129E-E271F, T22A-L111V-G159E, S101A-S103G-V104L-Y209E, S101A-S103G-V104L-G159E, S101A-S103G-V104L-S188D, S101G-S103A-V104I-G159D, T22A-S103G-G159E, T22A-S128N-E271F-Y209E, T22A-Y209E-E271F, T22A-S101A-Y209E, S101A-Y209E-E271F, T22A-L111V-S128N, T22A-S101A-G159E, S101A-S103G-V104L, T22A-S101A-S103G-V104L, S101A-S103G-V104L, S101G-S103A-V104I, S101A-S103G-V104L-S128N, S103A-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N248D, N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-S24R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-T253R, S101G-S103A-V104I-

A158E-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159E-A232V-Q245R-N248D-II249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N238R, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N76D, and S101G-S103A-V104I-G159E-A232V-Q245R-N248D-E271F, , wherein each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' protease set forth in SEQ ID NO:2. Such composition may be a fabric and home care product or fabric and home care composition. In another aspect, such composition may not be a fabric and home care product or fabric and home care composition.

A composition according to the seventeenth aspect of the invention may comprise a Series I GG36 protease variant which comprises an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% sequence identity to the subtilisin *Bacillus lentus* GG36 of SEQ ID NO:755 and (a) two or more substitutions selected from the group of consisting of V4R, H17R, N18R, G20R, T22R, S24R, S24W, G25R, N43R, N43A, G46R, P52F, P52N, T57R, Q59A, N62Q, T71G, L75R, N76D, S78R, L82R, P86W, E89P, E89W, E89T, E89I, E89H, E89V, V104L, S106V, S106G, G115R, G118I, V121F, S144R, N185I, D197F, Y209N, Y209S, L217E, A231I, P239R, P239S, W241R, S242R, S242L, N243R, V244R, N248I, H249R, N252R, T253R, E271T, E271V, E271L, E271H, E271F, E271P, A1R, S9A, S212F, and N269R; and/or (b) one or more sets of substitutions selected from the group consisting of T022R-S024R, S009A-E271L, N018R-W241R, N018R-G115R, N043R-H249R, G020R-H249R, V004R-H249R, G020R-S024R, N018R-H249R, S009A-G020R, G020R-W241R, S009A-S078R, G020R-G115R, N018R-S024R, S024R-S242R, T022R-G115R, N018R-N043R, G020R-N043R, N018R-S242R, S242R-N269R, N018R-V244R, S024R-N269R, G020R-E271L, S024R-E271L, V004R-S009A, G020R-N269R, A001R-S024R, V244R-E271L, S009A-N018R, W241R-E271L, V004R-S024R, S009A-H249R, S009A-T022R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-H249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-S24R, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K, and S101G-S103A-V104L-A232V-Q236H-Q245R-N248D-N252K, , wherein each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' protease set forth in SEQ ID NO:2. Such composition may be a fabric and home care product or fabric and home care composition. In another aspect, such composition may not be a fabric and home care product or fabric and home care composition.

A composition according to the seventeenth aspect of the invention may comprise at least one additional enzyme selected from the group consisting of hemicellulase, cellulase, amylase, peroxidase, protease, xylanase, lipase, phospholipase, esterase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase,

malanase, ß-glucanase, arabinosidase, hyaluronidase, chondroitinase, and laccase. A composition according to the seventeenth aspect of the invention may comprise two or more additional enzymes selected from the group consisting of hemicellulase, cellulase, amylase, peroxidase, protease, xylanase, lipase, phospholipase, esterase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, ß-glucanase, arabinosidase, hyaluronidase, chondroitinase, and laccase.

A composition according to the seventeenth aspect of the invention may comprise phosphate or may not contain phosphate. A composition according to the seventeenth aspect of the invention may comprise at least one builder and/or at least one surfactant.

As further elsewhere herein, the polypeptides of the invention, including the protease variants of the invention, are useful in a variety of cleaning applications, including laundry cleaning applications, automatic dishwashing applications, hand dishwashing applications, hard surface cleaning applications, personal care applications, and other applications described herein. Thus, for example, in one aspect, the invention provides cleaning compositions comprising at least one polypeptide (e.g., protease variant) of the invention. As noted above, such cleaning compositions include, but are not limited to, e.g., automatic and hand dishwashing detergent compositions, laundry detergent compositions (including, e.g., liquid and powder laundry detergent compositions), fabric cleaning compositions, hard surface cleaning compositions (including, but not limited to, e.g., hard surface of a non-dishware item, non-tableware item, table, table top, furniture item, wall, floor, ceiling, etc.). Such cleaning compositions, which are useful in methods of cleaning an item or a surface in need of cleaning, may comprise, e.g., but not limited to, at least one excipient, carrier, and/or other substituent, component, or material.

In another aspect, the invention provides a composition comprising any polypeptide of the invention (e.g., any protease variant or subtilisin variant of the invention) described herein, wherein said composition is a fabric and home care composition or a fabric and home care product.

In another aspect, the invention provides a composition comprising any polypeptide of the invention (e.g., any protease variant or subtilisin variant of the invention) described herein, wherein said composition is not a fabric and home care composition or not a fabric and home care product. A composition of the invention comprising a protease variant of the invention may further comprise at least one adjunct material selected from perfume encapsulate; fabric hueing agent; cold-water soluble brightener; a bleach catalyst that may comprise a material selected from an iminium cation, iminium polyion, iminium zwitterion; modified amine; modified amine oxide; N-sulphonyl imine; N-phosphonyl imine; N-acyl imine; thiadiazole dioxide; perfluoroimine; cyclic sugar ketone; first wash lipase; bacterial cleaning cellulase; Guerbet nonionic surfactant; and mixture of any thereof. Compositions of the invention may further comprise at least one additional non-immunoequivalent protease selected from subtilisins (EC 3.4.21.62); trypsin-like or chymotrypsin-like proteases; metalloproteases; and mixtures thereof.

Compositions of the invention may further comprise at least one additional non-immunoequivalent protease selected from: subtilisins (EC 3.4.21.62) derived from *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus* and *B. gibsonii*; trypsin proteases and/or chymotrypsin proteases derived from *Cellulomonas*; metalloproteases derived from *B. amyloliquefaciens*; and mixtures thereof.

Compositions of the invention further comprise at least one additional enzyme selected from first-wash lipases; alpha-amylases; bacterial cleaning cellulases; and mixtures thereof.

A composition of the invention may further comprise at least one of the following: an encapsulate comprising a perfume comprises a perfume micro capsule; a hueing agent comprising a material selected from basic, acid, hydrophobic, direct and polymeric dyes, and dye-conjugates having a peak absorption wavelength of from 550 nm to 650 nm and mixtures thereof; a detersive surfactant comprising a material selected from anionic detersive surfactants, non-ionic detersive surfactant, cationic detersive surfactants, zwitterionic detersive surfactants and amphoteric detersive surfactants and mixtures thereof; a builder comprising a material selected from zeolites, phosphates and mixtures thereof; a silicate salt comprising a material selected from sodium silicate, potassium silicate and mixtures thereof; a brightener comprising a material selected from cold-water soluble brightener and mixtures thereof; a carboxylate polymer comprising a material selected from maleate/acrylate random copolymer or polyacrylate homopolymer and mixtures thereof; a soil release polymer comprising a material selected from terephthalate co-polymer and mixtures thereof; a cellulosic polymer comprising a material selected from alkyl cellulose, alkyl alkoxyalkyl cellulose, carboxyalkyl cellulose, alkyl carboxyalkyl cellulose and mixtures thereof; a bleach catalyst comprising a material selected from an iminium cation; iminium polyion; iminium zwitterion; modified amine; modified amine oxide; N-sulphonyl imine; N-phosphonyl imine; N-acyl imine; thiadiazole dioxide; perfluoroimine; cyclic sugar ketone and any mixture thereof; a bleach activator comprising a material selected from dodecanoyl oxybenzene sulphonate, decanoyl oxybenzene sulphonate, decanoyl oxybenzoic acid or salt thereof, 3,5,5-trimethyl hexanoyloxybenzene sulphonate, tetraacetyl ethylene diamine (TAED), nonanoyloxybenzene sulphonate (NOBS) and mixtures thereof; a source of hydrogen peroxide comprising a material selected from an inorganic perhydrate salt, including an alkali metal salt, such as sodium salts of perborate (usually mono- or tetra-hydrate), percarbonate, persulphate, perphosphate, persilicate salt, and any mixture thereof; a chelant comprising a material selected from DTPA (diethylene triamine pentaacetic acid), HEDP (hydroxyethane diphosphonic acid), DTPMP (diethylene triamine penta(methylene phosphonic acid)), ethylenediaminedisuccinic acid (EDDS), 1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt hydrate, derivative of said chelant; and any mixture thereof.

A composition of the invention may comprise a fabric hueing agent selected from the group consisting of a dye; dye-clay conjugate comprising at least one cationic-basic dye and a smectite clay; and any mixture thereof.

A composition of the invention may comprise at least one fabric hueing agent selected from small molecule dye, polymeric dye, and any mixture thereof; dye-clay conjugate comprising at least one cationic-basic dye and a smectite clay; and any mixture thereof.

A composition comprising a protease of the invention may be provided in single or multiple-compartment unit doses. The composition may be a multi-compartment unit dose, wherein the protease variant is in a different compartment than any source of hydrogen peroxide and/or chelant and/or additional enzyme. A composition comprising at least one protease variant or polypeptide of the invention may comprise a wash liquor.

A composition comprising at least one protease variant of the invention may comprise one or more of the following ingredients (based on total composition weight): from about 0.0005 wt% to about 0.1 wt%, from about 0.001 wt% to about 0.05 wt%, or even from about 0.002 wt% to about 0.03 wt% of said protease variant; and one or more of the following: from about 0.00003 wt% to about 0.1 wt% fabric hueing agent; from about 0.001 wt% to about 5 wt %, perfume capsules; from about 0.001 wt% to about 1 wt%, cold-water soluble brighteners; from about 0.00003 wt% to about 0.1 wt% bleach catalysts; from about 0.00003 wt% to about 0.1 wt% first wash lipases; from about 0.00003 wt% to about 0.1 wt% bacterial cleaning cellulases; and/or from about 0.05 wt% to about 20 wt% Guerbet nonionic surfactants.

A composition may be a granular or powder laundry detergent comprising a cold water protease or comprising a protease variant that is not a cold water protease.

A composition of the invention may be provided in any suitable form, including a fluid or solid. The composition may be in the form of a unit dose pouch, especially when in the form of a liquid, and the composition may be at least partially, or even completely, enclosed by a water-soluble pouch. In addition, the composition may have any combination of parameters and/or characteristics detailed above.

Unless otherwise noted, all component or composition levels provided herein are made in reference to the active level of that component or composition, and are exclusive of impurities, for example, residual solvents or by-products, which may be present in commercially available sources. Enzyme components weights are based on total active protein. All percentages and ratios are calculated by weight unless otherwise indicated. All percentages and ratios are calculated based on the total composition unless otherwise indicated. In the exemplified detergent compositions, the enzymes levels are expressed by pure enzyme by weight of the total composition and, unless otherwise specified, the detergent ingredients are expressed by weight of the total compositions.

As indicated herein, in one aspect, the cleaning compositions of the present invention may further comprise one or more adjunct materials or ingredients including, but not limited to, e.g., one or more surfactants, builders, bleaches, bleach activators, bleach catalysts, other enzymes, enzyme stabilizing systems, chelants, optical brighteners, soil release polymers, dye transfer agents, dispersants, suds suppressors, dyes, perfumes, perfume capsules, colorants, filler salts, hydrotropes, photoactivators, fluorescers, fabric conditioners, fabric softeners, hydrolyzable surfactants, preservatives, anti-oxidants, anti-shrinkage agents, anti-wrinkle agents, germicides, fungicides, color speckles, silvercare, anti-tarnish

and/or anti-corrosion agents, alkalinity sources, solubilizing agents, carriers, excipients, processing aids, pigments, rinse aid (e.g., a rinse aid containing at least one surfactant to prevent water droplet formation by making water drain from the surface of the item being cleaned in a thin sheet, rather than forming droplets), solvents, and/or pH control agents (see, e.g., U.S. Pat. Nos. 6,610,642, 6,605,458, 5,705,464, 5,710,115, 5,698,504, 5,695,679, 5,686,014 and 5,646,101, all of which are incorporated herein by reference). Aspects of specific cleaning composition materials are exemplified in detail below. If a cleaning adjunct material(s) is not compatible with a protease variant of the present invention in a desired cleaning composition, then a suitable method of keeping the cleaning adjunct material(s) and the protease variant(s) separated (i.e., not in contact with one another) until combination of the two components is appropriate is used. Such separation methods include any suitable method known in the art (e.g., gelcaps, encapsulation, tablets, physical separation, etc.).

The cleaning compositions of the present invention are advantageously employed, for example, in laundry applications, hard surface cleaning applications, hand or manual dishwashing applications, automatic dishwashing applications, eyeglass cleaning applications, as well as cosmetic applications, such as for cleaning dentures, teeth, hair, and skin. Due to the unique advantages of increased effectiveness in lower temperature solutions, the protease variant enzymes of the present invention are suited for laundry applications and dishwashing applications, including hand and automatic dishwashing applications. Furthermore, the protease variant enzymes of the present invention find use in solid, gel, granular, and/or liquid compositions, including solid, gel, granular, and/or liquid detergent compositions and/or formulations.

The protease variants of the present invention also find use cleaning additive product compositions. In one aspect, a protease variant of the invention is useful in low temperature solution cleaning applications and methods. In one aspect, the invention provides cleaning additive product compositions which include at least one protease variant enzyme of the present invention and which are ideally suited for inclusion in a wash process when additional bleaching effectiveness is desired. Such instances include, but are not limited to, e.g., low temperature solution cleaning applications. In one aspect, the additive product composition is in its simplest form – i.e., one or more protease variants of the invention. In one aspect, the additive product composition is packaged in dosage form for addition to a cleaning process. In one aspect, the additive product composition is packaged in dosage form for addition to a cleaning process where a source of peroxygen is employed and increased bleaching effectiveness is desired. Any suitable single dosage unit form may be used, including but not limited to, e.g., pills, tablets, gelcaps, or other single dosage units, such as pre-measured powders or liquids. Thus, in one aspect, the invention provides a cleaning product composition comprising at least one protease variant of the invention, wherein the product is formulated in suitable form (e.g., as a liquid, powder solid, pill, tablet, gelcap or other suitable form) in a suitable single dosage unit such that a single dose of the protease variant is provided. Such cleaning products are useful in a variety of cleaning methods and applications, including but not limited to, e.g., machine or hand laundry methods and applications,

automatic dishwashing or hand dishwashing methods and applications, etc. Such cleaning methods and applications may be conducted at low temperature or low pH conditions. In one aspect, at least one filler and/or at least one carrier material is included to increase the volume of such compositions. Suitable filler or carrier materials include, but are not limited to, e.g., various salts of sulfate, carbonate and silicate as well as talc, clay and the like. Suitable filler or carrier materials for liquid compositions include, but are not limited to, e.g., water or low molecular weight primary and secondary alcohols including polyols and diols. Examples of such alcohols include, but are not limited to, e.g., methanol, ethanol, propanol and isopropanol. In one aspect, the compositions contain from about 5% to about 90% of such filler or carrier materials. Acidic fillers may be included in such compositions to reduce the pH of the resulting solution in the cleaning method or application. Alternatively, in one aspect, the cleaning additive includes one or more adjunct ingredients, as more fully described below.

The present cleaning compositions and cleaning additives require an effective amount of at least one protease variant of the invention, alone or in combination with other proteases and/or additional enzymes. The required level of enzyme is achieved by the addition of one or more protease variants of the invention. Typically, a cleaning composition comprises at least about 0.0001 weight percent to about 20 weight percent, from about 0.0001 to about 10 weight percent, from about 0.0001 to about 1 weight percent, from about 0.001 to about 1 weight percent, or from about 0.01 to about 0.1 weight percent of at least one protease variant of the invention. In one aspect, a composition of the invention (e.g., cleaning composition of the invention) comprises from about 0.01 milligram (mg) to about 10 mg, about 0.01 to about 5 mg, about 0.01 mg to about 2 mg, about 0.01 to about 1 mg, about 0.5 mg to about 10 mg, about 0.5 to about 5 mg, about 0.5 to about 4 mg, about 0.5 to about 4 mg, about 0.5 to about 3 mg, about 0.5 to about 2 mg, about 0.5 to about 1 mg, about 0.1 to about 10 mg, about 0.1 to about 5 mg, about 0.1 to about 4 mg, about 0.1 to about 3 mg, about 0.1 to about 2 mg, about 0.1 to about 2 mg, about 0.1 to about 1 mg, about 0.1 to about 0.5 mg of at least one active protease variant of the invention per gram of the composition.

The invention includes a cleaning composition comprising an amount of a protease variant of the invention (said composition optionally comprising one or more adjunct ingredients) such that when the cleaning composition is added to wash water the resultant protease concentration in the resultant wash liquor is 0.01 ppm to 10 ppm, including, e.g., 0.1 ppm to 1 ppm, 0.1 to 5 ppm, 1 to 5 ppm, 1 ppm to 10 ppm, 5 ppm to 10 ppm, 5 ppm to 7 ppm.

The cleaning compositions of the invention are typically formulated such that, during use in aqueous cleaning operations, the wash water will have a pH of from about 5.0 to about 11.5 or even from about 7.5 to about 10.5. Liquid product compositions or formulations are typically formulated to have a neat pH from about 3.0 to about 9.0 or from about 3.0 to about 5.0. Granular laundry product compositions are typically formulated to have a pH from about 9 to about 11. Hand dishwashing and automatic dishwashing detergent compositions are typically formulated to have a pH from about 8 to about 11.5, including, but not limited to, e.g., pH ranges of about 8 to about 10, from about 9 to about

11.5, and from about 9.5 to about 11.5 depending on the method and specific application. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

Suitable low pH cleaning compositions typically have a neat pH of from about 3 to about 5, and are typically free of surfactants that hydrolyze in such a pH environment. Such surfactants include sodium alkyl sulfate surfactants that comprise at least one ethylene oxide moiety or even from about 1 to about 16 moles of ethylene oxide. Such cleaning compositions typically comprise a sufficient amount of a pH modifier, such as sodium hydroxide, monoethanolamine, or hydrochloric acid, to provide such cleaning composition with a neat pH of from about 3 to about 5. Such compositions typically comprise at least one acid stable enzyme. In one aspect, the compositions are liquids, while in other aspects, they are solids. The pH of such liquid compositions is typically measured as a neat pH. The pH of such solid compositions is measured as a 10% solids solution of said composition wherein the solvent is distilled water. In these aspects, all pH measurements are taken at 20°C, unless otherwise indicated.

In one aspect, when the protease variant(s) of the invention is/are employed in a granular composition or liquid, it is desirable for the protease variant to be in the form of an encapsulated particle to protect the protease variant from other components of the granular composition during storage. In addition, encapsulation is also a means of controlling the availability of the protease variant during the cleaning process. In one aspect, encapsulation enhances the performance of the protease variant(s) and/or additional enzymes. In this regard, the protease variants of the present invention are encapsulated with any suitable encapsulating material known in the art. In one aspect, the encapsulating material typically encapsulates at least part of the catalyst for the protease variant(s) of the invention. Typically, the encapsulating material is water-soluble and/or water-dispersible. In one aspect, the encapsulating material has a glass transition temperature (Tg) of 0°C or higher. Glass transition temperature is described in more detail in WO 97/11151. The encapsulating material is typically selected from consisting of carbohydrates, natural or synthetic gums, chitin, chitosan, cellulose and cellulose derivatives, silicates, phosphates, borates, polyvinyl alcohol, polyethylene glycol, paraffin waxes, and combinations thereof. When the encapsulating material is a carbohydrate, it is typically selected from monosaccharides, oligosaccharides, polysaccharides, and combinations thereof. In some typical aspects, the encapsulating material is a starch (see, e.g., EP 0 922 499; U.S. Patent Nos. 4,977,252, 5,354,559, and 5,935,826). In one aspect, the encapsulating material is a microsphere made from plastic such as thermoplastics, acrylonitrile, methacrylonitrile, polyacrylonitrile, polymethacrylonitrile and mixtures thereof; commercially available microspheres that find use include, but are not limited to those supplied by EXPANCEL® (Stockviksverken, Sweden), and PM 6545, PM 6550, PM 7220, PM 7228, EXTENDOSPHERES®, LUXSIL®, Q-CEL®, and SPHERICEL® (PQ Corp., Valley Forge, PA).

As described herein, the protease variants of the invention find particular use in the cleaning methods and applications, including, but not limited to, e.g., cleaning, laundry, hand dishwashing, and automatic dishwashing detergent compositions. These applications place enzymes under various

environmental stresses. The protease variants of the invention provide advantages over many currently used enzymes in such cleaning applications due to their proteolytic activity and stability under various conditions.

There are a variety of wash conditions including varying detergent formulations, wash water volumes, wash water temperatures, and lengths of wash time, to which proteases involved in washing are exposed. In addition, detergent formulations used in different geographical areas have different concentrations of their relevant components present in the wash water. For example, European detergents typically have about 4500-5000 parts per million (ppm) of detergent components in the wash water, while Japanese detergents typically have approximately 667 ppm of detergent components in the wash water. In North America, particularly the United States, detergents typically have about 975 ppm of detergent components present in the wash water.

A low detergent concentration system includes detergents where less than about 800 ppm of the detergent components are present in the wash water. Japanese detergents are typically considered low detergent concentration system as they have approximately 667 ppm of detergent components present in the wash water.

A medium detergent concentration includes detergents where between about 800 ppm and about 2000 ppm of the detergent components are present in the wash water. North American detergents are generally considered to be medium detergent concentration systems as they have approximately 975 ppm of detergent components present in the wash water. Brazil typically has approximately 1500 ppm of detergent components present in the wash water.

A high detergent concentration system includes detergents where greater than about 2000 ppm of the detergent components are present in the wash water. European detergents are generally considered to be high detergent concentration systems as they have approximately 4500-5000 ppm of detergent components in the wash water.

Latin American detergents are generally high suds phosphate builder detergents and the range of detergents used in Latin America can fall in both the medium and high detergent concentrations as they range from 1500 ppm to 6000 ppm of detergent components in the wash water. As mentioned above, Brazil typically has approximately 1500 ppm of detergent components present in the wash water. However, other high suds phosphate builder detergent geographies, not limited to other Latin American countries, may have high detergent concentration systems up to about 6000 ppm of detergent components present in the wash water.

In light of the foregoing, it is evident that concentrations of detergent compositions in typical wash solutions throughout the world varies from less than about 800 ppm of detergent composition ("low detergent concentration geographies"), e.g., about 667 ppm in Japan, to between about 800 ppm to about 2000 ppm ("medium detergent concentration geographies"), e.g., about 975 ppm in U.S. and about 1500 ppm in Brazil, to greater than about 2000 ppm ("high detergent concentration geographies"), e.g., about 4500 ppm to about 5000 ppm in Europe and about 6000 ppm in high suds phosphate builder geographies.

The concentrations of the typical wash solutions are determined empirically. For example, in the U.S., a typical washing machine holds a volume of about 64.4 L of wash solution. Accordingly, in order to obtain a concentration of about 975 ppm of detergent within the wash solution about 62.79 g of detergent composition must be added to the 64.4 L of wash solution. This amount is the typical amount measured into the wash water by the consumer using the measuring cup provided with the detergent.

As a further example, different geographies use different wash temperatures. The temperature of the wash water in Japan is typically less than that used in Europe. For example, the temperature of the wash water in North America and Japan is typically between about 10 and about 30°C (e.g., about 20°C), whereas the temperature of wash water in Europe is typically between about 30 and about 60°C (e.g., about 40°C). However, in the interest of saving energy, many consumers are switching to using cold water washing. In addition, in some further regions, cold water is typically used for laundry, as well as in dishwashing applications. In one aspect, the "cold water washing" of the present invention utilizes washing at temperatures from about 4°C to about 10°C, from about 10°C to about 40°C, or from about 20°C to about 30°C, from about 15°C to about 25°C, from about 10°C to about 20°C, from about 14°C to about 18°C, as well as all other combinations within the range of about 15°C to about 35°C, and all ranges within 10°C to 40°C, and about 16°C.

As a further example, different geographies typically have different water hardness. Water hardness is usually described in terms of the grains per gallon mixed Ca^{2+}/Mg^{2+}. Hardness is a measure of the amount of calcium (Ca^{2+}) and magnesium (Mg^{2+}) in the water. Most water in the United States is hard, but the degree of hardness varies. Moderately hard (60-120 ppm) to hard (121-181 ppm) water has 60 to 181 parts per million (parts per million converted to grains per U.S. gallon is ppm # divided by 17.1 equals grains per gallon) of hardness minerals.

Water	Grains per gallon	Parts per million
Soft	less than 1.0	less than 17
Slightly hard	1.0 to 3.5	17 to 60
Moderately hard	3.5 to 7.0	60 to 120
Hard	7.0 to 10.5	120 to 180
Very hard	greater than 10.5	greater than 180

European water hardness is typically greater than about 10.5 (e.g., about 10.5 to about 20.0) grains per gallon mixed Ca^{2+}/Mg^{2+} (e.g., about 15 grains per gallon mixed Ca^{2+}/Mg^{2+}). North American water hardness is typically greater than Japanese water hardness, but less than European water hardness.

For example, North American water hardness can be between about 3 to about 10 grains, about 3 to about 8 grains or about 6 grains. Japanese water hardness is typically lower than North American water hardness, usually less than about 4, for example about 3 grains per gallon mixed Ca^{2+}/Mg^{2+}.

Accordingly, in one aspect, the invention provides protease variants that show surprising wash performance in at least one set of wash conditions (e.g., water temperature, water hardness, and/or detergent concentration). In one aspect, the protease variants of the invention are comparable in wash performance to other subtilisin proteases. In one aspect, the protease variants of the present invention exhibit enhanced wash performance as compared to subtilisin proteases currently commercially available. Thus, in one aspect of the invention, the protease variants provided herein exhibit enhanced oxidative stability, enhanced thermal stability, enhanced cleaning capabilities under various conditions, and/or enhanced chelator stability. In addition, the protease variants of the invention find use in cleaning compositions that do not include detergents, again either alone or in combination with builders and stabilizers.

In one aspect, the invention provides a cleaning composition comprising at least one protease variant of the present invention that is present at a level from about 0.00001 % to about 10% by weight of the composition with the balance (e.g., about 99.999% to about 90.0%) comprising one or more cleaning adjunct materials by weight of composition. In another aspect, the invention provides a cleaning composition comprising at least one protease variant of the invention that is present at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% by weight of the composition with the balance of the cleaning composition (e.g., about 99.9999% to about 90.0%, about 99.999 % to about 98%, about 99.995% to about 99.5% by weight) comprising one or more cleaning adjunct materials.

In one aspect, a cleaning composition of the invention comprises, in addition to at least one protease variant of the invention, one or more additional enzymes, which provide cleaning performance and/or fabric care and/or hand or manual dishwashing and/or automatic dishwashing benefits. Examples of suitable enzymes include, but are not limited to, e.g., hemicellulases, cellulases, peroxidases, proteases, xylanases, lipases, phospholipases, esterases, perhydrolases, cutinases, pectinases, pectate lyases, mannanases, keratinases, reductases, oxidases, phenoloxidases, lipoxygenases, ligninases, pullulanases, tannases, pentosanases, malanases, ß-glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, and/or amylases, neutral metalloprotease enzymes (abbreviated as "nprE"), or mixtures of any thereof. In one aspect, the cleaning composition comprises, in addition to at least one protease variant of the invention, a combination of additional enzymes (i.e., a "cocktail") comprising conventional applicable enzymes such as, e.g., at least one additional protease, lipase, cutinase, cellulose, and/or amylase.

In addition to the protease variants provided herein, any other suitable protease may find use and be included in a composition of the invention. In one aspect, the invention provides a composition (e.g., cleaning composition) comprising at least one protease variant of the invention and at least one additional

protease. Suitable proteases include those of animal, vegetable, or microbial origin. In one aspect, a microbial protease may be included. A chemically or genetically modified mutant of a protease may be included. In one aspect, the at least one additional protease is a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases include subtilisins, especially those derived from *Bacillus* (e.g., subtilisin, *B. lentus* subtilisin (i.e., GG36), *B. amyloliquefaciens* subtilisin (i.e., BPN'), subtilisin Carlsberg, subtilisin 309, subtilisin 147, PB92, and subtilisin 168). Additional examples include those mutant proteases (i.e., protease variants) described in U.S. Pat. Nos. RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628, all of which are incorporated herein by reference. Additional proteases include, but are not limited to trypsin (e.g., of porcine or bovine origin), and the *Fusarium* protease described in WO 89/06270. A composition of the invention comprising at least one protease variant of the invention may also comprise at least one commercially available protease enzyme. Commercially available protease enzymes that find use in compositions of the invention include, but are not limited to, e.g., MAXATASE®, MAXACAL™, MAXAPEM™, OPTICLEAN®, OPTIMASE®, PROPERASE®, PURAFECT®, PURAFECT® OXP, PURAMAX™, EXCELLASE™, and PURAFAST™ (Genencor); ALCALASE®, SAVINASE®, PRIMASE®, DURAZYM™, POLARZYME®, OVOZYME®, KANNASE®, LIQUANASE®, NEUTRASE®, RELASE® and ESPERASE® (Novozymes); KAP *Bacillus* alkalophilus subtilisin with A230V+S256G+S259N (Kao); and BLAP™ *B. lentus* protease, BLAP X, and BLAP S (Henkel Kommanditgesellschaft auf Aktien, Duesseldorf, Germany). Additional proteases that may be included in compositions of the invention include those described in WO95/23221, WO 92/21760, U.S. Pat. Pub. No. 2008/0090747, and U.S. Pat. Nos. 5,801,039, 5,340,735, 5,500,364, 5,855,625, RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628, and various other patents. Metalloproteases may be included in compositions of the invention. Such metalloproteases, include, but are not limited to, e.g., the neutral metalloprotease enzyme (nprE) described in WO 07/044993.

In one aspect, the invention provides a composition (e.g., cleaning composition) comprising at least one protease variant of the invention and at least one lipase. Suitable lipases include, but are not limited to, e.g., those of bacterial or fungal origin. A chemically or genetically modified mutant of a lipase may be included in the composition. Examples of useful lipases include *Humicola lanuginosa* lipase (see, e.g., EP 258 068, and EP 305 216), *Rhizomucor miehei* lipase (see, e.g., EP 238 023), *Candida* lipase, such as *C. antarctica* lipase (e.g., *C. antarctica* lipase A or B; see, e.g., EP 214 761), *Pseudomonas* lipases such as *P. alcaligenes* lipase and *P. pseudoalcaligenes* lipase (see, e.g., EP 218 272), *P. cepacia* lipase (see, e.g., EP 331 376), *P. stutzeri* lipase (see, e.g., GB 1,372,034), *P. fluorescens* lipase, *Bacillus* lipase (e.g., *B. subtilis* lipase (Dartois et al., Biochem. Biophys. Acta 1131:253-260 (1993)); *B. stearothermophilus* lipase (see, e.g., JP 64/744992); and *B. pumilus* lipase (see, e.g., WO 91/16422)).

Furthermore, a number of cloned lipases find use in compositions (e.g., cleaning compositions) of the present invention, including, but not limited to, e.g., *Penicillium camembertii* lipase (Yamaguchi et

al., Gene 103:61-67 (1991)), *Geotricum candidum* lipase (Schimada et al., J. Biochem. 106:383-388 (1989)), and various *Rhizopus* lipases such as *R. delemar* lipase (Hass et al., Gene 109:117-113 (1991)), a *R. niveus* lipase (Kugimiya et al., Biosci. Biotech. Biochem. 56:716-719 (1992)) and *R. oryzae* lipase.

Other types of lipolytic enzymes, such as cutinases, also find use in one aspect of the present invention, including, but not limited to, e.g., the cutinase derived from *Pseudomonas mendocina* (see WO 88/09367), and the cutinase derived from *Fusarium solani pisi* (see WO 90/09446).

Additional suitable lipases include commercially available lipases such as M1 LIPASE™, LUMA FAST™, and LIPOMAX™ (Genencor); LIPOLASE® and LIPOLASE® ULTRA (Novozymes); and LIPASE P™ "Amano" (Amano Pharmaceutical Co. Ltd., Japan).

In one aspect, the invention provides compositions (e.g., cleaning compositions) comprising at least one protease variant of the invention and at least one lipase that is present at a level from about 0.00001 % to about 10% of additional lipase by weight of the composition and the balance of one or more cleaning adjunct materials by weight of composition. In one aspect, a cleaning composition of the present invention comprises, in addition to at least one protease variant of the invention, at least one lipase at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% lipase by weight of the composition.

Also included is a composition (e.g., cleaning composition) comprising at least one variant of the invention and at least one amylase. Any amylase (e.g., alpha and/or beta) suitable for use in alkaline solutions may be useful to include in such a composition. Suitable amylases include, but are not limited to, e.g., those of bacterial or fungal origin. A chemically or genetically modified mutant of an amylase may be included. Amylases that find use in compositions of the invention, include, but are not limited to, e.g., α-amylases obtained from *B. licheniformis* (see, e.g., GB 1,296,839). Commercially available amylases that find use in compositions of the invention include, but are not limited to, e.g., DURAMYL®, TERMAMYL®, FUNGAMYL®, STAINZYME®, STAINZYME PLUS®, STAINZYME ULTRA®, and BAN™ (Novozymes), as well as POWERASE™, RAPIDASE® and MAXAMYL® P (Genencor).

In one aspect, the invention provides a cleaning composition comprising at least one protease variant or at least one amylase, wherein the amylase is present at a level from about 0.00001 % to about 10% of additional amylase by weight of the composition and the balance of one or more cleaning adjunct materials by weight of composition. In another aspect, the invention includes a cleaning composition comprising at least one protease variant and at least one amylase that is present at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% amylase by weight of the composition.

Any suitable cellulase may find used in a cleaning composition of the present invention. In one aspect, the invention provides a cleaning composition comprising at least one protease variant of the invention and one or at least one cellulase. Suitable cellulases include, but are not limited to, e.g., those of bacterial or fungal origin. A chemically or genetically modified mutant of a cellulase may be included

in a composition of the invention. Suitable cellulases include, but are not limited to, e.g., *Humicola insolens* cellulases (see, e.g., U.S. Pat. No. 4,435,307) and cellulases having color care benefits (see, e.g., EP 0 495 257). Additional suitable cellulases are known in the art. Commercially available cellulases that find use and may be included in a composition of the invention include, but are not limited to, e.g., CELLUZYME®, CAREZYME® (Novozymes), and KAC-500(B)™ (Kao Corporation), Puradax 7000L, Puradax HA 4000G (Genencor). In one aspect, cellulases are incorporated as portions or fragments of mature wild-type or variant cellulases, wherein a portion of the N-terminus is deleted (see, e.g., U.S. Pat. No. 5,874,276). In one aspect, a cleaning composition of the invention comprises at least one protease variant of the invention and at least one cellulase at a level from about 0.00001 % to about 10% of additional cellulase by weight of the composition and the balance of one or more cleaning adjunct materials by weight of composition. In another aspect, the invention provides a cleaning composition comprising at least one protease variant of the invention and at least one cellulase at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% cellulase by weight of the composition.

Any mannanase suitable for use in detergent compositions also finds use in and thus may be included in a cleaning composition of the invention. In one aspect, the invention provides a cleaning composition comprising at least one protease variant of the invention and least one mannanase. Suitable mannanases include, but are not limited to, e.g., those of bacterial or fungal origin. A chemically or genetically modified mutant of a mannanase may be included in a composition of the invention. Various mannanases are known which are useful and may be included in a composition of invention (see, e.g., mannanases described in U.S. Pat. Nos. 6,566,114, 6,602,842, and 6,440,991, all of which are incorporated herein by reference). In one aspect, the invention provides a cleaning composition comprising at least one protease variant of the invention and at least one mannanase at a level from about 0.00001 % to about 10% of additional mannanase by weight of the composition and the balance of one or more cleaning adjunct materials by weight of composition. In some such cleaning compositions, each mannanase is present at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% mannanase by weight of the composition.

A peroxidase may be used in combination with hydrogen peroxide or a source thereof (e.g., a percarbonate, perborate or persulfate) in a composition of the invention. An oxidase may be used in combination with oxygen in a composition of the invention. Both such types of enzymes are used for "solution bleaching" (i.e., to prevent transfer of a textile dye from a dyed fabric to another fabric when the fabrics are washed together in a wash liquor), preferably together with an enhancing agent (see, e.g., WO 94/12621 and WO 95/01426). Suitable peroxidases/oxidases that may be included in compositions of the invention include, but are not limited to, e.g., those of plant, bacterial, or fungal origin. A chemically or genetically modified mutant of a peroxidase or oxidase may be included in a composition of the invention. In one aspect, the invention provides a cleaning composition comprising at least one protease variant of the invention and at least one peroxidase and/or at least one oxidase enzyme. Each

such peroxidase or oxidase may be present in the composition at a level from about 0.00001 % to about 10% of peroxidase or oxidase by weight of the composition and the balance of one or more cleaning adjunct materials by weight of composition. In another aspect, the invention provides a cleaning composition comprising at least one protease variant of the invention and at least one peroxidase and/or at least oxidase enzyme, wherein each such peroxidase or oxidase is present at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, or about 0.005% to about 0.5% peroxidase or oxidase enzyme, respectively, by weight of the composition.

In one aspect, the invention provides a composition (e.g., cleaning composition) comprising at least one protease variant of the invention and one or more additional enzymes find use, including but not limited to, e.g., one or more perhydrolases (see, e.g., WO 05/056782).

In another aspect, the invention provides a composition (e.g., cleaning composition) comprising at least one protease variant of the invention and one or more mixtures of the above-mentioned enzymes are encompassed, such as, e.g., one or more additional proteases, amylases, lipases, mannanases, and/or cellulases. Indeed, it is contemplated that various mixtures of these enzymes will find use in compositions of the present invention. It is also contemplated that the varying levels of the protease variant(s) and one or more additional enzymes may both independently range to about 10%, the balance of the cleaning composition being one or more cleaning adjunct materials. The specific selection of a cleaning adjunct material is readily made by considering the surface or item (e.g., dishware item, tableware item, or fabric item) or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use (e.g., through the hand or automatic dishwashing detergent use).

In one aspect, the invention provides a composition (e.g., cleaning composition) comprising at least one protease variant of the invention (and optionally at least one additional enzyme, if desired) and one or more cleaning adjunct materials. Examples of suitable cleaning adjunct materials include, but are not limited to, e.g., surfactants, builders, bleaches, bleach activators, bleach catalysts, other enzymes, enzyme stabilizing systems, chelants, optical brighteners, soil release polymers, dye transfer agents, dye transfer inhibiting agents, catalytic materials, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil removal agents, structure elasticizing agents, dispersants, suds suppressors, dyes, perfumes, colorants, filler salts, hydrotropes, perfume, perfume capsule, photoactivators, fluorescers, fabric conditioners, fabric softeners, carriers, hydrotropes, processing aids, solvents, pigments, hydrolyzable surfactants, preservatives, anti-oxidants, anti-shrinkage agents, anti-wrinkle agents, germicides, fungicides, color speckles, silvercare, anti-tarnish and/or anti-corrosion agents, alkalinity sources, solubilizing agents, carriers, processing aids, pigments, and pH control agents (see, e.g., U.S. Pat. Nos. 6,610,642, 6,605,458, 5,705,464, 5,710,115, 5,698,504, 5,695,679, 5,686,014 and 5,646,101, all of which are incorporated herein by reference). Aspects of specific cleaning composition materials are exemplified in detail below. As noted above, if a cleaning adjunct material is not compatible with a protease variant of the present invention included in a desired cleaning composition, then a suitable method of keeping the cleaning adjunct material(s) and the

protease(s) separated (i.e., not in contact with each other) until combination of the components is appropriate is used. Such separation methods include any suitable method known in the art (e.g., gelcap, encapsulation, tablets, physical separation, etc.).

In one aspect, a composition (e.g., cleaning composition) of the invention comprises an effective amount of at least one protease variant of the invention that is useful or effective for cleaning a surface in need of proteinaceous stain removal. Such cleaning compositions include cleaning compositions for such applications as cleaning hard surfaces, laundry, fabrics, dishes, tableware, or dishware (e.g., by hand or manual dishwashing or automatic dishwashing). Indeed, in one aspect, the present invention provides fabric cleaning compositions, while in another aspect, the invention provides non-fabric cleaning compositions. Notably, the invention also provides cleaning compositions comprising at least one protease variant of the invention, wherein such cleaning compositions are suitable for personal care, including oral care (including dentifrices, toothpastes, mouthwashes, etc., as well as denture cleaning compositions), suitable for cleaning skin and hair, and suitable for cleaning eyeglasses. It is intended that the present invention encompass detergent compositions in any form (i.e., liquid, granular, bar, solid, semi-solid, gels, emulsions, tablets, capsules, etc.).

By way of example, several cleaning compositions wherein the protease variants of the invention find use are described in greater detail below. In one aspect in which the cleaning compositions of the invention are formulated as compositions suitable for use in laundry machine washing method(s), the compositions of the invention preferably contain at least one surfactant and at least one builder compound, as well as one or more cleaning adjunct materials, including, e.g., one or more cleaning adjunct materials selected from the group of organic polymeric compounds, bleaching agents, additional enzymes, suds suppressors, dispersants, lime-soap dispersants, soil suspension and anti-redeposition agents, and corrosion inhibitors. In one aspect, laundry compositions also contain one or more softening agents (i.e., as additional cleaning adjunct materials). Additional exemplary laundry or fabric cleaning compositions and formulations to which one or more protease variants of the invention can be added are presented in the Examples below.

The compositions of the invention also find use as detergent additive products in solid or liquid form. Such additive products are intended to supplement and/or boost the performance of conventional detergent compositions and can be added at any stage of the cleaning process. In one aspect, the density of the laundry detergent composition ranges from about 400 to about 1200 g/liter, while in other aspects, it ranges from about 500 to about 950 g/liter of composition measured at 20°C.

In one aspect, the invention provides cleaning compositions, such as those described in U.S. Pat. No. 6,605,458, comprising at least protease variant of the invention. In one aspect, the composition comprising at least one protease variant of the invention is a compact granular fabric cleaning composition, while in other aspects, the composition is a granular fabric cleaning composition useful in the laundering of colored fabrics; in another aspect, the composition is a granular fabric cleaning composition which provides softening through the wash capacity. In another aspect, the composition is a

heavy duty liquid fabric cleaning composition. In another aspect, the invention provides a composition comprising at least one protease variant of the invention, wherein the composition is a fabric cleaning composition, such as one described in U.S. Pat. Nos. 6,610,642 and 6,376,450. Also provided are granular laundry detergent compositions of particular utility under European or Japanese washing conditions (see, e.g., U.S. Pat. No. 6,610,642) which comprise at least one protease variant of the invention.

In one aspect, the invention provides hard surface cleaning compositions comprising at least one protease variant provided herein. Some such compositions comprise hard surface cleaning compositions such as those described in U.S. Pat. Nos. 6,610,642, 6,376,450, and 6,376,450 that include at least one such protease variant.

The invention includes hand dishwashing or automatic dishwashing detergent compositions comprising at least one protease variant provided herein. Some such compositions comprise hard surface cleaning compositions, such as those described in U.S. Pat. Nos. 6,610,642 and 6,376,450.

In one aspect, the invention provides cleaning compositions for use in manual or hand dishwashing or automatic dishwashing methods comprising at least one protease variant of the invention and/or at least one surfactant and/or at least one additional cleaning adjunct material selected from the group of organic polymeric compounds, suds enhancing agents, group II metal ions, solvents, hydrotropes, and additional enzymes.

In one aspect in which the cleaning compositions of the invention are formulated as compositions suitable for use in automatic dishwashing machine method(s), the compositions of the invention typically contain at least one surfactant and/or at least one builder compound and may contain one or more cleaning adjunct materials preferably selected from organic polymeric compounds, bleaching agents, additional enzymes, suds suppressors, dispersants, lime-soap dispersants, soil suspension and anti-redeposition agents and corrosion inhibitors. Additional exemplary dishwashing compositions and formulations to which one or more protease variants of the invention can be added are presented in the Examples below.

In another aspect, the invention provides oral care compositions comprising at least one protease variant of the present invention that are useful for oral care (e.g., cleaning teeth and dentures); components of oral care compositions that may be useful and included in such compositions include those described in U.S. Pat. No. 6,376,450. Compositions of the invention may further comprise cleaning adjunct materials and compounds described in the U.S. Pat. Nos. 6,376,450, 6,605,458, and 6,610,642, all of which are incorporated by reference herein.

The cleaning compositions of the invention are formulated into any suitable form and prepared by any process chosen by the formulator, non-limiting examples of which are described in U.S. Pat. Nos. 5,879,584, 5,691,297, 5,574,005, 5,569,645, 5,565,422, 5,516,448, 5,489,392, and 5,486,303, all of which are incorporated herein by reference. When a low pH cleaning composition is desired, the pH of

such composition is adjusted via the addition of a material such as monoethanolamine or an acidic material such as hydrogen chloride (HCl).

While not essential for the purposes of the present invention, the non-limiting list of adjuncts illustrated hereinafter are suitable for use in the instant cleaning compositions. In one aspect, these adjuncts are incorporated, e.g., to assist or enhance cleaning performance for treatment of the substrate to be cleaned or to modify the aesthetics of the cleaning composition as is the case with perfumes, colorants, dyes or the like. It is understood that such adjuncts are in addition to the protease variants of the present invention. The precise nature of these additional components, and levels of incorporation thereof, will depend on the physical form of the composition and the nature of the cleaning operation for which it is to be used. Suitable adjunct materials include, but are not limited to, e.g., surfactants, builders, chelating agents, dye transfer inhibiting agents, deposition aids, dispersants, additional enzymes, and enzyme stabilizers, catalytic materials, bleach activators, bleach boosters, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil removal/anti-redeposition agents, brighteners, suds suppressors, dyes, perfumes, structure elasticizing agents, fabric softeners, carriers, hydrotropes, processing aids and/or pigments. In addition to the disclosure below, suitable examples of such other adjuncts and levels of use are found in U.S. Patent Nos. 5,576,282, 6,306,812, and 6,326,348, incorporated by reference. The aforementioned adjunct ingredients may constitute the balance of the cleaning compositions of the present invention.

In one aspect, cleaning compositions of the invention comprise at least one surfactant and/or a surfactant system, wherein the surfactant is selected from nonionic surfactants, anionic surfactants, cationic surfactants, ampholytic surfactants, zwitterionic surfactants, semi-polar nonionic surfactants and mixtures thereof. In some low pH cleaning composition aspects (e.g., compositions having a neat pH of from about 3 to about 5), the composition typically does not contain alkyl ethoxylated sulfate, as it is believed that such surfactant may be hydrolyzed by such compositions the acidic contents. In one aspect, the surfactant is present at a level of from about 0.1% to about 60%, while in alternative aspects the level is from about 1% to about 50%, while in still further aspects the level is from about 5% to about 40%, by weight of the cleaning composition.

In one aspect, cleaning compositions of the invention comprise one or more detergent builders or builder systems. In some such compositions incorporating at least one builder, the cleaning compositions comprise at least about 1%, from about 3% to about 60% or even from about 5% to about 40% builder by weight of the cleaning composition. Builders include, but are not limited to, e.g., alkali metal, ammonium and alkanolammonium salts of polyphosphates, alkali metal silicates, alkaline earth and alkali metal carbonates, aluminosilicates, polycarboxylate compounds, ether hydroxypolycarboxylates, copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1, 3, 5-trihydroxy benzene-2, 4, 6-trisulphonic acid, and carboxymethyloxysuccinic acid, the various alkali metal, ammonium and substituted ammonium salts of polyacetic acids such as ethylenediamine tetraacetic acid and nitrilotriacetic acid, as well as polycarboxylates, such as mellitic acid, succinic acid, citric acid, oxydisuccinic acid, polymaleic

acid, benzene 1,3,5-tricarboxylic acid, carboxymethyloxysuccinic acid, and soluble salts thereof. It is contemplated that any suitable builder will find use in various compositions of the invention.

In some such compositions, the builders form water-soluble hardness ion complexes (e.g., sequestering builders), such as citrates and polyphosphates (e.g., sodium tripolyphosphate and sodium tripolyphospate hexahydrate, potassium tripolyphosphate, and mixed sodium and potassium tripolyphosphate, etc.). It is contemplated that any suitable builder will find use in the present invention, including those known in the art (see, e.g., EP 2 100 949).

Some cleaning compositions of the invention comprise at least one chelating agent in addition to at least one protease variant. Suitable chelating agents include, but are not limited to, e.g., copper, iron, and/or manganese chelating agents and mixtures thereof. In aspects in which at least one chelating agent is used, the cleaning compositions of the present invention comprise from about 0.1% to about 15% or even from about 3% to about 10% chelating agent by weight of the subject cleaning composition.

Some cleaning compositions provided herein comprise at least one deposition aid in addition to at least one protease variant. Suitable deposition aids include, but are not limited to, e.g., polyethylene glycol, polypropylene glycol, polycarboxylate, soil release polymers such as polytelephthalic acid, clays such as kaolinite, montmorillonite, atapulgite, illite, bentonite, halloysite, and mixtures thereof.

As indicated herein, in one aspect, anti-redeposition agents find use in one aspect of the present invention. In one aspect, non-ionic surfactants find use. These non-ionic surfactants also find use in preventing the re-deposition of soils. In one aspect, the anti-redeposition agent is a non-ionic surfactant as known in the art (see, e.g., EP 2 100 949).

In one aspect, the cleaning compositions of the present invention include one or more dye transfer inhibiting agents. Suitable polymeric dye transfer inhibiting agents include, but are not limited to, polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinyloxazolidones and polyvinylimidazoles or mixtures thereof. In aspects in which at least one dye transfer inhibiting agent is used, the cleaning compositions of the present invention comprise from about 0.0001% to about 10%, from about 0.01% to about 5%, or even from about 0.1% to about 3% by weight of the cleaning composition.

In one aspect, silicates are included within the compositions of the present invention. In some such aspects, sodium silicates (e.g., sodium disilicate, sodium metasilicate, and crystalline phyllosilicates) find use. In one aspect, silicates are present at a level of from about 1% to about 20%. In one aspect, silicates are present at a level of from about 5% to about 15% by weight of the composition.

In one aspect, the cleaning compositions of the invention also comprise dispersants. Suitable water-soluble organic materials include, but are not limited to, e.g., the homo- or co-polymeric acids or their salts, in which the polycarboxylic acid comprises at least two carboxyl radicals separated from each other by not more than two carbon atoms.

In one aspect, the enzymes (e.g., protease variants of the invention or other additional enzymes) used in the cleaning compositions are stabilized any suitable technique. In one aspect, the enzymes

employed herein are stabilized by the presence of water-soluble sources of calcium and/or magnesium ions in the finished compositions that provide such ions to the enzymes. In one aspect, the enzyme stabilizers include oligosaccharides, polysaccharides, and inorganic divalent metal salts, including alkaline earth metals, such as calcium salts. It is contemplated that various techniques for enzyme stabilization will find use in the present invention. For example, in one aspect, the enzymes employed herein are stabilized by the presence of water-soluble sources of zinc (II), calcium (II) and/or magnesium (II) ions in the finished compositions that provide such ions to the enzymes, as well as other metal ions (e.g., barium (II), scandium (II), iron (II), manganese (II), aluminum (III), Tin (II), cobalt (II), copper (II), nickel (II), and oxovanadium (IV). Chlorides and sulfates also find use in one aspect of the present invention. Examples of suitable oligosaccharides and polysaccharides (e.g., dextrins) are known in the art (see, e.g., WO 07/145964). In one aspect, reversible protease inhibitors also find use, such as boron-containing compounds (e.g., borate, phenyl boronic acid, 4-formyl phenyl boronic acid, other phenyl boronic acid derivatives, peptide inhibitors, and the like) and/or a tripeptide aldehyde find use in compositions of the invention to further improve stability, as desired.

In one aspect, one or more bleaches, bleach activators, and/or bleach catalysts are included in the compositions of the invention. In one aspect, the cleaning compositions of the invention comprise inorganic and/or organic bleaching compound(s). Inorganic bleaches include, but are not limited to perhydrate salts (e.g., perborate, percarbonate, perphosphate, persulfate, and persilicate salts). In one aspect, inorganic perhydrate salts are alkali metal salts. In one aspect, inorganic perhydrate salts are included as the crystalline solid, without additional protection, although in some other aspects, the salt is coated. Any suitable salt known in the art finds use in the compositions of the invention (see, e.g., EP 2 100 949).

In one aspect, bleach activators are used in the compositions of the invention. Bleach activators are typically organic peracid precursors that enhance the bleaching action in the course of cleaning at temperatures of 60°C and below. Bleach activators suitable for use herein include compounds which, under perhydrolysis conditions, give aliphatic peroxycarboxylic acids having preferably from about 1 to about 10 carbon atoms, in particular from about 2 to about 4 carbon atoms, and/or optionally substituted perbenzoic acid. Additional bleach activators are known in the art and find use in the composition of the invention (see, e.g., EP 2 100 949).

In one aspect and as further described herein, the cleaning compositions of the invention further comprise at least one bleach catalyst. In one aspect, the manganese triazacyclononane and related complexes find use, as well as cobalt, copper, manganese, and iron complexes. Additional bleach catalysts find use in the present invention (see, e.g., U.S. Pat Nos. 4,246,612, 5,227,084, and 4,810410; WO 99/06521; and EP 2 100 949).

In one aspect, the cleaning compositions of the invention comprise one or more catalytic metal complexes. In one aspect, a metal-containing bleach catalyst finds use. In some aspects, the metal bleach catalyst comprises a catalyst system comprising a transition metal cation of defined bleach catalytic

activity (e.g., copper, iron, titanium, ruthenium, tungsten, molybdenum, or manganese cations), an auxiliary metal cation having little or no bleach catalytic activity (e.g., zinc or aluminum cations), and a sequestrate having defined stability constants for the catalytic and auxiliary metal cations, particularly ethylenediaminetetraacetic acid, ethylenediaminetetra (methylenephosphonic acid) and water-soluble salts thereof are used (see, e.g., U.S. Pat. No. 4,430,243). In one aspect, the cleaning compositions of the invention are catalyzed by means of a manganese compound. Such compounds and levels of use are well known in the art (see, e.g., U.S. Patent No. 5,576,282). In additional aspects, cobalt bleach catalysts find use and are included in the cleaning compositions of the invention. Various cobalt bleach catalysts are known in the art (see, e.g., U.S. Patent Nos. 5,597,936 and 5,595,967) and are readily prepared by known procedures.

In one aspect, the cleaning compositions of the invention include a transition metal complex of a macropolycyclic rigid ligand (MRL). As a practical matter, and not by way of limitation, in one aspect, the compositions and cleaning processes provided by the invention are adjusted to provide on the order of at least one part per hundred million of the active MRL species in the aqueous washing medium, and in one aspect, provide from about 0.005 ppm to about 25 ppm, from about 0.05 ppm to about 10 ppm, and from about 0.1 ppm to about 5 ppm, of the MRL in the wash liquor.

In one aspect, transition-metals in the instant transition-metal bleach catalyst include, but are not limited to, e.g., manganese, iron and chromium. Preferred MRLs also include, but are not limited to, e.g., special ultra-rigid ligands that are cross-bridged (e.g., 5,12-diethyl-1,5,8,12-tetraazabicyclo(6.6.2)hexadecane). Suitable transition metal MRLs are readily prepared by known procedures (see, e.g., WO 2000/32601 and U.S. Pat. No. 6,225,464).

In one aspect, the invention provides an automatic dishwashing detergent composition formulated as a detergent tablet. Such tablet comprises at least one protease variant of the invention and a builder, such as, e.g., a builder salt. Some such tablets have an alkalinity of at least equivalent to 3 grams (g) of sodium hydroxide per 100 grams of the tablet composition and a density of at least 1.4 grams/cubic centimeter. The builder salt can comprise a mixture of a silicate salt and a phosphate salt, preferably with more silicate (e.g., sodium metasilicate) than phosphate (e.g., sodium tripolyphosphate). Some such tablets are free of surfactant materials and are especially adapted for use in automatic dishwashing machines.

In one aspect, the cleaning compositions of the present invention comprise metal care agents. Metal care agents are useful in preventing and/or reducing the tarnishing, corrosion, and/or oxidation of metals, including aluminum, stainless steel, and non-ferrous metals (e.g., silver and copper). Suitable metal care agents include those described in EP 2 100 949, WO 9426860, and WO 94/26859). In some such cleaning compositions, the metal care agent is a zinc salt. Some such cleaning compositions comprise from about 0.1% to about 5% by weight of one or more metal care agent.

As indicated above, the cleaning compositions of the present invention are formulated into any suitable form and prepared by any process chosen by the formulator, non-limiting examples of which are

described in U.S. Pat. Nos. 5,879,584, 5,691,297, 5,574,005, 5,569,645, 5,516,448, 5,489,392, and 5,486,303, all of which are incorporated herein by reference. In one aspect in which a low pH cleaning composition is desired, the pH of such composition is adjusted via the addition of an acidic material such as hydrogen chloride (HCl).

The cleaning compositions disclosed herein find use in cleaning a situs (e.g., a surface, dishware, tableware, or fabric). Typically, at least a portion of the situs is contacted with a present cleaning composition of the invention in neat form or diluted in a wash liquor, and then the situs is optionally washed and/or rinsed. For purposes of the present invention, "washing" includes, but is not limited to, e.g., scrubbing and mechanical agitation. In one aspect, the cleaning compositions are employed at concentrations of from about 500 ppm to about 15,000 ppm in solution. When the wash solvent is water, the water temperature typically ranges from about 5°C to about 90°C and when the situs comprises a fabric, the water to fabric mass ratio is typically from about 1:1 to about 30:1.

Processes of Making and Using Compositions

The compositions of the invention described herein and throughout, including, e.g., cleaning compositions, can be formulated into any suitable form and prepared by any suitable process chosen by the formulator (see, e.g., US Patent Nos. 5,879,584, 5,691,297, 5,574,005, 5,569,645, 5,565,422, 5,516,448, 5,489,392, 5,486,303, 4,515,705, 4,537,706, 4,515,707, 4,550,862, 4,561,998, 4,597,898, 4,968,451, 5,565,145, 5,929,022, 6,294,514 and 6,376,445).

In one aspect, the cleaning compositions of the invention are provided in unit dose form, including tablets, capsules, sachets, pouches, and multi-compartment pouches. In one aspect, the unit dose format is designed to provide controlled release of the ingredients within a multi-compartment pouch (or other unit dose format). Suitable unit dose and controlled release formats are known in the art (see, e.g., EP 2 100 949, WO 02/102955, U.S. Pat. Nos. 4,765,916 and 4,972,017, and WO 04/111178 for materials suitable for use in unit dose and controlled release formats). In one aspect, the unit dose form is provided by tablets wrapped with a water-soluble film or water-soluble pouches. Various formats for unit doses are provided in EP 2 100 947, and are known in the art.

Additional aspects of the invention relating to processes of making and using compositions of the invention are described elsewhere herein.

Methods of the Invention

The invention provides methods for cleaning or washing an item or surface (e.g., hard surface) in need of cleaning, including, but not limited to, e.g., methods for cleaning or washing a dishware item, a tableware item, a fabric item, a laundry item, personal care item, eye glass, etc., or the like, and methods for cleaning or washing a hard or soft surface, such as, e.g., a hard surface of an item.

In one aspect, the invention provides a method for cleaning an item, object, or surface in need of cleaning, the method comprising contacting the item or surface (or a portion of the item or surface

desired to be cleaned) with a protease variant of any of the invention or a composition of the invention for a sufficient time and/or under conditions suitable or effective to clean the item, object, or surface to a desired degree. Some such methods further comprise rinsing the item, object, or surface with water. For some such methods, the cleaning composition is a dishwashing detergent composition and the item or object to be cleaned is a dishware item or tableware item. A dishware item is a dish item generally used in serving or eating food. A dishware item can be, but is not limited to, e.g., a dish, plate, cup, bowl, etc., and the like. Tableware is a broader term that includes, but is not limited, to, e.g., dishes, cutlery, knives, forks, spoons, chopsticks, glassware, pitchers, sauce boats, drinking vessels, etc., and the like; a tableware item includes any of these or similar items for serving or eating food. For some such methods, the cleaning composition is an automatic dishwashing detergent composition or a hand dishwashing detergent composition and the item or object to be cleaned is a dishware or tableware item. For some such methods, the cleaning composition is a laundry detergent composition, such as, e.g., a power laundry detergent composition or a liquid laundry detergent composition, and the item to be cleaned is a fabric item.

In one aspect, the invention provides methods for cleaning or washing a fabric item optionally in need of cleaning or washing, respectively. Some such methods comprise providing a composition comprising the protease variant (such as, but not limited to, e.g., a fabric or laundry cleaning composition) and a fabric item or laundry item in need of cleaning, and contacting the fabric item or laundry item (or a portion of the item desired to be cleaned) with the composition under conditions sufficient or effective to clean or wash the fabric or laundry item to a desired degree.

In one aspect, the invention provides a method for cleaning or washing an item or surface (e.g., hard surface) optionally in need of cleaning, the method comprising providing an item or surface to be cleaned or washed and contacting the item or surface (or a portion of the item or surface desired to be cleaned or washed) with at least one protease variant of the invention or a composition of the invention comprising at least one such protease variant for a sufficient time and/or under conditions sufficient or effective to clean or wash the item or surface to a desired degree. Such compositions include, but are not limited to, e.g., a cleaning composition or detergent composition of the invention (including, but not limited to, e.g., hand dishwashing detergent composition, hand dishwashing cleaning composition, laundry detergent or fabric detergent or laundry or fabric cleaning composition, liquid laundry detergent, liquid laundry cleaning composition, powder laundry detergent composition, powder laundry cleaning composition, automatic dishwashing detergent composition, laundry booster cleaning or detergent composition, laundry cleaning additive, and laundry pre-spotter composition, etc.). In some instances, if desired, the method can be repeated one or more times, particularly if additional cleaning or washing is desired. For example, in some instance, the method optionally further comprises allowing the item or surface to remain in contact with the at least one protease variant or composition for a period of time sufficient or effective to clean or wash the item or surface to the desired degree. Some such methods further comprise rinsing the item or surface with water. Some such methods further comprise contacting

the item or surface with at least one protease variant of the invention or a composition of the invention again and allowing the item or surface to remain in contact with the at least one protease variant or composition for a period of time sufficient to clean or wash the item or surface to the desired degree. For some such methods, the cleaning composition is a dishwashing detergent composition and the item to be cleaned is a dishware or tableware item. For some such methods, the cleaning composition is an automatic dishwashing detergent composition or a hand dishwashing detergent composition and the item to be cleaned is a dishware or tableware item. For some such methods, the cleaning composition is a laundry detergent composition and the item to be cleaned is a fabric item.

In one aspect, the invention provides a method of cleaning a tableware or dishware item in an automatic dishwashing machine, the method comprising providing an automatic dishwashing machine, placing an amount of an automatic dishwashing composition comprising at least one protease variant of the invention or a composition of the invention sufficient to clean the tableware or dishware item in the machine (e.g., by placing the composition in an appropriate or provided detergent compartment or dispenser in the machine), putting a dishware or tableware item in the machine, and operating the machine so as to clean the tableware or dishware item (e.g., as per the manufacturer's instructions). Such method can include any automatic dishwashing composition described herein, which comprises, but is not limited to, e.g., any protease variant described herein. The amount of automatic dishwashing composition to be used can be readily determined according to manufacturer's instructions or suggestions and any form of automatic dishwashing composition comprising at least one protease variant of the invention (e.g., liquid, powder, solid, gel, table, etc.), including any described herein, may be employed.

In one aspect, the invention provides a method for cleaning a surface, item or object optionally in need of cleaning, the method comprises contacting the item or surface (or a portion of the item or surface desired to be cleaned) with at least one protease variant of the invention or a cleaning composition of the invention in neat form or diluted in a wash liquor for a sufficient time and/or under conditions sufficient or effective to clean or wash the item or surface to a desired degree. The surface, item, or object may then be (optionally) washed and/or rinsed if desired. For purposes of the present invention, "washing" includes, but is not limited to, e.g., scrubbing and mechanical agitation. In one aspect, the cleaning compositions are employed at concentrations of from about 500 ppm to about 15,000 ppm in solution (e.g., aqueous solution). When the wash solvent is water, the water temperature typically ranges from about 5°C to about 90°C and when the situs comprises a fabric, the water to fabric mass ratio is typically from about 1:1 to about 30:1.

In one aspect, the invention provides a method of cleaning a laundry or fabric item in an washing machine, the method comprising providing an washing machine, placing an amount of a laundry detergent composition comprising at least one protease variant of the invention sufficient to clean the laundry or fabric item in the machine (e.g., by placing the composition in an appropriate or provided detergent compartment or dispenser in the machine), putting the laundry or fabric item in the machine, and operating the machine so as to clean the tableware or dishware item (e.g., as per the manufacturer's

instructions). Such method can include any laundry washing detergent composition described herein, which comprises, but is not limited to, e.g., any protease variant described herein. The amount of laundry detergent composition to be used can be readily determined according to manufacturer's instructions or suggestions and any form of laundry detergent composition comprising at least one protease variant of the invention (e.g., solid, powder, liquid, tablet, gel, etc.), including any described herein, may be employed.

In an eighteenth aspect, the invention provides a method for cleaning an item, object, or surface in need of cleaning, the method comprising contacting the item, object, or surface with (i) a variant (or polypeptide as in the third aspect) of the first, second, third, fourth, fifth, sixth, seventh, eighth, or ninth aspect of the invention or (ii) a composition of the seventeenth aspect of the invention. A method of the eighteenth aspect of the invention may further comprise rinsing the item, object, or surface with water.

In a nineteenth aspect, the invention provides a method for cleaning an item or hard surface, the method comprising contacting at least a portion of the item or hard surface to be cleaned with (i) a variant (or polypeptide as in the third aspect) of the first, second, third, fourth, fifth, sixth, seventh, eighth, or ninth aspect of the invention or (ii) a composition of the seventeenth aspect of the invention for a sufficient time and/or under conditions sufficient or effective to clean or wash the item or hard surface to a desired degree, and optionally comprising rinsing the item or hard surface with water.

In a twentieth aspect, the invention provides a method of treating and/or cleaning a surface or fabric comprising the steps of optionally washing and/or rinsing said surface or fabric, contacting said surface or fabric with (i) a variant (or polypeptide as in the third aspect) of the first, second, third, fourth, fifth, sixth, seventh, eighth, or ninth aspect of the invention or (ii) a composition of the seventeenth aspect of the invention, and then optionally washing and/or rinsing said surface or fabric.

Included is a use of a protease variant or polypeptide of the invention in a detergent composition, including, but not limited to, in a laundry and/or dishwashing detergent composition.

Additional exemplary cleaning methods are provided throughout and in the Examples below.

EXPERIMENTAL

In the experimental disclosure of Part I Examples and Part I Examples which follow, the following abbreviations apply: PI (Performance Index), ppm (parts per million); M (molar); mM (millimolar); μM (micromolar); nM (nanomolar); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); gm (grams); mg (milligrams); μg (micrograms); pg (picograms); L (liters); ml and mL (milliliters); μl and μL (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); U (units); V (volts); MW (molecular weight); sec (seconds); min(s) (minute/minutes); h(s) and hr(s) (hour/hours); °C (degrees Centigrade); QS (quantity sufficient); ND (not done); rpm (revolutions per minute); GH (degrees German hardness); H_2O (water); dH_2O (deionized water); HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); cDNA (copy or complementary DNA); DNA (deoxyribonucleic acid); ssDNA (single stranded DNA); dsDNA (double

stranded DNA); dNTP (deoxyribonucleotide triphosphate); RNA (ribonucleic acid); $MgCl_2$ (magnesium chloride); NaCl (sodium chloride); w/v (weight to volume); v/v (volume to volume); w/w (weight to weight); *g* (gravity); OD (optical density); ppm (parts per million); Dulbecco's phosphate buffered solution (DPBS); SOC (2% Bacto-Tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl); Terrific Broth (TB; 12 g/l Bacto-Tryptone, 24 g/l glycerol, 2.31 g/l KH_2PO_4, and 12.54 g/l K_2HPO_4); OD_{280} (optical density at 280 nm); OD_{600} (optical density at 600 nm); A_{405} (absorbance at 405 nm); Vmax (the maximum initial velocity of an enzyme catalyzed reaction); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); PBST (PBS+0.25% TWEEN®-20); PEG (polyethylene glycol); PCR (polymerase chain reaction); RT-PCR (reverse transcription PCR); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); HEPES (N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]); HBS (HEPES buffered saline); Tris-HCl (tris[Hydroxymethyl]aminomethane-hydrochloride); Tricine (N-[tris-(hydroxymethyl)-methyl]-glycine); CHES (2-(N-cyclo-hexylamino) ethane-sulfonic acid); TAPS (3-{[tris-(hydroxymethyl)-methyl]-amino}-propanesulfonic acid); CAPS (3-(cyclo-hexylamino)-propane-sulfonic acid; DMSO (dimethyl sulfoxide); DTT (1,4-dithio-DL-threitol); SA (sinapinic acid (s,5-dimethoxy-4-hydroxy cinnamic acid); TCA (trichloroacetic acid); Glut and GSH (reduced glutathione); GSSG (oxidized glutathione); TCEP (Tris[2-carboxyethyl] phosphine); Ci (Curies); mCi (milliCuries); µCi (microCuries); HPLC (high-performance liquid chromatography); RP-HPLC (reverse phase high pressure liquid chromatography); TLC (thin layer chromatography); MALDI-TOF (matrix-assisted laser desorption/ionization--time of flight); Ts (tosyl); Bn (benzyl); Ph (phenyl); Ms (mesyl); Et (ethyl), Me (methyl); *Taq* (*Thermus aquaticus* DNA polymerase); Klenow (DNA polymerase I large (Klenow) fragment); EGTA (ethylene glycol-bis(ß-aminoethyl ether) N, N, N', N'-tetraacetic acid); EDTA (ethylenediaminetetracetic acid); bla (β-lactamase or ampicillin-resistance gene); HDL (heavy duty liquid); HDD (heavy duty powder detergent); HSG (high suds granular detergent); CFE (Central and Eastern Europe); WE (Western Europe); NA, when used in reference to detergents (North America); Japan and JPN, when used in reference to detergents (Japan); MTP (microtiter plate); MJ Research (MJ Research, Reno, NV); Baseclear (Baseclear BV, Inc., Leiden, The Netherlands); PerSeptive (PerSeptive Biosystems, Framingham, MA); ThermoFinnigan (ThermoFinnigan, San Jose, CA); Argo (Argo BioAnalytica, Morris Plains, NJ);Seitz EKS (SeitzSchenk Filtersystems GmbH, Bad Kreuznach, Germany); Pall (Pall Corp., East Hills, NY and Bad Kreuznach, Germany); Spectrum (Spectrum Laboratories, Dominguez Rancho, CA); Molecular Structure (Molecular Structure Corp., Woodlands, TX); Accelrys (Accelrys, Inc., San Diego, CA); Chemical Computing (Chemical Computing Corp., Montreal, Canada); New Brunswick (New Brunswick Scientific, Co., Edison, NJ); CFT (Center for Test Materials, Vlaardingen, The Netherlands); P&G and Procter & Gamble (Procter & Gamble, Inc., Cincinnati, OH); GE Healthcare (GE Healthcare, Chalfont St. Giles, United Kingdom); DNA2.0 (DNA2.0, Menlo Park, CA); OXOID (Oxoid, Basingstoke, Hampshire, UK); Megazyme (Megazyme International Ireland Ltd., Bray Business Park, Bray, Co., Wicklow, Ireland); Finnzymes (Finnzymes Oy,

Espoo, Finland); Kelco (CP Kelco, Wilmington, DE); Corning (Corning Life Sciences, Corning, NY); (NEN (NEN Life Science Products, Boston, MA); Pharma AS (Pharma AS, Oslo, Norway); Dynal (Dynal, Oslo, Norway); Bio-Synthesis (Bio-Synthesis, Lewisville, TX); ATCC (American Type Culture Collection, Rockville, MD); Gibco/BRL (Gibco/BRL, Grand Island , NY); Sigma (Sigma Chemical Co., St. Louis, MO); Pharmacia (Pharmacia Biotech, Piscataway, NJ); NCBI (National Center for Biotechnology Information); Applied Biosystems (Applied Biosystems, Foster City, CA); BD Biosciences and/or Clontech (BD Biosciences CLONTECH Laboratories, Palo Alto, CA); Operon Technologies (Operon Technologies, Inc., Alameda, CA); MWG Biotech (MWG Biotech, High Point, NC); Oligos Etc (Oligos Etc. Inc, Wilsonville, OR); Bachem (Bachem Bioscience, Inc., King of Prussia, PA); Difco (Difco Laboratories, Detroit, MI); Mediatech (Mediatech, Herndon, VA; Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); Oxoid (Oxoid Inc., Ogdensburg, NY); Worthington (Worthington Biochemical Corp., Freehold, NJ); GIBCO BRL or Gibco BRL (Life Technologies, Inc., Gaithersburg, MD); Millipore (Millipore, Billerica, MA); Bio-Rad (Bio-Rad, Hercules, CA); Invitrogen (Invitrogen Corp., San Diego, CA); NEB (New England Biolabs, Beverly, MA); Sigma (Sigma Chemical Co., St. Louis, MO); Pierce (Pierce Biotechnology, Rockford, IL); Takara (Takara Bio Inc. Otsu, Japan); Roche (Hoffmann-La Roche, Basel, Switzerland); Gene Oracle (Gene Oracle, Inc., Mountain View, CA); EM Science (EM Science, Gibbstown, NJ); Qiagen (Qiagen, Inc., Valencia, CA); Biodesign (Biodesign Intl., Saco, Maine); Aptagen (Aptagen, Inc., Herndon, VA); Sorvall (Sorvall brand, from Kendro Laboratory Products, Asheville, NC); Molecular Devices (Molecular Devices, Corp., Sunnyvale, CA); R&D Systems (R&D Systems, Minneapolis, MN); Siegfried Handel (Siegfried Handel AG, Zofingen, Switzerland); Stratagene (Stratagene Cloning Systems, La Jolla, CA); Marsh (Marsh Biosciences, Rochester, NY); Geneart (Geneart GmbH, Regensburg, Germany); Bio-Tek (Bio-Tek Instruments, Winooski, VT); (Biacore (Biacore, Inc., Piscataway, NJ); PeproTech (PeproTech, Rocky Hill, NJ); SynPep (SynPep, Dublin, CA); New Objective (New Objective brand; Scientific Instrument Services, Inc., Ringoes, NJ); Waters (Waters, Inc., Milford, MA); Matrix Science (Matrix Science, Boston, MA); Dionex (Dionex, Corp., Sunnyvale, CA); Monsanto (Monsanto Co., St. Louis, MO); Wintershall (Wintershall AG, Kassel, Germany); BASF (BASF Co., Florham Park, NJ); Huntsman (Huntsman Petrochemical Corp., Salt Lake City, UT); Shell Chemicals (Shell Chemicals, Inc., London, UK); Stepan (Stepan, Northfield, IL); Clariant (Clariant, Sulzbach, Germany); Industrial Zeolite (Industrial Zeolite Ltd., Grays, Essex, UK); Jungbunzlauer (Jungbunzlauer, Basel, Switzerland); Solvay (Solvay, Brussels, Belgium); 3V Sigma (3V Sigma, Bergamo, Italy); Innospec (Innospec, Ellesmere Port, UK); Thermphos (Thermphos, Vlissiggen-Ost, The Netherlands); Ciba Specialty (Ciba Specialty Chemicals, Basel, Switzerland); Dow Corning (Dow Corning, Barry, UK); Enichem (Enichem Iberica, Barcelona, Spain); Fluka Chemie AG (Fluka Chemie AG, Buchs, Switzerland); Gist-Brocades (Gist-Brocades, NV, Delft, The Netherlands); Dow Corning (Dow Corning Corp., Midland, MI); Mettler-Toledo (Mettler-Toledo Inc, Columbus, OH); RB (Reckitt-Benckiser, Slough, UK); and Microsoft (Microsoft, Inc., Redmond, WA).

In the exemplified detergent compositions provided herein, the enzymes levels are expressed by pure enzyme by weight of the total composition and unless otherwise specified, the detergent ingredients are expressed by weight of the total compositions. The abbreviated component identifications therein have the following meanings:

Abbreviation		Ingredient
LAS	:	Sodium linear C_{11-13} alkyl benzene sulfonate
NaC16-17HSAS	:	Sodium C_{16-17} highly soluble alkyl sulfate
TAS	:	Sodium tallow alkyl sulphate
CxyAS	:	Sodium C_{1x} - C_{1y} alkyl sulfate
CxyEz	:	C_{1x} - C_{1y} predominantly linear primary alcohol condensed with an average of z moles of ethylene oxide
CxyAEzS	:	C_{1x} - C_{1y} sodium alkyl sulfate condensed with an average of z moles of ethylene oxide. Added molecule name in the examples.
Nonionic	:	Mixed ethoxylated/propoxylated fatty alcohol e.g. Plurafac LF404 being an alcohol with an average degree of ethoxylation of 3.8 and an average degree of propoxylation of 4.5.
QAS	:	$R_2.N^+(CH_3)_2(C_2H_4OH)$ with $R_2 = C_{12}$-C_{14}
Silicate	:	Amorphous Sodium Silicate (SiO_2:Na_2O ratio = 1.6-3.2:1)
Metasilicate	:	Sodium metasilicate (SiO_2:Na_2O ratio = 1.0)
Zeolite A	:	Hydrated aluminosilicate of formula $Na_{12}(AlO_2SiO_2)_{12}\cdot 27H_2O$
SKS-6	:	Crystalline layered silicate of formula δ-$Na_2Si_2O_5$
Sulfate	:	Anhydrous sodium sulphate
STPP	:	Sodium Tripolyphosphate
MA/AA	:	Random copolymer of 4:1 acrylate/maleate, average molecular weight about 70,000-80,000
AA	:	Sodium polyacrylate polymer of average molecular weight (MW) 4,500

Abbreviation		Ingredient
Polycarboxylate	:	Copolymer comprising mixture of carboxylated monomers such as acrylate, maleate and methyacrylate with a MW ranging between 2,000-80,000 such as Sokolan commercially available from BASF, being a copolymer of acrylic acid, MW 4,500
BB1	:	3-(3,4-Dihydroisoquinolinium)propane sulfonate
BB2		1-(3,4-dihydroisoquinolinium)-decane-2-sulfate
PB1	:	Sodium perborate monohydrate
PB4	:	Sodium perborate tetrahydrate of nominal formula $NaBO_3 \cdot 4H_2O$
Percarbonate	:	Sodium percarbonate of nominal formula $2Na_2CO_3 \cdot 3H_2O_2$
TAED	:	Tetraacetyl ethylene diamine
NOBS	:	Nonanoyloxybenzene sulfonate in the form of the sodium salt
DTPA	:	Diethylene triamine pentaacetic acid
HEDP	:	1,1-hydroxyethane diphosphonic acid
DETPMP	:	Diethyltriamine penta (methylene) phosphonate, marketed by Monsanto under the Trade name Dequest 2060
EDDS	:	Ethylenediamine-N,N'-disuccinic acid, (S,S) isomer in the form of its sodium salt
Diamine	:	Dimethyl aminopropyl amine; 1,6-hezane diamine; 1,3-propane diamine; 2-methyl-1,5-pentane diamine; 1,3-pentanediamine; 1-methyl-diaminopropane
DETBCHD		5, 12- diethyl-1,5,8,12-tetraazabicyclo [6,6,2] hexadecane, dichloride, Mn(II) SALT
PAAC	:	Pentaamine acetate cobalt(III) salt
Paraffin	:	Paraffin oil sold under the tradename Winog 70 by Wintershall
Paraffin Sulfonate	:	A Paraffin oil or wax in which some of the hydrogen atoms have been replaced by sulfonate groups
Aldose oxidase	:	Oxidase enzyme sold under the tradename Aldose Oxidase by Novozymes A/S
Galactose oxidase	:	Galactose oxidase from Sigma
nprE	:	The recombinant form of neutral metalloprotease expressed in *Bacillus subtilis* (see, e.g., WO 07/044993)

Abbreviation		Ingredient
PMN	:	Purified neutral metalloprotease from *Bacillus amyloliquefaciens*
Amylase	:	A suitable amylolytic enzyme, such as those sold under the tradenames PURAFECT® Ox described in WO 94/18314, WO96/05295 sold by Genencor; NATALASE®, TERMAMYL®, FUNGAMYL® and DURAMYL™, all available from Novozymes A/S.
Lipase	:	A suitable lipolytic enzyme such as those sold under the tradenames LIPEX®, LIPOLASE®, LIPOLASE® Ultra by Novozymes A/S and Lipomax™ by Gist-Brocades.
Cellulase	:	A suitable cellulytic enzyme such as those sold under the tradenames CAREZYME®, CELLUZYME®, and/or ENDOLASE® by Novozymes A/S.
Pectin Lyase	:	A suitable pectin lyase, such as those sold under the tradenames XPECT®, PECTAWAY® and PECTAWASH® available from Novozymes A/S.
PVP	:	Polyvinylpyrrolidone with an average molecular weight of 60,000
PVNO	:	Polyvinylpyridine-N-Oxide, with an average molecular weight of 50,000
PVPVI	:	Copolymer of vinylimidazole and vinylpyrrolidone, with an average molecular weight of 20,000
Brightener 1	:	Disodium 4,4'-bis(2-sulphostyryl)biphenyl
Silicone antifoam	:	Polydimethylsiloxane foam controller with siloxane-oxyalkylene copolymer as dispersing agent with a ratio of said foam controller to said dispersing agent of 10:1 to 100:1
Suds Suppressor	:	12% Silicone/silica, 18% stearyl alcohol, 70% starch in granular form
SRP 1	:	Anionically end capped poly esters
PEG X	:	Polyethylene glycol of a molecular weight of x
PVP K60®	:	Vinylpyrrolidone homopolymer (average MW 160,000)
Jeffamine® ED-2001	:	Capped polyethylene glycol from Huntsman
Isachem® AS	:	A branched alcohol alkyl sulphate from Enichem
MME PEG (2000)	:	Monomethyl ether polyethylene glycol (MW 2000) from Fluka Chemie AG

Abbreviation		Ingredient
DC3225C	:	Silicone suds suppresser, mixture of Silicone oil and Silica from Dow Corning
TEPAE	:	Tetreaethylenepentaamine ethoxylate
BTA	:	Benzotriazole
Betaine	:	$(CH_3)_3N^+CH_2COO^-$
Sugar	:	Industry grade D-glucose or food grade sugar
CFAA	:	C_{12}-C_{14} alkyl N-methyl glucamide
TPKFA	:	C_{12}-C_{14} topped whole cut fatty acids
Clay	:	A hydrated aluminumu silicate in a general formula $Al_2O_3SiO_2 \cdot xH_2O$. Types: Kaolinite, montmorillonite, atapulgite, illite, bentonite, halloysite
pH	:	Measured as a 1% solution in distilled water at 20°C

For North American (NA) and Western European (WE) heavy duty liquid laundry (HDL) detergents, heat inactivation of the enzymes present in commercially-available detergents is performed by placing pre-weighed liquid detergent (in a glass bottle) in a water bath at 95°C for 2 hours. The incubation time for heat inactivation of NA and WE auto dish washing (ADW) detergents is 8 hours. Both un-heated and heated detergents are assayed within 5 minutes of dissolving the detergent to accurately determine percentage deactivated. Enzyme activity is tested by the AAPF assay.

For testing of enzyme activity in heat-inactivated detergents, working solutions of detergents are made from the heat inactivated stocks. Appropriate amounts of water hardness (e.g., 6 gpg or 12 gpg) and buffer are added to the detergent solutions to match the desired conditions. The solutions are mixed by vortexing or inverting the bottles. The following Table provides information regarding some of the commercially-available detergents and test conditions used herein. In some experiments, additional and/or other commercially available detergents find use in the following Examples.

Table 1.1. Laundry and Dish Washing Conditions							
Region	**Form**	**Dose**	**Detergent***	**Buffer**	**gpg**	**pH**	**T (°C)**
Laundry (Heavy Duty Liquid and Granular)							
NA	HDL	0.78 g/l	P&G TIDE® 2X	5 mM HEPES	6	8.0	20
WE	HDL	5.0 g/L	Henkel PERSIL™	5 mM HEPES	12	8.2	40
WE	HDG	8.0 g/L	P&G ARIEL®	2 mM Na_2CO_3	12	10.5	40
JPN	HDG	0.7 g/L	P&G TIDE®	2 mM Na_2CO_3	6	10.0	20
NA	HDG	1.0 g/L	P&G TIDE®	2 mM Na_2CO_3	6	10.0	20
Automatic Dish Washing							
WE	ADW	3.0 g/L	RB CALGONIT™	2 mM Na_2CO_3	21	10.0	40
NA	ADW	3.0 g/L	P&G CASCADE®	2 mM Na_2CO_3	9	10.0	40

In some additional aspects, the following solutions find use:

Table 1-2. Working Detergent Solutions					
Detergent	**Temp (C)**	**Detergent g/L**	**pII**	**Buffer**	**gpg**
TIDE® 2X Cold	16	0.98	8	5mM HEPES	6
TIDE® 2X Cold	32	0.98	8	5mM HEPES	6
TIDE® 2X Cold	16	0.98	7	5mM MOPS	6

The examples, which are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way, also describe and detail aspects and embodiments of the invention discussed above. The foregoing examples and detailed description are offered by way of illustration and not by way of limitation.

PART I

Table of Detergents

The compositions of the detergents used in the assays in Part I Examples are shown in Table 1-3. BPN' variant protein samples were added to the detergent compositions as described in Part I Example 1 to assay for the various properties tested.

The following are liquid laundry detergent compositions suitable for top-loading automatic washing machines (1, 2 & 4) and front loading washing machines (3).

Table 1-3. Composition of Detergents Used in the Assays to Test BPN' Variants				
Ingredient	**Composition (wt% of composition)**			
	1	**2**	**3**	**4**
C_{12-15} Alkylethoxy(1.8)sulfate	14.7	11.6		16.31
$C_{11.8}$ Alkylbenzene sulfonate	4.3	11.6	8.3	7.73
C_{16-17} Branched alkyl sulfate	1.7	1.29		3.09
C_{12-14} Alkyl -9-ethoxylate	0.9	1.07		1.31
C_{12} dimethylamine oxide	0.6	0.64		1.03
Citric acid	3.5	0.65	3	0.66
C_{12-18} fatty acid	1.5	2.32	3.6	1.52
Sodium Borate (Borax)	2.5	2.46	1.2	2.53
Sodium C_{12-14} alkyl ethoxy 3 sulfate			2.9	
C_{14-15} alkyl 7-ethoxylate			4.2	
C_{12-14} Alkyl -7-ethoxylate			1.7	
Ca formate	0.09	0.09		0.09
A compound having the following general structure: bis($(C_2H_5O)(C_2H_4O)n$)(CH_3)-N^+-C_xH_{2x}-N^+-(CH_3)-bis($(C_2H_5O)(C_2H_4O)n$), wherein n = from 20 to 30, and x = from 3 to 8, or sulphated or sulphonated variants thereof			1.2	
Random graft co-polymer[1]		1.46	0.5	
Ethoxylated Polyethylenimine [2]	1.5	1.29		1.44
Diethylene triamine pentaacetic acid	0.34	0.64		0.34
Diethylene triamine penta(methylene phosphonic acid)			0.3	
Tinopal AMS-GX		0.06		
Tinopal CBS-X	0.2	0.17		0.29
Amphiphilic alkoxylated grease cleaning polymer [3]	1.28	1	0.4	1.93

Ethanol	2	1.58	1.6	5.4
Propylene Glycol	3.9	3.59	1.3	4.3
Diethylene glycol	1.05	1.54		1.15
Polyethylene glycol	0.06	0.04		0.1
Monoethanolamine	3.05	2.41	0.4	1.26
NaOH	2.44	1.8		3.01
Sodium Cumene Sulphonate			1	
Sodium Formate		0.11		0.09
Water, Aesthetics (Dyes, perfumes) and Minors (Enzymes, solvents, structurants)	balance	balance	balance	

[1] Random graft copolymer is a polyvinyl acetate grafted polyethylene oxide copolymer having a polyethylene oxide backbone and multiple polyvinyl acetate side chains. The molecular weight of the polyethylene oxide backbone is about 6000 and the weight ratio of the polyethylene oxide to polyvinyl acetate is about 40 to 60 and no more than 1 grafting point per 50 ethylene oxide units.

[2] Polyethylenimine (MW = 600) with 20 ethoxylate groups per -NH.

[3] Amphiphilic alkoxylated grease cleaning polymer is a polyethylenimine (MW = 600) with 24 ethoxylate groups per –NH and 16 propoxylate groups per –NH

PART I EXAMPLES

EXAMPLE 1

Assays

Various assays were used as set forth below. Any deviations from the protocols provided below are indicated in the subsequent Examples.

A. TCA Assay for Protein Content Determination in 96-well Microtiter Plates

For BPN' and BPN' variants, this assay was started using filtered *B. subtilis* bacterial culture supernatant from microtiter plates grown 3-4 days at 33-37°C with shaking at 230-250 rpm and humidified aeration. A fresh 96-well flat bottom microtiter plate (MTP) was used for the assay. First, 100 µL/well of 0.25 N HCl was placed in each well. Then, 25 µL of filtered culture broth was added. The light scattering/absorbance at 405 nm (use 5 sec mixing mode in the plate reader) was then determined in order to provide the "blank" reading. For the test, 100 µL/well of 30% (w/v) trichloroacetic acid (TCA) was placed in the plates and incubated for 10 minutes at room temperature. The light scattering/absorbance at 405 nm (use 5 sec mixing mode in the plate reader) was then determined. The

equipment used was a Biomek FX Robot (Beckman Coulter) and a SpectraMAX (type 340; Molecular Devices) MTP Reader; the MTPs were from Costar (type 9017).

The calculations were performed by subtracting the blank (no TCA) from the test reading with TCA to provide a relative measure of the protein content in the samples. If desired, a standard curve can be created by calibrating the TCA readings with AAPF assays of clones with known conversion factors. However, the TCA results are linear with respect to protein concentration from 250 to 2500 micrograms protein per ml (ppm) and can thus be plotted directly against enzyme performance for the purpose of choosing good-performing variants. The turbidity/light scatter increase in the samples correlates to the total amount of precipitable protein in the culture supernatant.

B. AAPF Protease Assay in 96-well Microtiter Plates

In order to determine the protease activity of the proteases and variants thereof of the present invention, the hydrolysis of N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-*p*-nitroanilide (suc-AAPF-pNA) was measured. The reagent solutions used were: 100 mM Tris/HCl, pH 8.6, containing 0.005% TWEEN®-80 (Tris dilution buffer); 100 mM Tris buffer, pH 8.6, containing 10 mM $CaCl_2$ and 0.005% TWEEN®-80 (Tris/Ca buffer); and 160 mM suc-AAPF-pNA in DMSO (suc-AAPF-pNA stock solution) (Sigma: S-7388). To prepare a suc-AAPF-pNA working solution, 1 ml suc-AAPF-pNA stock solution was added to 100 ml Tris/Ca buffer and mixed well for at least 10 seconds. The assay was performed by adding 10 µl of diluted protease solution to each well, immediately followed by the addition of 190 µl 1 mg/ml suc-AAPF-pNA working solution. The solutions were mixed for 5 sec., and the absorbance change in kinetic mode (25 readings in 5 minutes) was read at 405 nm in an MTP reader, at 25°C. The protease activity was expressed as AU (activity = $\Delta OD \cdot min^{-1} ml^{-1}$).

C. BMI Microswatch Assay

Blood, milk and ink (BMI) stained microswatches of 5.5 millimeter circular diameter were obtained from CFT. Before cutting the swatches, the fabric (EMPA 116) was washed with water. One microswatch was placed in each well of a 96-well non-binding microtiter plate (Corning 3641). The detergents used for the assays included Detergent Composition 1, Detergent Composition 2, and Detergent Composition 4. The detergents were diluted in Milli-Q (deionized) water to a working strength concentration of 0.788 g/L. These detergents were buffered with 5 mM HEPES pH 8.2 or pH 7.2, which upon addition to detergent, buffers at pH 8 or pH 7, respectively. Additionally, 6 grains per gallon (gpg) water hardness (3:1 Ca:Mg - $CaCl_2$: $MgCl_2 \cdot 6H_2O$) was added. The detergent solution was pre-equilibrated in an ice-water bath for 16°C assays (room temperature for 32°C assays) and pumped into a circulating reservoir (Beckman FX). Then, 190 µl of the desired detergent solution was added to each well of the MTP that contained microswatches. To this mixture, 10 µl of the diluted enzyme master dilution solution was added, providing an approximate enzyme concentration of 0.4 -0.5 µg/mL. The master dilution was prepared from the culture supernatants at 8 µg/mL, where the approximate enzyme

concentrations of the culture supernatants and BPN'-v3 or BPN'-v36 parent controls were determined using the AAPF protease activity assay, basing the concentration on a purified BPN'-v3 or BPN'-v36 standard of known concentration. The MTP was sealed with tape and placed in the iEMS incubator/shaker (Thermo/Labsystems) pre-set at 16°C in a refrigerated dairy case or at 32°C on the benchtop for 20 minutes, with agitation at 1400 rpm. Following incubation under the appropriate conditions, the sealing tape was removed from each plate and 125 µl (150 µl if pipetting by hand for smaller screens) of the solution from each well was transferred into a fresh MTP (Corning 9017). The new MTP containing 125 µl – 150 µl of solution/well was read at 600 nm (with 5 sec mixing mode in the plate reader) using a MTP SpectraMax reader (type 340; Molecular Devices). Blank controls containing a microswatch and detergent but no enzyme were also included. The absorbance value obtained was corrected for the blank value (substrate without enzyme), providing a measure of hydrolytic activity. For each sample (variant), the performance index was calculated as described below. This BMI Microswatch Assay, run at 60°F (16°C) and pH 8, is referred to herein as the "Test Method."

D. Egg Microswatch Assay

CS38 aged egg yolk with pigment stained cotton microswatches of 5.5 millimeter circular diameter were obtained from CFT. These swatches were not pre-rinsed in water. One microswatch was placed in each well of a 96-well non-binding microtiter plate (Corning 3641). Detergent Composition 4 was diluted in Milli-Q (deionized) water to a working strength concentration of 0.788 g/L. The detergents were buffered with 5 mM HEPES pH 8.2 which upon addition to detergent, buffers at pH 8. Additionally 6 grains per gallon (gpg) water hardness (3:1 Ca:Mg - $CaCl_2$: $MgCl_2 \cdot 6H_2O$); was added. The detergent solution was pre-equilibrated in an ice-water bath for 16°C assays (room temperature for 32°C assays) and pumped into a circulating reservoir (Beckman FX). Then, 185 µl of the desired detergent solution was added to each well of the MTP, containing microswatches. To this mixture, 15 µl of the diluted enzyme master dilution solution was added, providing an approximate enzyme concentration of 0.6 µg/mL in the reaction. The master dilution was prepared from the culture supernatants at 8 µg/mL, where the approximate enzyme concentration of the culture supernatants and BPN'-v3 or BPN'-v36 parent control was determined using the AAPF protease activity assay, basing the concentration on a purified BPN'-v3 or BPN'-v36 standard of known concentration. The MTP was sealed with tape and placed in the iEMS incubator/shaker (Thermo/Labsystems) pre-set at 16°C in a refrigerated dairy case or at 32°C on the benchtop for 30 minutes, with agitation at 1400 rpm. Following incubation under the appropriate conditions, the sealing tape was removed from each plate and 125 µl (150 µl if pipetting by hand for smaller screens) of the solution from each well was transferred into a fresh MTP (Corning 9017). The new MTP containing 125 µl – 150 µl of solution/well was read at 405 nm (with 5 sec mixing mode in the plate reader) using a MTP SpectraMax reader (type 340; Molecular Devices). Blank controls containing a microswatch and detergent but no enzyme were also included. The absorbance value obtained was

corrected for the blank value (substrate without enzyme), providing a measure of hydrolytic activity. For each sample (variant), the performance index was calculated as described below.

E. Grass Microswatch Assay

Warwick Equest scrubbed grass on woven cotton swatches were obtained from Warwick. These swatches were cut into 5.5 millimeter circular diameter microswatches using a custom made 96-well punch machine that places one microswatch in each well of a 96-well non-binding microtiter plate (Corning 3641). After cutting of the swatches, the fabric was washed in the wells (pre-rinsed) with 100 µL per well of 50% working strength Detergent Composition 4 diluted in water. After 20 minutes of pre-rinsing the 100 µl of 50% detergent rinse was removed carefully pipetting by hand. Detergent Composition 4 was diluted in Milli-Q (deionized) water to a working strength concentration of 0.788 g/L. These detergents were buffered with 5 mM HEPES pH 8.2, which upon addition to detergent, buffers at pH 8. Additionally 6 grains per gallon (gpg) water hardness (3:1 Ca:Mg - $CaCl_2$: $MgCl_2 \cdot 6H_2O$); was added. The detergent solution was pre-equilibrated in an ice-water bath for 16°C assays (room temperature for 32°C assays). Then, 180 µl of the desired detergent solution was added to each well of the MTP containing the microswatches, immediately after the pre-rinsing was complete. To this mixture, 20 µl of the diluted enzyme master dilution solution was added making the approximate enzyme in the reaction at 0.8 µg/mL. The master dilution was prepared from the culture supernatants at 8 µg/mL where the approximate enzyme concentration of the culture supernatants and BPN'-v36 parent control was determined using the AAPF protease assay basing the concentration on a purified BPN'-v36 standard of known concentration. The MTP was sealed with tape and placed in the iEMS incubator/shaker (Thermo/Labsystems) pre-set at 16°C in a refrigerated dairy case or at 32°C on the benchtop for 30 minutes, with agitation at 1400 rpm. Following incubation under the appropriate conditions, the sealing tape was removed from each plate and 125 µl (150 µl if pipetting by hand for smaller screens) of the solution from each well was transferred into a fresh MTP (Corning 9017). The new MTP containing 125 µl – 150 µl of solution/well was read at both 430 nm and 670 nm (with 5 sec mixing mode in the plate reader) using a MTP SpectraMax reader (type 340; Molecular Devices). Blank controls containing a microswatch and detergent but no enzyme were also included. The absorbance value obtained was corrected for the blank value (substrate without enzyme), providing a measure of hydrolytic activity. For each sample (variant), the performance index was calculated as described below.

F. Stability Assay

The stability of protease variants was determined in the presence of 40% concentrated Detergent Composition 3 diluted in water. The reagents used were Detergent Composition 3 diluted to 50% in Milli-Q water, 10 mM MES 0.01 % TWEEN®-80 pH 5.8 master dilution buffer, AAPF reagents: see protocol AAPF assay. The equipment used was F-bottom MTP (Corning 9017) for dilution of diluted enzyme into detergent as well as for suc-AAPF-pNA plates, Biomek FX (Beckman Coulter), Spectramax

Plus 384 MTP Reader (Molecular Devices), iEMS Incubator/Shaker (1 mm amplitude) (Thermo Electron Corporation), sealing tape: Nunc (236366), circulating reservoir (Beckman Fx).

Detergent Composition 3 was initially diluted to 50% in water. This detergent was kept at room temperature and cycled through the circulating reservoir. The iEMS incubators/shakers (Thermo/Labsystems) were pre-set at 43°C. Culture supernatants were diluted into plates containing master dilution buffer to a concentration of ~ 20 ppm (master dilution plate). Then, 40 µl of sample from the master dilution plate was added to plates containing 160 µl 50% Detergent Composition 3 to give a final incubation concentration of 4 ppm. The contents were mixed and kept at room temperature and triplicate AAPF assays were performed immediately on these plates and recorded as unstressed reads. The AAPF assay was modified such that 20 µL of sample from the step above was added to 190 µL of suc-AAPF-pNA working solution. The plates were immediately covered with sealing tape and placed in 43°C iEMS shakers for 30 min at 650 rpm. Following 30 minutes of incubation, triplicate AAPF assays were performed on these stress plates and recorded as stressed reads. The stability of the samples was determined by calculating the ratio of the residual and initial AAPF activity as follows: Residual Activity (%) = |mOD.min^{-1} stressed|*100 / |mOD. min^{-1} unstressed|. For each sample (variant), the performance index was calculated as described below.

G. LAS/EDTA Stability Assay

The stability of protease variants in the presence of a representative anionic surfactant (LAS=linear alkylbenzene sulfonate, specifically, sodium dodecylbenzenesulfonate-DOBS) and di-sodium EDTA was measured after incubation under defined conditions and the residual activity was determined using the AAPF assay. The reagents used were dodecyllbenzene sulfonate, sodium salt (DOBS, Sigma No. D-2525), TWEEN®-80 (Sigma No. P-8074), di-sodium EDTA (Siegfried Handel No. 164599-02), HEPES (Sigma No. H-7523), unstressed buffer: 50 mM HEPES (11.9 g/l) + 0.005% TWEEN®-80, pH 8.0, Stress buffer: 50 mM HEPES (11.9 g/l), 0.1% (w/v) DOBS (1 g/l), 10 mM EDTA (3.36 g/l), pH 8.0, reference protease and protease variant culture supernatants, containing 200 - 400 µg/ml protein. The equipment used was V- or U-bottom MTPs as dilution plates (Greiner 651101 and 650161, respectively), F-bottom MTPs (Corning 9017) for unstressed and LAS/EDTA buffer as well as for suc-AAPF-pNA plates, Biomek FX (Beckman Coulter), Spectramax Plus 384 MTP Reader (Molecular Devices), iEMS Incubator/Shaker (1 mm amplitude) (Thermo Electron Corporation), and Nunc sealing tape (236366).

The iEMS incubator/shaker (Thermo/Labsystems) was set at 29°C. Culture supernatants were diluted into plates containing unstressed buffer to a concentration of ~ 25 ppm (master dilution plate). Then, 20 µl of sample from the master dilution plate was added to plates containing 180 µl unstressed buffer to give a final incubation concentration of 2.5 ppm. The contents were mixed and kept at room temperature and an AAPF assay was performed on this plate. Then, 20 µl of sample from the master

dilution plate was also added to plates containing 180 µl stress buffer (50 mM HEPES (11.9 g/l), 0.1% (w/v) DOBS (1 g/l), 10 mM EDTA (3.36 g/l), pH 8.0). The solutions were mixed and immediately placed in 29°C iEMS shaker for 30 min at 400 rpm. Following 30 minutes of incubation, an AAPF assay was performed on the stress plate. The stability of the samples was determined by calculating the ratio of the residual and initial AAPF activity as follows: Residual Activity (%) = [mOD.min-1 stressed]*100 / [mOD. min-1 unstressed]. For each sample (variant), the performance index was calculated as described below.

Performance Index

The performance index provides a comparison of the performance of a variant (actual value) and a standard or reference protease enzyme (theoretical value) at the same protein concentration. The theoretical values can be calculated using the parameters of a performance dose response curve (i.e. using a Langmuir equation to generate the performance curve) of the standard/reference protease. A performance index (PI) that is greater than 1 (PI>1) identifies a better variant as compared to the standard or reference protease (which may be, e.g., wild-type protease or another protease variant), while a PI of 1 (PI=1) identifies a variant that performs the same as the standard or reference protease, and a PI that is less than 1 (PI<1) identifies a variant that performs worse than the standard or reference protease. Thus, the PI identifies winners (e.g., variants having enhanced proteolytic activity compared to that of the standard/reference protease) as well as variants that may be less desirable for use under certain circumstances (e.g., variants having proteolytic activity lower than the proteolytic activity of the standard/reference protease).

It is important to note that protease variants of the invention having performance index values lower than that of a reference or standard protease are nevertheless useful in the applications and methods described herein. For example, protease variants of the invention having performance index values lower than that of a reference or standard protease have proteolytic activity and thus are useful in the compositions of the invention, such as, but not limited to, e.g., cleaning compositions (including, but not limited, to, e.g., detergent cleaning compositions) for cleaning a variety of surfaces and items, including, but not limited to, e.g., laundry, fabrics, and dishware, and in personal care applications and compositions as described elsewhere herein; such protease variants are also useful in fabric and home care products and compositions and in non-fabric and home care products and compositions described elsewhere herein and in methods of the invention, including, but not limited, to, e.g., cleaning methods, methods for personal care, etc., described elsewhere herein.

Various terms set forth below are used to describe the variant: non-deleterious variants have a PI >0.05; deleterious variants have a PI less than or equal to 0.05; combinable variants are those for which the variant has performance index values greater than or equal to 0.2 for at least one property, and >0.05 for all properties. Combinable variants are those that can be combined to deliver proteins with appropriate performance indices for one or more desired properties. These data find use in engineering

any subtilisin/subtilase or protease. Even if the subtilase or protease to be engineered has an amino acid different from that of subtilisin BPN' at one or more particular positions, these data find use in identifying amino acid substitutions that alter the desired properties by identifying the best choices for substitutions, including substitutions of the BPN' wild type amino acid.

EXAMPLE 2

Construction of BPN' Library and Cleaning Performance of BPN' Variants

a) Description of the BPN'-v3 expression cassette used for library construction

The BPN'-v3 (BPN' protease containing G097A-G128A-Y217Q substitutions) expression cassette used for combinatorial library construction was generated using the BPN' expression cassette, which comprises the aprE-BPN' hybrid leader sequence (i.e., signal sequence), BPN' pro and BPN' mature sequence from *B. amyloliquefaciens*. The DNA sequence is shown below as SEQ ID NO:1 and encodes the BPN' precursor protein shown below as SEQ ID NO:168.

GTGAGAAGCAAAAAATTGTGGATCAGTTTGCTGTTTGCTTTAGCGTTAATCTTTACGATGGC
GTTCGGCAGCACATCCTCTGCCCAGGCGGCAGGGAAATCAAACGGGGAAAAGAAATATATT
GTCGGGTTTAAACAGACAATGAGCACGATGAGCGCCGCTAAGAAGAAAGATGTCATTTCTG
AAAAAGGCGGGAAAGTGCAAAAGCAATTCAAATATGTAGACGCAGCTTCAGCTACATTAAA
CGAAAAAGCTGTAAAAGAATTGAAAAAAGACCCGAGCGTCGCTTACGTTGAAGAAGATCAC
GTAGCACATGCGTAC**GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGC**
TCTGCACTCTCAAGGCTACACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGT
ATCGACTCGAGCCATCCAGATCTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCG
AAACAAACCCGTTTCAAGATAACAATTCTCATGGCACACACGTCGCAGGAACGGTTGC
GGCGTTAAACAATTCTATTGGCGTGCTTGGTGTAGCCCCGTCTGCTTCGCTCTACGCC
GTTAAAGTTCTTGGCGCAGACGGATCAGGCCAATACTCATGGATTATCAACGGCATCG
AATGGGCCATCGCGAATAACATGGATGTAATCAACATGAGCCTGGGAGGACCAAGCG
GCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTGCATCTGGTGTCGTCGTAGT
AGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTACCGTCGGTTATCCAGG
CAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATCAACGTGCCTCT
TTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTCAATCGA
CGCTTCCAGGGAACAAGTATGGTGCGTATAACGGGACTTCCATGGCCTCGCCGCATGT
AGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTC
CGCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAG
GGCTGATCAACGTACAGGCGGCAGCTCAG (SEQ ID NO:1)

In the nucleotide sequence of SEQ ID NO:1, the DNA sequence encoding the mature protease is shown in bold, the nucleotide sequence encoding leader sequence (aprE-BPN' hybrid leader sequence) is shown in standard (non-underlined) text, and the nucleotide sequence encoding the pro sequence (BPN') is underlined. In the amino acid sequence (aprE-BPN' hybrid leader sequence, BPN' pro sequence, and BPN' mature protein sequence) of the BPN' precursor protein set forth in SEQ ID NO:168, the bolded portion indicates the mature BPN' subtilisin protease.

VRSKKLWISLLFALALIFTMAFGSTSSAQAAGKSNGEKKYIVGFKQTMSTMSAAKKKDVISEKG
GKVQKQFKYVDAASATLNEKAVKELKKDPSVAYVEEDHVAHAY**AQSVPYGVSQIKAPALHS**
QGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSHGTHVAGTVAALNNSI
GVLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMDVINMSLGGPSGSAALKAAVD
KAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPELDVMAP
GVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTTTKLGDSFY
YGKGLINVQAAAQ (SEQ ID NO:168)

Thus, the amino acid sequence of the mature BPN' subtilisin protease is:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSH
GTHVAGTVAALNNSIGVLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMDVINMSLG
GPSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFS
SVGPELDVMAPGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:2)

The nucleotide sequence of the mature BPN'-v3 gene is shown below (the signal sequence and propeptide sequence used in the BPN'-v3 expression cassette is the same as that for BPN' shown in SEQ ID NO:1):

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTCAAGATAACA
ATTCTCATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATTCTATTGGCGTGCTT
GGTGTAGCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGATCAGGCCA
ATACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAAC
ATGAGCCTGGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTG
CATCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTAC
CGTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATC
AACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTC
AATCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGCA
TGTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCC
GCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTG
ATCAACGTACAGGCGGCAGCTCAG (SEQ ID NO:3)

The protein sequence of the mature BPN'-v3 protease variant is shown below (the signal sequence and propeptide sequence used in the BPN'-v3 expression cassette is the same as that for BPN' shown in SEQ ID NO:168):

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSH
GTHVAGTVAALNNSIGVLGVAPSASLYAVKVLAADGSGQYSWIINGIEWAIANNMDVINMSLG
APSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFS
SVGPELDVMAPGVSIQSTLPGNKYGAQNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:4)

b) Construction of combinatorial library using pHPLT-BPN'-v3 plasmid

The pHPLT-BPN'-v3 plasmid (see Figure 1) containing the BPN'-v3 expression cassette described above served as template DNA for cloning to provide variants derived from BPN'-v3. The vector pHPLT (Figure 4 in US Patent No. 6,566,112) contains the *B. licheniformis* LAT promoter ("Plat"); a sequence encoding the LAT signal peptide ("preLAT"). Additional plasmid elements from plasmid pUB110 disclosed in McKenzie et al., *Plasmid* 15(2): 93-103 (1986): "ori-pUB" is the origin of replication from pUB110; "neo" is the neomycin/kanamycin resistance gene from pUB110; "Terminator" is the transcriptional terminator from *B. licheniformis* amylase.

A combinatorial DNA library was synthesized at DNA 2.0 and delivered as individual ligation reactions. In some instances for efficient transformation of *B. subtilis*, the DNA from the ligation reaction mixtures was amplified by rolling circle amplification (RCA) using the Illustra Templiphi kit (GE Healthcare). The reaction was performed according to the manufacturer's protocol. One microliter of ten-fold diluted amplified DNA was used to transform 50 µL of competent *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). The transformation mixture was shaken at 37°C for 1 hour. Ten micro-liter aliquots of the transformation mixture were plated on skim milk (1.6%) Luria agar plates supplemented with 10 µg/ml of neomycin (Teknova).

The transformants that formed halos on the skim milk plates were picked into microtiter plates containing 150µl Luria broth (LB) medium supplemented with 10µg/ml neomycin. Plates were grown overnight at 37°C with 250-300 rpm shaking and 70-80% humidity using Enzyscreen lids for microtiter plates (Enzyscreen). Using a 96 pin replicating tool, (Enzyscreen) the overnight culture plate was used to inoculate a new microtiter plate containing 180 µl of MBD medium (a MOPS based defined medium) with 2.5 µg/ml neomycin. MBD medium was prepared essentially as known in the art (see Neidhardt et al., J. Bacteriol. 119:736-747 [1974]), except that NH_4Cl_2, $FeSO_4$, and $CaCl_2$ were omitted from the base medium, 3 mM K_2HPO_4 was used, and the base medium was supplemented with 60 mM urea, and 100ml of a solution made of 210 g/L glucose, and 350 g/L maltodextrin. 1 g/L of BD Bacto Yeast Extract was added and the pH was adjusted to 7.4 with KOH. The micronutrients were made up as a 100X stock solution containing in one liter, 400 mg $FeSO_4 \cdot 7H_2O$, 100 mg $MnSO_4 \cdot H_2O$, 100 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $CuCl_2 \cdot 2H_2O$, 100 mg $CoCl_2 \cdot 6H_2O$, 100 mg $NaMoO_4 \cdot 2H_2O$, 100 mg $Na_2B_4O_7 \cdot 10H_2O$, 10 ml of 1M $CaCl_2$, and 10 ml of 0.5 M sodium citrate. The MBD medium containing microtiter plates were grown for 64 hours at 37°C, 250-300 rpm, and 70-80% humidity using Enzyscreen lids (Enzyscreen) for protease variant expression. The next day, cultures were filtered through a micro-filter plate (0.22um; Millipore) and the resulting filtrates containing protease variants were used for biochemical analysis.

The protease variants were tested for cleaning performance using a BMI microswatch assay in Detergent Composition 1 at 16°C and pH 8 and BMI microswatch assay in Detergent Composition 2 at 16°C and pH 8. Protein content was determined using the TCA assay. Assays were performed as

described in Example 1 and Performance Indices were calculated relative to BPN'-v3 (with a PI value of 1.0).

The following BPN' subtilisin protease variant was determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2 to about 5, or from greater than 1.0 to about 5 relative to BPN'-v3 (SEQ ID NO:4) in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising the set of amino acid substitutions G097A-G128A-P210S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variant has a PI value of 1.1 relative to BPN'-v3 in this BMI microswatch cleaning assay, and enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) in this assay, the variant having an amino acid sequence comprising amino acid substitutions G097A-G128A-P210S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Also included is a protease variant having enhanced proteolytic activity compared to SEQ ID NO:2 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising amino acid substitutions G097A-G128A-P210S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value greater than that of BPN' (SEQ ID NO:2) and/or BPN'-v3 in a BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to SEQ ID NO:2, wherein the variant comprises substitutions X097A-X128A-X210S-X217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2, and optionally wherein the variant comprises at least one substitution selected from the group of G097A, G128A, P210S, and Y217Q. Such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising such protease variant and methods for cleaning utilizing such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q, G097A-G128A-E156S-P210S-Y217Q, G097A-G128A-P210S-Y217Q-N218A, G097A-G128A-P210S-Y217Q-N218S, and G097A-Y104F-G128A-E156S-P210I-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having

enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) or a PI value of about 1.0 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value of about 1.0 compared to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2, wherein the variant comprises at least one substitution selected from the group of X097A, X104F, X128A, X156A/S, X210I/S, X217Q, X218A/S, and optionally at least one substitution selected from the group of G097A, Y104F, G128A, E156A/S, P210I/S, Y217Q, and N218A/S, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-E156A-P210S-Y217Q-N218S and G097A-G128A-Y217Q-N218A, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-P210S-Y217Q-N218A and G097A-G128A-P210S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least

60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value of greater than 1, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9 to about 5 compared to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2, wherein the variant comprises at least one substitution selected from the group of X097A, X104F, X128A, X156A/S, X210I/S, X217Q, X218A/S, and optionally at least one substitution selected from the group of G097A, Y104F, G128A, E156A/S, P210I/S, Y217Q, and N218A/S, wherein amino acid positions of the variant are numbered by correspondence with position of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q (i.e., BPN'-v3), G097A-G128A-E156S-P210S-Y217Q, and G097A-G128A-P210S-Y217Q-N218S, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity and enhanced proteolytic activity compared to BPN' in this assay. The invention includes a protease variant having proteolytic activity, PI value of 1.0 relative to BPN'-v3, and/or enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-E156A-P210S-Y217Q-N218S, G097A-G128A-Y217Q-N218A, and G097A-Y104F-G128A-E156S-P210I-Y217Q, wherein positions of the variant are numbered by

correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 3

Generation of Combinatorial Libraries and Cleaning Performance of Variants of BPN'-v3 + S78N

a) Description of BPN'-v3 + S78N variant and synthetic gene sequences derived from this variant

Gene Oracle synthesized and cloned eight genes into the pHPLT-BPN'-v3 + S78N (BPN'-S78N-G97A-G128A-Y217Q) parent plasmid (see Figure 2). Some of these genes were used as templates (parents) to create combinatorial libraries. The BPN'-v3 + S78N variant was generated using standard molecular biology methods known in the art. The nucleotide sequence encoding the BPN'-v3 + S78N variant is shown below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATTGATTCGAGCCATCCAGATC
TTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTCAAGATAACAAT
TCTCATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTTGG
TGTAGCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGATCAGGCCAAT
ACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAACAT
GAGCCTGGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTGCA
TCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTACCG
TCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATCAA
CGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTCAA
TCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGCATG
TAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCCGC
AGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTGAT
CAACGTACAGGCGGCAGCTCAG (SEQ ID NO:7)

The amino acid sequence of the BPN'-v3 + S78N variant is shown below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSH
GTHVAGTVAALNNNIGVLGVAPSASLYAVKVLAADGSGQYSWIINGIEWAIANNMDVINMSLG
APSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFS
SVGPELDVMAPGVSIQSTLPGNKYGAQNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:8)

The nucleotide and protein sequences of genes GcM90-96, and GcM100 are shown below. The nucleotide sequence of synthesized gene GcM90 is:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGGGAGAAACAAACCCGTTTCAAGATAACA
ATTCTCATGGCACACACGCAGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTT
GGTGTAGCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGATCAGCACA
ATACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAAC
ATGAGCCTGGGAGCAACAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTG
CATCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTAC
CGTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATA
CACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTC
AATCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGCA
TGTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCC
GCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTG
ATCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:9)

The amino acid sequence of GcM90 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPGETNPFQDNNSH
GTHAAGTVAALNNNIGVLGVAPSASLYAVKVLAADGSAQYSWIINGIEWAIANNMDVINMSLG
ATSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNTRASFS
SVGPELDVMAPGVSIQSTLPGNKYGAQNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:10)

The nucleotide sequence of synthesized gene GcM91 is provided below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTGTCGATAACAA
TTCTCATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTTG
GTGTAGCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGATCAGGCCAA
TACTCATGGATTGTCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAACA
TGAGCCTGGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTGC
ATCTGGTCAAGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTACC
GTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATCA
ACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTCA
ATCGACGCTTCCAGCAAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGCAT
GTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCCG
CAGCAGTTTAGAACAAACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTGA
TCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:11)

The amino acid sequence of GcM91 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFVDNNSH
GTHVAGTVAALNNNIGVLGVAPSASLYAVKVLAADGSGQYSWIVNGIEWAIANNMDVINMSL
GAPSGSAALKAAVDKAVASGQVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASF
SSVGPELDVMAPGVSIQSTLPANKYGAQNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLEQT
TTKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:12)

The nucleotide sequence of synthesized gene GcM92 is provided below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTCAAGATGCAA
ATTCTCATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTT
GGTGTAGCCCCGGAAGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGATCAGGCCA
ATACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAAC
ATCAGCCTGGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTG
CATCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGACCTTCGAGTAC
CGTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATC
AACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTC
AATCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCGCACCGCA
TGTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCC
GCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTG
ATCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:13)

The amino acid sequence of GcM92 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDANSH
GTHVAGTVAALNNNIGVLGVAPEASLYAVKVLAADGSGQYSWIINGIEWAIANNMDVINISLGA
PSGSAALKAAVDKAVASGVVVVAAAGNEGTSGPSSTVGYPGKYPSVIAVGAVDSSNQRASFSS
VGPELDVMAPGVSIQSTLPGNKYGAQNGTSMAAPHIVAGAAALILSKHPNWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:14)

The nucleotide sequence of synthesized gene of GcM93 is provided below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTCAAGATAACCA
ATCTCATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTTG
GTGTAGCCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACAACTCAGGCCAA
TACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAACA
TGGCACTGGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTGC
ATCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACAGATGGATCATCGAGTACC
GTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATCA
ACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTCA
ATCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGCAT
GTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGTCATGGACAAACACTCAAGTCCG
CAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTGA
TCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:15)

The amino acid sequence of GcM93 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNQSH
GTHVAGTVAALNNNIGVLGVAPSASLYAVKVLAADNSGQYSWIINGIEWAIANNMDVINMALG
APSGSAALKAAVDKAVASGVVVVAAAGNEGTDGSSSTVGYPGKYPSVIAVGAVDSSNQRASFS
SVGPELDVMAPGVSIQSTLPGNKYGAQNGTSMASPHVAGAAALILSKHPSWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:16)

The nucleotide sequence of synthesized gene GcM94 is provided below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTCAAGATAACA
ATACACATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCT
TGGTGTAGCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGAGCAGGCC
AATACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAA
CATGAGCGTCGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTT
GCATCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTA
CCGTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTACAAAT
CAACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATT
CAATCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGC
ATGTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACAACCAAGT
CCGCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGC
TGATCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:17)

The amino acid sequence of GcM94 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNTH
GTHVAGTVAALNNNIGVLGVAPSASLYAVKVLAADGAGQYSWIINGIEWAIANNMDVINMSVG
APSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSTNQRASFS
SVGPELDVMAPGVSIQSTLPGNKYGAQNGTSMASPHVAGAAALILSKHPNWTNNQVRSSLENT
TTKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:18)

The nucleotide sequence of synthesized gene GcM 95 is provided below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCTGGATC
TTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGGGAGAAACAAACCCGTTTGTCGATGCACA
AACACATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTT
GGTGTAGCCCCGGAAGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACAACGCAGCAC
AATACTCATGGATTGTCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAA
CATGAGCCTGGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTT
GCATCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTA
CCGTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAAT
CAACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATT
CAATCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGC
ATGTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTC
CGCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCT
GATCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:19)

The amino acid sequence of GcM 95 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHLDLKVAGGASMVPGETNPFVDAQTH
GTIIVAGTVAALNNNIGVLGVAPEASLYAVKVLAADNAAQYSWIVNGIEWAIANNMDVINMSL
GAPSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASF
SSVGPELDVMAPGVSIQSTLPGNKYGAQNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENT
TTKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:20)

The nucleotide sequence of synthesized gene GcM96 is provided below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTCAAGATAACA

ATTCTCATGGCACACACGTCGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTT
GGTGTAGCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGATCAGGCCA
ATACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAAC
ATGAGCCTGGGAGCAACAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTG
CATCTGGTCAAGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACAGATGGACCTTCGAGTAC
CGTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTACAAATA
CACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTC
AATCGACGCTTCCAGCAAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCGCACCGCA
TGTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGTCATGGACAAACAACCAAGTCC
GCAGCAGTTTAGAACAAACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTG
ATCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:21)

The amino acid sequence of GcM96 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSH
GTHVAGTVAALNNNIGVLGVAPSASLYAVKVLAADGSGQYSWIINGIEWAIANNMDVINMSLG
ATSGSAALKAAVDKAVASGQVVVVAAAGNEGTDGPSSTVGYPGKYPSVIAVGAVDSTNTRASFS
SVGPELDVMAPGVSIQSTLPANKYGAQNGTSMAAPHVAGAAALILSKHPSWTNNQVRSSLEQT
TTKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:22)

The nucleotide sequence of synthesized gene GcM100 is provided below:

GCGCAGTCCGTGCCTTACGGCGTATCACAAATTAAAGCCCCTGCTCTGCACTCTCAAGGCTA
CACTGGATCAAATGTTAAAGTAGCGGTTATCGACAGCGGTATCGACTCGAGCCATCCAGAT
CTTAAAGTCGCTGGAGGGGCTTCTATGGTGCCGTCCGAAACAAACCCGTTTCAAGATAACA
ATTCTCATGGCACACACGCAGCAGGAACGGTTGCGGCGTTAAACAATAATATTGGCGTGCTT
GGTGTAGCCCCGTCTGCTTCGCTCTACGCCGTTAAAGTTCTTGCAGCAGACGGATCAGCACA
ATACTCATGGATTATCAACGGCATCGAATGGGCCATCGCGAATAACATGGATGTAATCAAC
ATGGCACTGGGAGCACCAAGCGGCAGTGCGGCACTTAAAGCAGCAGTTGATAAAGCTGTTG
CATCTGGTGTCGTCGTAGTAGCGGCAGCTGGGAATGAGGGAACATCCGGATCATCGAGTAC
CGTCGGTTATCCAGGCAAGTACCCTTCAGTGATTGCAGTGGGCGCTGTAGACTCTTCAAATC
AACGTGCCTCTTTTTCCTCCGTGGGACCGGAGCTGGATGTCATGGCCCCTGGCGTTTCTATTC
AATCGACGCTTCCAGCAAACAAGTATGGTGCGCAAAACGGGACTTCCATGGCCTCGCCGCA
TGTAGCTGGGGCGGCCGCATTGATTCTTTCTAAGCACCCGAACTGGACAAACACTCAAGTCC
GCAGCAGTTTAGAAAACACCACTACAAAACTTGGTGATTCTTTCTACTATGGAAAAGGGCTG
ATCAACGTACAGGCGGCAGCTCAGTAA (SEQ ID NO:23)

The amino acid sequence of GcM100 is provided below:

AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLKVAGGASMVPSETNPFQDNNSH
GTHAAGTVAALNNNIGVLGVAPSASLYAVKVLAADGSAQYSWIINGIEWAIANNMDVINMALG
APSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFS
SVGPELDVMAPGVSIQSTLPANKYGAQNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:24)

b) Construction of combinatorial libraries CG1-CG5 and CG8 using the synthetic genes GcM90-94 and GcM100

Table 3-1. List of Possible Substitutions Introduced and Primers Used for the Construction of Combinatorial Libraries CG1-CG5 and CG8

Synthesized Genes (template or parent)	Library Name	Substitutions Introduced	Primer Name	Primer Sequence
GcM90	CG1	G53S	1 S53 f	/5Phos/CTTCTATGGTGCCGTCCGAAACA AACCCGTTTCAAG (SEQ ID NO:25)
		A68V	1 V68 f	/5Phos/TCATGGCACACACGTCGCAGGAA CGGTTGCGGCG (SEQ ID NO:26)
		A102G	1 G102 f	/5Phos/AGCAGACGGATCAGGCCAATACT CATGGATTATCAAC (SEQ ID NO:27)
		T129P	1 P129 f	/5Phos/TGAGCCTGGGAGCACCAAGCGGC AGTGCGGCACTTAAAG (SEQ ID NO:28)
		T185Q	1 Q185 f	/5Phos/TAGACTCTTCAAATCAACGTGCC TCTTTTTCCTCCGTG (SEQ ID NO:29)
GcM91	CG2	V59Q	2 Q59 f	/5Phos/GAAACAAACCCGTTTCAAGATAA CAATTCTCATG (SEQ ID NO:30)
		V108I	2 I108 f	/5Phos/ATACTCATGGATTATCAACGGCA TCGAATGGGCCATC (SEQ ID NO:31)
		Q147V	2 V147 f	/5Phos/TGTTGCATCTGGTGTCGTCGTAGT AGCGGCAGCTGG (SEQ ID NO:32)
		A211G	2 G211 f	/5Phos/ATCGACGCTTCCAGGGAACAAGT ATGGTGCGCAAAAC (SEQ ID NO:33)
		Q252N	2 N252 f	/5Phos/CAGCAGTTTAGAAAACACCACTA CAAAACTTGGTG (SEQ ID NO:34)
GcM92	CG3	A61N	3 N61 f	/5Phos/CAAACCCGTTTCAAGATAACAAT TCTCATGGCACACAC (SEQ ID NO:35)
		E87S	3 S87 f	/5Phos/TTGGTGTAGCCCCGTCTGCTTCGC TCTACGCCGTTAAAG (SEQ ID NO:36)
		I124M	3 M124 f	/5Phos/TGGATGTAATCAACATGAGCCTG GGAGCACCAAGCG (SEQ ID NO:37)
		P161S	3 S161 f	/5Phos/AGGGAACATCCGGATCATCGAGT ACCGTCGGTTATCCAG (SEQ ID NO:38)
		A224S	3 S224 f	/5Phos/GACTTCCATGGCCTCGCCGCATG TAGCTGGGGCGGC (SEQ ID NO:39)
GcM93	CG4	Q62N	4 N62 f	/5Phos/GTTTCAAGATAACAATTCTCATG GCACACACGTCGC (SEQ ID NO:40)
		N100G	4 G100 f	/5Phos/GTTCTTGCAGCAGACGGATCAGG CCAATACTCATG (SEQ ID NO:41)
		A125S	4 S125 f	/5Phos/ATGTAATCAACATGAGCCTGGGA GCACCAAGCGGCAG (SEQ ID NO:42)
		D159S	4 S159 f	/5Phos/GGAATGAGGGAACATCCGGATCA TCGAGTACCGTCGG (SEQ ID NO:43)
		S240N	4 N240 f	/5Phos/CTTTCTAAGCACCCGAACTGGAC AAACACTCAAGTCCG (SEQ ID NO:44)
GcM94	CG5	T63S	5 S63 f	/5Phos/TCAAGATAACAATTCTCATGGCA CACACGTCGCAGG (SEQ ID NO:45)

Table 3-1. List of Possible Substitutions Introduced and Primers Used for the Construction of Combinatorial Libraries CG1-CG5 and CG8

Synthesized Genes (template or parent)	Library Name	Substitutions Introduced	Primer Name	Primer Sequence
		A101S	5 S101 f	/5Phos/TGCAGCAGACGGATCAGGCCAATACTCATGGATTATC (SEQ ID NO:46)
		V126L	5 L126 f	/5Phos/AATCAACATGAGCCTGGGAGCACCAAGCGGCAGTG (SEQ ID NO:47)
		T183S	5 S183 f	/5Phos/CGCTGTAGACTCTTCAAATCAACGTGCCTCTTTTTCC (SEQ ID NO:48)
		N244T	5 T244 f	/5Phos/GAACTGGACAAACACTCAAGTCCGCAGCAGTTTAG (SEQ ID NO:49)
GcM100	CG8	A68V	1 V68 f	/5Phos/TCATGGCACACACGTCGCAGGAACGGTTGCGGCG (SEQ ID NO:50)
		A102G	1 G102 f	/5Phos/AGCAGACGGATCAGGCCAATACTCATGGATTATCAAC (SEQ ID NO:51)
		A211G	2 G211 f	/5Phos/ATCGACGCTTCCAGGGAACAAGTATGGTGCGCAAAAC (SEQ ID NO:52)
		A125S	4 S125 f	/5Phos/ATGTAATCAACATGAGCCTGGGAGCACCAAGCGGCAG (SEQ ID NO:53)

Each synthesized gene was built into the pIIPLT-BPN'-S78N-G97A-G128A-Y217Q parent molecule. Resulting plasmids containing the six synthesized genes GcM90-94, and GcM100 served as templates to make combinatorial libraries at the respective positions (Table 3-1). Two additional genes, GcM95 and GcM96, were also synthesized for analysis, but did not serve as parental DNA for libraries. These genes each have nine mutations on top of the pHPLT-BPN'- S78N-G97A-G128A-Y217Q parent molecule.

The parent plasmids (template DNA) containing the synthetic genes GcM90-94, and GcM100 were methylated were methylated using two micrograms of DNA and methylase (NEB), according to the NEB protocol. Methylated DNA was then purified using DNA Clean and Concentrator kit (Zymo Research). Combinatorial libraries CG1-5 and CG8 were made using a QUIKCHANGE® Multi Site-Directed Mutagenesis kit ("QCMS kit"; Stratagene) following the manufacturer's protocol (see Table 3-1 for respective template and primer combinations), with the exception of libraries CG3 and CG4, which used 86.5ng of each primer in place of the 50ng suggested in the protocol. All primers used for introducing the desired substitutions in each library are listed in Table 3-1. They were synthesized and provided by Integrated DNA Technologies. After the QCMS reactions were completed for each library, the template DNA was digested by the addition of 0.5 -1 µl *Dpn*I (from the QCMS kit) and incubated at 37°C for 1 - 4 hours, followed by another addition of 0.5 - 1 µl *Dpn*I and another incubation at 37°C for 1-4 hours. For efficient transformation of *B. subtilis*, DNA from the QCMS reaction mixtures were amplified before transformation and transformants grown as described in Example 2.

Additional variants of BPN'-v3+S78N were produced by DNA2.0. The following substitutions were introduced individually into the BPN'-v3+S78N parent molecule: Q59G, N62Q, V68A, S89Y, A92G, I108V, I115V, M124T, P129L, A138T, V147L, S161P, Y167A, P172V, G211T, L267V, and A273S.

All of the combinatorial library variants described above and the variants synthesized at DNA2.0 were tested for cleaning performance using a BMI microswatch assay in Detergent Composition 1 at 16°C and pH 8 and BMI microswatch assay in Detergent Composition 2 at 16°C and pH 8. Protein content was determined using the TCA assay. Assays were performed as described in Example 1 and Performance Indices were calculated relative to BPN'-v3 (with a PI value of 1.0).

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of S063T-S078N-G097A-S101A-G128A-S183T-Y217Q-T244N, N061A-S078N-G097A-G128A-Y217Q-S224A, S053G-S078N-G097A-G128A-P129T-Q185T-Y217Q, S063T-S078N-G097A-S101A-G128A-S183T-Y217Q, S063T-S078N-G097A-S101A-G128A-Y217Q, S063T-S078N-G097A-S101A-G128A-Y217Q-T244I, and S078N-G097A-G128A-P129T-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' and BPN'-v3 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2, wherein the variant comprises at least one substitution selected from the group of X040E, X053G, X059V, X061A, X062H/Q, X068A, X078N, X087E, X101A, X102A, X108V, X124I, X125A, X126V, X129T, X147Q, X159D, X183T, X185T, X211A, X224A, X244I/N, X252Q, and X274D, and optionally at least one substitution selected

from the group of X040E, X053G, X059V, X061A, X062H/Q, X068A, X078N, X087E, X101A, X102A, X108V, M124I, S125A, L126V, P129T, V147Q, S159D, S183T, Q185T, G211A, S224A, T244I/N, N252Q, and A274D, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q, N061A-S078N-S087E-G097A-G128A-Y217Q-S224A, Q059V-S078N-G097A-G128A-G211A-Y217Q, Q059V-S078N-G097A-G128A-V147Q-Y217Q, Q059V-S078N-G097A-G128A-Y217Q, Q059V-S078N-G097A-I108V-G128A-Y217Q-N252Q, S053G-S078N-G097A-G128A-P129T-Y217Q, S078N-G097A-G128A-G211A-Y217Q, S078N-G097A-G128A-Q185T-Y217Q, S078N-G097A-G128A-V147Q-Y217Q, S078N-G097A-G128A-Y217Q, S078N-G097A-G128A-Y217Q-S224A, and S078N-G097A-G128A-Y217Q-S224A-A274D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variant was determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising the set of amino acid substitutions S078N-G097A-I108V-G128A-V147Q-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising said set of amino acid substitutions above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such

variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of Q059V-S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q, S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-Y217Q-N252Q, and S078N-S087E-G097A-M124I-G128A-Y217Q-S224A, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of S063T-S078N-G097A-S101A-G128A-S183T-Y217Q, S063T-S078N-G097A-S101A-G128A-S183T-Y217Q-T244N, S063T-S078N-G097A-S101A-G128A-Y217Q, and S063T-S078N-G097A-S101A-G128A-Y217Q-T244I, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than about 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pII 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q, N061A-S078N-G097A-G128A-Y217Q-S224A, N061A-S078N-S087E-G097A-G128A-Y217Q-S224A, Q059V-S078N-G097A-G128A-G211A-Y217Q, Q059V-S078N-G097A-G128A-V147Q-Y217Q, Q059V-S078N-G097A-G128A-Y217Q, Q059V-S078N-G097A-I108V-G128A-Y217Q-N252Q, S053G-S078N-G097A-G128A-P129T-Q185T-Y217Q, S053G-S078N-G097A-G128A-P129T-Y217Q, S078N-G097A-G128A-G211A-Y217Q, S078N-G097A-G128A-P129T-Y217Q, S078N-G097A-G128A-Q185T-Y217Q, S078N-G097A-G128A-Y217Q, S078N-G097A-G128A-Y217Q-S224A, and S078N-G097A-G128A-Y217Q-S224A-A274D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of S078N-G097A-G128A-V147Q-Y217Q and S078N-G097A-I108V-G128A-V147Q-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including cleaning compositions, comprising at least one such variant and methods for cleaning an item or surface in need of cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8

and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of Q059V-S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q, S078N-G097A-I108V-G128A-V147Q-G211A-Y217Q-N252Q, S078N-G097A-I108V-G128A-V147Q-Y217Q-N252Q, and S078N-S087E-G097A-M124I-G128A-Y217Q-S224A, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including cleaning compositions, comprising at least one such variant and methods for cleaning an item or surface in need of cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 4

Generation and Cleaning Performance of BPN' Variants

Generation of BPN' variants LC1-LC4 via QUIKCHANGE® Multi Site-Directed Mutagenesis

BPN' variants were constructed from different parental plasmids using QUIKCHANGE® Multi Site-Directed Mutagenesis kits. The parental plasmids (Table 4-1) were methylated using a NEB Dam Methylase Kit in a reaction containing 77.5 µL H20 + 10 µL Buffer 10X + 0.25 µL SAM + 2 µL DAM methylase + 10 µL miniprep DNA (~150 ng/µL) at 37°C overnight. The methylated plasmid DNA was purified using a QIAGEN® PCR purification kit. QUIKCHANGE® Multi Site-Directed Mutagenesis reactions were set up for each of the DNA templates in a reaction mix containing 2.5 µL Buffer 5X + 0.5 µL primer 1 (25 µM) + 0.5 µL primer 2 (25 µM) + 1 µL dNTP's + 1 µL enzyme blend + 18 µL H_2O + 1.5 µL DNA. The PCR program used was: 95°C for 1 min; (95°C for 1 min, 53°C for 1 min, 65°C for 9:39 min) x 29 cycles; 65°C for 10 min, 4°C hold. Primer sequences are shown in Table 4-2. In all reactions, PCR was performed using a MJ Research PTC-200 Peltier thermal cycler. Parental DNA from the PCR samples was removed by addition of 1 µL of *Dpn*I to QUIKCHANGE® Multi Site-Directed Mutagenesis reactions at 37°C overnight. To increase transformation frequency, the *Dpn*I-digested reactions were amplified using rolling circle amplification (RCA) using the Illustra TempliPhi kit according to the manufacturer's protocol. *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo) were transformed with1 µL each of the RCA reaction and the transformed cells were plated onto LA + 1.6% skim milk plates containing 10 ppm neomycin and incubated at 37°C overnight. Colonies from overnight growth were selected to perform colony PCR for sequencing using "puReTaq Ready-To-Go PCR Beads" (Amersham). The PCR and sequencing primers used were pHPLT F1 (/5PHOS/TACATATGAGTTATGCAGTTTG (SEQ ID NO:54)) and pHPLT seq R1 (/5PHOS/TTATCCTTTACCTTGTCTC (SEQ ID NO:55)). Clones with appropriate sequences were

frozen. BPN' variant proteins were produced by growing *B. subtilis* transformants in 96 well microtiter plates at 37°C for 68 hours in a MOPS based medium containing urea as described in Example 2.

Table 4-1. Parental Plasmids and Primers Used for Generation of BPN' Variants LC1-LC4

Parental Plasmid	Mutations Introduced	Primers Used
BPN'-G097A-G128A-Y217Q-S024G-N025G-N061P-S101N (termed LC1)	A128S	A128Sf, A128Sr
BPN'-G097A-G128A-Y217Q-S053G-N061P-S101N-V203Y (termed LC2)	A128S	A128Sf, A128Sr
BPN'-G097A-G128A-Y217Q-S024G-N025G-S053G-T055P-N061P-S101N-V203Y (termed LC3)	A128S	A128Sf, A128Sr
BPN'-G097A-G128A-Y217Q-S024G-N025G-S053G-T055P-N061P-S101N-V203Y (termed LC4)	P55T	P55Tf, P55Tr

Table 4-2. Sequences of Primers Used for QUIKCHANGE® Multi Site-Directed Mutagenesis Reactions to Make BPN' variants LC1-LC4

Primer Name	Primer Sequence (5' to 3')
A128Sf	/5Phos/CAACATGAGCCTGGGATCACCAAGCGGCAGTGCGG (SEQ ID NO:56)
A128Sr	/5Phos/CCGCACTGCCGCTTGGTGATCCCAGGCTCATGTTG (SEQ ID NO:57)
P55Tf	/5Phos/CTATGGTGCCGGGCGAAACAAACCCGTTTCAAGATCCG (SEQ ID NO:58)
P55Tr	/5Phos/CGGATCTTGAAACGGGTTTGTTTCGCCCGGCACCATAG (SEQ ID NO:59)

Generation of Additional BPN' Variants LC5-LC37

An additional 33 BPN' variants termed successively LC5 through LC37 were produced by DNA 2.0 using the BPN' nucleic acid as the parent gene contained in the expression plasmid pHPLT-BPN' partial opt (see Figure 3). LC5 through LC37 BPN' variants are as follows, respectively: BPN'-P52L-V68A-G97A-I111V, BPN'-I111V-M124V-Y167A-Y217Q, BPN'-Y104N-G128A-Y217Q, BPN'-M124V-Y167A-Y217Q, BPN'-I111V-M124V-Y217Q, BPN'-P52L-V68A-G97A, BPN'-G97A-I111V-M124V, BPN'-V68A-A92G-G97A, BPN'-G97A-I111V-M124V-Y167A-Y217Q, BPN'-P52L-V68A-I111V-Y217Q, BPN'-P52L-V68A-I111V, BPN'-V68A-A92G-I111V, BPN'-P52L-V68A-G97A-I111V-Y217Q, BPN'-V68A-G97A-I111V, BPN'-G97A-I111V-Y217Q, BPN'-G97A-I111V-M124V-Y167A, BPN'-S89Y-I111V-M124V, BPN'-V68A-S89Y-I111V, BPN'-V68A-A92G-Y217Q, BPN'-I111V-Y167A-Y217Q, BPN'-G97A-I111V-Y167A-Y217Q, BPN'-G97A-I111V-M124V-Y217Q, BPN'-V68A-I111V-Y167A-Y217Q, BPN'-I111V-G128A-Y217Q, BPN'-G97A-M124V-Y217Q, BPN'-V68A-Y167A-Y217Q, BPN'-I111V-M124V-Y167A, BPN'-N62Q-G97A-I111V, BPN'-G97A-M124V-Y167A-Y217Q, BPN'-G97A-L126A-Y217Q, BPN'-V68A-I111V-Y217Q, BPN'-S89Y-M124V-Y217Q, and BPN'-L96T-G97A-Y217Q. Plasmid pHPLT-BPN' partial opt was also created by DNA 2.0.

Transformants were picked into microtiter plates and grown as described in Example 2. The variants were assayed for cleaning performance using a BMI microswatch assay in Detergent Composition 2 at 16°C and pH 8. Protein content was determined using the TCA assay. The assays were

performed as described in Example 1 and the Performance Indices were calculated relative to BPN'-v3 (with a PI value of 1.0).

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-I111V-M124V-Y217Q, G097A-I111V-Y167A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128A-V203Y-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128S-V203Y-Y217Q, and V068A-A092G-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2, wherein the variant comprises at least one substitution selected from the group of X024G, X025G, X052L, X053G, X055P, X061P, X062Q, X068A, X089Y, X092G, X096T, X097A, X101N, X104N, X111V, X124V, X126A, X128A/S, X167A, X203Y, and X217Q, and optionally at least one substitution selected from the group of S024G, N025G, P052L, S053G, T055P, N061P, N062Q, V068A, S089Y, A092G, L096T, G097A, S101N, Y104N, I111V, M124V, L126A, G128A/S, Y167A, V203Y, and Y217Q, and wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q, G097A-G128S-Y217Q, G097A-I111V-Y217Q, I111V-G128A-Y217Q, I111V-M124V-Y167A, I111V-M124V-Y217Q, L096T-G097A-Y217Q, N062Q-G097A-I111V,

S053G-N061P-G097A-S101N-G128S-V203Y-Y217Q, S089Y-M124V-Y217Q, and V068A-I111V-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a greater PI value than that of BPN', and a PI value of about 1.0 compared to BPN'-v3 in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-I111V-M124V, G097A-L126A-Y217Q, G097A-M124V-Y217Q, I111V-Y167A-Y217Q, M124V-Y167A-Y217Q, P052L-V068A-G097A, S089Y-I111V-M124V, V068A-A092G-G097A, V068A-A092G-I111V, V068A-G097A-I111V, V068A-S089Y-I111V, and Y104N-G128A-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 2 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-M124V-Y167A-Y217Q, V068A-Y167A-Y217Q, G097A-I111V-M124V-Y167A, I111V-M124V-Y167A-Y217Q, V068A-I111V-Y167A-Y217Q, G097A-I111V-M124V-Y167A-Y217Q, and P052L-V068A-I111V, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, the variant comprising an amino

acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Such variants have proteolytic activity. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 5

Cleaning Performance of BPN' Variants

Variants based on parent BPN' were made by DNA 2.0. The variants were grown as described in Example 2 and tested for cleaning performance on BMI microswatch assay in Detergent Composition 1 at 16°C and pH 8, BMI microswatch assay in Detergent Composition 4 at 16°C and pH 8, and egg microswatch assay in Detergent Composition 4 at 16°C and pH 8. The protein content was determined using the TCA assay. The assays were performed as described in Example 1 and the Performance Indices were calculated relative to BPN'-v3 (with a PI value of 1.0).

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128S-Y217Q, and S024G-N025G-S053G-N061P-S078N-G097A-S101N-I111V-G128S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid

sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q, G097A-G128A-P210S-Y217Q, G097A-G128S-P210S-Y217Q, G097A-I111V-M124I-Y217Q, G097A-I111V-M124V-P210S-Y217Q, G097A-N123Q-P210S-Y217Q, G097A-N123Q-Y217Q, N061P-G097A-G128A-P210S-Y217Q, N061P-G097A-G128S-Y217Q, N061P-G097A-I111V-M124V-Y217Q, N061P-G097A-N123Q-Y217Q, N061P-G097A-S101N-I111V-M124V-Y217Q, N061P-G097A-S101N-N123Q-Y217Q, N061P-G102A-P129S-Y217Q, N061P-N062Q-G097A-G100N-S101N-Y217Q, N061P-N062Q-G097A-G100N-Y217Q, N061P-N062Q-G097A-G100Q-P210S-Y217Q, N061P-N062Q-G097A-I111V-Y217Q, N061P-N062Q-G097A-S101N-I111V-Y217Q, N061P-S078N-G097A-I111V-M124I-Y217Q, N061P-S078N-G102A-I111V-P129S-Y217Q, N062Q-G097A-I111V-P210S-Y217Q, N062Q-G097A-I111V-Y217Q, N062Q-S078N-G097A-I111V-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-N123Q-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, S024G-N025G-S053G-N061P-S101N-G102A-P129S-Y217Q, S053G-N061P-G097A-G128S-Y217Q, S053G-N061P-G097A-M124I-Y217Q, S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, S053G-N061P-G102A-P129S-P210S-Y217Q, S053G-N061P-G102A-P129S-Y217Q, S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, S053-N061P-S101N-G102A-P129S-Y217Q, S053G-S078N-G097A-I111V-G128S-Y217Q, S078N-G097A-G128S-Y217Q, and S078N-G097A-I111V-M124V-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of N061P-G097A-M124I-Y217Q, N061P-G097A-M124V-Y217Q, N061P-N062Q-G097A-G100D-Y217Q, N061P-N062Q-G097A-G100Q-S101N-Y217Q, N061P-N062Q-G097A-G100Q-Y217Q, N061P-N062Q-G100N-G102A-Y217Q, N061P-N062Q-S078N-G097A-G100N-I111V-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-M124I-Y217Q, S053G-N061P-G097A-S101N-M124I-

Y217Q, and S053G-N061P-G097A-S101N-N123Q-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 or 4 at pH 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2, wherein the variant comprises at least one substitution selected from the group of X024G, X025G, X053G, X061P, X062Q, X078N, X097A, X100D/N/Q, X101N, X102A, X111V, X123A/Q/V, X124I/V, X128A/S, X129S, X210S, X217Q, and optionally at least one substitution selected from the group of S024G, N025G, S053G, N061P, N062Q, S078N, G097A, G100D/N/Q, S101N, G102A, I111V, N123A/Q/V, M124I/V, G128A/S, P129S, P210S, and Y217Q, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-N123A-Y217Q, G097A-N123V-Y217Q, N061P-G102A-G128S-Y217Q, N061P-S101N-G102A-G128S-Y217Q, Y217Q, S078N-G097A-I111V-N123Q-Y217Q, and G102A-N123Q-Y217Q, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g.,

cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of N061P-G097A-G128S-Y217Q, N061P-G097A-S101N-G128A-P210S-Y217Q, N061P-N062Q-G097A-S101N-I111V-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128S-Y217Q, and S024G-N025G-S053G-N061P-S078N-G097A-S101N-I111V-G128S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q, G097A-G128A-P210S-Y217Q, G097A-G128S-P210S-Y217Q, G097A-I111V-M124I-Y217Q, G097A-I111V-M124V-P210S-Y217Q, G097A-N123Q-P210S-Y217Q, G097A-N123Q-Y217Q, N061P-G097A-G128A-P210S-Y217Q, N061P-G097A-I111V-M124V-Y217Q, N061P-G097A-M124V-Y217Q, N061P-G097A-N123Q-Y217Q, N061P-G097A-S101N-I111V-M124V-Y217Q, N061P-G102A-P129S-Y217Q, N061P-N062Q-G097A-G100N-S101N-Y217Q, N061P-N062Q-G097A-G100Q-Y217Q, N061P-N062Q-G097A-I111V-Y217Q, N061P-N062Q-S078N-G097A-G100N-I111V-Y217Q, N061P-S078N-G097A-I111V-M124I-Y217Q, N061P-S078N-G102A-I111V-P129S-Y217Q, N062Q-G097A-I111V-P210S-Y217Q, N062Q-G097A-I111V-Y217Q, N062Q-S078N-G097A-I111V-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-N123Q-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, S024G-N025G-

S053G-N061P-S101N-G102A-P129S-Y217Q, S053G-N061P-G097A-G128S-Y217Q, S053G-N061P-G102A-P129S-P210S-Y217Q, S053G-N061P-G102A-P129S-Y217Q, S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, S053G-N061P-S101N-G102A-P129S-Y217Q, S053G-S078N-G097A-I111V-G128S-Y217Q, S078N-G097A-G128S-Y217Q, and S078N-G097A-I111V-M124V-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of N061P-G097A-M124I-Y217Q, N061P-G097A-S101N-N123Q-Y217Q, N061P-N062Q-G097A-G100N-Y217Q, N061P-N062Q-G097A-G100Q-P210S-Y217Q, N061P-N062Q-G097A-G100Q-S101N-Y217Q, N061P-N062Q-G100N-G102A-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-M124I-Y217Q, S053G-N061P-G097A-M124I-Y217Q, S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, S053G-N061P-G097A-S101N-M124I-Y217Q, S053G-N061P-G097A-S101N-N123Q-Y217Q, and S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-N123A-Y217Q, G097A-N123V-Y217Q,

N061P-N062Q-G097A-G100D-Y217Q, N061P-S101N-G102A-G128S-Y217Q, Y217Q, N061P-G102A-G128S-Y217Q, S078N-G097A-I111V-N123Q-Y217Q, and G102A-N123Q-Y217Q, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value greater than 1.0 to about 5 relative to BPN'-v3 in an egg microswatch cleaning assay in Detergent Composition 4 at 16°C and pH 8: BPN' amino acid sequence (SEQ ID NO:2) comprising the set of amino acid substitutions N061P-G097A-S101N-G128A-P210S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising amino acid substitutions N061P-G097A-S101N-G128A-P210S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128A-Y217Q, N061P-G102A-P129S-Y217Q, N062Q-G097A-I111V-P210S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128A-P210S-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-P210S-Y217Q, N061P-G097A-G128A-P210S-Y217Q, G097A-G128S-P210S-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-I111V-G128S-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-G128S-Y217Q, N061P-G097A-G128S-Y217Q, and G097A-G128A-P210S-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0

relative to BPN'-v3 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value equal to or greater than 0.5 and equal to or less than 0.9 relative to BPN'-v3 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of N061P-G097A-M124I-Y217Q, S053G-N061P-G097A-S101N-N123Q-Y217Q, S053G-N061P-G102A-P129S-P210S-Y217Q, G097A-I111V-M124V-P210S-Y217Q, G097A-N123Q-P210S-Y217Q, S053G-N061P-S101N-G102A-P129S-Y217Q, S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, N061P-N062Q-G097A-S101N-I111V-Y217Q, N061P-N062Q-G097A-I111V-Y217Q, N062Q-G097A-I111V-Y217Q, N061P-G097A-S101N-I111V-M124V-Y217Q, G097A-N123Q-Y217Q, N061P-G097A-I111V-M124V-Y217Q, S078N-G097A-I111V-M124V-Y217Q, S053G-S078N-G097A-I111V-G128S-Y217Q, S078N-G097A-G128S-Y217Q, S053G-N061P-G097A-G128S-Y217Q, N061P-N062Q-G097A-G100N-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-N123Q-Y217Q, N061P-G097A-S101N-N123Q-Y217Q, N061P-N062Q-G097A-G100Q-P210S-Y217Q, N061P-G097A-N123Q-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-M124I-Y217Q, S053G-N061P-G097A-S101N-M124I-Y217Q, S053G-N061P-G097A-M124I-Y217Q, N061P-S078N-G097A-I111V-M124I-Y217Q, N061P-G097A-M124V-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, S024G-N025G-S053G-N061P-S101N-G102A-P129S-Y217Q, N061P-S078N-G102A-I111V-P129S-Y217Q, S053G-N061P-G102A-P129S-Y217Q, S024G-N025G-S053G-N061P-N062Q-G097A-S101N-I111V-Y217Q, N062Q-S078N-G097A-I111V-Y217Q, S024G-N025G-S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, S053G-N061P-G097A-S101N-I111V-M124V-Y217Q, G097A-I111V-M124I-Y217Q, Y217Q, N061P-N062Q-G100N-G102A-Y217Q, S053G-N061P-N062Q-G097A-G100N-S101N-Y217Q, N061P-N062Q-G097A-G100N-S101N-Y217Q, N061P-N062Q-S078N-G097A-G100N-I111V-Y217Q, N061P-N062Q-G097A-G100Q-Y217Q, N061P-S101N-G102A-G128S-Y217Q, G097A-N123V-Y217Q, G097A-N123A-Y217Q, G102A-N123Q-Y217Q, N061P-N062Q-G097A-G100Q-S101N-Y217Q, S078N-G097A-I111V-N123Q-Y217Q, N061P-N062Q-G097A-G100D-Y217Q, and N061P-G102A-G128S-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity.

The invention includes a protease variant having proteolytic activity and/or a PI value of equal to or greater than 0.5 and equal to or less than 0.9 relative to BPN'-v3 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%,

93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v3 in this egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2, wherein the variant comprises at least one substitution selected from the group of X024G, X025G, X053G, X061P, X062Q, X078N, X097A, X100D/N/Q, X101N, X102A, X111V, X123A/Q/V, X124I/V, X128A/S, X129S, X210S, and X217Q, and optionally at least one substitution selected from the group of S024G, N025G, S053G, N061P, N062Q, S078N, G097A, G100D/N/Q, S101N, G102A, I111V, N123A/Q/V, M124I/V, G128A/S, P129S, P210S, and Y217Q, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 6

Construction and Cleaning Performance of BPN' variants

A BPN' combinatorial library based on BPN' parent was made by DNA2.0 and delivered as a ligation reaction. For efficient transformation of *B. subtilis*, DNA from the ligation reaction mixtures was amplified before transformation and transformants grown as described in Example 2. These variants were tested for cleaning performance using BMI microswatch assay in Detergent Composition 1 and Detergent Composition 4 at 16°C and pH 8 as well as egg microswatch assay in Detergent Composition 4 at 16°C and pH 8. Protein content was determined using the TCA assay and protease activity was assayed using the AAPF assay. The assays were performed as described in Example 1 and the Performance Indices were calculated relative to BPN'-v3 (with a PI value of 1.0).

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128A-

Y217Q, N025G-G097A-S101N-G128A-Y217Q, N025G-S038G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-G128A-Y217Q, N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, N025G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, N025G-S078N-G097A-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-S101N-G128A-Y217Q, N061P-S101N-G128A-Y217Q, S024G-N025G-N061P-G097A-G128A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G128A-Y217Q, S024G-N025G-T055P-G097A-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-N061P-G097A-G128A-Y217Q, S024G-S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-T055P-N061P-G097A-G128A-Y217Q, S053G-G097A-S101N-G128A-Y217Q, S053G-N061P-G097A-S101N-G128A-Y217Q-S249N, S053G-N061P-S078N-G097A-G128A-Y217Q, S053G-S078N-G097A-S101N-G128A-Y217Q, S053G-T055P-G097A-S101N-G128A-Y217Q, S053G-T055P-N061P-S101N-G128A-Y217Q, S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, T055P-G097A-S101N-G128A-Y217Q, and T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128S-Y217Q, G097A-G128A-Y217Q, N025G-S078N-G097A-G128A-Y217Q, N025G-T055P-G097A-G128A-Y217Q, S024G-G097A-S101N-G128A-Y217Q, S024G-I035V-T055P-N061P-S078N-G097A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A, S024G-N025G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-G097A-G128A-S130G-Y217Q, S024G-N025G-S053G-N061P-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-

S078N-G097A-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G128A-Y217Q, S024G-N025G-S053G-T055P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S101N-G128A-Y217Q, S024G-N025G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-Y217Q, S024G-N061P-G097A-S101N-G128A-Y217Q, S024G-S038G-S053G-S078N-S101N-G128A-Y217Q, S024G-S053G-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-S078N-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S024G-T055P-G097A-G128A-Y217Q, S024G-T055P-N061P-G097A-S101N-G128A, S024G-T055P-N061P-S078N-S101N-G128A-Y217Q, S024G-T055P-S078N-G097A-S101N-G128A-Y217Q, S101N-G128A-Y217Q, T055P-N061P-G097A-A116S-G128A, and T055P-N061P-S078N-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN' and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v3 in this BMI microswatch cleaning assay in Detergent Composition 1 at pH 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2, wherein the variant comprises at least one substitution selected from the group of X024G, X025G, X035V, X038G, X053G, X055P, X061P, X078N, X097A, X101N, X116S, X128A/S, X130G, X216Q, X217Q, and X249N, and optionally at least one substitution selected from the group of S024G, N025G, I035V, S038G, S053G, T055P, N061P, S078N, G097A, S101N, A116S, G128A/S, S130G, Y216Q, Y217Q, and S249N, and wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions

selected from the group consisting of S024G-N025G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-T055P-N061P-S078N-S101N-G128A-Y217Q, S053G-G097A-S101N-G128A-Y217Q, and T055P-N061P-S078N-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128S-Y217Q, G097A-G128A-Y217Q, N025G-G097A-S101N-G128A-Y217Q, N025G-S038G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-G128A-Y217Q, N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, N025G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, N025G-S078N-G097A-G128A-Y217Q, N025G-S078N-G097A-S101N-G128A-Y217Q, N025G-T055P-G097A-G128A-Y217Q, N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-S101N-G128A-Y217Q, N061P-S101N-G128A-Y217Q, S024G-G097A-S101N-G128A-Y217Q, S024G-I035V-T055P-N061P-S078N-G097A-Y217Q, S024G-N025G-N061P-G097A-G128A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A, S024G-N025G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-G097A-G128A-S130G-Y217Q, S024G-N025G-S053G-N061P-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G128A-Y217Q, S024G-N025G-S053G-T055P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S101N-G128A-Y217Q, S024G-N025G-T055P-G097A-G128A-Y217Q, S024G-N025G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N061P-G097A-S101N-G128A-Y217Q, S024G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S038G-S053G-S078N-S101N-G128A-Y217Q, S024G-S053G-N061P-G097A-G128A-Y217Q, S024G-S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-

S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-S078N-S101N-G128A-Y217Q, S024G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-T055P-G097A-G128A-Y217Q, S024G-T055P-N061P-G097A-G128A-Y217Q, S024G-T055P-N061P-G097A-S101N-G128A, S024G-T055P-S078N-G097A-S101N-G128A-Y217Q, S053G-N061P-G097A-S101N-G128A-Y217Q-S249N, S053G-N061P-S078N-G097A-G128A-Y217Q, S053G-S078N-G097A-S101N-G128A-Y217Q, S053G-T055P-G097A-S101N-G128A-Y217Q, S053G-T055P-N061P-S101N-G128A-Y217Q, S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, S101N-G128A-Y217Q, T055P-G097A-S101N-G128A-Y217Q, and T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pII 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of T055P-N061P-G097A-A116S-G128A and S024G-N025G-T055P-N061P-S078N-G097A-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity and may have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) in this assay. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2,

from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in an egg microswatch cleaning assay in Detergent Composition 4 at pII 8 and 16ºC: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N061P-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-G097A-S101N-G128A-Y217Q, S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-N025G-T055P-G097A-G128A-Y217Q, and S024G-N025G-S053G-T055P-G097A-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence.

The following BPN' variants were determined to have a PI value of about 1.0 relative to BPN'-v3 in an egg microswatch cleaning assay in Detergent Composition 4 at pII 8 and 16ºC: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of G097A-G128S-Y217Q, G097A-G128A-Y217Q, S024G-G097A-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-S101N-G128A-Y217Q, S053G-T055P-N061P-S101N-G128A-Y217Q, S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, N025G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, T055P-N061P-S078N-G128A-Y217Q, N025G-S038G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-G128A-Y217Q, S024G-N025G-S053G-T055P-S078N-S101N-G128A-Y217Q, T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G128A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-Y217Q, S024G-T055P-G097A-G128A-Y217Q, T055P-N061P-G097A-A116S-G128A, S053G-T055P-G097A-S101N-G128A-Y217Q, T055P-G097A-S101N-G128A-Y217Q, S024G-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-G097A-G128A-Y217Q, S024G-S053G-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S053G-G097A-S101N-G128A-

Y217Q, N025G-T055P-G097A-G128A-Y217Q, S024G-T055P-S078N-G097A-S101N-G128A-Y217Q, N025G-S078N-G097A-S101N-G128A-Y217Q, N025G-G097A-S101N-G128A-Y217Q, S024G-S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-Y217Q, and S024G-I035V-T055P-N061P-S078N-G097A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning comprising utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of S101N-G128A-Y217Q, S024G-T055P-N061P-G097A-S101N-G128A, S024G-N025G-N061P-S078N-G097A-S101N-G128A, S024G-T055P-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S053G-N061P-G097A-S101N-G128A-Y217Q-S249N, N025G-S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-G097A-S101N-G128A-Y217Q, S024G-S053G-N061P-G097A-G128A-Y217Q, S024G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S024G-T055P-N061P-G097A-G128A-Y217Q, S024G-S038G-S053G-S078N-S101N-G128A-Y217Q, S053G-S078N-G097A-S101N-G128A-Y217Q, N025G-S078N-G097A-G128A-Y217Q, and S024G-N025G-S053G-N061P-G097A-G128A-S130G-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variant was determined to have a PI value of about 0.8 relative to BPN'-v3 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising amino acid substitutions S024G-S053G-S078N-G097A-S101N-G128A-Y217Q, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. The invention includes a protease variant having proteolytic activity, a PI value of about 0.8 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising amino acid substitutions S024G-S053G-S078N-G097A-S101N-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1 to about 12, from greater than 4 to about 12, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v3 in an AAPF proteolytic assay: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of S024G-G097A-S101N-G128A-Y217Q, S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-S101N-G128A-Y217Q, S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-T055P-N061P-G097A-S101N-G128A, S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A, N061P-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-S101N-G128A-Y217Q, S024G-T055P-N061P-S078N-S101N-G128A-Y217Q, N025G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S101N-G128A-Y217Q, N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-G128A-Y217Q, S024G-S053G-T055P-N061P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, T055P-N061P-S078N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, N025G-S038G-S053G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-N061P-G128A-Y217Q, N025G-S053G-N061P-S078N-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-S078N-S101N-G128A-Y217Q, T055P-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G128A-Y217Q, S024G-N025G-N061P-G097A-S101N-G128A-Y217Q, S053G-N061P-G097A-S101N-G128A-Y217Q-S249N, N025G-S053G-T055P-S078N-G097A-S101N-G128A-Y217Q, S024G-T055P-G097A-G128A-Y217Q,

T055P-N061P-G097A-A116S-G128A, S024G-N025G-T055P-G097A-S101N-G128A-Y217Q, S024G-N025G-N061P-S078N-G097A-S101N-G128A-Y217Q, S053G-T055P-G097A-S101N-G128A-Y217Q, T055P-G097A-S101N-G128A-Y217Q, S024G-N061P-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-G097A-S101N-G128A-Y217Q, G097A-G128S-Y217Q, S024G-S053G-N061P-G097A-G128A-Y217Q, S024G-N025G-N061P-G097A-G128A-Y217Q, S024G-N061P-S078N-G097A-S101N-G128A-Y217Q, S024G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-S078N-S101N-G128A-Y217Q, S024G-N025G-S053G-T055P-N061P-S078N-G097A-G128A-Y217Q, S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-T055P-N061P-G097A-G128A-Y217Q, S024G-S038G-S053G-S078N-S101N-G128A-Y217Q, S053G-G097A-S101N-G128A-Y217Q, N025G-T055P-G097A-G128A-Y217Q, S024G-T055P-S078N-G097A-S101N-G128A-Y217Q, S053G-S078N-G097A-S101N-G128A-Y217Q, S024G-N025G-T055P-G097A-G128A-Y217Q, S024G-N025G-S053G-T055P-G097A-G128A-Y217Q, N025G-S078N-G097A-S101N-G128A-Y217Q, N025G-G097A-S101N-G128A-Y217Q, S024G-S053G-N061P-S078N-G097A-G128A-Y217Q, S024G-S053G-S078N-G097A-S101N-G128A-Y217Q, N025G-S078N-G097A-G128A-Y217Q, and S024G-N025G-S053G-N061P-G097A-G128A-S130G-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' protease (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v3 in this AAPF assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence.

The following BPN' variant was determined to have a PI value of about 1.0 relative to BPN'-v3 in an AAPF proteolytic assay: BPN' amino acid sequence (SEQ ID NO:2) comprising amino acid substitutions G097A-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' protease (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and/or a PI value of about 1.0 relative to BPN'-v3 in this AAPF assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and comprising amino acid substitutions G097A-G128A-Y217Q, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 7

Construction of Site Evaluation Libraries of BPN'-v36 and Cleaning Performance of BPN'-v36 Variants

Construction of the site evaluation libraries of BPN'-v36

The amino acid sequence of BPN'-v36 is set forth in SEQ ID NO:6 below:

AQSVPYGVSQIKAPALHSQGYTGGNVKVAVIDSGIDSSHPDLKVAGGASMVPGETNPFQDNNSH
GTHVAGTVAALNNNIGVLGVAPSASLYAVKVLGADGNGQYSWIINGIEWAIANNMDVINMSLG
APSGSAALKAAVDKAVASGVVVVAAAGNEGTSGSSSTVGYPGKYPSVIAVGAVDSSNQRASFS
SVGPELDVMAPGVSIQSTLPGNKYGAQNGTSMASPHVAGAAALILSKHPNWTNTQVRSSLENTT
TKLGDSFYYGKGLINVQAAAQ (SEQ ID NO:6)

The nucleic acid sequence encoding the BPN'-v36 protease variant is:

Gcgcagtccgtgccttacggcgtatcacaaattaaagcccctgctctgcactctcaaggctacactggaggcaatgttaaagtagcggttatcgacagc
ggtatcgactcgagccatccagatcttaaagtcgctggaggggcttctatggtgccgggcgaaacaaacccgtttcaagataacaattctcatggcacac
acgtcgcaggaacggttgcggcgttaaacaataatattggcgtgcttggtgtagccccgtctgcttcgctctacgccgttaaagttcttggcgcagacgga
aatggccaatactcatggattatcaacggcatcgaatgggccatcgcgaataacatggatgtaatcaacatgagcctgggagcaccaagcggcagtgc
ggcacttaaagcagcagttgataaagctgttgcatctggtgtcgtcgtagtagcggcagctgggaatgagggaacatccggatcatcgagtaccgtcgg
ttatccaggcaagtacccttcagtgattgcagtgggcgctgtagactcttcaaatcaacgtgcctcttttttcctccgtgggaccggagctggatgtcatggc
ccctggcgtttctattcaatcgacgcttccagggaacaagtatggtgcgcaaaacgggacttccatggcctcgccgcatgtagctggggcggccgcatt
gattctttctaagcacccgaactggacaaacactcaagtccgcagcagtttagaaaacaccactacaaaacttggtgattctttctactatggaaaagggct
gatcaacgtacaggcggcagctcag (SEQ ID NO:5)

The amino acid sequence of BPN'-v36 may be represented by reference to the subtilisin BPN' amino acid sequence of SEQ ID NO:2. That is, BPN'-v36 may be represented as the subtilisin BPN' sequence of SEQ ID NO:2 with the six amino acid substitutions S024G-S053G-S078N-S101N-G128A-Y217Q. The BPN'-v36 amino acid sequence may be conveniently designated as BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q or BPN'+S024G+S053G+S078N+S101N+G128A+Y217Q. Throughout this specification, unless otherwise indicated, each amino acid position of an amino acid sequence is numbered according to the numbering of a corresponding amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin BPN' shown in SEQ ID NO:2 as determined by alignment of the variant amino acid sequence with the *Bacillus amyloliquefaciens* subtilisin BPN' amino acid sequence.

Site evaluation libraries (SELs) were created at every single amino acid position in mature BPN'-v36 (i.e., BPN'-S24G-S53G-S78N-S101N-G128A-Y217Q) protein by PCR fusion.

For each codon to be mutated in the BPN'-v36 protease, a pair of partially overlapping, complementary (mutagenic forward and reverse) primers were designed. Each mutagenic primer contained the NNS (N=A,C,G, or T and S=G or C) mutagenic codon in the center flanked by at least 15 nucleotides on each side. To create a library at a given position, two PCR reactions were carried out using either a common forward gene-flanking primer (P4974, GCC1CACATT1G1GCCACCTA; SEQ

ID NO:60) and a mutagenic NNS reverse primer, or the common reverse gene-flanking primer (P4976, CCTCTCGGTTATGAGTTAGTTC; SEQ ID NO:61) and a mutagenic NNS forward primer. These PCR reactions generated two PCR fragments, one encoding the 5' half of the mutant BPN'-v36 gene (5' gene fragment) and the other encoding the 3' half of the mutant BPN'-v36 gene (3' gene fragment).

Each PCR amplification reaction contained 30 pmol of each primer and 100 ng of the BPN'-v36 parent template DNA (plasmid pHPLT-BPN'-v36, see Figure 4). Amplifications were carried out using Vent DNA polymerase (NEB). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 40 sec. Following amplification, the 5' and 3' gene fragments were gel-purified by the QIAGEN® gel-band purification kit, mixed (50 ng of each fragment), mixed and amplified by PCR once again using the primers P4973 (AAAGGATCCTAATCGGCGCTTTTC; SEQ ID NO:62) and P4950 (CTTGTCTCCAAGCTTAAAATAAAA; SEQ ID NO:63) to generate the full-length gene fragment. The PCR conditions were same as described above, except the extension phase, which was carried out at 72°C for 2 min. The full-length DNA fragment was gel-purified by the QIAGEN® gel-band purification kit, digested by the *Bam*HI and *Hin*dIII restriction enzymes and ligated with the pHPLT-BPN' partial opt vector that also was digested with the same restriction enzymes. Ligation mixtures were amplified using rolling circle amplification in an Illustra Templiphi kit according to the manufacturer's recommendation (GE Healthcare) to generate multimeric DNA for transformation into *Bacillus subtilis*. For this purpose, 1µl of the ligation mixture was mixed with 5 µl of the sample buffer, heated to 95°C for 3 min and cooled on ice. Next, 5 µl of the reaction buffer and 0.2 µl of the enzyme were added to each tube, followed by incubation at 30°C for 10 hours. Products of the rolling circle amplification were diluted 100 times and used to transform *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and incubated overnight at 37°C. Subsequently, the colonies with halos were inoculated in 150 µl of LB media containing 10 µg/mL neomycin. The next day, cultures were either frozen with 15% glycerol or grown in MBD medium for biochemical analysis as described in Example 2.

Cleaning Performance of the BPN'-v36 Variants

Protein variants from BPN'-v36 SEL were tested for cleaning performance using a BMI microswatch assay in Detergent Composition 4 at 16°C and pH 8 and egg microswatch assay in Detergent Composition 4 at 16°C and pH 8. Protein content was determined using the TCA assay. The assays were performed as described in Example 1 and the Performance Indices were calculated relative to BPN'-v36 (i.e., BPN'-S24G-S53G-S78N-S101N-G128A-Y217Q) (with a PI value of 1.0).

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8

and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q (SEQ ID NO:6) (i.e., BPN'-v36) comprising at least one amino acid substitution selected from the group consisting of A116V, G160S, I111L, I115V, N109S, N117M, P005G, Q059V, T164S, Y262M, A015Q, A015S, A098E, A098N, A098S, A098T, A098V, A098Y, A114S, A114T, A116G, A116L, A116S, A116T, A116W, A133G, A133H, A133T, A133V, A137G, A137I, A137L, A137S, A137T, A138S, A216E, A216F, A216V, D099S, D181E, F261A, F261Q, G024F, G024I, G024Q, G024Y, G097S, G160T, G211L, G211V, H017F, H017W, H039V, H226A, I031V, I111V, I268V, K170R, K265R, L016Q, L016T, L135M, L209T, L209V, L233M, L257T, L257V, L267A, L267V, N025A, N025I, N025Q, N025R, N025T, N025V, N101I, N101Q, N101S, N109A, N109G, N109H, N109L, N109M, N109Q, N109T, N117Q, N184A, N184L, N184T, N184W, N212G, N212L, N212V, N243P, N252G, N252M, P005T, P014S, P040G, P040L, P040Q, P129A, P129S, P172G, P172S, P194Q, P210A, P210S, Q185F, Q185G, Q185I, Q185M, Q185N, Q185S, Q275H, R186K, S009A, S009G, S009H, S009M, S018T, S130T, S132N, S145K, S159T, S161I, S161K, S161N, S161T, S162I, S162M, S162Y, S163G, S182F, S182G, S182V, S182W, S183F, S183L, S183M, S183T, S183V, S183W, S224A, S236T, S249V, T022A, T022G, T022Q, T022V, T208V, T242S, T253N, T253S, T254A, T254S, T255L, T255S, T255V, V004A, V004P, V004W, V084C, V139C, V165M, V203F, Y021K, Y021N, Y021T, Y021V, Y167F, Y171F, Y214F, Y262F, and Y262T, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN'-v3 and BPN'-v36, a PI value greater than that of BPN'-v3, and/or a PI value greater than 1 to about 5 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one, two, three, four, five, six or more amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one amino acid substitution selected from the group consisting of A001G, A001Y, A013G, A013V, A015F, A015G, A015K, A015M, A015P, A015T, A015W, A015Y, A029G, A073S, A088C, A088I, A088L, A088T, A088V, A098D, A098K, A098P, A098R, A098W, A116D, A116E, A116R, A128S, A133L, A133M, A133S, A134G, A134S, A137N, A137V, A144M, A144Q, A144S, A144T, A144V, A151C, A176S, A176T, A179S, A216G, A216L, A216P, A216Q, A216S, A216T, A216Y, A228T,

A230C, A231C, A272I, A272L, A272Q, A272S, A272T, A272W, A273S, A274G, A274L, A274Q, A274T, A274V, D041E, D099G, D099N, D120A, D120K, D120Q, D120R, D120S, D140E, D181S, D259E, E054D, E156D, E156T, E251V, F261G, F261H, F261L, F261R, F261S, F261T, F261V, F261W, G007A, G007S, G020A, G020D, G020S, G024N, G024R, G024S, G024T, G024V, G024W, G053H, G053K, G053N, G053T, G097A, G097D, G097T, G131A, G131H, G131P, G131Q, G131T, G131V, G160H, G160P, G166A, G166S, G166T, G211A, G211D, G211M, G211N, G211P, G211Q, G211R, G211W, G215A, G215N, G215V, H017L, H017M, H017T, H017V, H017Y, H039A, H039C, H039N, H226F, H226I, H226M, H226S, H226V, H226Y, I035V, I079A, I079S, I079T, I079V, I079W, I108V, I115L, I122A, I234L, I234V, K012R, K027R, K136R, K141F, K213W, K237R, K256R, K265Q, L016A, L016F, L016I, L016S, L016V, L042I, L075A, L075M, L075Q, L075V, L075Y, L082K, L082M, L082Q, L082V, L090I, L196I, L196V, L209Q, L209W, L233A, L233Q, L233V, L235I, L235K, L250I, L257A, L257H, L257Q, L257S, L257Y, L267Q, L267R, L267S, L267T, M199V, N025F, N025G, N025H, N025K, N025L, N025M, N025S, N025Y, N061F, N061H, N061P, N061S, N061T, N061V, N061W, N076G, N076W, N078S, N078T, N078V, N101A, N101H, N101L, N101T, N109K, N109R, N117A, N117E, N117H, N117K, N118G, N184G, N184H, N184I, N184S, N184V, N212A, N212F, N212I, N212K, N212P, N212Q, N212S, N212Y, N218A, N218H, N218L, N218S, N240E, N240H, N240L, N240R, N240T, N243A, N243Q, N243T, N243V, N252A, N252K, N252L, N252Q, N252R, N252S, N252T, N269Q, N269S, P014G, P014Q, P014T, P040A, P040H, P040S, P040T, P040V, P040Y, P086A, P086C, P086F, P086H, P086S, P129D, P129G, P129K, P129T, P172A, P172Q, P194A, P194G, P194L, P194M, P194S, P194V, P194Y, P210G, P210R, P210V, Q002A, Q002S, Q010A, Q010F, Q010H, Q010I, Q010L, Q010N, Q010S, Q010T, Q019A, Q019G, Q019N, Q019S, Q019T, Q019V, Q019W, Q059I, Q103L, Q103S, Q185A, Q185H, Q185L, Q185T, Q185Y, Q206P, Q206S, Q206Y, Q217I, Q217N, Q217S, Q217T, Q245K, Q275D, Q275S, Q275W, S003A, S003G, S003H, S003M, S003P, S003Q, S003T, S003V, S009I, S009L, S009P, S009T, S009W, S018A, S018G, S018I, S018L, S018M, S018N, S018P, S018V, S018W, S033T, S037Q, S037T, S037V, S038G, S038H, S038K, S038Q, S038T, S063K, S063N, S063Q, S063T, S087A, S087F, S087G, S087Q, S087T, S089L, S089M, S089N, S089Q, S089T, S089W, S130A, S130F, S130G, S130L, S130V, S145A, S145H, S145M, S145V, S159A, S159G, S159H, S159Q, S159R, S161A, S161G, S161H, S161L, S161M, S161P, S161Q, S161W, S162A, S162F, S162G, S162L, S162N, S162P, S162R, S162V, S163P, S173A, S173G, S182A, S182H, S182K, S182L, S182N, S182P, S182Q, S182T, S183A, S183G, S183H, S183Q, S188A, S188G, S188T, S188V, S191A, S204A, S204I, S204L, S204Q, S204V, S224C, S236A, S236N, S236Q, S248A, S248F, S248G, S248I, S248K, S248L, S248M, S248N, S248Q, S248T, S248V, S249A, S249C, S249H, S249Q, S249T, S249W, S249Y, S260H, S260N, S260P, S260T, T022H, T022K, T022N, T022R, T022S, T022Y, T055A, T055G, T055L, T055N, T055P, T055Q, T071S, T158H, T158S, T164N, T208C, T208L, T220S, T242N, T244A, T244G, T244H, T244I, T244Q, T244S, T244V, T244W, T253A, T253G, T253H, T253Q, T254V, T255A, T255G, T255H, T255I, T255Q, T255Y, V004G, V004N, V004R, V008A, V008C, V008M, V026I, V044I, V044L, V045H, V045K, V045L, V045M, V045Q, V045S,

V045V, V045W, V045Y, V051I, V081L, V081Q, V081T, V084A, V084S, V084T, V093I, V121I, V143N, V143S, V143Y, V147C, V147I, V147L, V147T, V180I, V180L, V180T, V192A, V192S, V192T, V198I, V198L, V198M, V203H, V203I, V203L, V203N, V203Q, V203T, V203W, V203Y, V270A, V270S, V270T, W241M, W241Y, Y006G, Y006H, Y006I, Y006K, Y006L, Y006P, Y006Q, Y006T, Y006V, Y006W, Y021A, Y021D, Y021E, Y021L, Y021Q, Y021R, Y021S, Y104F, Y104I, Y214L, Y214V, Y214W, Y262A, Y262G, Y262L, Y262N, Y262S, Y262W, Y263G, and Y263W, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Thus, e.g., the invention includes BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising substitution A128S, e.g., BPN'-S024G-S053G-S078N-S101N-G128S-Y217Q. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and/or a PI value of about 1.0 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one, two, three, four, five, six or more amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one amino acid substitution selected from the group consisting of A001F, A001K, A001L, A001M, A001Q, A001R, A001S, A001T, A001V, A013C, A013S, A015D, A015E, A015L, A015R, A048S, A073N, A073T, A074G, A074S, A085C, A085G, A085S, A085V, A088M, A088S, A092S, A098G, A114G, A133P, A137E, A137H, A144G, A144H, A144K, A144L, A144N, A153S, A153V, A176C, A179G, A187G, A187S, A200G, A216W, A223S, A228S, A230T, A230V, A231V, A232C, A232V, A272E, A272G, A272K, A272P, A273G, A273L, A273V, A274M, A274R, D036E, D099A, D099Q, D120E, D181A, D181G, D259A, D259G, D259Q, D259T, E156A, E156S, E251I, E251L, E251Q, E251T, F058Y, F261C, F261D, F261K, F261P, G020E, G020F, G020H, G020L, G020N, G020Q, G020R, G020T, G020Y, G024A, G024P, G053A, G053D, G053E, G053F, G053L, G053Q, G053S, G053Y, G097K, G097M, G157A, G157S, G160A, G160L, G166C, G166I, G166Q, G169A, G211K, G215H, G215L, G215S, G215T, G215W, G258S, H017I, H039S, H226L, H238N, H238Y, I011L, I011V, I031L, I079F, I079K, I079L, I079M, I079Q, I205A, I205V, I268L, I268M, K012G, K043F, K043H, K043I, K043N, K043Q, K043T, K141A, K141R, K141W, K170A, K213A, K213G, K213H, K213I, K213L, K213N, K213Q, K213R, K213S, K213T, K213V, K237A, K237H, K237I, K237L,

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comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S24G-S53G-S78N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one amino acid substitution selected from the group consisting of A001D, A001H, A001N, A015C, A048C, A048E, A085T, A133R, A137R, A142C, A144D, A144R, A152S, A153G, A187P, A187Q, A187T, A187V, A216R, A230S, A272R, A273H, A273T, A274H, D036N, D036S, D181H, D181T, D259N, D259P, D259S, E156G, E156H, E156L, E156Q, E156V, E251C, F189S, F189T, F189W, F189Y, F261E, G020C, G024D, G053M, G053R, G097R, G131D, G131R, G157N, G160R, G160V, G166L, G166W, G211E, G215D, G258A, G258D, G258P, I011T, I031C, I079E, I079R, I175L, I205C, K012H, K012N, K027A, K027N, K027S, K043A, K043D, K043E, K043G, K043L, K043M, K043P, K043V, K043W, K043Y, K136H, K141H, K141L, K141M, K141N, K141Q, K141T, K141V, K170G, K170S, K237T, K237V, K256D, K256S, K256T, K256V, K265N, K265S, L042F, L042M, L082E, L209A, L209E, L209G, L209R, L233G, L235V, L257D, L257E, L257P, L257R, L257W, L267E, M050L, N056D, N056S, N061C, N061D, N062A, N062H, N062L, N062V, N062Y, N076D, N076L, N076M, N078D, N078F, N101D, N101R, N118A, N212C, N212E, N218C, N218D, N218E, N252D, N252E, P014F, P014K, P057A, P057W, P172R, P194E, P201T, P210E, Q059C, Q059D, Q059R, Q185E, Q206D, Q217A, Q217K, Q217R, Q245A, Q245D, Q245E, Q245H, Q245R, Q271E, Q271F, Q271R, Q271W, Q275G, Q275I, R186I, R186L, R186V, R186W, S003E, S009C, S009E, S018C, S037D, S037E, S037H, S037R, S037Y, S038D, S038P, S038R, S038Y, S063L, S087D, S087R, S089C, S089E, S130C, S130R, S145D, S159C, S159P, S161C, S173T, S182C, S188E, S188F, S188K, S188L, S188R, S188W, S190A, S190G, S190T, S204R, S236D, S236E, S248C, S248E, S260C, S260E, S260R, T022P, T055C, T055W, T071A, T158D, T158E, T158P, T158R, T158Y, T164R, T242D, T242G, T255C, V004E, V004T, V045C, V045D, V045R, V045T, V051H, V081R, V143C, V143E, V143G, V192G, V203C, V203D, V203E, V203M, V203R, V270G, W241L, Y214H, Y214Q, A001E, A133E, A187L, A187N, A216C, A216H, A273Q, D099H, D259H, E156C, E195G, F189H, G131C, G146A, G166V, G215C, G215E, I107L, K012A, K012S, K012T, K043C, K170C, K256C, K256E, K265G, K265Y, L233E, M222F, M222S, N062Q, N076E, N078E, N184P, N218R, P005V, P014D, Q002K, Q002L, Q002R, Q010W, Q271C, R186H, S049C, S063C, S063D, S105T, S188C, S190C, S204E, T055R, T164G, V004D, V044T, V045I, V165C, V180S, Y006C, Y006D, Y006E, Y104T, A001C, A187C, A230G, A273D, A273P, D036Q, F189G, F189L, F189R, G157T, G178A, I031F, I111M, K012F, K012L, K027T, K043R, K136G, K141G, K170Q, M222A, M222L, N062R, N117G, N269C, P005W, P129V, P239A, P239H, P239T, Q059W, Q217G, Q275A, R186A, S191G, T164A, T220A, A001P, A187F, A187W, A273R, D041C, D060G, D197T, F189A, G046D, G157P, K012C, K012E, K012W, L042C, M222T, N062C, P239G, P239N, Q217C, R186M, S049T, S089P, S125A, S173V, and V044A, wherein amino acid positions of the variant are numbered by

correspondence with amino acid positions of the SEQ ID NO:2 sequence. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one, two, three, four, five, six or more amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at 16°C and pH 8: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one amino acid substitution selected from the group consisting of A216E, L090I, A098R, A098W, A098Y, A116G, A116R, A116S, A133M, I107L, I115V, M124L, N101I, N109H, N109S, N109T, N117R, P005G, Q185L, S089V, V095A, A015Y, A029G, A098D, A098E, A098G, A098N, A098S, A098T, A098V, A114S, A114T, A116E, A116L, A116T, A116V, A133H, A133L, A133S, A137G, A137I, A137L, A137S, A137V, A138S, A144S, A144V, A176S, A176T, A187T, A216F, A216P, A216Q, A216R, A216S, A216T, A216V, A216Y, D041E, D120A, D120E, D120Q, D120R, D120S, D181S, G020A, G020S, G024A, G097A, G097D, G097S, G131Q, G160S, G166I, G211L, G215N, H039N, H238N, I111L, I111V, I122A, L075I, L075Q, L135M, L209T, L209V, L233V, L235M, L235R, L257A, M119I, N025A, N025G, N025T, N061K, N101F, N101H, N101L, N101Q, N101R, N101S, N101T, N109A, N109G, N109K, N109L, N117E, N117H, N117K, N117S, N212G, N212S, N218F, N218G, N218H, N218L, N218S, N218W, N240Q, N252M, N252R, N252S, P005T, P040A, P040G, P040T, P129D, P129S, P194S, P210R, Q019R, Q019W, Q103L, Q103W, Q185A, Q185G, Q185M, Q185R, Q185T, Q206G, Q206Y, Q217A, Q217E, Q217R, Q217S, Q217T, S003Q, S009H, S018M, S033T, S130A, S130F, S130G, S130T, S130V, S145T, S159A, S161N, S161T, S162V, S162Y, S182L, S182W, S183F, S183L, S183V, S183W, S188K, S188W, S236Q, S236T, S248L, T022H, T022K, T208C, T253H, T255V, V044I, V121I, V139C, V143H, V143Q, V143T, V143W, V143Y, Y006K, Y021A, Y104W, A001F, A001G, A001H, A001K, A001L, A001Q, A001S, A001Y, A013V, A015G, A015K, A015R, A015S, A015T, A015W, A048S, A073N, A073S, A092S, A098K, A098P, A116D, A116W, A128S, A133P, A133T, A133V, A134G, A134S, A137H, A137N, A137T, A144D, A144K, A144L, A144M, A144N, A144R, A179G, A179S, A187V, A216G, A216L, A216W, A223S, A230C, A272K, A272L, A272P, A272S, A272T, A272W, A273G, A273S, A274G, A274M, A274T, D120K, D140E, D181A, D181E, D181G, D181H, D181T, D259E, D259N, D259Q, E054D,

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The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one amino acid substitution selected from the group consisting of A001D, A001M, A001N, A001R, A001T, A001V, A013C, A013G, A013S, A015D, A015F, A015L, A015M, A015P, A015Q, A074G, A074S, A085S, A085T, A085V, A088C, A088L, A088S, A088T, A088V, A133E, A133G, A133R, A137E, A144G, A144H, A144Q, A144T, A151C, A152S, A153G, A153S, A153V, A176C, A187G, A187Q, A187S, A200G, A228S, A228T, A230T, A230V, A231C, A231V, A232C, A232V, A272E, A272G, A272I, A272Q, A272R, A273L, A273V, A274L, A274Q, A274R, A274V, D036E, D259A, D259G, D259P, D259S, D259T, E156A, E156S, E156V, E195G, E251C, E251I, E251Q, F189Y, F261A, F261G, F261H, F261L, F261P, F261S, F261T, F261V, F261W, G020C, G020D, G020E, G020L, G020R, G024D, G024I, G024N, G024P, G024S, G053A, G053D, G053F, G053H, G053K, G053N, G053S, G053Y, G157A, G157S, G160A, G160L, G160P, G160R, G166A, G166L, G166V, G166W, G169A, G211E, G211P, G215A, G215D, G215E, G215H, G215V, G258D, G258S, H017F, H017I, H017L, H017M, H017V, H039C, H226S, H226Y, I011T, I011V, I031C, I031L, I031V, I079F, I079K, I079L, I079M, I079Q, I079R, I079T, I079V, I079W, I115L, I205A, I205C, I268L, K012R, K012T, K027R, K043A, K043E, K043F, K043H, K043I, K043M, K043N, K043Q, K043T, K043V, K043Y, K141H, K141Q, K141V, K170S, K213G, K213H, K213I, K213L, K213N, K213Q, K213T, K213W, K237I, K237N, K237R, K237T, K256C, K256E, K256S, K256T, K256V, K256W, K265H, K265R, L016E, L016F, L016I, L016S, L042I, L042M, L075A, L075H, L075Y, L082K, L082Q, L082T, L196M, L196V, L209A, L209C, L209E, L209G, L235W, L257C, L257H, L257Q, L257Y, L267E,

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included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 0.9 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one amino acid substitution selected from the group consisting of A001E, A015C, A015E, A048C, A048E, A073T, A085C, A085G, A088I, A088M, A114G, A137R, A187L, A187N, A187P, A187W, A216C, A230S, A273D, A273H, A273T, D036N, D099N, D259H, E156C, E156G, E156H, E156Q, F189H, F189R, F189S, F189T, F261C, F261D, F261E, G053E, G053M, G053Q, G131C, G131D, G157N, G160V, G215C, G215L, G258A, H039A, I011L, I079E, I268M, K012A, K012G, K012H, K012N, K027N, K043C, K043D, K043G, K043L, K043W, K136G, K141G, K141L, K141M, K141N, K141R, K170C, K170Q, K213V, K256D, K265G, K265N, K265Q, K265S, L082A, L082F, L082H, L082R, L082S, L090M, L233S, L235V, L257E, L257G, L257R, L257W, M222S, N025R, N062H, N062T, N076D, N076P, N078D, N078E, N078F, N078R, N078V, N269H, N269Q, P014K, P057A, P086F, P201T, Q002D, Q002I, Q002P, Q002V, Q019L, Q019P, Q059C, Q059D, Q059E, Q185W, Q271C, Q271D, Q271E, Q271L, Q271W, Q275G, R186M, S009C, S009L, S018D, S037E, S037H, S037K, S037L, S037P, S038P, S063C, S063D, S063F, S063L, S063Y, S087L, S087N, S087R, S087Y, S089D, S089F, S089G, S089W, S105T, S125A, S130C, S159D, S159P, S163A, S182P, S183P, S190A, S190G, S204E, S224G, S248E, S248H, S249E, S260V, S260Y, T055M, T055R, T055W, T158D, T158E, T164G, T164K, T164Q, T220A, T242G, T253E, T255C, T255G, V004D, V044L, V044P, V045C, V045G, V045L, V045N, V045R, V045V, V081A, V081G, V081H, V084S, V147A, V203D, V203G, V270C, V270P, V270S, W241L, Y104T, Y214Q, Y262D, Y262E, Y262G, Y262H, Y262L, Y262N, Y263G, and Y263W, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), and/or a PI value of about 0.9 relative to BPN'-v36 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one, two, three, four, five, six or more amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID

NO:6) comprising at least one amino acid substitution selected from the group consisting of A001C, A142C, A187C, A216H, A273Q, A274H, D036Q, D036S, D099S, D197T, E156L, F189A, F189L, G053L, G053R, G157P, G178A, G258P, H039S, H238Y, K012C, K012E, K012L, K012W, K136E, K265Y, L075G, L075V, L082E, L126W, L257D, L257P, M050L, M222A, M222F, M222L, N056S, N062C, N062L, N062Y, N269C, P057W, Q002K, Q002L, Q217C, Q245A, Q245H, S018C, S038Y, S049C, S087C, S087K, S145D, S191G, T022P, T055E, T164A, T164R, V045K, V051H, V081R, V143G, V148L, V180S, V203S, V270G, Y214H, A187F, A273P, F189G, G046D, G146A, G157T, I031F, I175L, K012F, K027T, L042F, L233E, L233G, M222T, N062R, N184P, P005V, P005W, P129V, P239N, P239T, Q010W, Q059W, Q275A, V004T, V165C, A128H, A230G, D041C, H067T, K027S, K043R, L090T, N062Q, N117G, P225G, P225S, P239G, P239H, Q002R, S089E, V044A, V045I, A001P, A273R, D041N, D099A, D099H, D099Q, F058G, I111M, L042C, N118L, P239A, S049N, S089P, S173V, T242P, V044T, and V045T, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value of equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one, two, three, four, five, six or more amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 8

Cleaning Performance of Additional Combinatorial Variants Based on BPN'-v36 Parent

Additional combinatorial variants based on parent BPN'-v36 (BPN'-S24G-S53G-S78N-S101N-G128A-Y217Q) were made and provided by DNA 2.0. These variants were tested for their cleaning performance using BMI microswatch assay in Detergent Composition 4 at 16°C and pH 8, BMI microswatch assay in Detergent Composition 4 at 16°C and pH 7, Egg microswatch assay in Detergent Composition 4 at 16°C and pH 8, and Grass microswatch assay in Detergent Composition 4 at 16°C and pH 8. Protein content was determined using TCA assay and protease activity was assayed using AAPF assay. All assays were performed as described in Example 1 and the Performance Indices were calculated relative to BPN'-v36 (with a PI value of 1.0).

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to

about 5 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T-L257G, A116T-A128S, N061S-N109G-A128S-N243V-S260P, S009T-N109G-A128S-K141R-N243V, S009T-S018T-Y021N-N109G-A128S-K141R, and S162G-K256R, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T, A088T-A116T, A088T-G131H, A088T-K256R, A088T-N109G, A088T-N243V, A088T-Q103H, A088T-S162G, A088T-S248N, A088T-S249A, A088T-T158S, A116T, A116T-G131H, A116T-K256R, A116T-L257G, A116T-N243V, A116T-S162G, A116T-S248N, A116T-S249A, A116T-T158S, A128S-K256R, A128S-L257G, A128S-N243V-S248N-K256R, A128S-S162G, A128S-S248N, A128S-S249A, A128S-T158S, G024E-A116T, G024E-K256R, G024E-L257G, G024E-N109G, G024E-N243V, G024E-T158S, G131H, G131H-K256R, G131H-L257G, G131H-N243V-K256R, G131H-S162G, G131H-S248N, G131H-S249A, G131H-T158S, K043Y-A088T, K043Y-K256R, K043Y-N243V, K256R, K256R-L257G, L257G, N061G-N109G-N243V, N061P-N109G-G131H-N243V, N061P-N109G-N243V, N061S-A128S-N243V-S260P, N061S-N109G-A128S-N243V-S248N-K256R-S260P, N061S-N109G-A128S-S260P, N061S-N109G-N243V, N076D-K256R, N076D-L257G, N076D-N109G, N076D-T158S, N109A-A128S-N243V-K256R, N109G, N109G-A116T, N109G-A128S, N109G-A128S-G131H-N243V-S248N-K256R, N109G-A128S-N243V-K256R, N109G-A128S-N243V-S248A, N109G-A128S-N243V-S248A-K256R, N109G-A128S-N243V-S248N, N109G-A128S-N243V-S248N-K256R, N109G-A128S-N243V-S248N-K256R-L257G, N109G-A128S-S162G-N243V-S248N-K256R, N109G-A128S-S248N-K256R, N109G-A128S-T158S-N243V-S248N-K256R, N109G-G131H, N109G-K256R, N109G-L257G, N109G-N218S, N109G-N243P-S248A-K256R, N109G-N243P-S248N-K256R, N109G-N243V, N109G-N243V-K256R, N109G-N243V-S248A-K256R, N109G-N243V-S248N, N109G-N243V-S248N-K256R, N109G-S162G, N109G-S248N-K256R, N109G-S249A, N109G-T158S, N109Q-A128S-

N243V-K256R, N109S-A128S-N243V-K256R, N218S-N243V, N243V, N243V-K256R, N243V-L257G, N243V-S248N, N243V-S248N-K256R, N243V-S249A, P040A-N109G-A128S-N243V-S248N-K256R, Q103H-A116T, Q103H-A128S, Q103H-G131H, Q103H-K256R, Q103H-L257G, Q103H-N109G, Q103H-N218S, Q103H-N243V, Q103H-S162G, Q103H-S248N, Q103H-S249A, Q103H-T158S, S009T-A128S-K141R-N243V, S009T-N109G-A128S-K141R, S009T-N109G-A128S-K141R-N243V-S248N-K256R, S009T-S018T-Y021N-A128S-K141R-N243V, S018T-Y021N-A128S-N243V, S018T-Y021N-N061S-A128S-N243V-S260P, S018T-Y021N-N061S-N109G-A128S-S260P, S018T-Y021N-N109G-A128S, S018T-Y021N-N109G-A128S-N243V, S018T-Y021N-N109G-A128S-N243V-S248N-K256R, S033T-N109G-A128S-N243P-S248N-K256R, S033T-N243V, S033T-Q103H, S033T-T158S, S063G, S063G-A088T, S063G-A128S, S063G-K256R, S063G-L257G, S063G-N076D, S063G-N109G, S063G-Q103H, S063G-S162G, S063G-S248N, S063G-T158S, S162G, S162G-L257G, S162G-N243V, S162G-S248N, S248N, S248N-L257G, S249A, T158S, T158S-L257G, T158S-N218S, T158S-N243V, T158S-S248N, and T158S-S249A, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and a PI value of about 1.0 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E-A088T, A001E-A116T, A001E-A128S-G131H-N243V, A001E-G131H-G169A-N243V, A001E-K256R, A001E-N109G, A001E-N243V, A001E-S033T, A001E-S033T-N109G-N218S, A001E-S033T-N109G-N243V, A001E-S162G, A001E-T158S, A088T-A128S, A088T-G169A, A088T-N218S, A088T-Q206D, A116T-G169A, A116T-N218S, A116T-Q206D, A128S, A128S-G131H, A128S-G169A, A128S-N218S, A128S-N243V, A128S-Q206D, G024E, G024E-A088T, G024E-A128S, G024E-G131H, G024E-K043Y, G024E-N218S, G024E-Q103H, G024E-S033T, G024E-S063G, G024E-S162G, G024E-S248N, G024E-S249A, G131H-G169A, G131H-N218S, G131H-N243V, G131H-Q206D, G169A, G169A-K256R, G169A-L257G, G169A-N218S, G169A-N243V, G169A-Q206D, G169A-S248N, G169A-S249A, K043Y, K043Y-A116T, K043Y-A128S, K043Y-G131H, K043Y-G169A, K043Y-L257G, K043Y-N109G, K043Y-

N218S, K043Y-Q103H, K043Y-S063G, K043Y-S162G, K043Y-S248N, K043Y-S249A, K043Y-T158S, N076D, N076D-A088T, N076D-A128S, N076D-G131H, N076D-N218S, N076D-N243V, N076D-Q103H, N076D-S162G, N076D-S248N, N076D-S249A, N109G-G169A, N109G-Q206D, N109G-S248N, N218S, N218S-K256R, N218S-L257G, N218S-S248N, N218S-S249A, P040E-N109G-A128S-G131H, Q103H, Q103H-G169A, Q206D, Q206D-K256R, Q206D-L257G, Q206D-N218S, Q206D-N243V, Q206D-S248N, Q206D-S249A, S018T-Y021N-S033T-N109G-A128S-N243V-S248N-K256R, S033T, S033T-A088T, S033T-A116T, S033T-A128S, S033T-A128S-G131H-N243P, S033T-G131H, S033T-K043Y, S033T-K256R, S033T-L257G, S033T-N076D, S033T-N076D-A128S-N218S, S033T-N076D-N109G-A128S-N218S-N243V-S248N-K256R, S033T-N109G, S033T-N109G-A128S-N243V-S248N-K256R, S033T-N218S, S033T-P040E-Q103H-N109G, S033T-Q103H-A128S-G131H, S033T-Q206D, S033T-S063G, S033T-S162G, S033T-S248N, S033T-S249A, S063G-A116T, S063G-G131H, S063G-G169A, S063G-N109G-A128S-G131H, S063G-N218S, S063G-N243V, S063G-Q206D, S063G-S249A, S162G-G169A, S162G-N218S, S162G-Q206D, S162G-S249A, S248N-K256R, S248N-S249A, S249A-K256R, S249A-L257G, T158S-G169A, T158S-K256R, T158S-Q206D, and T158S-S162G, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), and/or a PI value of about 0.9 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E, A001E-A128S, A001E-G024E, A001E-G131H, A001E-G169A, A001E-L257G, A001E-N218S, A001E-Q103H, A001E-S063G, A001E-S248N, A001E-S249A, G024E-N076D, K043Y-N076D, K043Y-Q206D, N076D-A116T, N076D-G169A, N076D-Q206D, Q103H-Q206D, S033T-G169A, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243V, A001E-K043Y, A001E-N076D, A001E-N076D-N109G-A128S, A001E-Q206D, and G024E-Q206D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity.

The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A116T, A088T-N243V, G024E-A116T, K043Y, N076D-A116T, N218S-S248N, S033T-N243V, S033T-S063G, S248N-L257G, A001E-S249A, A088T-A116T, A088T-A128S, A088T-G131H, A088T-L257G, A088T-N109G, A088T-S248N, A088T-S249A, A116T-N243V, A116T-T158S, A128S, A128S-K256R, A128S-L257G, A128S-N243V, A128S-S248N, A128S-T158S, G024E-A088T, G024E-A128S, G024E-G131H, G024E-K256R, G024E-L257G, G024E-N218S, G024E-N243V, G024E-S162G, G024E-S249A, G024E-T158S, G131H, G131H-K256R, G131H-S249A, K043Y-A088T, K043Y-A116T, K256R, N076D-K256R, N109G, N109G-A116T, N109G-A128S, N109G-A128S-N243V-K256R, N109G-A128S-N243V-S248A, N109G-G131H, N109G-K256R, N109G-L257G, N109G-N218S, N109G-N243V, N109G-S248N, N218S-L257G, N243V, N243V-K256R, N243V-L257G, N243V-S248N, N243V-S249A, Q103H-A128S, Q103H-G131H, Q103H-K256R, Q103H-L257G, Q103H-N243V, Q103H-S248N, Q103H-S249A, Q103H-T158S, Q206D-N243V, S033T-A128S, S033T-K256R, S033T-N076D, S033T-N218S, S033T-S248N, S033T-T158S, S063G-A128S, S063G-K256R, S063G-N243V, S063G-S162G, S063G-T158S, S162G-K256R, S248N-K256R, S249A, T158S-N243V, and T158S-S249A, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said

group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E-A128S, A001E-G131H, A001E-K256R, A001E-N218S, A001E-N243V, A001E-S033T, A001E-S063G, A001E-S162G, A088T, A088T-K256R, A088T-N218S, A088T-Q103H, A088T-S162G, A088T-T158S, A116T-A128S, A116T-G131H, A116T-K256R, A116T-L257G, A116T-S162G, A116T-S248N, A116T-S249A, A128S-G169A, A128S-N218S, A128S-S162G, A128S-S249A, G024E, G024E-N109G, G024E-Q103H, G024E-S033T, G024E-S063G, G024E-S248N, G131H-L257G, G131H-N243V, G131H-S162G, G131H-T158S, G169A, G169A-L257G, G169A-S248N, K043Y-A128S, K043Y-G131H, K043Y-K256R, K043Y-L257G, K043Y-N109G, K043Y-N243V, K043Y-Q103H, K043Y-S063G, K043Y-S162G, K043Y-S248N, K043Y-S249A, K043Y-T158S, K256R-L257G, L257G, N061G-N109G-N243V, N061S-A128S-N243V-S260P, N061S-N109G-A128S-N243V-S260P, N061S-N109G-A128S-S260P, N076D-A088T, N076D-A128S, N076D-G169A, N076D-N218S, N076D-N243V, N076D-S162G, N076D-S248N, N076D-T158S, N109A-A128S-N243V-K256R, N109G-A128S-G131H-N243V-S248N-K256R, N109G-A128S-N243V-S248A-K256R, N109G-A128S-N243V-S248N-K256R-L257G, N109G-A128S-S162G-N243V-S248N-K256R, N109G-A128S-T158S-N243V-S248N-K256R, N109G-Q206D, N109G-S162G, N109G-S249A, N109G-T158S, N109Q-A128S-N243V-K256R, N109S-A128S-N243V-K256R, N218S, N218S-K256R, N218S-N243V, P040A-N109G-A128S-N243V-S248N-K256R, Q103H, Q103H-A116T, Q103H-G169A, Q103H-N109G, Q103H-N218S, Q103H-S162G, S009T-A128S-K141R-N243V, S009T-N109G-A128S-K141R, S009T-N109G-A128S-K141R-N243V, S009T-S018T-Y021N-N109G-A128S-K141R, S018T-Y021N-N109G-A128S, S018T-Y021N-N109G-A128S-N243V, S018T-Y021N-N109G-A128S-N243V-S248N-K256R, S033T-A088T, S033T-A116T, S033T-G131H, S033T-K043Y, S033T-L257G, S033T-N109G, S033T-Q103H, S033T-Q206D, S033T-S162G, S033T-S249A, S063G, S063G-A088T, S063G-A116T, S063G-L257G, S063G-N076D, S063G-N109G, S063G-N218S, S063G-Q103H, S063G-S248N, S063G-S249A, S162G, S162G-G169A, S162G-L257G, S162G-N218S, S162G-N243V, S162G-S248N, S162G-S249A, S248N, S248N-S249A, S249A-K256R, S249A-L257G, T158S, T158S-G169A, T158S-K256R, T158S-L257G, T158S-N218S, and T158S-S248N, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' protease (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of 1.0 relative to BPN'-v3, and a PI value of about 1.0 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant

comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E, A001E-A088T, A001E-A116T, A001E-G169A, A001E-L257G, A001E-N109G, A001E-S033T-N109G-N243V, A001E-T158S, A088T-G169A, A088T-Q206D, A116T-N218S, A128S-G131H, A128S-N243V-S248N-K256R, A128S-Q206D, G024E-K043Y, G024E-N076D, G024E-Q206D, G131H-G169A, G131H-N218S, G131H-N243V-K256R, G131H-Q206D, G131H-S248N, G169A-K256R, G169A-N218S, G169A-N243V, G169A-Q206D, K043Y-N076D, K043Y-N218S, N061P-N109G-G131H-N243V, N061P-N109G-N243V, N061S-N109G-A128S-N243V-S248N-K256R-S260P, N061S-N109G-N243V, N076D, N076D-G131H, N076D-L257G, N076D-N109G, N076D-Q103H, N076D-S249A, N109G-A128S-N243V-S248N, N109G-A128S-N243V-S248N-K256R, N109G-A128S-S248N-K256R, N109G-N243P-S248A-K256R, N109G-N243P-S248N-K256R, N109G-N243V-K256R, N109G-N243V-S248A-K256R, N109G-N243V-S248N, N109G-N243V-S248N-K256R, N109G-S248N-K256R, N218S-S249A, N243V-S248N-K256R, Q103H-Q206D, Q206D, Q206D-K256R, Q206D-N218S, Q206D-S248N, Q206D-S249A, S009T-N109G-A128S-K141R-N243V-S248N-K256R, S009T-S018T-Y021N-A128S-K141R-N243V, S018T-Y021N-A128S-N243V, S018T-Y021N-N061S-A128S-N243V-S260P, S018T-Y021N-N061S-N109G-A128S-S260P, S018T-Y021N-S033T-N109G-A128S-N243V-S248N-K256R, S033T, S033T-G169A, S033T-N076D-A128S-N218S, S033T-N076D-N109G-A128S-N218S-N243V-S248N-K256R, S033T-N109G-A128S-N243P-S248N-K256R, S033T-N109G-A128S-N243V-S248N-K256R, S063G-G131H, S063G-G169A, S162G-Q206D, and T158S-S162G, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value of about 0.9 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such

variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E-A128S-G131H-N243V, A001E-G024E, A001E-G131H-G169A-N243V, A001E-Q103H, A001E-S033T-N109G-N218S, A001E-S248N, A116T-G169A, A116T-Q206D, G169A-S249A, K043Y-G169A, N109G-G169A, P040E-N109G-A128S-G131H, Q206D-L257G, S033T-A128S-G131H-N243P, S033T-A128S-G131H-N243V, S033T-P040E-Q103H-N109G, S033T-Q103H-A128S-G131H, S063G-N109G-A128S-G131H, S063G-Q206D, T158S-Q206D, A001E-K043Y, A001E-N076D, A001E-Q206D, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243V, A001E-N076D-N109G-A128S, K043Y-Q206D, and N076D-Q206D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T-L257G, G024E-K256R, G024E-L257G, N109G-A116T, N109G-L257G, N243V-K256R, S033T-N109G, S033T-T158S, S063G-L257G, A001E-L257G, A088T-A128S, A088T-G169A, A088T-K256R, A088T-N109G, A088T-N218S, A088T-N243V, A088T-S248N, A088T-T158S, A116T, A116T-A128S, A116T-G131H, A116T-K256R, A116T-L257G, A116T-N218S, A116T-S162G, A116T-T158S, A128S, A128S-G169A, A128S-K256R, A128S-L257G, A128S-N218S, G024E, G024E-A128S, G024E-G131H, G024E-N109G, G024E-N243V, G024E-S033T, G024E-S063G, G024E-S248N, G024E-S249A, G024E-T158S, G131H, G131H-G169A, G131H-K256R, G131H-N218S, G131H-S249A, G169A, G169A-L257G, G169A-N243V, K043Y-A088T, K043Y-N109G, K256R, K256R-L257G, N061G-N109G-

N243V, N076D-N109G, N109G, N109G-A128S, N109G-G131H, N109G-K256R, N109G-N218S, N109G-S162G, N109G-S248N, N109G-S249A, N109G-T158S, N218S, N218S-K256R, N218S-L257G, N218S-S248N, N243V, N243V-L257G, N243V-S248N, N243V-S249A, P040A-N109G-A128S-N243V-S248N-K256R, Q103H-K256R, Q103H-L257G, Q103H-N109G, S009T-S018T-Y021N-N109G-A128S-K141R, S033T-A088T, S033T-A116T, S033T-A128S, S033T-G131H, S033T-K043Y, S033T-K256R, S033T-L257G, S033T-N076D, S033T-N218S, S033T-N243V, S033T-Q103H, S033T-S063G, S033T-S162G, S033T-S248N, S033T-S249A, S063G, S063G-A088T, S063G-A116T, S063G-A128S, S063G-G131H, S063G-K256R, S063G-N109G, S063G-N218S, S063G-N243V, S063G-S248N, S063G-S249A, S063G-T158S, S162G-K256R, S162G-N218S, S162G-N243V, S162G-S248N, S162G-S249A, S248N, S249A, S249A-L257G, T158S, T158S-L257G, and T158S-N243V, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E, A001E-A116T, A001E-G131H, A001E-G169A, A001E-K256R, A001E-N109G, A001E-S033T-N109G-N243V, A001E-S063G, A001E-S248N, A001E-S249A, A001E-T158S, A088T, A088T-A116T, A088T-G131H, A088T-Q103H, A088T-Q206D, A088T-S162G, A088T-S249A, A116T-G169A, A116T-N243V, A116T-S248N, A116T-S249A, A128S-G131H, A128S-N243V, A128S-S162G, A128S-S248N, A128S-S249A, A128S-T158S, G024E-A088T, G024E-A116T, G024E-K043Y, G024E-N076D, G024E-N218S, G024E-Q103H, G024E-S162G, G131H-L257G, G131H-N243V, G131H-N243V-K256R, G131H-S162G, G131H-S248N, G131H-T158S, G169A-K256R, G169A-N218S, G169A-Q206D, G169A-S248N, G169A-S249A, K043Y, K043Y-A116T, K043Y-A128S, K043Y-G169A, K043Y-K256R, K043Y-L257G, K043Y-N076D, K043Y-N218S, K043Y-N243V, K043Y-S063G, K043Y-S248N, K043Y-S249A, K043Y-T158S, L257G, N061P-N109G-G131H-N243V, N061P-N109G-N243V, N061S-A128S-N243V-

S260P, N061S-N109G-A128S-N243V-S248N-K256R-S260P, N061S-N109G-A128S-S260P, N061S-N109G-N243V, N076D, N076D-A088T, N076D-A116T, N076D-G131H, N076D-G169A, N076D-K256R, N076D-L257G, N076D-N218S, N076D-N243V, N076D-Q103H, N076D-S249A, N076D-T158S, N109A-A128S-N243V-K256R, N109G-A128S-G131H-N243V-S248N-K256R, N109G-A128S-N243V-K256R, N109G-A128S-N243V-S248A, N109G-A128S-N243V-S248A-K256R, N109G-A128S-N243V-S248N, N109G-A128S-N243V-S248N-K256R, N109G-A128S-N243V-S248N-K256R-L257G, N109G-A128S-S162G-N243V-S248N-K256R, N109G-G169A, N109G-N243P-S248A-K256R, N109G-N243V-K256R, N109G-N243V-S248A-K256R, N109G-N243V-S248N, N109G-N243V-S248N-K256R, N109G-S248N-K256R, N109Q-A128S-N243V-K256R, N109S-A128S-N243V-K256R, N218S-N243V, N218S-S249A, Q103H, Q103H-A116T, Q103H-A128S, Q103H-G131H, Q103H-G169A, Q103H-N218S, Q103H-N243V, Q103H-S162G, Q103H-S248N, Q103H-S249A, Q103H-T158S, Q206D, Q206D-L257G, Q206D-N218S, S009T-A128S-K141R-N243V, S009T-N109G-A128S-K141R, S009T-N109G-A128S-K141R-N243V, S009T-N109G-A128S-K141R-N243V-S248N-K256R, S009T-S018T-Y021N-A128S-K141R-N243V, S018T-Y021N-A128S-N243V, S018T-Y021N-N109G-A128S, S018T-Y021N-N109G-A128S-N243V, S018T-Y021N-N109G-A128S-N243V-S248N-K256R, S018T-Y021N-S033T-N109G-A128S-N243V-S248N-K256R, S033T, S033T-A128S-G131H-N243V, S033T-G169A, S033T-N109G-A128S-N243P-S248N-K256R, S033T-N109G-A128S-N243V-S248N-K256R, S033T-Q103H-A128S-G131H, S033T-Q206D, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243V, S063G-G169A, S063G-N076D, S063G-N109G-A128S-G131H, S063G-Q103H, S063G-S162G, S162G, S162G-G169A, S162G-L257G, S248N-K256R, S248N-L257G, S248N-S249A, S249A-K256R, T158S-G169A, T158S-K256R, T158S-N218S, T158S-S162G, T158S-S248N, and T158S-S249A, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' protease (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and a PI value of 1.0 relative to BPN'-v36 in this egg microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of

A001E-A088T, A001E-A128S, A001E-A128S-G131H-N243V, A001E-G024E, A001E-G024E-S204E-Q206D, A001E-G131H-G169A-N243V, A001E-K043Y, A001E-N076D, A001E-N076D-N109G-A128S, A001E-N218S, A001E-N243V, A001E-Q103H, A001E-Q206D, A001E-S033T, A001E-S033T-N109G-N218S, A001E-S162G, A116T-Q206D, A128S-N243V-S248N-K256R, A128S-Q206D, G024E-Q206D, G131H-Q206D, K043Y-G131H, K043Y-Q103H, K043Y-Q206D, K043Y-S162G, N061S-N109G-A128S-N243V-S260P, N076D-A128S, N076D-Q206D, N076D-S162G, N076D-S248N, N109G-A128S-S248N-K256R, N109G-A128S-T158S-N243V-S248N-K256R, N109G-N243P-S248N-K256R, N109G-Q206D, N243V-S248N-K256R, P040E-N109G-A128S-G131H, Q103H-Q206D, Q206D-K256R, Q206D-N243V, Q206D-S248N, Q206D-S249A, S018T-Y021N-N061S-A128S-N243V-S260P, S018T-Y021N-N061S-N109G-A128S-S260P, S033T-A128S-G131H-N243P, S033T-N076D-A128S-N218S, S033T-N076D-N109G-A128S-N218S-N243V-S248N-K256R, S033T-P040E-Q103H-N109G, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S063G-Q206D, S162G-Q206D, and T158S-Q206D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in a grass microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of T158S-L257G, K256R, L257G, S033T-N109G, S162G-K256R, S162G-L257G, G024E-K256R, G024E-L257G, G024E-S033T, N109G-A116T, N218S-L257G, S033T-A088T, S033T-A116T, S033T-N243V, S033T-Q103H, S162G-N218S, S162G-N243V, T158S, T158S-N218S, T158S-N243V, A088T, A088T-G169A, A088T-K256R, A088T-L257G, A088T-S162G, A088T-T158S, A116T-K256R, A116T-L257G, A116T-N243V, A128S-L257G, A128S-N218S, A128S-N243V, A128S-S248N, G024E-A116T, G024E-A128S, G024E-G131H, G024E-N243V, G024E-S248N, G024E-S249A, G024E-T158S, G131H-N243V, G131H-T158S, G169A-N218S, G169A-N243V, G169A-S248N, K256R-L257G, N109G-A128S, N109G-G131H, N109G-N218S, N109G-N243V, N109G-S249A, N218S, N218S-K256R, N218S-N243V, N218S-S249A, N243V, N243V-K256R, N243V-L257G, N243V-S248N, Q103H-N109G,

Q103H-N218S, S033T-A128S, S033T-L257G, S033T-N218S, S033T-S162G, S033T-S248N, S033T-T158S, S063G-K256R, S063G-L257G, S162G, S162G-G169A, S162G-S248N, S248N, S248N-K256R, S248N-L257G, S249A, T158S-S162G, and T158S-S248N, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in a grass microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E-A088T, A001E-A116T, A088T-A128S, A088T-N243V, A088T-Q103H, A088T-S248N, A088T-S249A, A116T, A116T-G169A, A116T-N218S, A116T-S162G, A116T-S249A, A116T-T158S, A128S-G169A, A128S-K256R, A128S-S162G, A128S-S249A, A128S-T158S, G024E-A088T, G024E-K043Y, G024E-N218S, G024E-Q103H, G024E-S063G, G024E-S162G, G131H-G169A, G131H-K256R, G131H-N218S, G131H-S162G, G131H-S248N, G131H-S249A, G169A, G169A-L257G, G169A-S249A, N076D, N076D-K256R, N076D-L257G, N076D-S162G, N076D-S249A, N109G-K256R, N109G-L257G, N109G-S248N, N243V-S249A, Q103H-A116T, Q103H-G169A, Q103H-K256R, Q103H-L257G, Q103H-N243V, Q103H-S162G, S033T-G131H, S033T-G169A, S033T-K043Y, S033T-N076D, S033T-Q206D, S063G, S063G-A116T, S063G-A128S, S063G-N243V, S063G-S162G, S063G-S248N, S063G-S249A, S063G-T158S, S249A-L257G, T158S-G169A, and T158S-K256R, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of 1.0 relative to BPN'-v3, and a PI value of about 1.0 relative to BPN'-v36 in this BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions

selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a grass microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E-G169A, A001E-K256R, A001E-N109G, A001E-N218S, A088T-A116T, A088T-G131H, A088T-N109G, A088T-N218S, A116T-A128S, A116T-G131H, A116T-S248N, A128S, A128S-G131H, G024E, G024E-N109G, G131H, G131H-L257G, G169A-K256R, G169A-Q206D, K043Y, K043Y-A088T, K043Y-L257G, K043Y-N109G, N076D-A088T, N076D-G131H, N076D-G169A, N076D-N243V, N076D-T158S, N109G, N109G-S162G, N109G-T158S, N218S-S248N, Q103H-A128S, Q103H-S248N, Q103H-S249A, Q103H-T158S, Q206D-K256R, Q206D-L257G, Q206D-N218S, Q206D-N243V, Q206D-S248N, S033T, S033T-K256R, S033T-S063G, S033T-S249A, S063G-A088T, S063G-G131H, S063G-G169A, S063G-N109G, S162G-Q206D, S162G-S249A, S248N-S249A, S249A-K256R, T158S-Q206D, T158S-S249A, A001E-L257G, A001E-N243V, A001E-Q103H, A001E-S063G, A001E-S162G, A001E-T158S, G024E-N076D, G131H-Q206D, K043Y-A116T, K043Y-G169A, K043Y-K256R, K043Y-N076D, K043Y-S063G, K043Y-S162G, K043Y-S248N, K043Y-S249A, K043Y-T158S, N076D-A116T, N076D-A128S, N076D-S248N, N109G-G169A, Q103H, Q103H-G131H, Q206D-S249A, S063G-N076D, S063G-N218S, S063G-Q103H, A001E-A128S, A001E-G024E, A001E-G131H, A001E-N076D, A001E-Q206D, A001E-S033T, A001E-S248N, A001E-S249A, A088T-Q206D, A116T-Q206D, A128S-Q206D, G024E-Q206D, K043Y-A128S, K043Y-G131H, K043Y-N218S, K043Y-Q103H, N076D-N109G, N076D-N218S, N076D-Q103H, N109G-Q206D, Q103H-Q206D, Q206D, A001E, A001E-K043Y, K043Y-N243V, and S063G-Q206D, N076D-Q206D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this grass microswatch cleaning assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an AAPF proteolytic assay: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of S033T-N076D-A128S-N218S, A001E-S033T-N109G-N218S, S033T-N218S, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243V, A128S-G169A, S033T-S063G-Q103H-N109Q-A128S-G131H-G169A-N243P, S018T-Y021N-S033T-N109G-A128S-N243V-S248N-K256R, S033T-A128S-G131H-N243P, P040E-N109G-A128S-G131H, S033T-A128S, S033T-N109G-A128S-N243V-S248N-K256R, N109G-G169A, S063G-N109G-A128S-G131H, G169A, N109G-A128S-G131H-N243V-S248N-K256R, S033T-A128S-G131H-N243V, A128S-N218S, A001E-G169A, A088T-G169A, G169A-L257G, N109G-N218S, S033T-N109G-A128S-N243P-S248N-K256R, G169A-K256R, N076D-G169A, A001E-G131H-G169A-N243V, G169A-S249A, S033T-N109G, G169A-S248N, K043Y-G169A, K043Y-N218S, N218S-L257G, N218S-N243V, S063G-G169A, A001E-A128S-G131H-N243V, A001E-S033T-N109G-N243V, A088T-N218S, G024E-N218S, G024E-S033T, G169A-Q206D, N076D-N218S, S033T-L257G, S162G-G169A, A001E-N218S, A116T-N218S, G169A-N243V, N218S, P040A-N109G-A128S-N243V-S248N-K256R, S033T-N076D, A001E-S033T, A128S-G131H, N218S-S248N, S018T-Y021N-N109G-A128S, S033T-K043Y, S033T-N243V, S033T-Q206D, S063G-N218S, S162G-N218S, T158S-G169A, A116T-G169A, G131H-G169A, N061S-N109G-A128S-S260P, N109G-A128S-N243V-K256R, N109G-A128S-N243V-S248A, N109G-A128S-N243V-S248A-K256R, N109G-A128S-N243V-S248N-K256R-L257G, N218S-K256R, S009T-N109G-A128S-K141R, S009T-S018T-Y021N-N109G-A128S-K141R, S033T-A088T, S033T-S063G, S033T-S162G, T158S-N218S, A001E-N076D-N109G-A128S, N109G-A128S-N243V-S248N-K256R, N109G-A128S-S248N-K256R, S009T-N109G-A128S-K141R-N243V, S018T-Y021N-N061S-N109G-A128S-S260P, S033T-A116T, S033T-S248N, S033T-S249A, S033T-T158S, G131H-N218S, N109A-A128S-N243V-K256R, N109G-A128S, N109G-A128S-S162G-N243V-S248N-K256R, N109G-A128S-T158S-N243V-S248N-K256R, N218S-S249A, Q206D-N218S, S018T-Y021N-N109G-A128S-N243V, S018T-Y021N-N109G-A128S-N243V-S248N-K256R, S033T-K256R, A116T-A128S, N061S-N109G-A128S-N243V-S260P, N109G-A128S-N243V-S248N, S009T-N109G-A128S-K141R-N243V-S248N-K256R, G024E-A128S, N061S-N109G-A128S-N243V-S248N-K256R-S260P, N109S-A128S-N243V-K256R, S033T, S033T-G131H, A001E-A128S, A128S, A128S-L257G, A128S-Q206D, N109Q-A128S-N243V-K256R, S009T-A128S-K141R-N243V, S009T-S018T-Y021N-A128S-K141R-N243V, A088T-A128S, A128S-K256R, A128S-N243V, N061P-N109G-N243V, N061S-A128S-N243V-S260P, S018T-Y021N-A128S-N243V, A128S-N243V-S248N-K256R, A128S-S248N, A128S-S249A, N076D-A128S, S063G-A128S, A128S-S162G, A128S-T158S, S018T-Y021N-N061S-A128S-N243V-S260P, S033T-Q103H-A128S-G131H, N061S-N109G-N243V, K043Y-A128S, N061P-N109G-G131H-N243V, N109G-L257G, A001E-G024E-S204E-Q206D, A001E-L257G, A088T-N109G, G024E-N109G, K043Y-N109G,

N061G-N109G-N243V, N076D-N109G, N109G, N109G-A116T, N109G-K256R, N109G-N243V-K256R, N109G-N243V-S248A-K256R, N109G-Q206D, S063G-N109G, A001E-A116T, A001E-N109G, A001E-Q206D, A088T-A116T, A088T-N243V, A116T-L257G, G024E-A116T, G024E-L257G, G024E-N243V, G024E-Q206D, N109G-G131H, N109G-N243V, N109G-S162G, N109G-S248N, N109G-S248N-K256R, N109G-S249A, N109G-T158S, N243V-L257G, A001E-A088T, A001E-G024E, A001E-K256R, A001E-N076D, A001E-N243V, A088T, A088T-L257G, A088T-Q206D, A116T, A116T-K256R, A116T-N243V, G024E-A088T, G024E-K043Y, G024E-K256R, G024E-N076D, G024E-S162G, G024E-S248N, K043Y-A088T, K043Y-A116T, K043Y-L257G, K043Y-N243V, K043Y-Q206D, K256R-L257G, N076D-A116T, N076D-L257G, N076D-N243V, N076D-Q206D, N109G-N243V-S248N, N109G-N243V-S248N-K256R, N243V-K256R, Q206D, Q206D-L257G, Q206D-N243V, Q206D-S248N, S063G-K256R, S063G-L257G, T158S-L257G, A001E, A001E-K043Y, A001E-S162G, A001E-S248N, A001E-S249A, A001E-T158S, A088T-K256R, A088T-S162G, A088T-S248N, A088T-S249A, A116T-Q206D, A116T-S248N, A116T-S249A, G024E, G024E-G131H, G024E-S249A, G024E-T158S, G131H, G131H-K256R, G131H-L257G, K043Y-K256R, K043Y-N076D, K256R, L257G, N076D-A088T, N076D-K256R, N076D-S162G, N076D-S248N, N076D-S249A, N109G-N243P-S248A-K256R, N109G-N243P-S248N-K256R, N243V, Q206D-K256R, S033T-P040E-Q103H-N109G, S063G, S063G-A116T, S063G-Q206D, S162G-K256R, S162G-L257G, S162G-N243V, S162G-Q206D, S162G-S248N, S248N, S248N-L257G, S249A, S249A-L257G, T158S, T158S-N243V, and T158S-Q206D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in an AAPF proteolytic assay: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A001E-G131H, A001E-S063G, A088T-G131H, A088T-T158S, A116T-G131H, A116T-S162G, A116T-T158S, G024E-S063G, G131H-N243V, G131H-N243V-K256R, G131H-

Q206D, G131H-S249A, K043Y, K043Y-S063G, K043Y-S248N, K043Y-S249A, K043Y-T158S, N076D, N076D-G131H, N076D-T158S, N243V-S248N, N243V-S248N-K256R, N243V-S249A, Q103H-G169A, Q206D-S249A, S063G-N076D, S063G-N243V, S063G-S162G, S063G-S249A, S063G-T158S, S162G, S162G-S249A, S248N-K256R, S248N-S249A, S249A-K256R, T158S-K256R, T158S-S248N, and T158S-S249A, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and a PI value of 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 0.9 relative to BPN'-v36 in an AAPF proteolytic assay: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of G131H-S162G, G131H-S248N, G131H-T158S, K043Y-G131H, K043Y-S162G, S063G-A088T, S063G-G131H, S063G-S248N, T158S-S162G, Q103H-N218S, S033T-Q103H, and Q103H-A128S, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value of about 0.9 relative to BPN'-v36 in this proteolytic assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having proteolytic activity, enhanced proteolytic activity compared to BPN', or a PI value greater than that of BPN' (SEQ ID NO:2) in a BMI microswatch cleaning assay, the variant comprising an amino acid sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:6, wherein the variant comprises at least one substitution selected from the group of X001E, X009T, X018T, X021N, X024G, X033T, X040A, X043Y, X061G/P/S, X063G, X076D, X088T, X103H, X109A/G/Q/S, X116T,

X128S, X131H, X141R, X158S, X162G, X169A, X204E, X206D, X218S, X243P/V, X248A/N, X249A, X256R, X257G, and X260P, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2, and optionally wherein the variant comprises at least one substitution selected from the group of A001E, S009T, S018T, Y021N, S024G, S033T, P040A, K043Y, N061G/P/S, S063G, N076D, A088T, Q103H, N109A/G/Q/S, A116T, G128S, G131H, K141R, T158S, S162G, G169A, S204E, Q206D, N218S, N243P/V, S248A/N, S249A, K256R, L257G, and S260P, and wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

EXAMPLE 9

Construction and Cleaning Performance of Variants from a Combinatorial Library Based on BPN'-v36 Parent

A BPN' combinatorial library based on the BPN'-v36 parent molecule was made by DNA 2.0 and delivered as a ligation reaction. For efficient transformation of *B. subtilis*, DNA from the ligation reaction mixture was amplified before transformation and transformants grown as described in Example 2. The variants were tested for cleaning performance using BMI microswatch assay in Detergent Composition 4 at 16°C and pH 8 and egg microswatch assay in Detergent Composition 4 at 16°C and pH 8. Protein content was determined using the TCA assay. Assays were performed as in Example 1 and Performance Indices were calculated relative to BPN'-v36 (i.e., BPN'-S24G-S53G-S78N-S101N-G128A-Y217Q) (with a PI value of 1.0).

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T-A116T-N243V-K256R-L257G, A088T-A116T-N243V-L257G, A088T-T158S-N218S-K256R, A088T-T158S-N218S-N243V-L257G, A088T-A116T-T158S-N218S-N243V-K256R-L257G, A088T-N109G-A116T-G131H-A153S-N218S-S248N-L257G, A088T-N109G-A116T-T158S-S248N-K256R-L257G, A088T-N109G-T158S-L257G, A114S-A116T-N218S-N243V-S248N-K256R-L257G, A116T-T158S-K256R, A088T-A116T-G131H-T158S-S248N-L257G, A088T-A116T-T158S, A088T-N109G-A116T-G131H-L257G, A088T-N109G-A116T-T158S-N243V-S248N-L257G, A088T-N109G-N243V-L257G, A088T-N109G-N243V-S248N, A088T-N109G-T158S-N243V-L257G, A088T-N109G-T158S-N243V-S248N-L257G, A116T-T158S-S248N-L257G, Y006H-A116T-G131H-S248N, A088T-A116T-G131H-T158S-N218S-N243V, A088T-A116T-G131H-T158S-N243V, A088T-A116T-G131H-T158S-N243V-

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G131H-T158S-N218S-N243V-K256R, G131H-T158S-N243V-K256R-L257G, G131H-T158S-N243V-S248N-L257G, N109G-A116T-G131H-N218S-K256R-L257G, N109G-A116T-G131H-N218S-L257G, N109G-A116T-G131H-N218S-N243V-K256R-L257G, N109G-A116T-G131H-N218S-S248N-K256R, N109G-A116T-G131H-N243V-K256R, N109G-A116T-G131H-N243V-L257G, N109G-A116T-G131H-N243V-S248N-K256R-L257G, N109G-A116T-G131H-S248N, N109G-A116T-G131H-S248N-I268V, N109G-A116T-G131H-T158S-N218S-N243V-S248N-K256R, N109G-A116T-G131H-T158S-N218S-S248N-L257G, N109G-A116T-G131H-T158S-S248N, N109G-A116T-G131H-T158S-S248N-K256R, N109G-A116T-N218S, N109G-A116T-N218S-N243V-K256R, N109G-A116T-N218S-N243V-K256R-L257G, N109G-A116T-N218S-S248N-L257G, N109G-A116T-N243V-K256R, N109G-A116T-N243V-S248N-K256R-L257G, N109G-A116T-S248N-L257G, N109G-A116T-T158S-G211V-N243V-S248N-K256R, N109G-A116T-T158S-K256R-L257G, N109G-A116T-T158S-N218S, N109G-A116T-T158S-N218S-N243V-K256R-L257G, N109G-A116T-T158S-N218S-N243V-L257G, N109G-A116T-T158S-N218S-N243V-S248N-L257G, N109G-A116T-T158S-N218S-S248N-K256R-L257G, N109G-A116T-T158S-N243V, N109G-A116T-T158S-Q275R, N109G-G131H-A137V-T158S-N218S-S248N, N109G-G131H-N218S-K237N, N109G-G131H-N218S-N243V-K256R-L257G, N109G-G131H-N218S-S248N-K256R, N109G-G131H-N243V-K256R-L257G, N109G-G131H-S145F-N218S-N243V-K256R-L257G, N109G-G131H-S248N-K256R, N109G-G131H-S248N-L257G, N109G-G131H-T158S-K256R, N109G-G131H-T158S-N218S-N243V-K256R, N109G-G131H-T158S-N243V, N109G-G131H-T158S-N243V-K256R-L257G, N109G-G131H-T158S-N243V-L257G, N109G-G131H-T158S-S248N-L257G, N109G-G131H-T158S-S248N-Q271R, N109G-N218S-L257G, N109G-N218S-N243V, N109G-N243V-K256R-L257G, N109G-N243V-S248N-K256R-L257G, N109G-T158S-I268V, N109G-T158S-K256R, N109G-T158S-N218S-N243V-K256R-L257G, N109G-T158S-N218S-S248N-L257G, N109G-T158S-N243V, N109G-T158S-N243V-K256R-L257G, N109G-T158S-N243V-S248N, N109S-A116T-S248N, N218S, N218S-N243V-S248N-K256R-L257G, N218S-S248N-L257G, N243V, N243V-K256R, N243V-S248N-K256R, N243V-S248N-K256R-L257G, S105P-A116T-T158S-N218S-N243V-S248N-K256R, S248N, T158S-N243V-K256R, and T158S-N243V-L257G, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such

variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T, A088T-A116T-G131H-L257G, A088T-A116T-G131H-N218S-A274T, A088T-A116T-G131H-N218S-K256R, A088T-A116T-G131H-N218S-K256R-L257G, A088T-A116T-G131H-N218S-N243V, A088T-A116T-G131H-N218S-N243V-L257G, A088T-A116T-G131H-N218S-N243V-S248N-K256R, A088T-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-A116T-G131H-N218S-N243V-S248N-L257G, A088T-A116T-G131H-N218S-S248N, A088T-A116T-G131H-N218S-S248N-L257G, A088T-A116T-G131H-N243V, A088T-A116T-G131H-N243V-K256R, A088T-A116T-G131H-N243V-S248N, A088T-A116T-G131H-N243V-S248N-A274V, A088T-A116T-G131H-N243V-S248N-K256R-L257G, A088T-A116T-G131H-N243V-S248N-K256R-L257G, A088T-A116T-G131H-N243V-S248N-L257G, A088T-A116T-G131H-S248N-K256R, A088T-A116T-G131H-S248N-K256R-L257G, A088T-A116T-G131H-T158S-K256R, A088T-A116T-G131H-T158S-K256R-L257G, A088T-A116T-G131H-T158S-N218S-K256R, A088T-A116T-G131H-T158S-N218S-K256R-L257G, A088T-A116T-G131H-T158S-N218S-L257G, A088T-A116T-G131H-T158S-N218S-N243V-K256R, A088T-A116T-G131H-T158S-N218S-N243V-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-A116T-G131H-T158S-N243V-K256R, A088T-A116T-G131H-T158S-N243V-K256R-L257G, A088T-A116T-G131H-T158S-N243V-L257G, A088T-A116T-G131H-T158S-N243V-S248N, A088T-A116T-G131H-T158S-N243V-S248N-K256R-L257G, A088T-A116T-G131H-T158S-N243V-S248N-K256R-L257G, A088T-A116T-G131H-T158S-N243V-S248N-L257G, A088T-A116T-G131H-T158S-S248N-K256R, A088T-A116T-G131H-T158S-S248N-K256R-L257G, A088T-A116T-K256R, A088T-A116T-L257G, A088T-A116T-N218S, A088T-A116T-N218S-N243V-K256R, A088T-A116T-N218S-N243V-N269D, A088T-A116T-N218S-N243V-S248N, A088T-A116T-N218S-N243V-S248N-K256R-L257G, A088T-A116T-N218S-N243V-S248N-L257G, A088T-A116T-N218S-S248N, A088T-A116T-N218S-S248N-L257G, A088T-A116T-N243V-K256R-L257G, A088T-A116T-N243V-S248N-K256R, A088T-A116T-S248N, A088T-A116T-S248N-K256R, A088T-A116T-T158S-A216S-N218S-N243V-K256R-L257G, A088T-A116T-T158S-N218S-K256R, A088T-A116T-T158S-N218S-N243V-K256R, A088T-A116T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-A116T-T158S-N218S-S248N, A088T-A116T-T158S-N243V-K256R, A088T-A116T-T158S-N243V-S248N, A088T-A116T-T158S-N243V-S248N-K256R, A088T-A116T-T158S-N243V-S248N-L257G, A088T-A116T-T158S-S248N, A088T-G131D-T158S-N243V-S248N, A088T-G131H-A138V-N218S-L257G, A088T-G131H-K256R, A088T-G131H-N218S-N243V-K256R, A088T-G131H-N218S-N243V-K256R, A088T-G131H-N218S-S248N, A088T-G131H-

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N243V-L257G, N218S-N243V-S248N-K256R, N243V-K256R-L257G, N243V-S248N-L257G-Q271R, P057Q-A088T-N109G-A116T-G131H-T158S-N218S-S248N, S003P-A116T-N218S-K256R, S003P-N109G-G131H-N218S-N243V-S248N-K256R-L257G, S248N-K256R-L257G, T158S-K256R-L257G, T158S-N218S-A272V, T158S-N218S-K256R-L257G, T158S-N218S-L233S, T158S-N218S-N243V, T158S-N218S-N243V-K256R-L257G, T158S-N218S-N243V-L257G, T158S-N218S-N243V-S248N-K256R, T158S-N218S-S248N-K256R, T158S-N243V, T158S-N243V-K256R-L257G, T158S-N243V-S248N, T158S-N243V-S248N-K256R-N269D, and V004A-N109G-A116T-T158S-N218S-S248N-L257G, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of 1.0 relative to BPN'-v3, and a PI value of about 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T-A098S-N218S-K256R, A088T-A116T-G131H-K256R, A088T-A116T-G131H-K256R-L257G-L267M, A088T-A116T-G131H-N218S-N243V-K256R, A088T-A116T-G131H-N218S-N243V-K256R-L257G, A088T-A116T-G131H-N218S-N243V-S248N, A088T-A116T-G131H-N218S-S248N, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-N243V, A088T-A116T-G131H-S248N, A088T-A116T-G131H-S248N-L257G, A088T-A116T-G131H-S248N-L257G, A088T-A116T-G131H-T158S-N218S, A088T-A116T-G131H-T158S-N218S-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-A116T-G131H-T158S-N218S-S248N, A088T-A116T-G131H-T158S-N218S-S248N-K256R, A088T-A116T-K256R-L257G, A088T-A116T-N218S-I268V, A088T-A116T-N218S-K256R, A088T-A116T-N218S-N243V-Q271R, A088T-A116T-N218S-N243V-S248N-K256R, A088T-A116T-N218S-N243V-S248N-K256R-Q275R, A088T-A116T-N218S-S248N, A088T-A116T-N218S-S248N-K256R, A088T-A116T-N243V-S248N-K256R, A088T-A116T-T158S, A088T-A116T-T158S-N218S-K256R, A088T-A116T-T158S-N218S-N243V-L257G, A088T-A116T-T158S-N218S-N243V-S248N-L257G, A088T-A116T-T158S-N218S-S248N, A088T-A116T-T158S-N218S-S248N-K256R, A088T-

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S248N-K256R, A088T-N109G-G131H-T158S-N243V-S248N-K256R, A088T-N109G-G131H-T158S-S248N-K256R, A088T-N109G-G131H-T158S-S248N-K256R-L257G, A088T-N109G-G131H-V149A-K256R-L257G, A088T-N109G-N218S-K256R-L257G, A088T-N109G-N218S-N243V-K256R, A088T-N109G-N218S-N243V-L257G, A088T-N109G-N218S-N243V-S248N, A088T-N109G-N218S-N243V-S248N-K256R-L257G, A088T-N109G-N218S-S248N-K256R, A088T-N109G-N243V-K256R, A088T-N109G-N243V-S248N-K256R, A088T-N109G-S248N-K256R-L257G, A088T-N109G-T158S, A088T-N109G-T158S-K256R-L257G, A088T-N109G-T158S-N218S-N243V-K256R, A088T-N109G-T158S-N218S-N243V-K256R-L257G, A088T-N109G-T158S-N218S-N243V-S248N-K256R, A088T-N109G-T158S-N218S-N243V-S248N-L257G, A088T-N109G-T158S-N218S-S248N, A088T-N109G-T158S-S248N, A088T-N109G-T158S-S248N, A088T-N218S-N243V-K256R, A088T-N218S-N243V-L257G, A088T-N218S-N243V-S248N, A088T-N218S-N243V-S248N-K256R, A088T-N218S-N243V-S248N-K256R, A088T-N218S-S248N, A088T-N218S-S248N-L257G, A088T-S248N-K256R-L257G, A088T-T158S-K256R, A088T-T158S-N218S-N243V-K256R, A088T-T158S-N218S-N243V-L257G, A088T-T158S-N218S-N243V-S248N, A088T-T158S-N218S-N243V-S248N-K256R, A088T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-T158S-N218S-N243V-S248N-L257G, A088T-T158S-N218S-S248N, A088T-T158S-N218S-S248N-K256R, A088T-T158S-N218S-S248N-L257G, A088T-T158S-N218S-S248N-L257G-Q275K, A088T-T158S-N243V, A088T-T158S-N243V-K256R, A088T-T158S-N243V-S248N, A088T-T158S-S248N-K256R-L257G, A088T-T158S-S248N-L257G, A088T-V147I-N218S-N243V-K256R-L257G, A116T-G131H-L257G, A116T-G131H-N218S-N243V, A116T-G131H-N218S-N243V-K256R-L257G, A116T-G131H-N218S-N243V-S248N-K256R-L257G, A116T-G131H-N218S-S248N, A116T-G131H-N218S-S248N-K256R, A116T-G131H-N243V-S248N-K256R, A116T-G131H-T158S-N218S-N243V-S248N, A116T-G131H-T158S-N218S-S248N, A116T-G131H-T158S-N243V-K256R, A116T-G131H-V139I-N218S-N243V-S248N, A116T-K141E-N218S-N243V-S248N-K256R-L257G, A116T-K256R, A116T-N218S-N243V-S248N, A116T-N218T-N243V-S248N, A116T-N243V-K256R-L257G, A116T-S248N-K256R, A116T-T158S-N218S-K256R, A116T-T158S-N218S-K256R-L257G, A116T-T158S-N218S-N243V-L257G, A116T-T158S-N218S-N243V-S248N, A116T-T158S-N218S-S248N-L257G, G024S-G053S-N078S-G097A-N101S, G053S-A088T-N109G-A116T-G131H-T158S-G169S-N218S-S248N-K256R-L257G, G131H-K141R-T158S-N218S-K256R, G131H-K256R, G131H-N218S-K256R-L257G, G131H-N218S-N243V-S248N-L257G, G131H-N218S-S248N-K256R, G131H-N243V-S248N, G131H-N243V-S248N-L257G, G131H-T158S-N218S, G131H-T158S-N218S-N240H-N243V-S248N-K256R-L257G, G131H-T158S-N218S-N243V, G131H-T158S-N218S-S248N-K256R-L257G-N269S, G131H-T158S-N243V-L257G, G131H-T158S-N243V-S248N, G131H-T158S-S248N, K256R, K256R-L257G, N109G-A116T-G131H-N218S-N243V, N109G-A116T-G131H-N218S-N243V-L257G, N109G-A116T-G131H-N218S-S248N-L257G, N109G-A116T-G131H-N218S-W241R-N243V-K256R, N109G-A116T-G131H-S248N-L257G, N109G-A116T-G131H-T158S-N218S-K256R, N109G-A116T-G131H-T158S-N218S-N243V-K256R-L257G, N109G-A116T-G131H-T158S-N218S-N243V-S248N, N109G-A116T-G131H-

T158S-N218S-N243V-S248N-L257G, N109G-A116T-G131H-T158S-N243V-K256R-L257G, N109G-A116T-G131H-T158S-N243V-S248N-K256R, N109G-A116T-G131H-T158S-S248N-K256R-L257G, N109G-A116T-I234T-N243V-S248N-K256R-L257G, N109G-A116T-N218S-N243V-S248N, N109G-A116T-N243V-K256R-L257G, N109G-A116T-T158S-N218S-K237R-N243V-S248N, N109G-G131H-N218S-N243V-S248N, N109G-G131H-S248N, N109G-G131H-T158S, N109G-G131H-T158S-L257G, N109G-G131H-T158S-N218S-N243V-S248N-K256R, N109G-G131H-T158S-N218S-S248N-K256R, N109G-G131H-T158S-N218S-S248N-L257G, N109G-G131H-T158S-N243V-S248N-K256R-L257G, N109G-G131H-T158S-S248N-K256R, N109G-N218S-K256R-L257G, N109G-N218S-N243V-S248N-K256R, N109G-N218S-S248N-L257G, N109G-S248N, N109G-T158S-N218S, N109G-T158S-N218S-N243V, N109G-T158S-N243V-L257G, N218S-K256R, N218S-N243V-K256R, N218S-N243V-S248N, N218S-S248N, N218S-S248N-K256R, N243V-L257G, S003P-N109G-A116T-G131H-N218S-N243V-S248N, S003P-N109G-A116T-G131H-T158S-N218S-K256R, S105H-W106G-I107L-I108S-N109A-G110A-I111S-E112N-W113G-A114P, S248N-L257G, T158S, T158S-K256R, T158S-N218S, T158S-N218S-K256R, T158S-N218S-L233S-S248N, T158S-N218S-L257G, T158S-N218S-N243V-K256R, T158S-N218S-N243V-S248N, T158S-N218S-N243V-S248N-L257G, T158S-N218S-S248N-K256R-L257G, T158S-N218S-S248N-L257G, T158S-N243V-S248N-L257G, T158S-S248N, and V004L-A088T-G131H-T158S-N218S-S248N-L257G, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value of about 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A015S-A088T-N109G-G131H-T158S-N218S-S248N, A088T-A098S-G131H-S248N-K256R-L257G, A088T-A116T-G131H-N218S-N243V-K256R-L257G, A088T-A116T-G131H-T158S-L257G, A088T-A116T-G131H-T158S-N218S-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-A116T-N218S-L257G, A088T-A116T-T158S-K256R, A088T-A116T-T158S-S248N-K256R-L257G, A088T-G131H-K141E-N218S-N243V-S248N-L257G, A088T-G131H-K256R, A088T-G131H-N218S-K256R, A088T-G131H-N218S-N243V-S248N-K256R, A088T-G131H-N218S-N243V-S248N-L257G, A088T-G131H-N218S-

S248N-K256R, A088T-G131H-N218S-S248N-K256R, A088T-G131H-T158S-S248N-K256R, A088T-G131H-T158S-S248N-K256R-L257G, A088T-I107T-N109G-G131H-N218S-S248N-K256R, A088T-N109G-A116T-G131H-D140G-T158S-N218S-N243V-K256R, A088T-N109G-A116T-G131H-N218S-N243V-K256R, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R, A088T-N109G-A116T-G131H-T158S-N218S-S248N-L257G, A088T-N109G-A116T-G131H-T158S-N243V-S248N-K256R-I268V, A088T-N109G-A116T-G131H-V149A-N218S-S248N-K256R-L257G, A088T-N109G-A116T-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-T158S-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-L257G, A088T-N109G-D140G-N243V, A088T-N109G-G131H-D140G-T158S-N243V-S248N-K256R, A088T-N109G-G131H-K141E-T158S-N218S-K256R, A088T-N109G-G131H-N218S-S248N, A088T-N109G-G131H-N218S-S248N-K256R-Q271R, A088T-N109G-G131H-N218S-S248N-L257G, A088T-N109G-G131H-T158S-K256R, A088T-N109G-G131H-T158S-N218S-S248N-K256R, A088T-N109G-G131H-V149L-T158S-K256R-L257G, A088T-N109G-T158S-N218S, A088T-N109G-T158S-N218S-K256R-L257G-Q271K, A088T-N109G-T158S-N218S-L257G, A088T-N109G-T158S-S248N-K256R, A088T-N218S-S248N-L257G-Q271R, A088T-T158S-N218S-K256R-L257G, A088T-T158S-N218S-N243V-K256R, A088T-Y104H-A116T-G131H-N218S-N243V, A116T-G131H-K141E-N218S-N243V-S248N-L257G, A116T-G131H-N218S-N243V-S248N-K256R, A116T-G131H-T158S-N218S-S248N-L257G-N269D, A116T-G131H-T158S-N218S-S248N-Q271R, A116T-G131H-T158S-N243V-S248N, A116T-G157E-T158S-N243V-S248N-K256R, A116T-T158S-N218S, G131H-N218S-L257G, G131H-N218S-S248N, G131H-T158S-N218S-N243V-S248N-K256R-L257G, G131H-T158S-N218S-N243V-S248N-L257G, G131H-T158S-N218S-S248N-I268V, I107T-N109G-G131H-N218S-L257G, L090I-N109G-T158S-N243V, L257G, N109G-A116T-G131H-T158S-N218S-K256R-L257G-Q271R, N109G-A116T-N218S-W241R-N243V-S248N-K256R-L257G, N109G-G131H-K141E-L257G, N109G-G131H-N218S-N243V, N109G-T158S-N218S-N243V-L257G, N109G-T158S-N218S-S248N-K256R, N109G-T158S-N243V-S248N-K256R-L257G, N218S-S248N-K256R-L257G, S003P-N109G-G131H-T158S-L257G, S003P-S248N-L257G, T158S-S248N-K256R-L257G, V004A-A088T-G131H-N218S-N243V-S248N-L257G, Y006H-N218S-N243V-S248N, Y104H-N109G-G131H-N243V-S248N, A088T-A116T-T158S-N218S-N243V-S248N-K256R, A088T-A116T-T158S-N243V, A088T-G131H-T158S-N218S-I234T-S248N-L257G, A088T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-G131H-V149L-T158S-N243V-S248N-K256R-L257G, A088T-I107T-N109G-G131H-N218S-A223G-S248N-K256R, A088T-K213N-N243V-S248N-K256R, A088T-K256R-L257G, A088T-N109G-A116T-G131H-A232S-N243V-K256R, A088T-N109G-A116T-G131H-D140G-S248N-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N243V-S248N-L257G, A088T-N109G-A116T-M124I-G131H-T158S-N218S-S248N-L257G, A088T-N109G-A116T-V148A-N218S-N243V, A088T-N109G-G131H-N218S-N243V-S248N, A088T-N109G-N218S-S248N-T255K-K256R-L257G, A088T-T158S-N218S-L257G, A088T-T158S-N218S-Q245K-S248N-K256R, A088T-T158S-N218S-S248N-K256R, A116T-G131H-N218S-N243V-K256R, A116T-G131H-N218S-W241R-N243V-

S248N-K256R-L257G, A116T-G131H-T158S-N218S-L257G, A116T-G131H-V150A-T158S-N243V-S248N-K256R-L257G, I107T-G131H-T158S-N243V-S248N-K256R-L257G, N109G-A116T-K141E-T158S-N218S-N243V-L257G, N109G-A116T-T158S-N218S-N243V-S248N, T158S-N243V-S248N-K256R, T158S-N243V-S248N-K256R-L257G, A088T-A116T-G131H-G146C, A088T-A116T-N218S, A088T-A116T-T158S-N243V-K256R-L257G, A088T-A138E-N218S-N243V-K256R, A088T-N109G-A116T-G131H-T158S-N218S-N243F-S248N, A088T-T158S-V203I-N218S-K256R-L257G, A116T-D140G-T158S-N218S-N243V-S248N, A088T-A116T-T158S-K256R-L257G, A088T-A116T-T158S-N218S-N243V-S248N-E251K-K256R-L257G, A088T-I108T-N109G-G131H-T158S-N218S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-K141E-N218S, A088T-N109G-W241R-S248N-K256R, and G065D-A088T-G131H-N243V-S248N, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T-N109G-A116T-T158S-N243V-L257G, A116T-N218S-N243V-L257G-N269S, A088T-A116T-K256R, A088T-G131H-K256R, A088T-N109G-A116T-T158S-S248N-K256R-L257G, A088T-N109G-T158S-L257G, A088T-A116T-G131H-T158S-N218S-N243V-K256R-A273T, A088T-A116T-N243V-L257G, A088T-A116T-S248N-K256R-L257G, A088T-A116T-T158S-N243V-L257G, A088T-A116T-T158S-N243V-S248N-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N243V-L257G, A088T-N109G-A116T-G131H-T158S-L257G, A088T-N109G-A116T-T158S-N218S-L257G, A088T-N109G-A116T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-G131H-N218S-K256R-L257G, A088T-N109G-N218S-S248N-L257G, A088T-T158S-N218S-N243V-K256R-I268V, A088T-T158S-N218S-S248N-L257G, A116T-N218S-K256R-L257G, N109G-A116T, N109G-A116T-G131H-T158S-L257G, N109G-A116T-N243V, N109G-A116T-N243V-K256R, N109G-A116T-T158S-L257G, N109G-K256R, N109G-N243V-K256R-L257G, S003P-N109G-

G131H-T158S-K256R, A088T-A116T, A088T-A116T-G131H-N218S-K256R-L257G, A088T-A116T-G131H-N218S-L257G, A088T-A116T-G131H-N218S-N243V-S248N-L257G, A088T-A116T-G131H-N243V-K256R-L257G, A088T-A116T-G131H-N243V-S248N-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V, A088T-A116T-G131H-T158S-N218S-N243V-K256R-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N, A088T-A116T-G131H-T158S-S248N-K256R-L257G, A088T-A116T-G131H-T158S-S248N-L257G, A088T-A116T-N218S-N243V-L257G, A088T-A116T-N218S-N243V-S248N-K256R-L257G, A088T-A116T-N218S-N243V-S248N-K256R-Q275R, A088T-A116T-T158S-A216S-N218S-N243V-K256R-L257G, A088T-A116T-T158S-K256R, A088T-A116T-T158S-N218S-L257G, A088T-A116T-T158S-N218S-N243V, A088T-A116T-T158S-N218S-N243V-K256R, A088T-A116T-T158S-N218S-N243V-K256R-L257G, A088T-A116T-T158S-N218S-N243V-K256R-N269S, A088T-A116T-T158S-N243V, A088T-A116T-T158S-N243V-K256R, A088T-A116T-V147I-T158S-N218S-N243V-L257G, A088T-G131H-K256R-L257G, A088T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-G131H-S248N-K256R-L257G, A088T-G131H-T158S-N218S-L257G, A088T-G131H-T158S-N218S-N243V-L257G, A088T-I107T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-I107T-N109G-G131H-N218S-S248N-K256R, A088T-N109G-A116T-G131H-A153S-N218S-S248N-L257G, A088T-N109G-A116T-G131H-K256R-L257G, A088T-N109G-A116T-G131H-N218S-K256R-L257G, A088T-N109G-A116T-G131H-N218S-L257G, A088T-N109G-A116T-G131H-N218S-N243V-K256R, A088T-N109G-A116T-G131H-N218S-N243V-L257G, A088T-N109G-A116T-G131H-N243V-L257G, A088T-N109G-A116T-G131H-S248N-L257G, A088T-N109G-A116T-G131H-T158S-N218S, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-N109G-A116T-N218S-K256R-L257G, A088T-N109G-A116T-N218S-L257G, A088T-N109G-A116T-N218S-N243V, A088T-N109G-A116T-N218S-N243V-L257G, A088T-N109G-A116T-N218T-K256R, A088T-N109G-A116T-N218T-K256R-L257G, A088T-N109G-A116T-N243V, A088T-N109G-A116T-N243V-K256R-L257G, A088T-N109G-A116T-N243V-K256R-L257G-N269D, A088T-N109G-A116T-T158S, A088T-N109G-A116T-T158S, A088T-N109G-A116T-T158S-L257G, A088T-N109G-A116T-T158S-N218S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N218S-N243V-S248N, A088T-N109G-A116T-T158S-N243V-S248N-L257G, A088T-N109G-G131H-A138V-T158S-N218S-N243V-S248N-L257G, A088T-N109G-G131H-L257G, A088T-N109G-G131H-N218S, A088T-N109G-G131H-N218S-N243V-L257G, A088T-N109G-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-G131H-N218S-S248N-K256R-L257G, A088T-N109G-G131H-T158S-N218S-K256R, A088T-N109G-G131H-T158S-N218S-N243V, A088T-N109G-G131H-T158S-N243V-K256R-L257G, A088T-N109G-G131H-T158S-N243V-L257G, A088T-N109G-G131H-T158S-N243V-S248N-L257G, A088T-N109G-G131H-V149A-K256R-L257G, A088T-N109G-N218S-N243V-L257G, A088T-N109G-N218S-N243V-S248N-L257G, A088T-N109G-N218S-S248N-K256R-L257G, A088T-N109G-N243V, A088T-N109G-N243V-K256R-L257G, A088T-N109G-N243V-L257G, A088T-N109G-N243V-S248N-L257G, A088T-N109G-S248N-K256R-L257G, A088T-N109G-T158S-K256R, A088T-

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N218S-N243V-S248N-S260F, N109G-N218S-S248N, N109G-N243V-K256R, N109G-N243V-L257G, N109G-N243V-S248N, N109G-N243V-S248N-K256R-L257G, N109G-S182F-S204F-S207L-N218S-S236F-S248N-L257G, N109G-S248N-K256R, N109G-T158S-K256R, N109G-T158S-N218S-N243V-K256R-L257G, N109G-T158S-N218S-S248N-L257G, N109G-T158S-N243V, N109G-T158S-N243V-K256R, N109G-T158S-N243V-S248N, N109G-T158S-N243V-S248N-K256R, N109G-T158S-S248N-K256R, N109G-T158S-S248N-L257G, N218S, N218S-N243V-S248N-K256R, N218S-S248N-L257G, N243V-K256R, N243V-S248N-K256R, N243V-S248N-K256R-L257G, N243V-S248N-L257G-Q271R, S003P-A116T-T158S-S248N-K256R, S248N, T158S-N218S, T158S-N218S-A272V, T158S-N218S-L257G, T158S-N218S-N243V-K256R-L257G, T158S-N218S-N243V-L257G, T158S-N218S-S248N-K256R-L257G, T158S-N243V-K256R, T158S-N243V-K256R-L257G, T158S-N243V-S248N-K256R, V004A-N109G-A116T-G131H-S248N-K256R-L257G, and Y006H-A116T-G131H-S248N, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A015S-A088T-N109G-G131H-T158S-N218S-S248N, A088T, A088T-A116T-G131H-N218S-N243V-S248N, A088T-A116T-G131H-N218S-S248N, A088T-A116T-G131H-N218S-S248N, A088T-A116T-G131H-N218S-S248N-K256R-L257G, A088T-A116T-G131H-N243V, A088T-A116T-G131H-N243V-K256R, A088T-A116T-G131H-N243V-S248N, A088T-A116T-G131H-S248N-K256R, A088T-A116T-G131H-T158S-N218S-N243V-S248N, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-A116T-G131H-T158S-N218S-S248N, A088T-A116T-G131H-T158S-N218S-S248N-K256R, A088T-A116T-G131H-T158S-N243V, A088T-A116T-G131H-T158S-N243V-K256R, A088T-A116T-G131H-T158S-N243V-S248N, A088T-A116T-G131H-T158S-N243V-S248N-K256R-L257G, A088T-A116T-

G131H-T158S-S248N, A088T-A116T-G131H-T158S-S248N-K256R, A088T-A116T-G131H-T158S-S248N-L257G, A088T-A116T-G131H-V147A-T158S-N218S-N243V-S248N-L257G, A088T-A116T-N218S-I268V, A088T-A116T-N218S-L257G, A088T-A116T-N218S-N243V-N269D, A088T-A116T-N218S-S248N, A088T-A116T-N218S-S248N, A088T-A116T-N218S-S248N-L257G, A088T-A116T-N243V-K256R-L257G, A088T-A116T-N243V-S248N-K256R, A088T-A116T-S248N-K256R, A088T-A116T-T158S-N218S, A088T-A116T-T158S-N218S-N243V-S248N-K256R-L257G, A088T-A116T-T158S-N218S-S248N, A088T-A116T-T158S-N218S-S248N-K256R-L257G, A088T-A116T-T158S-N243V-S248N, A088T-A116T-T158S-N243V-S248N-K256R, A088T-A116T-T158S-N243V-S248N-K256R-L257G, A088T-A116T-T158S-N243V-S248N-L257G, A088T-A116T-T158S-S248N, A088T-A116T-T158S-S248N-K256R-L257G, A088T-A116T-T158S-S248N-L257G, A088T-A116T-V143A-N218S-S248N-K256R, A088T-G131H-A138V-N218S-L257G, A088T-G131H-K141E-N218S-N243V-S248N-L257G, A088T-G131H-K256R, A088T-G131H-N218S-K256R, A088T-G131H-N218S-N243V-K256R-L257G, A088T-G131H-N218S-N243V-S248N, A088T-G131H-N218S-N243V-S248N, A088T-G131H-N218S-N243V-S248N-K256R, A088T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-G131H-N218S-S248N, A088T-G131H-N218S-S248N-K256R, A088T-G131H-N243V-K256R-L257G, A088T-G131H-N243V-S248N, A088T-G131H-N243V-S248N-K256R-L257G, A088T-G131H-S248N, A088T-G131H-S248N-K256R, A088T-G131H-T158S-K256R, A088T-G131H-T158S-L257G, A088T-G131H-T158S-N218S, A088T-G131H-T158S-N218S-N243V-S248N, A088T-G131H-T158S-N218S-N243V-S248N-K256R, A088T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-G131H-T158S-N218S-N243V-S248N-L257G, A088T-G131H-T158S-N218S-S248N, A088T-G131H-T158S-N218S-S248N-K256R-L257G, A088T-G131H-T158S-N243V, A088T-G131H-T158S-N243V-K256R, A088T-G131H-T158S-N243V-S248N-K256R, A088T-G131H-T158S-N243V-S248N-L257G, A088T-G131H-T158S-S248N-K256R-L257G, A088T-G131H-T158S-S248N-L257G, A088T-I107T-N109G-G131H-N218S-A223G-S248N-K256R, A088T-L257G, A088T-L257G, A088T-N109G-A116T-G131H-A232S-N243V-K256R, A088T-N109G-A116T-G131H-K256R-L257G, A088T-N109G-A116T-G131H-L257G, A088T-N109G-A116T-G131H-N218S-K256R, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R, A088T-N109G-A116T-G131H-N218S-N243V-S248N-N269D, A088T-N109G-A116T-G131H-N243V-K256R, A088T-N109G-A116T-G131H-S248N, A088T-N109G-A116T-G131H-T158S-L257G, A088T-N109G-A116T-G131H-T158S-N218S-N243F-S248N, A088T-N109G-A116T-G131H-T158S-N218S-N243V, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-N218T-K256R, A088T-N109G-A116T-G131H-T158S-N243V, A088T-N109G-A116T-G131H-T158S-N243V-K256R, A088T-N109G-A116T-G131H-T158S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N243V-S248N-K256R-I268V, A088T-N109G-A116T-G131H-T158S-N243V-S248N-L257G, A088T-N109G-A116T-G131H-T158S-S248N, A088T-N109G-A116T-G131H-T158S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-V149A-N218S-S248N-K256R-L257G, A088T-N109G-A116T-K256R, A088T-N109G-A116T-N218S-K256R, A088T-N109G-A116T-N218S-N243V-S248N-K256R, A088T-N109G-A116T-N218S-N243V-S248N-L257G, A088T-

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T158S-N218S-N243V-K256R, A116T-G131H-T158S-N218S-N243V-L257G, A116T-G131H-T158S-N218S-N243V-S248N, A116T-G131H-T158S-N218S-S248N-L257G-N269D, A116T-G131H-T158S-N218S-S248N-Q271R, A116T-G131H-T158S-N218T-L257G, A116T-G131H-T158S-N243V-K256R, A116T-G131H-V150A-T158S-N243V-S248N-K256R-L257G, A116T-K141E-N218S-N243V-S248N-K256R-L257G, A116T-L257G, A116T-N218S-N243V-S248N, A116T-N218T-N243V-S248N, A116T-N243V-S248N-L257G, A116T-S248N, A116T-S248N-L257G, A116T-T158S-K256R, A116T-T158S-N218S-L257G, A116T-T158S-N218S-N243V-K256R, A116T-T158S-N218S-N243V-L257G, A116T-T158S-N243V, A116T-T158S-N243V-K256R-L257G, A116T-T158S-N243V-S248N-K256R, A116T-T158S-N243V-S248N-K256R-L257G, A116T-V149I-T158S-N243V-S248N-K256R-Q271H, G024S-G053S-N078S-G097A-N101S, G024S-G053S-N078S-G097A-N101S-A128S, G131H-K256R, G131H-N218S-K256R-L257G, G131H-N218S-N243V-L257G, G131H-N218S-N243V-S248N-K256R, G131H-N218S-S248N-K256R, G131H-N218S-S248N-L257G, G131H-N243V, G131H-N243V-S248N, G131H-N243V-S248N-L257G, G131H-T158S-K256R-L257G, G131H-T158S-N218S, G131H-T158S-N218S-K256R, G131H-T158S-N218S-N240H-N243V-S248N-K256R-L257G, G131H-T158S-N218S-N243V-K256R, G131H-T158S-N218S-N243V-K256R-L257G, G131H-T158S-N218S-N243V-S248N, G131H-T158S-N218S-N243V-S248N-K256R-L257G, G131H-T158S-N218S-S248N-I268V, G131H-T158S-N218S-S248N-K256R-L257G-N269S, G131H-T158S-N243V-K256R-L257G, G131H-T158S-N243V-S248N, G131H-T158S-N243V-S248N-K256R, G131H-T158S-S248N, G131H-T158S-S248N-L257G, G131H-W241L-N243V-S248N-K256R, I107T-G131H-T158S-N243V-S248N-K256R-L257G, I107T-N109G-G131H-N218S-L257G, N109G-A116T-G131H-L257G, N109G-A116T-G131H-N218S-L257G, N109G-A116T-G131H-N218S-N243V, N109G-A116T-G131H-N218S-N243V-S248N-K256R, N109G-A116T-G131H-N218S-W241R-N243V-K256R, N109G-A116T-G131H-N243V-S248N-K256R, N109G-A116T-G131H-S248N-I268V, N109G-A116T-G131H-T158S-N218S, N109G-A116T-G131H-T158S-N218S-S248N-K256R, N109G-A116T-G131H-T158S-N218S-S248N-K256R-L257G, N109G-A116T-G131H-T158S-N218S-S248N-L257G, N109G-A116T-G131H-T158S-N243V-K256R-L257G, N109G-A116T-G131H-T158S-S248N-L257G, N109G-A116T-K141E-T158S-N218S-N243V-L257G, N109G-A116T-K256R, N109G-A116T-N218S-N243V, N109G-A116T-N218S-N243V-S248N, N109G-A116T-N218S-S248N-L257G, N109G-A116T-N243V-S248N-K256R-L257G, N109G-A116T-S248N, N109G-A116T-T158S-N218S-N243V-S248N-K256R, N109G-A116T-T158S-N218S-W241R-N243V, N109G-A116T-T158S-N243V-S248N, N109G-A116T-T158S-S248N-L257G, N109G-G131H-K256R, N109G-G131H-N218S-K256R, N109G-G131H-N218S-N243V, N109G-G131H-N218S-N243V-K256R-L257G, N109G-G131H-N218S-N243V-S248N-K256R, N109G-G131H-N218S-S248N-K256R, N109G-G131H-N243V, N109G-G131H-N243V-S248N, N109G-G131H-N243V-S248N-K256R-L257G, N109G-G131H-S248N, N109G-G131H-T158S-L257G, N109G-G131H-T158S-N218S-N243V-S248N, N109G-G131H-T158S-N218S-N243V-S248N-K256R, N109G-G131H-T158S-N218S-N243V-S248N-K256R-L257G, N109G-G131H-T158S-N243V-L257G, N109G-G131H-T158S-N243V-S248N-K256R, N109G-G131H-T158S-S248N-K256R, N109G-K141E-N218S-S248N-L257G, N109G-N218S-N243V,

N109G-N218S-N243V-S248N-K256R, N109G-N218S-S248N-K256R-L257G, N109G-N218S-S248N-L257G, N109G-N243V-S248N-L257G-Q275R, N109G-T158S-I268V, N109G-T158S-N218S, N109G-T158S-N218S-N243V, N109G-T158S-N218S-N243V-S248N, N109G-T158S-N218S-S248N-K256R, N109S-A116T-S248N, N218S-K256R, N218S-N243V-K256R, N218S-N243V-L257G, N218S-N243V-S248N, N218S-S248N, N218S-S248N-K256R, N218S-S248N-K256R-L257G, S003P-N109G-G131H-N218S-N243V-S248N-K256R-L257G, S248N-K256R-L257G, T158S-K256R, T158S-K256R-L257G, T158S-N218S-K256R-L257G, T158S-N218S-L233S-S248N, T158S-N243V, T158S-N243V-S248N-K256R-N269D, T158S-N243V-S248N-L257G, V004L-A116T-N218S-N243V-S248N-L257G, and Y006H-N218S-N243V-S248N, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and a PI value of 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A088T-A098S-G131H-S248N-K256R-L257G, A088T-A116T-G131H-K256R, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-N243V-S248N-L257G, A088T-A116T-G131H-S248N, A088T-A116T-G131H-S248N-K256R-L257G, A088T-A116T-G131H-T158S-K256R, A088T-A116T-G131H-T158S-L257G, A088T-A116T-G131H-T158S-N218S-L257G, A088T-A116T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-A116T-K256R-L257G, A088T-A116T-K256R-L257G, A088T-A116T-N218S-N243V-Q271R, A088T-A116T-N218S-S248N-K256R, A088T-A116T-T158S, A088T-A116T-T158S, A088T-A116T-T158S-K256R, A088T-A116T-T158S-N218S-N243V-L257G, A088T-A116T-T158S-N218S-S248N-K256R, A088T-G131H, A088T-G131H-L257G, A088T-G131H-N218S-N243V-S248N-L257G, A088T-G131H-N243V-K256R, A088T-G131H-N243V-L257G, A088T-G131H-N243V-S248N, A088T-G131H-N243V-S248N-K256R, A088T-G131H-T158S-N218S-I234T-S248N-L257G, A088T-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-G131H-T158S-N243V-K256R-L257G, A088T-G131H-T158S-S248N, A088T-G131H-T158S-S248N-K256R, A088T-G131H-T158S-S248N-K256R-L257G, A088T-G131H-V149L-T158S-N243V-S248N-K256R-L257G,

A088T-N109G-A116T-G131H-K141E-N218S, A088T-N109G-A116T-G131H-N218S-N243V-S248N-Q275R, A088T-N109G-A116T-G131H-N218S-S248N, A088T-N109G-A116T-G131H-N243V-S248N-K256R, A088T-N109G-A116T-G131H-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-K256R, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-G131H-T158S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-S248N, A088T-N109G-A116T-G131H-V149A-T158S-N218S-K256R, A088T-N109G-A116T-N218S-S248N-K256R-L257G, A088T-N109G-A116T-N243V-K256R, A088T-N109G-A116T-N243V-S248N-L257G, A088T-N109G-A116T-S248N-L257G, A088T-N109G-A116T-T158S-N212D-N243V-K256R-L257G, A088T-N109G-A137E-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-D140G-N243V, A088T-N109G-G131H-A152S-T158S-N218S-S248N-K256R, A088T-N109G-G131H-D140G-T158S-N243V-S248N-K256R, A088T-N109G-G131H-K256R-L257G, A088T-N109G-G131H-N218S, A088T-N109G-G131H-N218S-N243V, A088T-N109G-G131H-T158S-L257G, A088T-N109G-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A088T-N109G-G131H-T158S-N218S-W241R-S248N-L257G, A088T-N109G-G131H-T158S-S248N-K256R, A088T-N109G-N218S-S248N-K256R, A088T-N109G-N243V-S248N-K256R, A088T-N109G-T158S, A088T-N109G-T158S-N243V-S248N-K256R, A088T-N109G-T158S-N243V-S248N-Q275R, A088T-N109G-T158S-S248N, A088T-N218S-N243V-S248N-K256R-L257G, A088T-N243V-L257G, A088T-S248N, A088T-T158S-K256R, A088T-T158S-N218S-K256R, A088T-T158S-N218S-L257G, A088T-T158S-N218S-L257G, A088T-T158S-N218S-N243V-S248N, A088T-T158S-N218S-N243V-S248N-L257G, A088T-T158S-N218S-Q245K-S248N-K256R, A088T-T158S-N218S-S248N-L257G-Q275K, A088T-T158S-N243V-K256R, A088T-T158S-N243V-K256R-L257G, A088T-T158S-N243V-S248N-K256R, A088T-T158S-S248N, A088T-T158S-S248N-K256R-L257G, A088T-T158S-S248N-L257G, A088T-T158S-S248N-L257G, A098S-G131H-T158S-N218S-N243V-S248N-K256R-L257G, A116T-G131H-K141E-N218S-N243V-S248N-L257G, A116T-G131H-N218S-W241R-N243V-S248N-K256R-L257G, A116T-G131H-N243V, A116T-G131H-T158S-N218S-S248N-K256R, A116T-G131H-V139I-N218S-N243V-S248N, A116T-N218S-S248N-K256R, A116T-T158S-L257G-Q271R, A116T-T158S-N218S-N243V-K256R-L257G, G053S-A088T-N109G-A116T-G131H-T158S-G169S-N218S-S248N-K256R-L257G, G131H-N218S-L257G, G131H-T158S, G131H-T158S-K256R, K256R, L090I-N109G-T158S-N243V, L257G, N109G-A116T-G131H, N109G-A116T-G131H-N243V, N109G-A116T-G131H-T158S-N218S-N243V-S248N-K256R, N109G-A116T-S248N-K256R, N109G-A116T-T158S-N218S-K237R-N243V-S248N, N109G-A116T-T158S-S248N, N109G-G131H-T158S, N109G-G131H-T158S-N243V-K256R-L257G, N109G-G131H-T158S-S248N-Q271R, N109G-N218S-S248N-K256R, N109G-N243V-S248N-L257G, N109G-S248N, N109G-T158S-N218S-N243V-L257G, N109G-T158S-N243V-K256R-L257G, N109G-T158S-N243V-S248N-K256R-L257G, N218S-N243V-S248N-K256R-L257G, S003P-N109G-A116T-G131H-T158S-N218S-K256R, S003P-N109G-G131H-T158S-L257G, S105H-W106G-I107L-I108S-N109A-G110A-I111S-E112N-W113G-A114P, T158S-N218S-S248N-K256R, T158S-N243V-S248N, T158S-S248N, T158S-S248N-K256R-L257G, V004A-A088T-A116T-T158S-N218S, V004A-A088T-G131H-N218S-N243V-S248N-L257G,

Y006H-N109G-N218S-N243V-S248N, Y104H-A116T-T158S-S248N, A088T-A116T-G131H-N218S-S248N-L257G, A088T-A116T-G131H-T158S-N218S-N243V-L257G, A088T-A116T-G131H-T158S-N243V-K256R-L257G, A088T-A116T-N218S, A088T-G131D-T158S-N243V-S248N, A088T-G131H-N218S-K237R-K256R-L257G, A088T-G131H-N218S-K256R, A088T-K213N-N243V-S248N-K256R, A088T-K256R-L257G, A088T-N109G-A116T-G131H-D140G-S248N-L257G, A088T-N109G-A116T-G131H-L257G, A088T-N109G-A116T-G131H-N218S-N243V-S248N-K256R-L257G, A088T-N109G-A116T-G131H-T158S-N218S-N243V-S248N, A088T-N109G-A116T-M124I-G131H-T158S-N218S-S248N-L257G, A088T-N109G-A116T-T158S-N218S-N243V-K256R, A088T-N109G-A116T-T158S-N218S-N243V-L257G, A088T-N109G-A116T-T158S-N218S-S248N, A088T-N109G-G131H-K256R-L257G, A088T-N109G-G131H-N218S-N243V-S248N-L257G, A088T-N109G-G131H-N243V-S248N, A088T-N109G-G131H-T158S-L233S-N243V-S248N, A088T-N109G-G131H-T158S-N218S-S248N-K256R, A088T-N109G-L257G, A088T-N109G-N218S-K256R-L257G, A088T-N109G-N218S-S248N-T255K-K256R-L257G, A088T-N109G-S248N-K256R-L257G, A088T-N109G-T158S-N218S-N243V, A088T-N109G-T158S-N218S-N243V-L257G, A088T-N109G-T158S-N218S-N243V-S248N-L257G, A088T-N109G-T158S-N218S-S248N, A088T-S248N-K256R-L257G, A088T-S248N-L257G-I268V, A088T-T158S-N243V-L257G, A088T-T158S-V203I-N218S-K256R-L257G, A088T-Y104H-A116T-G131H-N218S-N243V, A116T-D140G-T158S-N218S-N243V-S248N, A116T-G131H-T158S-K256R-L257G, A116T-G131H-T158S-N243V-S248N, A116T-G157E-T158S-N243V-S248N-K256R, A116T-S248N-K256R, G131H-N218S-S248N, G131H-N243V-K256R, G131H-T158S-N243V-L257G, K256R-L257G, N109G-A116T-G131H-T158S-N218S-N243V-S248N, N109G-A116T-N218S-N243V-L257G, N109G-A116T-N218S-W241R-N243V-S248N-K256R-L257G, N109G-A116T-T158S-N243V, N109G-A116T-T158S-S248N-K256R, N109G-N243V, N109G-T158S-K256R-L257G, N243V, P014L-A015L-L016C-H017T-S018L-Q019K-G020A-Y021T-T022L-G023E, S003P-S248N-L257G, T158S-N218S-N243V-S248N, T158S-S248N-K256R, Y104H-N109G-G131H-N243V-S248N, A088T-A116T-G131H-L257G, A088T-A116T-G131H-N218S-N243V, A088T-A116T-G131H-N218S-S248N-K256R, A088T-A116T-G131H-T158S-N243V-S248N-L257G, A088T-A116T-L257G, A088T-A116T-N218S, A088T-A116T-N218S-N243V-S248N, A088T-A116T-T158S-N218S-S248N, A088T-A116T-T158S-N243V-S248N-K256R, A088T-A138E-N218S-N243V-K256R, A088T-G131H-N218S-N243V-S248N-K256R, A088T-G131H-T158S-S248N-K256R, A088T-N109G-A116T-G131H-N218S-S248N-K256R-L257G, A088T-N109G-A116T-G131H-N243V-S248N-K256R, A088T-N109G-A116T-G131H-T158S-N218S-L257G-I268V, A088T-N109G-A116T-G131H-W241L-S248N-K256R-L257G, A088T-N109G-A116T-N218S-N243V-S248N-K256R, A088T-N109G-A116T-T158S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-N243V-S248N, A088T-N109G-G131H-N218S-L257G, A088T-N109G-G131H-N218S-S248N, A088T-N109G-G131H-T158S-N243V-S248N-L257G, A088T-N109G-N243V-K256R-L257G, A088T-N109G-T158S-S248N-K256R, A088T-N109G-W241R-S248N-K256R, A088T-N218S-S248N-L257G, A088T-T158S-N218S-S248N-K256R, A088T-T158S-N218S-S248N-L257G, A116T-G131H-T158S-N218S-N243V, A116T-N218S-S248N, A116T-N243V-K256R, A116T-T158S, G131H-S248N-

K256R, N109G-A116T-G131H-N243V-K256R-L257G, N109G-A116T-I234T-N243V-S248N-K256R-L257G, N109G-A116T-T158S-K256R-L257G, N109G-A116T-T158S-N218S-S248N-K256R, N109G-G131H, N109G-G131H-A137V-T158S-N218S-S248N, N109G-G131H-K141E-L257G, N109G-G131H-T158S-N243V-S248N-L257G, T158S-N218S-N243V-K256R, A088T-A116T-T158S-N243V-K256R-L257G, A088T-N109G-A116T-T158S-K256R-L257G, A088T-N109G-T158S-N218S-S248N, A088T-S248N-L257G, A088T-Y104H-N109G-G131H-A137E-T158S-N218S-N243V-S248N-K256R, G065D-A088T-G131H-N243V-S248N, Y104H-N218S-L257G, A088T-A116T-G131H-V150A-N218S-S248N-L257G, A088T-A116T-T158S-K256R-L257G, A088T-I108T-N109G-G131H-T158S-N218S-S248N-K256R-L257G, A088T-N109G-A116T-T158S-S248N-L257G-Q271P, A088T-N109G-A116T-V148A-N218S-N243V, A088T-Y104H-N109G-A116T-A153S-N218S-N243V-S248N-L257G-N269D, and V004M-A116T-V148A-T158S-N243V-S248N-K256R, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN'-v36 and/or BPN-'v3 and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v36 in a BMI microswatch or egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:6, wherein the variant comprises at least one substitution selected from the group of X001R/T, X002R, X003F/P, X004A/L/M/P, X005S, X006H, X014L, X015L/S, X016C, X017T, X018L, X019K, X020A, X021T, X022L, X023E, X024S, X034S, X053S, X057Q, X065D, X078S, X086L, X088T, X090I, X097A, X098S, X101S, X104H, X105H/P, X106G, X107L/T, X108S/T, X109A/G/S, X110A, X111S, X112N, X113G, X114P/S, X116T, X124I, X128S, X131D/H, X137E/V, X138E/V, X139I, X140G, X141E/R, X143A/F, X144V, X145F/P/T, X146C, X147A/I, X148A, X149A/I/L, X150A, X151S, X152S, X153S, X154A, X155P, X156T, X157E/L, X158M/S, X159E, X160E, X161L, X169S, X182F, X203I, X204F, X207L, X211V, X212D, X213N, X216S, X218S/T, X223G, X231V, X232S, X233S, X234T, X235P, X236F/P, X237N/R, X240H, X241L/R, X243F/V, X245K, X248N, X251K, X255K, X256R, X257G, X260F, X267M, X268V, X269D/S, X271H/K/P/R, X272G/V, X273T, X274D/L/T/V, and X275K/R/S, and optionally at least one substitution selected from the group of A001R/T, Q002R, S003F/P, V004A/L/M/P, P005S,

Y006H, P014L, A015L/S, L016C, H017T, S018L, Q019K, G020A, Y021T, T022L, G023E, G024S, G034S, G053S, P057Q, G065D, N078S, P086L, A088T, L090I, G097A, A098S, N101S, Y104II, S105H/P, W106G, I107L/T, I108S/T, N109A/G/S, G110A, I111S, E112N, W113G, A114P/S, A116T, M124I, A128S, G131D/H, A137F/V, A138F/V, V139I, D140G, K141E/R, V143A/F, A144V, S145F/P/T, G146C, V147A/I, V148A, V149A/I/L, V150A, A151S, A152S, A153S, G154A, N155P, E156T, G157E/L, T158M/S, S159E, G160E, S161L, G169S, S182F, V203I, S204F, S207L, G211V, N212D, K213N, A216S, N218S/T, A223G, A231V, A232S, L233S, I234T, L235P, S236F/P, K237N/R, N240H, W241L/R, N243F/V, Q245K, S248N, E251K, T255K, K256R, L257G, S260F, L267M, I268V, N269D/S, Q271H/K/P/R, A272G/V, A273T, A274D/I/T/V, Q275K/R/S, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) BPN'-v3, and BPN'-v36 and a PI value greater than that of BPN', BPN'-v3, and BPN'-v36 in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 10

Construction and Cleaning Performance of Additional Variants of BPN'-v36

The DNA from the site evaluation libraries of the BPN'-v36 (described in Example 7) was further mutagenized by error-prone PCR. These libraries were amplified with primers P4973 and P4950 (described in Example 7) using *Taq* DNA polymerase (Promega). Each PCR amplification reaction contained 30 pmol of each primer, 100 ng of the template DNA (SELs of the BPN'-v36) and various amount of $MnCl_2$. The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 2 min. The DNA fragment was gel-purified by the QIAGEN® gel-band purification kit, digested by the *Bam*HI and *Hin*dIII restriction enzymes and ligated with the pHPLT-BPN' partial opt vector that also was digested with the same restriction enzymes. Ligation mixtures were amplified using rolling circle amplification in an Illustra Templiphi kit according to the manufacturer's recommendation (GE Healthcare) to generate multimeric DNA for transformation into *Bacillus subtilis*. For this purpose, 1µl of the ligation mixture was mixed with 5 µl of the sample buffer, heated to 95°C for 3 min and cooled on ice. Next, 5 µl of the reaction buffer and 0.2 µl of the enzyme were added to each tube, followed by incubation at 30°C for 10 hours. Products of the rolling circle amplification were diluted 100 times and used to transform *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and incubated overnight at 37°C.

About 500,000 clones were pre-screened on skim milk plates. Very few of them formed halos (i.e., indicative of the presence of functional protease). Colonies with halos were picked, inoculated in

150 µl of LB media containing 10µg/mL neomycin and sequenced (Quintara). Sequences of these clones were analyzed by looking for combination of mutations, which occurred in this pool multiple times and might provide performance benefits. In order to assess the performance of these mutation combinations, double mutants were created in the BPN'-v36 background by PCR fusion as described below. For this purpose, two or three partially overlapping fragments were amplified by mutagenic primers. Primer combinations used to generate the respective variants are shown in Table 10-1 and primer sequences are shown in Table 10-2.

Table 10-1. Primers Pairs Used to Amplify Fragments

Variant	Mutation 1	Mutation 2	Fragment 1	Fragment 2	Fragment 3
1	A45S	S236Y	P4974, P6645	P6644, P6647	P6646, P4976
2	A45S	S236G	P4974, P6645	P6644, P6649	P6648, P4976
3	I115T	S183T	P4974, P6651	P6650, P6655	P6654, P4976
4	I115V	N184Y	P4974, P6653	P6652, P6657	P6656, P4976
5	I31T	S37P	P4974, P6659	P6658, P4976	
6	I31T	I35L	P4974, P6661	P6660, P4976	
7	I31V	S38W	P4974, P6663	P6662, P4976	
8	*N25K*	P129R	P4974, P6665	P6664, P6667	P6666, P4976
9	*N25K*	P129K	P4974, P6665	P6664, P6669	P6668, P4976
10	P14T	S37T	P4974, P6671	P6670, P6673	P6672, P4976
11	P5L	Q217K	P4974, P6679	P6678, P6681	P6680, P4976
12	P5L	Q217G	P4974, P6679	P6678, P6683	P6682, P4976
13	Q10L	S37P	P4974, P6685	P6684, P6675	P6674, P4976
14	Q10R	S37T	P4974, P6687	P6686, P6673	P6672, P4976
15	S37P	T254S	P4974, P6675	P6674, P6689	P6688, P4976
16	*N25K*	S37P	P4974, P6665	P6664, P6675	P6674, P4976
17	G24A	S37W	P4974, P6691	P6690, P6677	P6676, P4976
18	*N25K*	P129R	P4974, P6665	P6664, P6667	P6666, P4976
20	S161P	S162L	P4974, P6695	P6694, P4976	
21	S161P	T253A	P4974, P6693	P6692, P6701	P6700, P4976
22	S161P	S260P	P4974, P6693	P6692, P6703	P6702, P4976
23	S162L	D181H	P4974, P6697	P6696, P6711	P6710, P4976
24	S162L	D181G	P4974, P6697	P6696, P6713	P6712, P4976
25	S18F	S162L	P4974, P6715	P6714, P6697	P6696, P4976
26	S18T	S162P	P4974, P6717	P6716, P6699	P6698, P4976
27	S18P	D120N	P4974, P6719	P6718, P6727	P6726, P4976
28	S18Y	K213R	P4974, P6721	P6720, P6729	P6728, P4976
29	S18L	Y21S	P4974, P6731	P6730, P4976	
30	S18T	Y21N	P4974, P6733	P6732, P4976	
31	S9T	K141F	P4974, P6635	P6734, P6737	P6736, P4976
32	S9T	K141R	P4974, P6635	P6734, P6739	P6738, P4976
33	Q19L	S260N	P4974, P6725	P6724, P6705	P6704, P4976
34	Q19L	S260P	P4974, P6725	P6724, P6703	P6702, P4976

Table 10-1. Primers Pairs Used to Amplify Fragments					
35	N61S	S260P	P4974, P6741	P6740, P6703	P6702, P4976
36	N61D	S260I	P4974, P6743	P6742, P6707	P6706, P4976
37	T253A	S260P	P4974, P6701	P6700, P6703	P6702, P4976
38	A134T	S260G	P4974, P6745	P6744, P6709	P6708, P4976
39	A133V	S260N	P4974, P6648	P6746, P6705	P6704, P4976

Table 10-2. Primer Sequences Used for Generation of Double Mutants of BPN'-v36		
Primer Name	**Primer Sequence**	**SEQ ID NO:**
P6644	CAGATCTTAAAGTCTCTGGAGGGGCTTCTATGGTGC	SEQ ID NO:64
P6645	CATAGAAGCCCCTCCAGAGACTTTAAGATCTGGATGGCTC	SEQ ID NO:65
P6646	GCATTGATTCTTTACAAGCACCCGAACTGGACAAAC	SEQ ID NO:66
P6647	CAGTTCGGGTGCTTGTAAAGAATCAATGCGGCCGCCCCA	SEQ ID NO:67
P6648	GCATTGATTCTTGGTAAGCACCCGAACTGGACAAAC	SEQ ID NO:68
P6649	CCAGTTCGGGTGCTTACCAAGAATCAATGCGGCCGCCCCA	SEQ ID NO:69
P6650	CATCGAATGGGCCACAGCGAATAACATGGATGTAATCAAC	SEQ ID NO:70
P6651	CATCCATGTTATTCGCTGTGGCCCATTCGATGCCGTTGAT	SEQ ID NO:71
P6652	CATCGAATGGGCCGTAGCGAATAACATGGATGTAATCAAC	SEQ ID NO:72
P6653	CATCCATGTTATTCGCTACGGCCCATTCGATGCCGTTGAT	SEQ ID NO:73
P6654	CTGTAGACTCTACAAATCAACGTGCCTCTTTTTCCT	SEQ ID NO:74
P6655	AAAGAGGCACGTTGATTTGTAGAGTCTACAGCGCCCACTG	SEQ ID NO:75
P6656	CTGTAGACTCTTCATACCAACGTGCCTCTTTTTCCTCC	SEQ ID NO:76
P6657	GAAAAAGAGGCACGTTGGTATGAAGAGTCTACAGCGCCCA	SEQ ID NO:77
P6658	TAGCGGTTACAGACAGCGGTATCGACCCAAGCCATCCAGATC TTAAAGTCG	SEQ ID NO:78
P6659	ATGGCTTGGGTCGATACCGCTGTCTGTAACCGCTACTTTAACA TTGCCTC	SEQ ID NO:79
P6660	TAAAGTAGCGGTTACAGACAGCGGTTTAGACTCGAGCCATCC AGATCTT	SEQ ID NO:80

Table 10-2. Primer Sequences Used for Generation of Double Mutants of BPN'-v36		
P6661	ATGGCTCGAGTCTAAACCGCTGTCTGTAACCGCTACTTTAACA TTGCCTC	SEQ ID NO:81
P6662	GGTTGTAGACAGCGGTATCGACTCGTGGCATCCAGATCTTAA AGTCGCTG	SEQ ID NO:82
P6663	ATGCCACGAGTCGATACCGCTGTCTACAACCGCTACTTTAACA TTGCCTC	SEQ ID NO:83
P6664	CTACACTGGAGGCAAAGTTAAAGTAGCGGTTATCGACA	SEQ ID NO:84
P6665	ATAACCGCTACTTTAACTTTGCCTCCAGTGTAGCCTTGAG	SEQ ID NO:85
P6666	GAGCCTGGGAGCACGTAGCGGCAGTGCGGCACTTAAA	SEQ ID NO:86
P6667	GTGCCGCACTGCCGCTACGTGCTCCCAGGCTCATGTTGAT	SEQ ID NO:87
P6668	TGAGCCTGGGAGCAAAGAGCGGCAGTGCGGCACTTAAA	SEQ ID NO:88
P6669	GTGCCGCACTGCCGCTCTTTGCTCCCAGGCTCATGTTGAT	SEQ ID NO:89
P6670	ATCACAAATTAAAGCCACAGCTCTGCACTCTCAAGGCTAC	SEQ ID NO:90
P6671	AGAGTGCAGAGCTGTGGCTTTAATTTGTGATACGCCGTAA	SEQ ID NO:91
P6672	GACAGCGGTATCGACACAAGCCATCCAGATCTTAAAGTCG	SEQ ID NO:92
P6673	TAAGATCTGGATGGCTTGTGTCGATACCGCTGTCGATAAC	SEQ ID NO:93
P6674	GACAGCGGTATCGACCCAAGCCATCCAGATCTTAAAGTCG	SEQ ID NO:94
P6675	TAAGATCTGGATGGCTTGGGTCGATACCGCTGTCGATAAC	SEQ ID NO:95
P6676	GACAGCGGTATCGACTGGAGCCATCCAGATCTTAAAGTCG	SEQ ID NO:96
P6677	TAAGATCTGGATGGCTCCAGTCGATACCGCTGTCGATAAC	SEQ ID NO:97
P6678	ACGCGCAGTCCGTGTTATACGGCGTATCACAAATTAAAGC	SEQ ID NO:98
P6679	ATTTGTGATACGCCGTATAACACGGACTGCGCGTACGCAT	SEQ ID NO:99
P6680	ACAAGTATGGTGCGAAAAACGGGACTTCCATGGCCTC	SEQ ID NO:100
P6681	CATGGAAGTCCCGTTTTTCGCACCATACTTGTTCCCTG	SEQ ID NO:101

Table 10-2. Primer Sequences Used for Generation of Double Mutants of BPN'-v36		
P6682	ACAAGTATGGTGCGGGAAACGGGACTTCCATGGCCTC	SEQ ID NO:102
P6683	CCATGGAAGTCCCGTTTCCCGCACCATACTTGTTCCCTG	SEQ ID NO:103
P6684	CTTACGGCGTATCATTAATTAAAGCCCCTGCTCTGCAC	SEQ ID NO:104
P6685	GAGCAGGGGCTTTAATTAATGATACGCCGTAAGGCACGGA	SEQ ID NO:105
P6686	CTTACGGCGTATCACGTATTAAAGCCCCTGCTCTGCAC	SEQ ID NO:106
P6687	GAGCAGGGGCTTTAATACGTGATACGCCGTAAGGCACGGA	SEQ ID NO:107
P6688	TTTAGAAAACACCTCTACAAAACTTGGTGATTCTTTCTAC	SEQ ID NO:108
P6689	TCACCAAGTTTTGTAGAGGTGTTTTCTAAACTGCTGCGGA	SEQ ID NO:109
P6690	AGGCTACACTGGAGCAAATGTTAAAGTAGCGGTTATCGAC	SEQ ID NO:110
P6691	GCTACTTTAACATTTGCTCCAGTGTAGCCTTGAGAGTG	SEQ ID NO:111
P6692	GAGGGAACATCCGGACCATCGAGTACCGTCGGTTATCCA	SEQ ID NO:112
P6693	ACCGACGGTACTCGATGGTCCGGATGTTCCCTCATTCCCA	SEQ ID NO:113
P6694	AGGGAACATCCGGACCATTAAGTACCGTCGGTTATCCAGG	SEQ ID NO:114
P6695	ACCGACGGTACTTAATGGTCCGGATGTTCCCTCATTCCCA	SEQ ID NO:115
P6696	GAACATCCGGATCATTAAGTACCGTCGGTTATCCAGGCA	SEQ ID NO:116
P6697	ATAACCGACGGTACTTAATGATCCGGATGTTCCCTCATTC	SEQ ID NO:117
P6698	GAACATCCGGATCACCAAGTACCGTCGGTTATCCAGGCA	SEQ ID NO:118
P6699	ATAACCGACGGTACTTGGTGATCCGGATGTTCCCTCATTC	SEQ ID NO:119
P6700	GTTTAGAAAACGCAACTACAAAACTTGGTGATTCTTTC	SEQ ID NO:120
P6701	CACCAAGTTTTGTAGTTGCGTTTTCTAAACTGCTGCGGAC	SEQ ID NO:121
P6702	CAAAACTTGGTGATCCATTCTACTATGGAAAAGGGCTGAT	SEQ ID NO:122

Table 10-2. Primer Sequences Used for Generation of Double Mutants of BPN'-v36		
P6703	TTTCCATAGTAGAATGGATCACCAAGTTTTGTAGTGGTGT	SEQ ID NO:123
P6704	CAAAACTTGGTGATAACTTCTACTATGGAAAAGGGCTGAT	SEQ ID NO:124
P6705	TTTCCATAGTAGAAGTTATCACCAAGTTTTGTAGTGGTGT	SEQ ID NO:125
P6706	CAAAACTTGGTGATATCTTCTACTATGGAAAAGGGCTGAT	SEQ ID NO:126
P6707	TTTCCATAGTAGAAGATATCACCAAGTTTTGTAGTGGTGT	SEQ ID NO:127
P6708	CAAAACTTGGTGATGGATTCTACTATGGAAAAGGGCTGAT	SEQ ID NO:128
P6709	TTTCCATAGTAGAATCCATCACCAAGTTTTGTAGTGGTGT	SEQ ID NO:129
P6710	GTGGGCGCTGTACACTCTTCAAATCAACGTGCCTCTT	SEQ ID NO:130
P6711	CACGTTGATTTGAAGAGTGTACAGCGCCCACTGCAATCAC	SEQ ID NO:131
P6712	GTGGGCGCTGTAGGATCTTCAAATCAACGTGCCTCTT	SEQ ID NO:132
P6713	CACGTTGATTTGAAGATCCTACAGCGCCCACTGCAATCAC	SEQ ID NO:133
P6714	CCTGCTCTGCACTTCCAAGGCTACACTGGAGGCAATG	SEQ ID NO:134
P6715	CTCCAGTGTAGCCTTGGAAGTGCAGAGCAGGGGCTTTAAT	SEQ ID NO:135
P6716	CCTGCTCTGCACACACAAGGCTACACTGGAGGCAATG	SEQ ID NO:136
P6717	CTCCAGTGTAGCCTTGTGTGTGCAGAGCAGGGGCTTTAAT	SEQ ID NO:137
P6718	CCTGCTCTGCACCCACAAGGCTACACTGGAGGCAATG	SEQ ID NO:138
P6719	CTCCAGTGTAGCCTTGTGGGTGCAGAGCAGGGGCTTTAAT	SEQ ID NO:139
P6720	CCTGCTCTGCACTACCAAGGCTACACTGGAGGCAATG	SEQ ID NO:140
P6721	CTCCAGTGTAGCCTTGGTAGTGCAGAGCAGGGGCTTTAAT	SEQ ID NO:141
P6722	CCTGCTCTGCACTTACAAGGCTACACTGGAGGCAATG	SEQ ID NO:142
P6723	CTCCAGTGTAGCCTTGTAAGTGCAGAGCAGGGGCTTTAAT	SEQ ID NO:143

Table 10-2. Primer Sequences Used for Generation of Double Mutants of BPN'-v36		
P6724	TGCTCTGCACTCTTTAGGCTACACTGGAGGCAATGTTA	SEQ ID NO:144
P6725	TTGCCTCCAGTGTAGCCTAAAGAGTGCAGAGCAGGGGCTT	SEQ ID NO:145
P6726	ATCGCGAATAACATGAACGTAATCAACATGAGCCTGGGA	SEQ ID NO:146
P6727	CTCATGTTGATTACGTTCATGTTATTCGCGATGGCCCAT	SEQ ID NO:147
P6728	CTTCCAGGGAACCGTTATGGTGCGCAAAACGGGACTT	SEQ ID NO:148
P6729	GTTTTGCGCACCATAACGGTTCCCTGGAAGCGTCGATTG	SEQ ID NO:149
P6730	CTGCACTTACAAGGCTCTACTGGAGGCAATGTTAAAGTAG	SEQ ID NO:150
P6731	TAACATTGCCTCCAGTAGAGCCTTGTAAGTGCAGAGCAGGGG CTTTAAT	SEQ ID NO:151
P6732	GCTCTGCACTTACAAGGCAACACTGGAGGCAATGTTAAAGTA G	SEQ ID NO:152
P6733	AACATTGCCTCCAGTGTTGCCTTGTAAGTGCAGAGCAGGGGC TTTAAT	SEQ ID NO:153
P6734	CTTACGGCGTAACACAAATTAAAGCCCCTGCTCTG	SEQ ID NO:154
P6735	AGGGGCTTTAATTTGTGTTACGCCGTAAGGCACGGACT	SEQ ID NO:155
P6736	TTAAAGCAGCAGTTGATTTCGCTGTTGCATCTGGTGTCGT	SEQ ID NO:156
P6737	AGATGCAACAGCGAAATCAACTGCTGCTTTAAGTGCCGCA	SEQ ID NO:157
P6738	TTAAAGCAGCAGTTGATCGTGCTGTTGCATCTGGTGTCGT	SEQ ID NO:158
P6739	AGATGCAACAGCACGATCAACTGCTGCTTTAAGTGCCGCA	SEQ ID NO:159
P6740	AAACCCGTTTCAAGATTCTAATTCTCATGGCACACACGTC	SEQ ID NO:160
P6741	TGTGCCATGAGAATTAGAATCTTGAAACGGGTTTGTTTCG	SEQ ID NO:161
P6742	AAACCCGTTTCAAGATGATAATTCTCATGGCACACACGTC	SEQ ID NO:162
P6743	TGTGCCATGAGAATTATCATCTTGAAACGGGTTTGTTTCG	SEQ ID NO:163
P6744	AAGCGGCAGTGCGACACTTAAAGCAGCAGTTGATAAAGC	SEQ ID NO:164

Table 10-2. Primer Sequences Used for Generation of Double Mutants of BPN'-v36		
P6745	TCAACTGCTGCTTTAAGTGTCGCACTGCCGCTTGGTGCTC	SEQ ID NO:165
P6746	CAAGCGGCAGTGTTGCACTTAAAGCAGCAGTTGATAA	SEQ ID NO:166
P6747	ACTGCTGCTTTAAGTGCAACACTGCCGCTTGGTGCTCCCA	SEQ ID NO:167

Each PCR amplification reaction contained 30 pmol of each primer and 100 ng of the BPN'-v36 parent template DNA (plasmid pHPLT-BPN'-v36) (see Figure 4). Amplifications were carried out using Vent DNA polymerase (NEB). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 40 sec. Following amplification, the 5' and 3' gene fragments were gel-purified by the QIAGEN® gel-band purification kit, mixed (50 ng of each fragment), mixed and amplified by PCR once again using the primers P4973 and P4950 to generate the full-length gene fragment. The PCR conditions were same as described above, except the extension phase, which was carried out at 72°C for 2 min. The full-length DNA fragment was gel-purified by the QIAGEN® gel-band purification kit, digested by the *Bam*HI and *Hin*dIII restriction enzymes and ligated with the pHPLT-BPN' partial opt vector that also was digested with the same restriction enzymes. Ligation mixtures were amplified using rolling circle amplification in an Illustra Templiphi kit according to the manufacturer's recommendation (GE Healthcare) to generate multimeric DNA for transformation into *Bacillus subtilis*. For this purpose, 1 µl of the ligation mixture was mixed with 5 µl of the sample buffer, heated to 95°C for 3 min and cooled on ice. Next, 5 µl of the reaction buffer and 0.2 µl of the enzyme were added to each tube, followed by incubation at 30°C for 10 hours. Products of the rolling circle amplification were diluted 100 times and used to transform *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and incubated overnight at 37°C. Subsequently, the colonies with halos were inoculated in 150 µl of LB media containing 10µg/mL neomycin. The next day, the cultures were either frozen with 15% glycerol or grown in MBD medium for biochemical analysis as described in Example 2.

The variants were tested for cleaning performance using BMI microswatch assay in Detergent Composition 4 at 16°C and pH 8, BMI microswatch assay in Detergent Composition 4 at 16°C and pH 7, and Egg microswatch assay in Detergent Composition 4 at 16°C and pH 8. Protein content was determined using TCA assay. All assays were performed as described in Example 1 and Performance Indices were calculated relative to BPN'-v36 (i.e., BPN'-S24G-S53G-S78N-S101N-G128A-Y217Q) (with a PI value of 1.0).

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-

S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A133V-S260N, N061S-S260P, P014T-S037T, S009T-K141F, S009T-K141R, S018F-S162L, S018L-Y021S, S018P-D120N, S018T-S162P, S018T-Y021N, S018Y-K213R, S161P-S162L, S161P-S260P, and T253A-S260P, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and a PI value of about 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A134T-S260G, I115V-N184Y, N025K-S037P, Q010L-S037P, Q019L-S260N, Q019L-S260P, S037P-T254S, and S161P-T253A, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value of about 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A045S-S236G, G024A-S037W, I031V-S038W, N061D-S260I, Q010R-S037T, I115T-S183T, N025K-P129K, N025K-P129R, A045S-S236Y, and S162L-D181H, wherein amino acid positions of the variant

are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of S018F-S162L, S018P-D120N, P014T-S037T, S009T-K141R, and S161P-S162L, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of N061S-S260P, Q010L-S037P, S009T-K141F, S018L-Y021S, S018T-S162P, S018T-Y021N, S018Y-K213R, S037P-T254S, S161P-S260P, S161P-T253A, and T253A-S260P, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity

compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and a PI value of about 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN' variants were determined to have a PI value of about 0.9 relative to BPN'-v3 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16°C: BPN' amino acid sequence (SEQ ID NO:2) comprising at least one set of amino acid substitutions selected from the group consisting of A133V-S260N, A134T-S260G, I115T-S183T, I115V-N184Y, N061D-S260I, Q019L-S260N, and Q019L-S260P, wherein positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, a PI value of about 0.9 relative to BPN'-v3, and/or an enhanced proteolytic activity compared to BPN' in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 7 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A045S-S236G, G024A-S037W, Q010R-S037T, A045S-S236Y, I031V-S038W, N025K-S037P, S162L-D181H, and N025K-P129R, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are

compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of I031V-S038W, P014T-S037T, S018F-S162L, S018P-D120N, and S162L-D181H, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value of about 1.0 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A133V-S260N, A134T-S260G, G024A-S037W, I115V-N184Y, N025K-P129K, N025K-P129R, N061D-S260I, Q019L-S260P, S009T-K141F, S009T-K141R, S018L-Y021S, S018T-S162P, S018T-Y021N, S018Y-K213R, S161P-S162L, S161P-T253A, and T253A-S260P, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of about 1.0 relative to BPN'-v3, and/or a PI value of about 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino

acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.8 and equal to or less than 0.9 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of A045S-S236G, A045S-S236Y, I115T-S183T, N025K-S037P, N061S-S260P, Q010L-S037P, Q010R-S037T, Q019L-S260N, S037P-T254S, and S161P-S260P, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.8 and equal to or less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN'-v36 and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v36 in a BMI or egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:6, wherein the variant comprises at least one substitution comprising at least one substitution selected from the group of X009T, X010L/R, X014T, X018F/L/P/T/Y, X019L, X021N/S, X024A, X025K, X031V, X037P/T/W, X038W, X045S, X061D/S, X115T/V, X120N, X129K/R, X133V, X134T, X141F/R, X161P, X162L/P, X181H, X183T, X184Y, X213R, X236G/Y, X253A, X254S, and X260G/I/N/P, and optionally at least one substitution selected from the group of S009T, Q010L/R, P014T, S018F/L/P/T/Y, Q019L, Y021N/S, G024A, N025K, I031V, S037P/T/W, S038W, A045S, N061D/S, I115T/V, D120N, P129K/R, A133V, A134T, K141F/R, S161P, S162L/P, D181H, S183T, N184Y, K213R, S236G/Y, T253A, T254S, and S260G/I/N/P, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) BPN'-v3, and BPN'-v36 and a PI value greater than that of BPN', BPN'-v3, and BPN'-v36 in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such

variant and methods for cleaning utilizing at least one such variant as described in greater detail elsewhere herein.

EXAMPLE 11

1. Generation of Combinatorial Libraries FS1-FS3

The pHPLT-BPN'-v3 plasmid containing the BPN' expression cassette served as template DNA (parent plasmid) for cloning. Three separate combinatorial libraries (FS1, FS2, and FS3) were synthesized by DNA2.0, and were delivered as individual ligation reactions. A list of libraries and possible substitutions are shown in Table 11-1. The libraries were designed to allow the incorporation of either the wild type residues or the substitutions at each site described in Table 11-1.

For efficient transformation of *B. subtilis*, the DNA from the ligation reaction was amplified by rolling circle amplification (RCA) using the Illustra Templiphi kit (GE Healthcare). Reactions were performed according to the manufacturer's protocol. One microliter of ten-fold diluted amplified DNA was used to transform 50 µL of competent *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). The transformation mixture was shaken at 37°C for 1 hour. Ten microliter aliquots of the transformation mixture were plated on skim milk (1.6%) Luria Agar plates supplemented with 10 µg/ml of neomycin (Teknova).

Transformants were picked into microtiter plates containing 125-150 µl Luria broth medium supplemented with 10µg/ml neomycin. Plates were grown overnight at 37°C with 250-300 rpm shaking and 70-80% humidity using Enzyscreen lids for microtiter plates (Enzyscreen). Between 7 and 10 microliters from the overnight culture plate were used to inoculate a new microtiter plate containing 190µl of MBD medium (a MOPS based defined medium) with 10µg/ml neomycin. MBD medium was prepared essentially as known in the art (see Neidhardt et al., J. Bacteriol. 119:736-747 (1974)), except that NH_4Cl_2, $FeSO_4$, and $CaCl_2$ were omitted from the base medium, 3 mM K_2HPO_4 was used, and the base medium was supplemented with 60 mM urea, and 100 ml of a solution made of 210 g/L glucose, and 350 g/L maltodextrin. The micronutrients were made up as a 100X stock solution containing in one liter, 400 mg $FeSO_4 \cdot 7H_2O$, 100 mg $MnSO_4 \cdot H_2O$, 100 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $CuCl_2 \cdot 2H_2O$, 100 mg $CoCl_2 \cdot 6H_2O$, 100 mg $NaMoO_4 \cdot 2H_2O$, 100 mg $Na_2B_4O_7 \cdot 10H_2O$, 10 ml of 1 M $CaCl_2$, and 10 ml of 0.5 M sodium citrate. The MBD medium containing microtiter plates were grown for 60-70 hours at 37°C, 250-300 rpm, and 70-80% humidity using Enzyscreen lids (Enzyscreen) for determining protein expression. The next day, cultures were filtered through a micro-filter plate (0.22µm; Millipore) and the resulting filtrate was used for biochemical analysis.

Table 11-1: Possible Substitutions for Combinatorial Libraries FS1-FS3			
	FS1	**FS2**	**FS3**
1	G102A	A97G	N61E
2	S130P	A128G	P129E
3	T55P	N123G	K213L
4	V203Y	G102A	S145D
5	N61P	L126V	Q275E
6	S101N	G100N	P40E
7	S53G	N62Q	S159K
8	S78N	M124I	S24R
9	S87T-A88L-S89G	N61P	A144K
10	S24G-N25G	S130P	N240K
11	L75S-N76Y	P129S	P239R

2. Generation of Variants to Improve BPN' Stability

To improve BPN' stability, variants were constructed using either parent molecules pHPLT-BPN' G97A-G128A-Y217Q-S87D or pHPLT-BPN' G97A-G128A-Y217Q-P40E, both synthesized by Gene Oracle, or parent molecules pHPLT-BPN' G97A-G128A-Y217Q-S78N and pHPLT-partial opt FNA (*B. amyloliquefaciens* subtilisin BPN'-Y217L) synthesized by GeneArt.

The information listed in Tables 11-2 and 11-3 summarizes the parent molecule used, the mutations added, and the primers used to construct variants provided herein.

Table 11-2: Primer Sequences Used for the Generation of BPN' Stability Mutants		
Primer	Primer Sequence 5' to 3'	SEQ ID NO:
p31	/5PHOS/CGGCGTTAAACAATAACATTGGCGTGCTTGGTGTAG	169
p32	/5PHOS/ACCAAGCACGCCAATGTTATTGTTTAACGCCGCAACC	170
p25	/5PHOS/GTATCGACTCGAGCCATGAAGATCTTAAAGTCGCTGGAG	171
p26	/5PHOS/CAGCGACTTTAAGATCTTCATGGCTCGAGTCGATACCG	172
p33	/5PHOS/TTGGTGTAGCCCCGGATGCTTCGCTCTACGCCGTTAAAG	173
p34	/5PHOS/CGTAGAGCGAAGCATCCGGGGCTACACCAAGCACG	174
p29	/5PHOS/GAACGGTTGCGGCGTTAGATAATTCTATTGGCGTGCTTG	175
p30	/5PHOS/AGCACGCCAATAGAATTATCTAACGCCGCAACCGTTC	176

Table 11-2: Primer Sequences Used for the Generation of BPN' Stability Mutants		
Primer	Primer Sequence 5' to 3'	SEQ ID NO:
p27	/5PHOS/AACGGTTGCGGCGTTAGATAATAACATTGGCGTGCTTGGTGTAG	177
p28	/5PHOS/ACACCAAGCACGCCAATGTTATTATCTAACGCCGCAACCGTTCCTG	178
p7	/5PHOS/CGGCGTTAAACAATAATATTGGCGTGCTTGG	179
p8	/5PHOS/CCAAGCACGCCAATATTATTGTTTAACGCCG	180
p21	/5PHOS/GGTGTAGCCCCGGATGCTTCGCTCTACG	181
p22	/5PHOS/CGTAGAGCGAAGCATCCGGGGCTACACC	182
p11	/5PHOS/GTATCGACTCGAGCCATGAAGATCTTAAAG	183
p12	/5PHOS/CTTTAAGATCTTCATGGCTCGAGTCGATAC	184
p13	/5PHOS/CGCTTCCAGGGAACAACTATGGTGCGTA	185
p14	/5PHOS/TACGCACCATAGTTGTTCCCTGGAAGCG	186
p15	/5PHOS/CACTCTCAAGGCTACGTTGGATCAAATGTTA	187
p16	/5PHOS/TAACATTTGATCCAACGTAGCCTTGAGAGTG	188
p17	/5PHOS/TGGCGTTTCTATTGAATCGACGCTTCCAG	189
p18	/5PHOS/ CTGGAAGCGTCGATTCAATAGAAACGCCA	190

Table 11-3: Templates Used, Mutations to be Incorporated, and Primers Used in the Generation of Variants for Improved Stability			
	Parent Molecules (Templates)	Mutation to Incorporate	Primers Used
1	pHPLT-BPN' G97A-G128A-Y217Q-S87D	S78N	p31, p32
2	pHPLT-BPN' G97A-G128A-Y217Q-S78N	P40E	p25, p26
3	pHPLT-BPN' G97A-G128A-Y217Q-P40E	S87D	p33, p34
4	pHPLT-BPN' G97A-G128A-Y217Q-S78N	P40E, S87D	p25, p33
5	pHPLT-BPN' G97A-G128A-Y217Q-S87D	N76D	p29, p30
6	pHPLT-BPN' G97A-G128A-Y217Q-S87D	N76D, S78N	p27, p28
7	pHPLT-partial opt FNA	S78N	p7, p8
8	pHPLT-partial opt FNA	S87D	p21, p22
9	pHPLT-partial opt FNA	P40E	p11, p12
10	pHPLT-partial opt FNA	K213N	p13, p14
11	pHPLT-partial opt FNA	T22V	p15, p16
12	pHPLT-partial opt FNA	Q206E	p17, p18
13	pHPLT-partial opt FNA	P40E, S87D, S78N	p11, p21, p7

Bacillus subtilis strains expressing plasmids were streaked onto 1.6% skim milk plates containing 10ppm neomycin and grown overnight at 37°C. Single colonies from the plates were grown overnight at 37°C with shaking at 250 rpm in 5 mL Luria broth containing 10 ppm neomycin. Plasmids were isolated using the QIAGEN® Miniprep kit protocol adding 1 microliter of Ready Lyse lysozyme (Epicentre) for 15 minutes at 37°C in buffer P1 to aid in cell lysis. The plasmids were sequenced to

ensure correct DNA sequences before proceeding. The plasmids were methylated using NEB's Dam Methylase Kit in a reaction containing 77.5 µL water + 10 µL Buffer 10X + 0.25 µL SAM + 2 µL DAM methylase + 10 µL miniprep DNA (~150ng/µL) at 37°C overnight. The methylated plasmid DNA was purified using a DNA Clean and Concentrator Kit (Zymo) or with a QIAGEN® PCR purification kit. Multi-Site QUIKCHANGE® mutagenesis reactions were set up for each of the DNA templates in a reaction mix containing 2.5 µL Buffer 5X +0.75 µL Quik Solution + 0.5 µL primer 1 (25 µM) + 0.5 µL primer 2 (25 µM) + 1.5 µL dNTP's + 1 µL enzyme blend + 16.25 µL H_2O + 2 µL DNA. The PCR program used was: 95°C for 1 min; (95°C for 1 min, 53°C for 1 min, 65°C for 10 min) x 29 cycles; 65°C for 10 min, 4°C hold. In all reactions, PCR was performed using a MJ Research PTC-200 Peltier thermal cycler. The parental DNA from the PCR samples was removed by addition of 1µL of *Dpn*I to QUIKCHANGE® mutagenesis kit reactions at 37°C overnight. To increase the transformation frequency, the *Dpn*I digested reactions were amplified using rolling circle amplification (RCA) using the Illustra TempliPhi kit according to the manufacturer's protocol. *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo) were transformed with 1 µL each of the RCA reaction and the transformed cells were plated onto LA + 1.6% skim milk plates containing 10 ppm neomycin and incubated at 37°C overnight. Colonies from overnight growth were selected to perform colony PCR for sequencing using "puReTaq Ready-To-Go PCR Beads" (Amersham). The PCR and sequencing primers used were pHPLT F1 (SEQ ID NO:54) and pHPLT seq R1 (SEQ ID NO:55). Clones with appropriate sequences were frozen. The BPN' variant proteins were produced by growing the *B. subtilis* transformants in 96 well microtiter plates at 37°C for 68 hours in a MOPS based medium containing urea.

3. Generation of BPN' Variants Derived From Five Different Parent Plasmids

BPN' variants were constructed using a total of five different templates: BPN'-v3 (G97A-G128A-Y217Q), BPN'-v4 (G97A-N123G-Y217Q), BPN' variant 8, (S87D-G97A-N109D-G128A-S188D-S248R-Y217Q), BPN' variant 16 (S87D-G97A-N109D-G128A-S188D-Y217Q), and BPN' variant 21 (S87R-G97A-N109R-G128A-S188R-Y217Q-S248R) as shown in Table 11-4. The generation of BPN'-v4 and BPN'-v3 are described in PCT App. No. PCT/US09/46066 (WO 09/149144), filed on June 3, 2009, hereby incorporated herein by reference for such description. BPN' variants 8, 16, 21 were synthesized by Gene Oracle and served as parent plasmids to build additional variants. All variants were generated using QUIKCHANGE® mutagenesis kits, except two (variants 5 and 33), which were generated using fusion PCR as described below. Primers (listed in Table 11-5) for the generation of variants were synthesized at Integrated DNA Technologies. The mutations introduced (shown in bold) and the primers and template used are shown in Table 11-4.

Table 11-4: Mutations Introduced (bold) & Parent Plasmids Used to Generate BPN' Variants			
Variant	Variants Constructed	Parent Plasmid	Primers Used
1	G97A-**N123C**-Y217Q	G97A-N123G-Y217Q	N123C f, N123C r
2	**N76D**-G97A-G128A-Y217Q	G97A-G128A-Y217Q	N76D f, N76D r
3	G97A-**N109D**-G128A-Y217Q	G97A-G128A-Y217Q	N109D f1, N109D r
4	G97A-G128A-**S188D**-Y217Q	G97A-G128A-Y217Q	S188D f, S188D r
5	G97A-G128A-**S248D**-Y217Q	G97A-G128A-Y217Q	pHPLT F1, S248D forfus, pHPLT R1, S248D revfus
6	G97A-G128A-**S188D-S248R**-Y217Q	G97A-G128A-Y217Q	S188D f, S248R f1
7	G97A-**N109D**-G128A-**S188D-S248R**-Y217Q	S87D-G97A-N109D-G128A-S188D-S248R-Y217Q	D87S f, D87S r
8	**S87D**-G97A-**N109D**-G128A-**S188D-S248R**-Y217Q	Synthesized by Gene Oracle	Synthesized by Gene Oracle
9	**S87R**-G97A-G128A-**S188D-S248D**-Y217Q	S87D-G97A-N109D-G128A-S188D-Y217Q	S87R f, S248D f1, D109N f
10	**S87R**-G97A-**N109D**-G128A-**S188D**-Y217Q	S87D-G97A-N109D-G128A-S188D-S248R-Y217Q	R248Sfor, R248Srev
11	G97A-**N109D**-G128A-**S188R**-Y217Q	G97A-G128A-Y217Q	N109D f2, S188R f
12	**S87R**-G97A-**N109D**-G128A-**S188D**-Y217Q-**S248R**	S87D-G97A-N109D-G128A-S188D-S248R-Y217Q	S87R f, S87R r
13	G97A-**N109D**-G128A-Y217Q-**S248R**	G97A-G128A-Y217Q	N109D f2, S248R f2
14	**S87D**-G97A-G128A-Y217Q-**S248R**	G97A-G128A-Y217Q	S87D f, S248R f1
15	**S87D**-G97A-**N109D**-G128A-**S188D**-Y217Q-**S248D**	S87D-G97A-N109D-G128A-S188D-Y217Q	S248D f1, S248D r
16	**S87D**-G97A-**N109D**-G128A-**S188D**-Y217Q	Synthesized by Gene Oracle	Synthesized by Gene Oracle
17	G97A-**N109D**-G128A-Y217Q-**S248D**	G97A-G128A-Y217Q	N109D f2, S248D f2
18	**S87R**-G97A-G128A-Y217Q	G97A-G128A-Y217Q	S87R f, S87R r
19	**S87R**-G97A-G128A-Y217Q-**S248R**	G97A-G128A-Y217Q	S87R f, S248R f1
20	**S87R**-G97A-G128A-**S188R**-Y217Q-**S248R**	S87R-G97A-N109R-G128A-S188R-Y217Q-S248R	D109N f, D109N r
21	**S87R**-G97A-**N109R**-G128A-**S188R**-Y217Q-**S248R**	Synthesized by Gene Oracle	Synthesized by Gene Oracle
22	G97A-**G102A**-G128A-Y217Q	G97A-G128A-Y217Q	G102A f, G102A r
23	G97A-G128A-**S130P**-Y217Q	G97A-G128A-Y217Q	S130P f, S130P r

Table 11-4: Mutations Introduced (bold) & Parent Plasmids Used to Generate BPN' Variants			
Variant	Variants Constructed	Parent Plasmid	Primers Used
24	G97A-**S101N**-G128A-Y217Q	G97A-G128A-Y217Q	S101N f, S101N r
25	G97A-**G100N**-G128A-Y217Q	G97A-G128A-Y217Q	G100N f, G100N r
26	**N61P**-G97A-G128A-Y217Q	G97A-G128A-Y217Q	N61P f, N61P r
27	G97A-G128A-**A187D**-Y217Q	G97A-G128A-Y217Q	A187D f, A187D r
28	G97A-G128A-**F189D**-Y217Q	G97A-G128A-Y217Q	f189D f, f189D r
29	G97A-G128A-**A137V**-Y217Q	G97A-G128A-Y217Q	A137V f, A137V r
30	**S63T**-G97A-G128A-Y217Q	G97A-G128A-Y217Q	S63T f, S63T r
31	G97A-**Q103N**-G128A-Y217Q	G97A-G128A-Y217Q	Q103N f, Q103N r
32	**N62D**-G97A-G128A-Y217Q	G97A-G128A-Y217Q	N62D f, N62D r
33	G97A-**G100E**-G128A-Y217Q	G97A-G128A-Y217Q	G100E Fsfor, pHPLT F1, G100E Fsrev, pHPLT R1

Generation of BPN' Variants via QUIKCHANGE® Mutagenesis

Bacillus subtilis strains containing plasmids expressing BPN'-v3, BPN'-v4, BPN' variant 8, BPN' variant 16, and BPN' variant 21 were streaked onto 1.6% skim milk plates containing 10 ppm neomycin and grown overnight at 37°C. Single colonies from the plates were grown overnight at 37°C with shaking at 250 rpm in 5 mL Luria broth containing 10 ppm neomycin. Plasmids expressing BPN'-v3, BPN'-v4, BPN' variant 8, BPN' variant 16, and BPN' variant 21 were isolated using QIAGEN® Miniprep kit protocol except following cell lysis, 1 microliter of Ready Lyse lysozyme was added and incubated for 15 minutes at 37°C. The plasmids were methylated using NEB's Dam Methylase Kit in a reaction containing 77.75 µL H_20 + 10 µL Buffer 10X + 0.25 µL SAM + 2 µL DAM methylase + 10µL miniprep DNA at 37°C overnight. The methylated DNA was purified using the QIAGEN® PCR purification kit. Variants 14, 18, and 19 listed in Table 11-4 were generated using QUIKCHANGE LIGHTNING™ Multi Site-Directed Mutagenesis kits (Stratagene) in a reaction mix containing 2.5 µL Buffer 5X + 0.75 µL Quik Solution + 0.5 µL primer 1 (25 µM) + 0.5 µL primer 2 (25 µM) + 1.5 µL dNTP's + 1 µL enzyme blend + 16.25 µL H_2O + 2 µL DNA. The PCR program used was as follows: 95°C for 2 min; (95°C for 20 sec, 55°C for 30 sec, 64°C for 2 min 30 sec) x 29 cycles; 64°C for 5 min, 4°C hold.

The remaining variants were created using QUIKCHANGE® Multi Site-Directed Mutagenesis kits in a reaction mix containing 2.5 µL Buffer 5X + 0.75 µL Quik Solution + 0.5 µL primer 1 (25 µM) + 0.5 µL primer 2 (25 µM) + 1.5 µL dNTP's + 1 µL enzyme blend + 16.25 µL H2O + 2 µL DNA. The PCR program used was as follows: 95°C for 1 min; (95°C for 1 min, 53°C for 1 min, 65°C for 10 min) x 29 cycles; 65°C for 10 min, 4°C hold. In all reactions, PCR was performed using a MJ Research PTC-200 Peltier thermal cycler. The primers used for the Quik-Change reactions are provided in Table 11-5.

Table 11-5: Primers Used for Quik-Change Reactions		
Primer Name	**Primer Sequence (5' to 3')**	**SEQ ID NO:**
N76D f	/5PHOS/GAACGGTTGCGGCGTTAGATAATTCTATTGGCGTGCTTG	191
N76D r	/5PHOS/AGCACGCCAATAGAATTATCTAACGCCGCAACCGTTC	192
S87D f	/5PHOS/TTGGTGTAGCCCCGGATGCTTCGCTCTACGCCGTTAAAG	193
S87D r	/5PHOS/CGTAGAGCGAAGCATCCGGGGCTACACCAAGCACG	194
G102A f	/5PHOS/GCAGCAGACGGATCAGCACAATACTCATGGATTAT	195
G102A r	/5PHOS/ATAATCCATGAGTATTGTGCTGATCCGTCTGCTGC	196
S130P f	/5PHOS/CAACATGAGCCTGGGAGCACCACCGGGCAGTGCGGCACTTAAAGC	197
S130P r	/5PHOS/GCTTTAAGTGCCGCACTGCCCGGTGGTGCTCCCAGGCTCATGTTG	198
S101N f	/5PHOS/GTTCTTGCAGCAGACGGAAATGGCCAATACTCATGGATT	199
S101N r	/5PHOS/AATCCATGAGTATTGGCCATTTCCGTCTGCTGCAAGAAC	200
G100N f	/5PHOS/TTAAAGTTCTTGCAGCAGACAATTCAGGCCAATACTCATGGA	201
G100N r	/5PHOS/TCCATGAGTATTGGCCTGAATTGTCTGCTGCAAGAACTTTAA	202
N61P f	/5PHOS/CCGTTTCAAGATCCGAATTCTCATGGCACACACGTC	203
N61P r	/5PHOS/TGCCATGAGAATTCGGATCTTGAAACGGGTTTGTTTCG	204
A187D f	/5PHOS/TTCAAATCAACGTGATTCTTTTTCCTCCGTGGGACCGGAG	205
A187D r	/5PHOS/ACGGAGGAAAAAGAATCACGTTGATTTGAAGAGTCTACAG	206
F189D f	/5PHOS/CAAATCAACGTGCCTCTGATTCCTCCGTGGGACCGGAG	207
F189D r	/5PHOS/CTCCGGTCCCACGGAGGAATCAGAGGCACGTTGATTTG	208
A137V f	/5PHOS/AGCGGCAGTGCGGCACTTAAAGTTGCAGTTGATAAAGCTGTTGC	209
A137V r	/5PHOS/GCAACAGCTTTATCAACTGCAACTTTAAGTGCCGCACTGCCGCT	210

Table 11-5: Primers Used for Quik-Change Reactions		
Primer Name	**Primer Sequence (5' to 3')**	**SEQ ID NO:**
S63T f	/5PHOS/TCAAGATAACAATACACATGGCACACACGTCGCAGGAAC	211
S63T r	/5PHOS/ACGTGTGTGCCATGTGTATTGTTATCTTGAAACGGGTTTG	212
Q103N f	/5PHOS/AGACGGATCAGGCAATTACTCATGGATTATCAACGGCATC	213
Q103N r	/5PHOS/TAATCCATGAGTAATTGCCTGATCCGTCTGCTGCAAG	214
N62D f	/5PHOS/ACCCGTTTCAAGATAACGATTCTCATGGCACACACGTC	215
N62D r	/5PHOS/GACGTGTGTGCCATGAGAATCGTTATCTTGAAACGGGT	216
N109D f1	/5PHOS/CAATACTCATGGATTATCGATGGCATCGAATGGGCCA	217
N109D r	/5PHOS/TGGCCCATTCGATGCCATCGATAATCCATGAGTATTG	218
S188D f	/5PHOS/CTCTTCAAATCAACGTGCCGATTTTTCCTCCGTGGGACC	219
S188D r	/5PHOS/GGTCCCACGGAGGAAAAATCGGCACGTTGATTTGAAGAG	220
S248R f1	/5PHOS/CAAACACTCAAGTCCGCAGAAGTTTAGAAAACACCAC	221
S87R f	/5PHOS/GTGCTTGGTGTAGCCCCGAGAGCTTCGCTCTACGCCGT	222
S87R r	/5PHOS/ACGGCGTAGAGCGAAGCTCTCGGGGCTACACCAAGCAC	223
S248D f1	/5PHOS/CAAACACTCAAGTCCGCGATAGTTTAGAAAACACCAC	224
S248D r	/5PHOS/GTGGTGTTTTCTAAACTATCGCGGACTTGAGTGTTTG	225
D87S f	/5PHOS/GTGCTTGGTGTAGCCCCGTCTGCTTCGCTCTACGCCGT	226
D87S r	/5PHOS/ACGGCGTAGAGCGAAGCAGACGGGGCTACACCAAGCAC	227
D109N f	/5PHOS/CAATACTCATGGATTATCAACGGCATCGAATGGGCCA	228
D109N r	/5PHOS/TGGCCCATTCGATGCCGTTGATAATCCATGAGTATTG	229
N109D f2	/5PHOS/ACTCATGGATTATCGATGGCATCGAATGGGCCATCGC	230
S248R f2	/5PHOS/CACTCAAGTCCGCAGAAGTTTAGAAAACACCACTAC	231

Table 11-5: Primers Used for Quik-Change Reactions		
Primer Name	**Primer Sequence (5' to 3')**	**SEQ ID NO:**
S248D f2	/5PHOS/CACTCAAGTCCGCGATAGTTTAGAAAACACCACTAC	232
S188R f	/5PHOS/CAAATCAACGTGCCAGATTTTCCTCCGTGGGACCGGAG	233
QC FUSION _For1	AAAGGGGAGGAAAATCGTGAAACA	234
QC FUSION _Rev1	GTTCTAAATCGTGTTTTTCTTG	235
S248D forfus	GAACTGGACAAACACTCAAGTCCGCGATAGTTTAGAAAACACCA CTAC	236
S248D revfus	GACTTGAGTGTTTGTCCAGTTCGGGTGCTTAGAAAG	237
R248Sfor	/5PHOS/CAAACACTCAAGTCCGCAGCAGTTTAGAAAACACCAC	238
R248S rev	/5PHOS/GTGGTGTTTTCTAAACTGCTGCGGACTTGAGTGTTTG	239
G100E_F sfor	ACGCCGTTAAAGTTCTTGCAGCAGACGAATCAGGCCAATACTCAT GGAT	240
G100E_F srev	TGCTGCAAGAACTTTAACGGCGTAGAGCGAAGCAGA	241
N123C f	/5PHOS/ACATGGATGTAATCTGCATGAGCCTGGGAGGACCAAG	242
N123C r	/5PHOS/TCCTCCCAGGCTCATGCAGATTACATCCATGTTATTCG	243
pHpLT R1	/5PHOS/GTTATGAGTTAGTTCAAATTCG	244

The parental DNA from the PCR samples was removed by addition of 1µL of *Dpn*I to Quik-Change reactions at 37°C overnight. One micro-liter of the *Dpn*I-digested reactions were amplified using rolling circle amplification (RCA) using the Illustra TempliPhi kit according to the manufacturer's protocol. *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo) were transformed with1 µL each of the RCA reaction and the transformed cells were plated onto LA + 1.6% skim milk plates containing 10 ppm neomycin and incubated at 37°C overnight. Colonies from overnight growth were selected to perform colony PCR using "puReTaq Ready-To-Go PCR Beads" (Amersham). The PCR primers used were pHPLT F1 (SEQ ID NO:54) and pHPLT seq R1 (SEQ ID NO:55). Clones with appropriate sequences were frozen. BPN' variants were expressed by growing the *B. subtilis* transformants in 96 well microtiter plates at 37°C for 68 hours in a MOPS based medium containing urea.

Generation of BPN' Variants via Fusion PCR

Variants 5 and 33 were generated using Fusion PCR, with fragments amplified from template pHPLT-BPN'-v3. The PCR primers used to generate these variants are included in Table 11-5. For Variant 5, a 5' fragment of the BPN' gene was amplified using forward primer pHPLT F1(SEQ ID NO:54), and reverse primer S248D revfus. The 3' fragment of the BPN' gene was amplified using the forward primer S248D forfus containing the mutation of interest and the reverse primer pHPLT R1. The two products contained 20 bp of overlapping sequence, and were fused by combining 1 µL of each fragment and fusion primers QC FUSION_For1 and QC FUSION_Rev1 in a final PCR reaction. All PCR reactions were performed using standard conditions of the Herculase II PCR Kit (Stratagene). The PCR mix contained 1 µL DNA polymerase, 1 µL plasmid DNA (or fragment DNA for fusion), 0.5 µL dNTP's, 1.25 µL 25 µM forward primer, 1.25 µL 25 µM reverse primer, 10 µL Buffer 5X, 35 µL H_2O and the PCR program used was as follows: 95°C for 2 min, (95°C for 30 sec, 55°C for 30 sec, 72°C for "X" sec) for 29 cycles, 72°C for 1 min, 4°C hold (the "X" is 15 seconds per 1 kB of DNA to amplify).

For Variant 33, a 5' fragment of the BPN' gene was amplified using the template pHPLT-BPN'-v3 and primers pHPLT F1 (SEQ ID NO:54), and G100E_Fsrev. The 3' fragment that contained the variant was amplified using primers G100E_Fsfor and pHPLT R1. The two products contained 20 bp of overlapping sequence, and were fused by combining 1 µl of each fragment and fusion primers QC FUSION_For1 and QC FUSION_Rev1 in a final PCR reaction. The PCR conditions were the same as listed above.

The two fusion products were purified using a QIAGEN® PCR purification column with conditions provided by the manufacturer, and digested overnight using *Bgl* I and *Hin*dIII enzymes. The plasmid pHPLT-BPN' partial opt was digested using the same enzymes and the vector band was gel extracted and purified over a QIAGEN® gel purification column using the manufacturer's recommendations. The restriction enzyme mix contained: 10 µL purified DNA, 5 µL Roche Buffer B, 0.5µL *Hin*dIII, 0.5µL *Bgl* I, 34µL H_2O and the reactions were carried out at 37°C for 8 hours followed by 65°C for 20 min. The digest was purified using a QIAGEN® PCR purification column and ligated to the cut vector backbone overnight at 16°C using the Mighty Mix Ligase kit (Tekara). Following incubation, 1µL of the ligation mix was amplified using the Illustra TempliPhi kit.

For the amplification reaction, 1µL of the ligation reaction mix was mixed with 5 µL of sample buffer from the TempliPhi kit and heated for 3 minutes at 95°C to denature the DNA. The reaction was placed on ice to cool for 2 minutes and then spun down briefly. Five microliters of reaction buffer and 0.2 µL of phi29 polymerase from the TempliPhi kit were added, and the reactions were incubated at 30°C in an MJ Research PCR machine for 4 hours. The phi29 enzyme was heat inactivated in the reactions by incubation at 65°C for 10 min in the PCR machine. *Bacillus subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylΛcomK-phleo) were transformed using 1µL of the reaction mix and the transformants were grown overnight at 37°C on 1.6% skim milk plates containing 10 ppm neomycin. Transformants

were selected to perform colony PCR and sequencing using "puReTaq Ready-To-Go PCR Beads" (Amersham) and primers pIIPLT F1 (SEQ ID NO:54) and pIIPLT seqR1 (SEQ ID NO:55).

4. Generation of BPN' Variants from Libraries RCL4-RCL7

RCL4 Library

"RCL4" refers to a group of site saturation libraries created by PCR fusion that simultaneously randomize three contiguous codons in the BPN'-v3-encoding (BPN"- G97A-G128A-Y217Q) gene. The amino acid positions corresponding to the three mutated codons in each library are provided in Table 11-6. Two partially overlapping, complementary mutagenic primers, each containing three degenerate codons were used to introduce mutations within each library as shown in Table 11-6 below. Only the first two nucleotides of each degenerate codon (NNX, N = A, C, T, or G and X is unchanged nucleotide) of interest were mutated in each primer (Table 11-6).

To create each library, two PCR reactions were carried out using either the common 3' gene-flanking primer (P4976, CCTCTCGGTTATGAGTTAGTTC; (SEQ ID NO:61)) and mutagenic primer, or the common 5'gene-flanking primer (P4974, GCCTCACATTTGTGCCACCTA; (SEQ ID NO:60)) and mutagenic primer as shown for each library in Table 11-6. These PCR reactions generated two PCR fragments, one encoding the 5' half of the mutant BPN'-v3 gene (5' gene fragment) and the other encoding the 3' half of the mutant BPN'-v3 gene (3' gene fragment). Each PCR amplification reaction contained 30 pmol of each primer and 100 ng of the BPN'-v3 parent template DNA (plasmid pHPLT-BPN'-v3) (see Figure 1). Amplifications were carried out using Vent DNA polymerase (NEB). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 40 sec. Following amplification, the 5' and 3' gene fragments were gel-purified by the QIAGEN® gel-band purification kit, mixed (50 ng of each fragment) and amplified by PCR once again using the primers P4973 (AAAGGATCCTAATCGGCGCTTTTC; SEQ ID NO:62) and P4950 (CTTGTCTCCAAGCTTAAAATAAAA; SEQ ID NO:63) to generate the full-length gene fragment. The PCR conditions were same as described above, except the extension phase, which was carried out at 72°C for 2 min. The full-length DNA fragment was gel-purified by the QIAGEN® gel-band purification kit, digested by the *Bam*HI and *Hin*dIII restriction enzymes and ligated with the pHPLT-BPN' partial opt vector that also was digested with the same restriction enzymes. Ligation mixtures were amplified using rolling circle amplification in an Illustra Templiphi kit according to the manufacturer's recommendation (GE Healthcare) to generate multimeric DNA for transformation into *Bacillus subtilis*. For this purpose, 1 µl of the ligation mixture was mixed with 5 µl of the sample buffer, heated to 95°C for 3 min and cooled on ice. Next, 5 µl of the reaction buffer and 0.2 µl of the enzyme were added to each tube, followed by incubation at 30°C for 10 hours. Products of the rolling circle amplification were diluted 100 times and used to transform *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and

incubated overnight at 37°C. Subsequently, the colonies with halos were inoculated in 120 µl of LB media containing 10µg/mL neomycin.

Table 11-6: List of Primers Used to Create RCL4 Libraries

Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
1	5-7	3'	P4976	P5119	TACGCGCAGTCCGTGNNTNNCNNCGTATCACAAATTAAAGCCCCTG	245
		5'	P4974	P5120	TTTAATTTGTGATACGNNGNNANNCACGGACTGCGCGTACGCAT	246
2	11-13	3'	P4976	P5121	TACGGCGTATCACAANNTNNANNCCCTGCTCTGCACTCTCAAG	247
		5'	P4974	P5122	AGAGTGCAGAGCAGGGNNTNNANNTTGTGATACGCCGTAAGGCAC	248
3	20-22	3'	P4976	P5123	GCTCTGCACTCTCAANNCNNCNNTGGATCAAATGTTAAAGTAGCGGT	249
		5'	P4974	P5124	TTTAACATTTGATCCANNGNNGNNTTGAGAGTGCAGAGCAGGGCTT	250
4	21-23	3'	P4976	P5125	CTGCACTCTCAAGGCNNCNNTNNATCAAATGTTAAAGTAGCGGTTATC	251
		5'	P4974	P5126	TACTTTAACATTTGATNNANNGNNGCCTTGAGAGTGCAGAGCAG	252
5	22-24	3'	P4976	P5127	CACTCTCAAGGCTACNNTNNANNAAATGTTAAAGTAGCGGTTATCGA	253
		5'	P4974	P5128	CGCTACTTTAACATTTNNTNNANNGTAGCCTTGAGAGTGCAGAG	254
6	23-25	3'	P4976	P5129	TCTCAAGGCTACACTNNANNANNTGTTAAAGTAGCGGTTATCGACA	255

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5130	AACCGCTACTTTAACANNTNNTNNAGTGTAGCCTTGAGAGTGCAG	256
7	24-26	3'	P4976	P5131	CAAGGCTACACTGGANNANNTNNTAAAGTAGCGGTTATCGACAGC	257
		5'	P4974	P5132	GATAACCGCTACTTTANNANNTNNTCCAGTGTAGCCTTGAGAGTG	258
8	25-27	3'	P4976	P5133	GGCTACACTGGATCANNTNNTNNAGTAGCGGTTATCGACAGCGGT	259
		5'	P4974	P5134	GTCGATAACCGCTACTNNANNANNTGATCCAGTGTAGCCTTGAGA	260
9	26-28	3'	P4976	P5135	TACACTGGATCAAATNNTNNANNAGCGGTTATCGACAGCGGTAT	261
		5'	P4974	P5136	GCTGTCGATAACCGCTNNTNNANNATTTGATCCAGTGTAGCCTTGA	262
10	27-29	3'	P4976	P5137	ACTGGATCAAATGTTNNANNANNGGTTATCGACAGCGGTATCGAC	263
		5'	P4974	P5138	ACCGCTGTCGATAACCNNTNNTNNAACATTTGATCCAGTGTAGCCT	264
11	28-30	3'	P4976	P5139	GGATCAAATGTTAAANNANNGNNTATCGACAGCGGTATCGACTC	265
		5'	P4974	P5140	GATACCGCTGTCGATANNCNNTNNTTTAACATTTGATCCAGTGTAGC	266
12	29-31	3'	P4976	P5141	TCAAATGTTAAAGTANNGNNTNNCGACAGCGGTATCGACTCGAGCCAT	267

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5142	GTCGATACCGCTGT CGNNANNCNNTACT TTAACATTTGATCC AGTGTA	268
13	30-32	3'	P4976	P5143	AATGTTAAAGTAGC GNNTNNCNNCAGCG GTATCGACTCGAGC CAT	269
		5'	P4974	P5144	CGAGTCGATACCGC TGNNGNNANNCGCT ACTTTAACATTTGA TCCAG	270
14	31-33	3'	P4976	P5145	GTTAAAGTAGCGGT TNNCNNCNNCGGTA TCGACTCGAGCCAT CCA	271
		5'	P4974	P5146	GCTCGAGTCGATAC CGNNGNNGNNAAC CGCTACTTTAACAT TTGATC	272
15	32-34	3'	P4976	P5147	AAAGTAGCGGTTAT CNNCNNCNNTATCG ACTCGAGCCATCCA GAT	273
		5'	P4974	P5148	ATGGCTCGAGTCGA TANNGNNGNNGAT AACCGCTACTTTAA CATTTG	274
16	33-35	3'	P4976	P5149	GTAGCGGTTATCGA CNNCNNTNNCGACT CGAGCCATCCAGAT CT	275
		5'	P4974	P5150	TGGATGGCTCGAGT CGNNANNGNNGTC GATAACCGCTACTT TAACA	276
17	34-36	3'	P4976	P5151	GCGGTTATCGACAG CNNTNNCNNCTCGA GCCATCCAGATCTT AAAG	277
		5'	P4974	P5152	ATCTGGATGGCTCG AGNNGNNANNGCT	278

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					GTCGATAACCGCTA CTTT	
18	35-37	3'	P4976	P5153	GTTATCGACAGCGG TNNCNNCNNGAGCC ATCCAGATCTTAAA GTC	279
		5'	P4974	P5154	AAGATCTGGATGGC TCNNGNNGNNACCG CTGTCGATAACCGC TA	280
19	36-38	3'	P4976	P5155	ATCGACAGCGGTAT CNNCNNGNNCCATC CAGATCTTAAAGTC GCTG	281
		5'	P4974	P5156	TTTAAGATCTGGAT GGNNCNNGNNGAT ACCGCTGTCGATAA CCGCTA	282
20	37-39	3'	P4976	P5157	GACAGCGGTATCGA CNNGNNCNNTCCAG ATCTTAAAGTCGCT GGA	283
		5'	P4974	P5158	GACTTTAAGATCTG GANNGNNCNNGTC GATACCGCTGTCGA TAAC	284
21	38-40	3'	P4976	P5159	AGCGGTATCGACTC GNNCNNTNNAGATC TTAAAGTCGCTGGA GG	285
		5'	P4974	P5160	AGCGACTTTAAGAT CTNNANNGNNCGA GTCGATACCGCTGT CGA	286
22	41-43	3'	P4976	P5161	GACTCGAGCCATCC ANNTNNTNNAGTCG CTGGAGGGGCTTCT AT	287
		5'	P4974	P5162	AGCCCCTCCAGCGA CTNNANNANNTGGA TGGCTCGAGTCGAT AC	288

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
23	43-45	3'	P4976	P5163	AGCCATCCAGATCT TNNANNCNNTGGAG GGGCTTCTATGGTG CCGT	289
		5'	P4974	P5164	CATAGAAGCCCCTC CNAGCGACTTTNAA GATCTGGATGGCTC GAGTC	290
24	44-46	3'	P4976	P5165	CATCCAGATCTTAA ANNCNNTNNAGGG GCTTCTATGGTGCC GT	291
		5'	P4974	P5166	CACCATAGAAGCCC CTNNANNGNNTTTA AGATCTGGATGGCT CGAG	292
25	51-53	3'	P4976	P5167	GGAGGGGCTTCTAT GNNGNNGNNCGAA ACAAACCCGTTTCA AGATAA	293
		5'	P4974	P5168	AAACGGGTTTGTTT CGNNCNNCNNCATA GAAGCCCCTCCAGC GA	294
26	52-54	3'	P4976	P5169	GGGGCTTCTATGGT GNNGNNCNNAACA AACCCGTTTCAAGA TAACAA	295
		5'	P4974	P5170	TTGAAACGGGTTTG TTNNGNNCNNCACC ATAGAAGCCCCTCC AG	296
27	53-55	3'	P4976	P5171	GCTTCTATGGTGCC GNNCNNANNAAAC CCGTTTCAAGATAA CAATTC	297
		5'	P4974	P5172	ATCTTGAAACGGGT TTNNTNNGNNCGGC ACCATAGAAGCCCC TC	298
28	54-56	3'	P4976	P5173	TCTATGGTGCCGTC CNNANNANNCCCGT	299

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					TTCAAGATAACAATTCTCA	
		5'	P4974	P5174	GTTATCTTGAAACGGGNNTNNTNNGGACGGCACCATAGAAGCCCCT	300
29	55-57	3'	P4976	P5175	ATGGTGCCGTCCGAANNANNCNNGTTTCAAGATAACAATTCTCATGG	301
		5'	P4974	P5176	ATTGTTATCTTGAAACNNGNNTNNTTCGGACGGCACCATAGAAG	302
30	56-58	3'	P4976	P5177	GTGCCGTCCGAAACANNCNNGNNTCAAGATAACAATTCTCATGGCAC	303
		5'	P4974	P5178	AGAATTGTTATCTTGANNCNNGNNTGTTTCGGACGGCACCATAGA	304
31	57-59	3'	P4976	P5179	CCGTCCGAAACAAACNNGNNTNNAGATAACAATTCTCATGGCACAC	305
		5'	P4974	P5180	ATGAGAATTGTTATCTNNANNCNNGTTTGTTTCGGACGGCACCA	306
32	58-60	3'	P4976	P5181	TCCGAAACAAACCCGNNTNNANNTAACAATTCTCATGGCACACAC	307
		5'	P4974	P5182	GCCATGAGAATTGTTANNTNNANNCGGGTTTGTTTCGGACGGCA	308
33	59-61	3'	P4976	P5183	GAAACAAACCCGTTTNNANNTNNCAATTCTCATGGCACACACGTC	309

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5184	TGTGCCATGAGAATTGNNANNTNNAAACGGGTTTGTTTCGGACG	310
34	60-62	3'	P4976	P5185	ACAAACCCGTTTCAANNTNNCNNTTCTCATGGCACACACGTCG	311
		5'	P4974	P5186	GTGTGTGCCATGAGAANNGNNANNTTGAAACGGGTTTGTTTCGGAC	312
35	61-63	3'	P4976	P5187	AACCCGTTTCAAGATNNCNNTNNTCATGGCACACACGTCGCAG	313
		5'	P4974	P5188	GACGTGTGTGCCATGANNANNGNNATCTTGAAACGGGTTTGTTTCG	314
36	62-64	3'	P4976	P5189	CCGTTTCAAGATAACNNTNNTNNTGGCACACACGTCGCAGGAA	315
		5'	P4974	P5190	TGCGACGTGTGTGCCANNANNANNGTTATCTTGAAACGGGTTTGTTT	316
37	67-69	3'	P4976	P5191	AATTCTCATGGCACANNCNNCNNAGGAACGGTTGCGGCGTTAAA	317
		5'	P4974	P5192	CGCCGCAACCGTTCCTNNGNNGNNTGTGCCATGAGAATTGTTATCTT	318
38	68-70	3'	P4976	P5193	TCTCATGGCACACACNNCNNANNAACGGTTGCGGCGTTAAACAAT	319
		5'	P4974	P5194	TAACGCCGCAACCGTTNNTNNGNNGTGT	320

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					GTGCCATGAGAATT GTTA	
39	71-73	3'	P4976	P5195	ACACACGTCGCAGG ANNGNNTNNGGCGT TAAACAATTCTATT GGCGT	321
		5'	P4974	P5196	AGAATTGTTTAACG CCNNANNCNNTCCT GCGACGTGTGTGCC AT	322
40	74-76	3'	P4976	P5197	GCAGGAACGGTTGC GNNGNNANNCAATT CTATTGGCGTGCTT GGTG	323
		5'	P4974	P5198	CACGCCAATAGAAT TGNNTNNCNNCGCA ACCGTTCCTGCGAC GT	324
41	77-79	3'	P4976	P5199	ACGGTTGCGGCGTT ANNCNNTNNTATTG GCGTGCTTGGTGTA GC	325
		5'	P4974	P5200	ACCAAGCACGCCAA TANNANNGNNTAAC GCCGCAACCGTTCC TG	326
42	81-83	3'	P4976	P5201	AACAATTCTATTGG CNNGNNTNNTGTAG CCCCGTCTGCTTCG CT	327
		5'	P4974	P5202	AGCAGACGGGGCTA CANNANNCNNGCC AATAGAATTGTTTA ACGCCGCAA	328
43	83-85	3'	P4976	P5203	TCTATTGGCGTGCT TNNTNNANNCCCGT CTGCTTCGCTCTAC G	329
		5'	P4974	P5204	GAGCGAAGCAGAC GGGNNTNNANNAA GCACGCCAATAGAA TTGTTTA	330

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
44	84-86	3'	P4976	P5205	ATTGGCGTGCTTGGTNNANNCNNGTCTGCTTCGCTCTACGCCGT	331
		5'	P4974	P5206	GTAGAGCGAAGCAGACNNGNNTNNACCAAGCACGCCAATAGAATTG	332
45	85-87	3'	P4976	P5207	TGGCGTGCTTGGTGTANNCNNGNNTGCTTCGCTCTACGCCGTTAA	333
		5'	P4974	P5208	GGCGTAGAGCGAAGCANNCNNGNNTACACCAAGCACGCCAATAGA	334
46	86-88	3'	P4976	P5209	GTGCTTGGTGTAGCCNNGNNTNNTTCGCTCTACGCCGTTAAAGTT	335
		5'	P4974	P5210	AACGGCGTAGAGCGAANNANNCNNGGCTACACCAAGCACGCCAA	336
47	87-89	3'	P4976	P5211	CTTGGTGTAGCCCCGNNTNNTNNGCTCTACGCCGTTAAAGTTCTT	337
		5'	P4974	P5212	TTTAACGGCGTAGAGCNNANNANNCGGGGCTACACCAAGCACGCCAAT	338
48	88-90	3'	P4976	P5213	GGTGTAGCCCCGTCTNNTNNGNNCTACGCCGTTAAAGTTCTTGCAG	339
		5'	P4974	P5214	AACTTTAACGGCGTAGNNCNNANNAGACGGGGCTACACCAAGCA	340
49	89-91	3'	P4976	P5215	GTAGCCCCGTCTGCTNNGNNCNNCGCCG	341

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					TTAAAGTTCTTGCAGCA	
		5'	P4974	P5216	AAGAACTTTAACGGCGNNGNNCNNAGCAGACGGGGCTACACCAA	342
50	90-92	3'	P4976	P5217	GCCCCGTCTGCTTCGNNCNNCNNCGTTAAAGTTCTTGCAGCAGAC	343
		5'	P4974	P5218	TGCAAGAACTTTAACGNNGNNGNNCGAAGCAGACGGGGCTACAC	344
51	91-93	3'	P4976	P5219	CCGTCTGCTTCGCTCNNCNNCNNTAAAGTTCTTGCAGCAGACGGA	345
		5'	P4974	P5220	TGCTGCAAGAACTTTANNGNNGNNGAGCGAAGCAGACGGGGCT	346
52	92-94	3'	P4976	P5221	TCTGCTTCGCTCTACNNCNNTNNAGTTCTTGCAGCAGACGGATC	347
		5'	P4974	P5222	GTCTGCTGCAAGAACTNNANNGNNGTAGAGCGAAGCAGACGGGGCTA	348
53	93-95	3'	P4976	P5223	GCTTCGCTCTACGCCNNTNNANNTCTTGCAGCAGACGGATCAG	349
		5'	P4974	P5224	TCCGTCTGCTGCAAGANNTNNANNGGCGTAGAGCGAAGCAGACG	350
54	94-96	3'	P4976	P5225	TCGCTCTACGCCGTTNNANNTNNTGCAGCAGACGGATCAGGCCA	351

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5226	TGATCCGTCTGCTGCANNANNTNNAACGGCGTAGAGCGAAGCAG	352
55	95-97	3'	P4976	P5227	CTCTACGCCGTTAAANNTNNTNNAGCAGACGGATCAGGCCAATA	353
		5'	P4974	P5228	GCCTGATCCGTCTGCTNNANNANNTTTAACGGCGTAGAGCGAAG	354
56	96-98	3'	P4976	P5229	TACGCCGTTAAAGTTNNTNNANNAGACGGATCAGGCCAATACTC	355
		5'	P4974	P5230	TTGGCCTGATCCGTCTNNTNNANNAACTTTAACGGCGTAGAGCGA	356
57	97-99	3'	P4976	P5231	GCCGTTAAAGTTCTTNNANNANNCGGATCAGGCCAATACTCATG	357
		5'	P4974	P5232	GTATTGGCCTGATCCGNNTNNTNNAAGAACTTTAACGGCGTAGAG	358
58	98-100	3'	P4976	P5233	GTTAAAGTTCTTGCANNANNCNNATCAGGCCAATACTCATGGATTA	359
		5'	P4974	P5234	TGAGTATTGGCCTGATNNGNNTNNTGCAAGAACTTTAACGGCGTAG	360
59	99-101	3'	P4976	P5235	AAAGTTCTTGCAGCANNCNNANNAGGCCAATACTCATGGATTATC	361
		5'	P4974	P5236	CCATGAGTATTGGCCTNNTNNGNNTGCT	362

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					GCAAGAACTTTAAC GGCGTA	
60	100-102	3'	P4976	P5237	GTTCTTGCAGCAGA CNNANNANNCCAAT ACTCATGGATTATC AAC	363
		5'	P4974	P5238	AATCCATGAGTATT GGNNTNNTNNGTCT GCTGCAAGAACTTT AACG	364
61	101-103	3'	P4976	P5239	CTTGCAGCAGACGG ANNANNCNNATACT CATGGATTATCAAC GGCA	365
		5'	P4974	P5240	GATAATCCATGAGT ATNNGNNTNNTCCG TCTGCTGCAAGAAC TTT	366
62	102-104	3'	P4976	P5241	GCAGCAGACGGATC ANNCNNANNCTCAT GGATTATCAACGGC ATC	367
		5'	P4974	P5242	TTGATAATCCATGA GNNTNNGNNTGATC CGTCTGCTGCAAGA AC	368
63	103-105	3'	P4976	P5243	GCAGACGGATCAGG CNNANNCNNATGG ATTATCAACGGCAT CGAAT	369
		5'	P4974	P5244	GCCGTTGATAATCC ATNNGNNTNNGCCT GATCCGTCTGCTGC AA	370
64	104-106	3'	P4976	P5245	GACGGATCAGGCCA ANNCNNANNGATTA TCAACGGCATCGAA TGG	371
		5'	P4974	P5246	GATGCCGTTGATAA TCNNTNNGNNTTGG CCTGATCCGTCTGC TG	372

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
65	105-107	3'	P4976	P5247	GGATCAGGCCAATACNNANNGNNTATCAACGGCATCGAATGGGCCAT	373
		5'	P4974	P5248	TTCGATGCCGTTGATANNCNNTNNGTATTGGCCTGATCCGTCTG	374
66	106-108	3'	P4976	P5249	TCAGGCCAATACTCANNGNNTNNCAACGGCATCGAATGGGCCAT	375
		5'	P4974	P5250	CCATTCGATGCCGTTGNNANNCNNTGAGTATTGGCCTGATCCGTC	376
67	107-109	3'	P4976	P5251	GGCCAATACTCATGGNNTNNCNNCGGCATCGAATGGGCCATCGCGAAT	377
		5'	P4974	P5252	GGCCCATTCGATGCCGNNGNNANNCCATGAGTATTGGCCTGATCC	378
68	108-110	3'	P4976	P5253	CAATACTCATGGATTNNCNNCNNCATCGAATGGGCCATCGCGAA	379
		5'	P4974	P5254	GATGGCCCATTCGATGNNGNNGNNAATCCATGAGTATTGGCCTGAT	380
69	109-111	3'	P4976	P5255	TACTCATGGATTATCNNCNNCNNCGAATGGGCCATCGCGAATAA	381
		5'	P4974	P5256	CGCGATGGCCCATTCGNNGNNGNNGATAATCCATGAGTATTGGCCT	382
70	110-112	3'	P4976	P5257	TCATGGATTATCAACNNCNNCNNATGGG	383

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					CCATCGCGAATAACATG	
		5'	P4974	P5258	ATTCGCGATGGCCCATNNGNNGNNGTTGATAATCCATGAGTATTGG	384
71	111-113	3'	P4976	P5259	TGGATTATCAACGGCNNCNNANNGGCCATCGCGAATAACATGGA	385
		5'	P4974	P5260	GTTATTCGCGATGGCCNNTNNGNNGCCGTTGATAATCCATGAGTAT	386
72	112-114	3'	P4976	P5261	ATTATCAACGGCATCNNANNGNNCATCGCGAATAACATGGATGTAA	387
		5'	P4974	P5262	CATGTTATTCGCGATGNNCNNTNNGATGCCGTTGATAATCCATGAG	388
73	113-115	3'	P4976	P5263	ATCAACGGCATCGAANNGNNCNNCGCGAATAACATGGATGTAATCAA	389
		5'	P4974	P5264	ATCCATGTTATTCGCGNNGNNCNNTTCGATGCCGTTGATAATCCAT	390
74	114-116	3'	P4976	P5265	AACGGCATCGAATGGNNCNNCNNGAATAACATGGATGTAATCAACAT	391
		5'	P4974	P5266	TACATCCATGTTATTCNNGNNGNNCCATTCGATGCCGTTGATAATC	392
75	115-117	3'	P4976	P5267	GGCATCGAATGGGCCNNCNNGNNTAACATGGATGTAATCAACATGAG	393

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5268	GATTACATCCATGTTANNCNNGNNGGCCCATTCGATGCCGTTGA	394
76	116-118	3'	P4976	P5269	ATCGAATGGGCCATCNNGNNTNNCATGGATGTAATCAACATGAGCCT	395
		5'	P4974	P5270	GTTGATTACATCCATGNNANNCNNGATGGCCCATTCGATGCCGT	396
77	117-119	3'	P4976	P5271	GAATGGGCCATCGCGNNTNNCNNGGATGTAATCAACATGAGCCTG	397
		5'	P4974	P5272	CATGTTGATTACATCCNNGNNANNCGCGATGGCCCATTCGATGC	398
78	118-120	3'	P4976	P5273	TGGGCCATCGCGAATNNCNNGNNTGTAATCAACATGAGCCTGGGA	399
		5'	P4974	P5274	GCTCATGTTGATTACANNCNNGNNATTCGCGATGGCCCATTCGA	400
79	119-121	3'	P4976	P5275	GCCATCGCGAATAACNNGNNTNNAATCAACATGAGCCTGGGAGCA	401
		5'	P4974	P5276	CAGGCTCATGTTGATTNNANNCNNGTTATTCGCGATGGCCCATTC	402
80	120-122	3'	P4976	P5277	ATCGCGAATAACATGNNTNNANNCAACATGAGCCTGGGAGCAC	403
		5'	P4974	P5278	TCCCAGGCTCATGTTGNNTNNANNCATG	404

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					TTATTCGCGATGGC CCAT	
81	121-123	3'	P4976	P5279	GCGAATAACATGGA TNNANNCNNCATGA GCCTGGGAGCACCA AG	405
		5'	P4974	P5280	TGCTCCCAGGCTCA TGNNGNNTNNATCC ATGTTATTCGCGAT GGCCCATT	406
82	122-124	3'	P4976	P5281	AATAACATGGATGT ANNCNNCNNGAGC CTGGGAGCACCAAG CGGCA	407
		5'	P4974	P5282	TGGTGCTCCCAGGC TCNNGNNGNNTACA TCCATGTTATTCGC GATG	408
83	123-125	3'	P4976	P5283	AACATGGATGTAAT CNNCNNGNNCCTGG GAGCACCAAGCGGC A	409
		5'	P4974	P5284	GCTTGGTGCTCCCA GGNNCNNGNNGATT ACATCCATGTTATT CGCGA	410
84	124-126	3'	P4976	P5285	ATGGATGTAATCAA CNNGNNCNNGGGA GCACCAAGCGGCAG TG	411
		5'	P4974	P5286	GCCGCTTGGTGCTC CCNNGNNCNNGTTG ATTACATCCATGTT ATTCG	412
85	125-127	3'	P4976	P5287	GATGTAATCAACAT GNNCNNGNNAGCA CCAAGCGGCAGTGC GGCA	413
		5'	P4974	P5288	ACTGCCGCTTGGTG CTNNCNNGNNCATG TTGATTACATCCAT GTTATT	414

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
86	126-128	3'	P4976	P5289	GTAATCAACATGAG CNNGNNANNACCA AGCGGCAGTGCGGC ACT	415
		5'	P4974	P5290	CGCACTGCCGCTTG GTNNTNNCNNGCTC ATGTTGATTACATC CATG	416
87	129-131	3'	P4976	P5291	ATGAGCCTGGGAGC ANNANNCNNCAGT GCGGCACTTAAAGC AGCA	417
		5'	P4974	P5292	TTTAAGTGCCGCAC TGNNGNNTNNTGCT CCCAGGCTCATGTT GAT	418
88	132-134	3'	P4976	P5293	GGAGCACCAAGCG GCNNTNNGNNACTT AAAGCAGCAGTTGA TAAAG	419
		5'	P4974	P5294	AACTGCTGCTTTAA GTNNCNNANNGCCG CTTGGTGCTCCCAG GCT	420
89	133-135	3'	P4976	P5295	GCACCAAGCGGCAG TNNGNNANNTAAA GCAGCAGTTGATAA AGCTG	421
		5'	P4974	P5296	ATCAACTGCTGCTT TANNTNNCNNACTG CCGCTTGGTGCTCC CA	422
90	134-136	3'	P4976	P5297	CCAAGCGGCAGTGC GNNANNTNNAGCA GCAGTTGATAAAGC TGTTG	423
		5'	P4974	P5298	TTTATCAACTGCTG CTNNANNTNNCGCA CTGCCGCTTGGTGC TC	424
91	135-137	3'	P4976	P5299	AGCGGCAGTGCGGC ANNTNNANNAGCA	425

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					GTTGATAAAGCTGT TGCAT	
		5'	P4974	P5300	AGCTTTATCAACTG CTNNTNNANNTGCC GCACTGCCGCTTGG TG	426
92	136-138	3'	P4976	P5301	GGCAGTGCGGCACT TNNANNANNAGTTG ATAAAGCTGTTGCA TCTG	427
		5'	P4974	P5302	AACAGCTTTATCAA CTNNTNNTNNAAGT GCCGCACTGCCGCT TG	428
93	137-139	3'	P4976	P5303	AGTGCGGCACTTAA ANNANNANNTGAT AAAGCTGTTGCATC TGGTG	429
		5'	P4974	P5304	TGCAACAGCTTTAT CANNTNNTNNTTTA AGTGCCGCACTGCC GCTT	430
94	138-140	3'	P4976	P5305	GCGGCACTTAAAGC ANNANNTNNTAAA GCTGTTGCATCTGG TGTC	431
		5'	P4974	P5306	AGATGCAACAGCTT TANNANNTNNTGCT TTAAGTGCCGCACT GC	432
95	139-141	3'	P4976	P5307	GCACTTAAAGCAGC ANNTNNTNNAGCTG TTGCATCTGGTGTC GT	433
		5'	P4974	P5308	ACCAGATGCAACAG CTNNANNANNTGCT GCTTTAAGTGCCGC AC	434
96	140-142	3'	P4976	P5309	CTTAAAGCAGCAGT TNNTNNANNTGTTG CATCTGGTGTCGTC GT	435

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5310	GACACCAGATGCAACANNTNNANNAACTGCTGCTTTAAGTGCCGCA	436
97	141-143	3'	P4976	P5311	AAAGCAGCAGTTGATNNANNTNNTGCATCTGGTGTCGTCGTAGT	437
		5'	P4974	P5312	GACGACACCAGATGCANNANNTNNATCAACTGCTGCTTTAAGTGC	438
98	142-144	3'	P4976	P5313	GCAGCAGTTGATAAANNTNNTNNATCTGGTGTCGTCGTAGTAGC	439
		5'	P4974	P5314	TACGACGACACCAGATNNANNANNTTTATCAACTGCTGCTTTAAGTG	440
99	143-145	3'	P4976	P5315	GCAGTTGATAAAGCTNNTNNANNTGGTGTCGTCGTAGTAGCGGCA	441
		5'	P4974	P5316	TACTACGACGACACCANNTNNANNAGCTTTATCAACTGCTGCTTTAA	442
100	144-146	3'	P4976	P5317	GTTGATAAAGCTGTTNNANNTNNTGTCGTCGTAGTAGCGGCAGCT	443
		5'	P4974	P5318	CGCTACTACGACGACANNANNTNNAACAGCTTTATCAACTGCTGCT	444
101	145-147	3'	P4976	P5319	GATAAAGCTGTTGCANNTNNTNNCGTCGTAGTAGCGGCAGCTG	445
		5'	P4974	P5320	TGCCGCTACTACGACGNNANNANNTGC	446

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					AACAGCTTTATCAACTGCT	
102	146-148	3'	P4976	P5321	AAAGCTGTTGCATCTNNTNNCNNCGTAGTAGCGGCAGCTGGGAA	447
		5'	P4974	P5322	AGCTGCCGCTACTACGNNGNNANNAGATGCAACAGCTTTATCAACTG	448
103	147-149	3'	P4976	P5323	GCTGTTGCATCTGGTNNCNNCNNAGTAGCGGCAGCTGGGAATGA	449
		5'	P4974	P5324	CCCAGCTGCCGCTACTNNGNNGNNACCAGATGCAACAGCTTTATCA	450
104	148-150	3'	P4976	P5325	GTTGCATCTGGTGTCNNCNNANNAGCGGCAGCTGGGAATGAGGGAA	451
		5'	P4974	P5326	ATTCCCAGCTGCCGCTNNTNNGNNGACACCAGATGCAACAGCTTT	452
105	149-151	3'	P4976	P5327	GCATCTGGTGTCGTCNNANNANNGGCAGCTGGGAATGAGGGAAC	453
		5'	P4974	P5328	CTCATTCCCAGCTGCCNNTNNTNNGACGACACCAGATGCAACAG	454
106	150-152	3'	P4976	P5329	TCTGGTGTCGTCGTANNANNGNNAGCTGGGAATGAGGGAACATC	455
		5'	P4974	P5330	TCCCTCATTCCCAGCTNNCNNTNNTACGACGACACCAGATGCAAC	456

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
107	151-153	3'	P4976	P5331	GGTGTCGTCGTAGTANNGNNANNTGGGAATGAGGGAACATCCGGAT	457
		5'	P4974	P5332	TGTTCCCTCATTCCCANNTNNCNNTACTACGACGACACCAGATGCA	458
108	158-160	3'	P4976	P5333	GCTGGGAATGAGGGANNANNCNNATCATCGAGTACCGTCGGTTAT	459
		5'	P4974	P5334	GACGGTACTCGATGATNNGNNTNNTCCCTCATTCCCAGCTGCCGCTA	460
109	159-161	3'	P4976	P5335	GGGAATGAGGGAACANNCNNANNATCGAGTACCGTCGGTTATCCA	461
		5'	P4974	P5336	ACCGACGGTACTCGATNNTNNGNNTGTTCCCTCATTCCCAGCTG	462
110	160-162	3'	P4976	P5337	AATGAGGGAACATCCNNANNANNGAGTACCGTCGGTTATCCAGG	463
		5'	P4974	P5338	ATAACCGACGGTACTCNNTNNTNNGGATGTTCCCTCATTCCCAG	464
111	163-165	3'	P4976	P5339	ACATCCGGATCATCGNNTNNCNNCGGTTATCCAGGCAAGTACCCTT	465
		5'	P4974	P5340	CTTGCCTGGATAACCGNNGNNANNCGATGATCCGGATGTTCCCT	466
112	164-166	3'	P4976	P5341	TCCGGATCATCGAGTNNCNNCNNTTATC	467

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					CAGGCAAGTACCCT TCA	
		5'	P4974	P5342	GTACTTGCCTGGAT AANNGNNGNNACT CGATGATCCGGATG TTCC	468
113	167-169	3'	P4976	P5343	TCGAGTACCGTCGG TNNTNNANNCAAGT ACCCTTCAGTGATT GCA	469
		5'	P4974	P5344	CACTGAAGGGTACT TGNNTNNANNACCG ACGGTACTCGATGA TC	470
114	168-170	3'	P4976	P5345	AGTACCGTCGGTTA TNNANNCNNGTACC CTTCAGTGATTGCA GTG	471
		5'	P4974	P5346	AATCACTGAAGGGT ACNNGNNTNNATAA CCGACGGTACTCGA TGA	472
115	169-171	3'	P4976	P5347	ACCGTCGGTTATCC ANNCNNGNNCCCTT CAGTGATTGCAGTG G	473
		5'	P4974	P5348	TGCAATCACTGAAG GGNNCNNGNNTGG ATAACCGACGGTAC TCGA	474
116	170-172	3'	P4976	P5349	GTCGGTTATCCAGG CNNGNNCNNTTCAG TGATTGCAGTGGGC GCT	475
		5'	P4974	P5350	CACTGCAATCACTG AANNGNNCNNGCCT GGATAACCGACGGT AC	476
117	171-173	3'	P4976	P5351	GGTTATCCAGGCAA GNNCNNTNNAGTGA TTGCAGTGGGCGCT GTA	477

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5352	GCCCACTGCAATCACTNNANNGNNCTTGCCTGGATAACCGACGGTA	478
118	172-174	3'	P4976	P5353	TATCCAGGCAAGTACNNTNNANNGATTGCAGTGGGCGCTGTAGA	479
		5'	P4974	P5354	AGCGCCCACTGCAATCNNTNNANNGTACTTGCCTGGATAACCGAC	480
119	182-184	3'	P4976	P5355	GTGGGCGCTGTAGACNNTNNANNTCAACGTGCCTCTTTTTCCTC	481
		5'	P4974	P5356	AAAAGAGGCACGTTGANNTNNANNGTCTACAGCGCCCACTGCAA	482
120	183-185	3'	P4976	P5357	GGCGCTGTAGACTCTNNANNTNNACGTGCCTCTTTTTCCTCCGT	483
		5'	P4974	P5358	GGAAAAAGAGGCACGTNNANNTNNAGAGTCTACAGCGCCCACTG	484
121	184-186	3'	P4976	P5359	GCTGTAGACTCTTCANNTNNANNTGCCTCTTTTTCCTCCGTGGGA	485
		5'	P4974	P5360	GGAGGAAAAAGAGGCANNTNNANNTGAAGAGTCTACAGCGCCCA	486
122	185-187	3'	P4976	P5361	GTAGACTCTTCAAATNNANNTNNCTCTTTTTCCTCCGTGGGAC	487
		5'	P4974	P5362	CACGGAGGAAAAAGAGNNANNTNNATT	488

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					TGAAGAGTCTACAG CGCCCA	
123	186-188	3'	P4976	P5363	GACTCTTCAAATCA ANNTNNCNNTTTTT CCTCCGTGGGACCG GA	489
		5'	P4974	P5364	TCCCACGGAGGAAA AANNGNNANNTTG ATTTGAAGAGTCTA CAGCGCCCA	490
124	192-194	3'	P4976	P5365	GCCTCTTTTTCCTCC NNGNNANNGGAGC TGGATGTCATGGCC CCT	491
		5'	P4974	P5366	CATGACATCCAGCT CCNNTNNCNNGGAG GAAAAAGAGGCAC GTTG	492
125	194-196	3'	P4976	P5367	TTTTCCTCCGTGGG ANNGNNGNNGGAT GTCATGGCCCCTGG CGTT	493
		5'	P4974	P5368	AGGGGCCATGACAT CCNNCNNCNNTCCC ACGGAGGAAAAAG AGG	494
126	195-197	3'	P4976	P5369	TCCTCCGTGGGACC GNNGNNGNNTGTCA TGGCCCCTGGCGTT TCTATT	495
		5'	P4974	P5370	GCCAGGGGCCATGA CANNCNNCNNCGGT CCCACGGAGGAAA AAG	496
127	196-198	3'	P4976	P5371	TCCGTGGGACCGGA GNNGNNTNNCATGG CCCCTGGCGTTTCT ATT	497
		5'	P4974	P5372	AACGCCAGGGGCCA TGNNANNCNNCTCC GGTCCCACGGAGGA AAAA	498

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
128	197-199	3'	P4976	P5373	GTGGGACCGGAGCTGNNTNNCNNGGCCCCTGGCGTTTCTATTCAA	499
		5'	P4974	P5374	AGAAACGCCAGGGGCCNNGNNANNCAGCTCCGGTCCCACGGAGGAAA	500
129	198-200	3'	P4976	P5375	GGACCGGAGCTGGATNNCNNGNNCCCTGGCGTTTCTATTCAATCGA	501
		5'	P4974	P5376	AATAGAAACGCCAGGGNNCNNGNNATCCAGCTCCGGTCCCACGGA	502
130	203-205	3'	P4976	P5377	GTCATGGCCCCTGGCNNTNNTNNTCAATCGACGCTTCCAGGGAA	503
		5'	P4974	P5378	TGGAAGCGTCGATTGANNANNANNGCCAGGGGCCATGACATCCA	504
131	210-212	3'	P4976	P5379	ATTCAATCGACGCTTNNANNGNNCAAGTATGGTGCGCAAAACGGGA	505
		5'	P4974	P5380	TTGCGCACCATACTTGNNCNNTNNAAGCGTCGATTGAATAGAAACG	506
132	211-213	3'	P4976	P5381	CAATCGACGCTTCCANNGNNCNNGTATGGTGCGCAAAACGGGACT	507
		5'	P4974	P5382	GTTTTGCGCACCATACNNGNNCNNTGGAAGCGTCGATTGAATAGAA	508

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
133	216-218	3'	P4976	P5383	GGGAACAAGTATGGTNNGNNANNCGGGACTTCCATGGCCTCGCCGCAT	509
		5'	P4974	P5384	GGCCATGGAAGTCCCGNNTNNCNNACCATACTTGTTCCCTGGAAG	510
134	217-219	3'	P4976	P5385	AACAAGTATGGTGCGNNANNCNNGACTTCCATGGCCTCGCCGCAT	511
		5'	P4974	P5386	CGAGGCCATGGAAGTCNNGNNTNNCGCACCATACTTGTTCCCTG	512
135	218-220	3'	P4976	P5387	AAGTATGGTGCGCAANNCNNGNNTTCCATGGCCTCGCCGCATG	513
		5'	P4974	P5388	CGGCGAGGCCATGGAANNCNNGNNTTGCGCACCATACTTGTTCCC	514
136	219-221	3'	P4976	P5389	TATGGTGCGCAAAACNNGNNTNNCATGGCCTCGCCGCATGTAG	515
		5'	P4974	P5390	ATGCGGCGAGGCCATGNNANNCNNGTTTTGCGCACCATACTTGTTC	516
137	230-232	3'	P4976	P5391	CCGCATGTAGCTGGGNNGNNCNNATTGATTCTTTCTAAGCACCCGAA	517
		5'	P4974	P5392	CTTAGAAAGAATCAATNNGNNCNNCCCAGCTACATGCGGCGAGGCCAT	518
138	231-233	3'	P4976	P5393	CATGTAGCTGGGGCGNNCNNANNGATTC	519

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
					TTTCTAAGCACCCG AACT	
		5'	P4974	P5394	GTGCTTAGAAAGAA TCNNTNNGNNCGCC CCAGCTACATGCGG CGAGGCCAT	520
139	232-234	3'	P4976	P5395	GTAGCTGGGGCGGC CNNANNGNNTCTTT CTAAGCACCCGAAC TG	521
		5'	P4974	P5396	CGGGTGCTTAGAAA GANNCNNTNNGGCC GCCCCAGCTACATG C	522
140	238-240	3'	P4976	P5397	TTGATTCTTTCTAAG NNCNNGNNCTGGAC AAACACTCAAGTCC GCA	523
		5'	P4974	P5398	TTGAGTGTTTGTCC AGNNCNNGNNCTTA GAAAGAATCAATGC GGC	524
141	240-242	3'	P4976	P5399	CTTTCTAAGCACCC GNNCNNGNNAAAC ACTCAAGTCCGCAG CAGT	525
		5'	P4974	P5400	GCGGACTTGAGTGT TTNNCNNGNNCGGG TGCTTAGAAAGAAT CAAT	526
142	246-248	3'	P4976	P5401	TGGACAAACACTCA ANNCNNCNNCAGTT TAGAAAACACCACT ACAAAA	527
		5'	P4974	P5402	GGTGTTTTCTAAAC TGNNGNNGNNTTGA GTGTTTGTCCAGTT CGGGT	528
143	255-257	3'	P4976	P5403	TTAGAAAACACCAC TNNANNANNTGGTG ATTCTTTCTACTATG GAAA	529

Table 11-6: List of Primers Used to Create RCL4 Libraries						
Library #	Mutated residues in BPN'-v3	Gene fragment	Common flanking primer names	Mutagenic primer names	Mutagenic primer sequence	SEQ ID NO:
		5'	P4974	P5404	GTAGAAAGAATCAC CANNTNNTNNAGTG GTGTTTTCTAAACT GCTG	530
144	258-260	3'	P4976	P5405	ACCACTACAAAACT TNNTNNTNNTTTCT ACTATGGAAAAGGG CTGA	531
		5'	P4974	P5406	TTTTCCATAGTAGA AANNANNANNAAG TTTTGTAGTGGTGTT TTCTAA	532
145	265-267	3'	P4976	P5407	GATTCTTTCTACTAT NNANNANNGCTGAT CAACGTACAGGCGG CA	533
		5'	P4974	P5408	CTGTACGTTGATCA GCNNTNNTNNATAG TAGAAAGAATCACC AAGTTT	534

RCL5 Variants

"RCL5" refers to a set of combinatorial variants created by PCR fusion using several BPN' mutants as parent (template) molecules. The mutations introduced in each parent plasmid are shown in Table 11-7 and the mutagenic primers used to create the mutants are indicated in Table 11-8.

Table 11-7: List of Parent Plasmids and Mutations Introduced in the RCL5 Variants		
Combinatorial Variant #	Parent Plasmids	Mutations Introduced
1	G97A-G128A-Y217Q-T22N-S24A	N61P-N62S
2	G97A-G128A-Y217Q-T22N-S24A	T55P
3	G97A-G128A-Y217Q-T22N-S24A	N61P-S63H
4	G97A-G128A-Y217Q-T22N-S24A	Q59S-N61P
5	G97A-G128A-Y217Q-T22N-S24A	L75S-N76Y
6	G97A-G128A-Y217Q-T22N-S24A	P86S-S87G-A88V
7	G97A-G128A-Y217Q-T22N-S24A	S87G-A88V-S89A
8	G97A-G128A-Y217Q-T22N-S24A	S87T-A88L-S89G
9	G97A-G128A-Y217Q-T22N-S24A	P129Q-S130G-G131S
10	G97A-G128A-Y217Q-T22N-S24A	V203Y
11	G97A-G128A-Y217Q-T22N-S24A	G211R-N212S-K213V

Table 11-7: List of Parent Plasmids and Mutations Introduced in the RCL5 Variants		
Combinatorial Variant #	**Parent Plasmids**	**Mutations Introduced**
12	G97A-G128A-Y217Q-S24G-N25G	N61P-N62S
13	G97A-G128A-Y217Q-S24G-N25G	T55P
14	G97A-G128A-Y217Q-S24G-N25G	N61P-S63H
15	G97A-G128A-Y217Q-S24G-N25G	Q59S-N61P
16	G97A-G128A-Y217Q-S24G-N25G	L75S-N76Y
17	G97A-G128A-Y217Q-S24G-N25G	P86S-S87G-A88V
18	G97A-G128A-Y217Q-S24G-N25G	S87G-A88V-S89A
19	G97A-G128A-Y217Q-S24G-N25G	S87T-A88L-S89G
20	G97A-G128A-Y217Q-S24G-N25G	P129Q-S130G-G131S
21	G97A-G128A-Y217Q-S24G-N25G	V203Y
22	G97A-G128A-Y217Q-S24G-N25G	G211R-N212S-K213V
23	G97A-G128A-Y217Q-S24R	N61P-N62S
24	G97A-G128A-Y217Q-S24R	T55P
25	G97A-G128A-Y217Q-S24R	N61P-S63H
26	G97A-G128A-Y217Q-S24R	Q59S-N61P
27	G97A-G128A-Y217Q-S24R	L75S-N76Y
28	G97A-G128A-Y217Q-S24R	P86S-S87G-A88V
29	G97A-G128A-Y217Q-S24R	S87G-A88V-S89A
30	G97A-G128A-Y217Q-S24R	S87T-A88L-S89G
31	G97A-G128A-Y217Q-S24R	P129Q-S130G-G131S
32	G97A-G128A-Y217Q-S24R	V203Y
33	G97A-G128A-Y217Q-S24R	G211R-N212S-K213V
34	G97A-G128A-Y217Q-G23A-S24G-N25G	N61P-N62S
35	G97A-G128A-Y217Q-G23A-S24G-N25G	T55P
36	G97A-G128A-Y217Q-G23A-S24G-N25G	N61P-S63H
37	G97A-G128A-Y217Q-G23A-S24G-N25G	Q59S-N61P
38	G97A-G128A-Y217Q-G23A-S24G-N25G	L75S-N76Y
39	G97A-G128A-Y217Q-G23A-S24G-N25G	P86S-S87G-A88V
40	G97A-G128A-Y217Q-G23A-S24G-N25G	S87G-A88V-S89A
41	G97A-G128A-Y217Q-G23A-S24G-N25G	S87T-A88L-S89G
42	G97A-G128A-Y217Q-G23A-S24G-N25G	P129Q-S130G-G131S
43	G97A-G128A-Y217Q-G23A-S24G-N25G	V203Y
44	G97A-G128A-Y217Q-G23A-S24G-N25G	G211R-N212S-K213V
45	G97A-G128A-Y217Q-N61P-N62S	L75S-N76Y
46	G97A-G128A-Y217Q-N61P-N62S	P86S-S87G-A88V
47	G97A-G128A-Y217Q-N61P-N62S	S87G-A88V-S89A
48	G97A-G128A-Y217Q-N61P-N62S	S87T-A88L-S89G
49	G97A-G128A-Y217Q-N61P-N62S	P129Q-S130G-G131S
50	G97A-G128A-Y217Q-N61P-N62S	V203Y
51	G97A-G128A-Y217Q-N61P-N62S	G211R-N212S-K213V
52	G97A-G128A-Y217Q-T55P	L75S-N76Y

Table 11-7: List of Parent Plasmids and Mutations Introduced in the RCL5 Variants		
Combinatorial Variant #	**Parent Plasmids**	**Mutations Introduced**
53	G97A-G128A-Y217Q-T55P	P86S-S87G-A88V
54	G97A-G128A-Y217Q-T55P	S87G-A88V-S89A
55	G97A-G128A-Y217Q-T55P	S87T-A88L-S89G
56	G97A-G128A-Y217Q-T55P	P129Q-S130G-G131S
57	G97A-G128A-Y217Q-T55P	V203Y
58	G97A-G128A-Y217Q-T55P	G211R-N212S-K213V
59	G97A-G128A-Y217Q-N61P-S63H	L75S-N76Y
60	G97A-G128A-Y217Q-N61P-S63H	P86S-S87G-A88V
61	G97A-G128A-Y217Q-N61P-S63H	S87G-A88V-S89A
62	G97A-G128A-Y217Q-N61P-S63H	S87T-A88L-S89G
63	G97A-G128A-Y217Q-N61P-S63H	P129Q-S130G-G131S
64	G97A-G128A-Y217Q-N61P-S63H	V203Y
65	G97A-G128A-Y217Q-N61P-S63H	G211R-N212S-K213V
66	G97A-G128A-Y217Q-Q59S-N61P	L75S-N76Y
67	G97A-G128A-Y217Q-Q59S-N61P	P86S-S87G-A88V
68	G97A-G128A-Y217Q-Q59S-N61P	S87G-A88V-S89A
69	G97A-G128A-Y217Q-Q59S-N61P	S87T-A88L-S89G
70	G97A-G128A-Y217Q-Q59S-N61P	P129Q-S130G-G131S
71	G97A-G128A-Y217Q-Q59S-N61P	V203Y
72	G97A-G128A-Y217Q-Q59S-N61P	G211R-N212S-K213V
73	G97A-G128A-Y217Q-L75S-N76Y	P86S-S87G-A88V
74	G97A-G128A-Y217Q-L75S-N76Y	S87G-A88V-S89A
75	G97A-G128A-Y217Q-L75S-N76Y	S87T-A88L-S89G
76	G97A-G128A-Y217Q-L75S-N76Y	P129Q-S130G-G131S
77	G97A-G128A-Y217Q-L75S-N76Y	V203Y
78	G97A-G128A-Y217Q-L75S-N76Y	G211R-N212S-K213V
79	G97A-G128A-Y217Q-P86S-S87G-A88V	P129Q-S130G-G131S
80	G97A-G128A-Y217Q-P86S-S87G-A88V	V203Y
81	G97A-G128A-Y217Q-P86S-S87G-A88V	G211R-N212S-K213V
82	G97A-G128A-Y217Q-S87G-A88V-S89A	P129Q-S130G-G131S
83	G97A-G128A-Y217Q-S87G-A88V-S89A	V203Y
84	G97A-G128A-Y217Q-S87G-A88V-S89A	G211R-N212S-K213V
85	G97A-G128A-Y217Q-S87T-A88L-S89G	P129Q-S130G-G131S
86	G97A-G128A-Y217Q-S87T-A88L-S89G	V203Y
87	G97A-G128A-Y217Q-S87T-A88L-S89G	G211R-N212S-K213V
88	G97A-G128A-Y217Q-P129Q-S130G-G131S	V203Y
89	G97A-G128A-Y217Q-P129Q-S130G-G131S	G211R-N212S-K213V
90	G97A-G128A-Y217Q-V203Y	G211R-N212S-K213V

Table 11-8: Primers Used to Create RCL5 Combinatorial Variants					
Mutations Introduced	**Gene Fragments**	**Common 3' & 5' Gene Flanking Primer Names**	**Primer Name**	**Primer Sequence**	**SEQ ID NO:**
T22N-S24A	3'	P4976	P5432	TCAAGGCTACAATGGAGCAAATG TTAAAGTAGCGGTTATCGA	535
	5'	P4974	P5433	TTTAACATTTGCTCCATTGTAGCC TTGAGAGTGCAGAG	536
S24G-N25G	3'	P4976	P5434	CTACACTGGAGGAGGTGTTAAAG TAGCGGTTATCGACA	537
	5'	P4974	P5435	CTACTTTAACACCTCCTCCAGTGT AGCCTTGAGAGTG	538
S24R	3'	P4976	P5436	AGGCTACACTGGAAGAAATGTTA AAGTAGCGGTTATCGAC	539
	5'	P4974	P5437	CTTTAACATTTCTTCCAGTGTAGC CTTGAGAGTG	540
G23A-S24G-N25G	3'	P4976	P5438	AAGGCTACACTGCAGGAGGTGTT AAAGTAGCGGTTATCGACA	541
	5'	P4974	P5439	CTACTTTAACACCTCCTGCAGTGT AGCCTTGAGAGTGCAG	542
N61P-N62S	3'	P4976	P5440	CGTTTCAAGATCCCTCTTCTCATG GCACACACGTCGC	543
	5'	P4974	P5441	TGTGCCATGAGAAGAGGGATCTT GAAACGGGTTTGTTTCG	544
T55P	3'	P4976	P5442	TGCCGTCCGAACCAAACCCGTTT CAAGATAACAATTCT	545
	5'	P4974	P5443	TCTTGAAACGGGTTTGGTTCGGA CGGCACCATAGAAG	546
N61P-S63H	3'	P4976	P5444	CCGTTTCAAGATCCCAATCATCA TGGCACACACGTCGCAG	547
	5'	P4974	P5445	TGTGTGCCATGATGATTGGGATC TTGAAACGGGTTTGTTTCG	548
Q59S-N61P	3'	P4976	P5446	ACAAACCCGTTTTCAGATCCCAA TTCTCATGGCACACACGTCGCA	549
	5'	P4974	P5447	CCATGAGAATTGGGATCTGAAAA CGGGTTTGTTTCGGACGGCA	550
L75S-N76Y	3'	P4976	P5448	GGTTGCGGCGTCATACAATTCTA TTGGCGTGCTTGGTG	551
	5'	P4974	P5449	GCCAATAGAATTGTATGACGCCG CAACCGTTCCTGCGA	552
P86S-S87G-A88V	3'	P4976	P5450	TGGTGTAGCCTCGGGTGTTTCGCT CTACGCCGTTAAAGTT	553
	5'	P4974	P5451	CGTAGAGCGAAACACCCGAGGCT ACACCAAGCACGCCAA	554
S87G-A88V-S89A	3'	P4976	P5452	GTGTAGCCCCGGGTGTTGCACTC TACGCCGTTAAAGTTCTTG	555

Table 11-8: Primers Used to Create RCL5 Combinatorial Variants					
Mutations Introduced	**Gene Fragments**	**Common 3' & 5' Gene Flanking Primer Names**	**Primer Name**	**Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5453	ACGGCGTAGAGTGCAACACCCGG GGCTACACCAAGCACGCCAA	556
S87T-A88L-S89G	3'	P4976	P5454	TGGTGTAGCCCCGACTCTTGGAC TCTACGCCGTTAAAGTTCTTG	557
	5'	P4974	P5455	ACGGCGTAGAGTCCAAGAGTCGG GGCTACACCAAGCACGCCAA	558
P129Q-S130G-G131S	3'	P4976	P5456	GCCTGGGAGCACAAGGCTCTAGT GCGGCACTTAAAGCAGCA	559
	5'	P4974	P5457	AGTGCCGCACTAGAGCCTTGTGC TCCCAGGCTCATGTTGAT	560
V203Y	3'	P4976	P5458	ATGGCCCCTGGCTATTCTATTCAA TCGACGCTTCCAG	561
	5'	P4974	P5459	TCGATTGAATAGAATAGCCAGGG GCCATGACATCCA	562
G211R-N212S-K213V	3'	P4976	P5460	TCGACGCTTCCAAGGTCCGTGTA TGGTGCGCAAAACGGGACT	563
	5'	P4974	P5461	TTGCGCACCATACACGGACCTTG GAAGCGTCGATTGAATAGAAA	564

To create each mutant, two PCR reactions were carried out using either the common 3' gene-flanking primer (P4976, CCTCTCGGTTATGAGTTAGTTC; SEQ ID NO:61) and the mutagenic primer, or the common 5'gene-flanking primer (P4974, GCCTCACATTTGTGCCACCTA; SEQ ID NO:60) and mutagenic primer as shown for each library in Table 11-8. These PCR reactions generated two PCR fragments, one encoding the 5' half of the mutant BPN' gene (5' gene fragment) and the other encoding the 3' half of the mutant BPN' gene (3' gene fragment). Each PCR amplification reaction contained 30 pmol of each primer and 100 ng of the parent molecules listed in Table 11-7. Amplifications were carried out using Vent DNA polymerase (NEB). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 40 sec. Following amplification, the 5' and 3' gene fragments were gel-purified by the QIAGEN® gel-band purification kit, mixed (50 ng of each fragment) and amplified by PCR once again using the primers P4973 (AAAGGATCCTAATCGGCGCTTTTC; SEQ ID NO:62) and P4950 (CTTGTCTCCAAGCTTAAAATAAAA; SEQ ID NO:63) to generate the full-length gene fragment. The PCR conditions were same as described above, except the extension phase, which was carried out at 72°C for 2 min. The full-length DNA fragment was gel-purified by the QIAGEN® gel-band purification kit, digested by the BamHI and HindIII restriction enzymes and ligated with the pHPLT-BPN' partial opt that also was digested with the same restriction enzymes. Ligation mixtures were amplified using rolling

circle amplification by Illustra Templiphi kit according to the manufacturer's instructions (GE Healthcare) to generate multimeric DNA for transformation into Bacillus subtilis. For this purpose, 1 µl of the ligation mixture was mixed with 5 µl of the sample buffer, heated to 95°C for 3 min and cooled on ice. Next, 5 µl of the reaction buffer and 0.2 µl of the enzyme were added to each tube, followed by incubation at 30°C for 10 hours. Products of the rolling circle amplification were diluted 100 times and used to transform B. subtilis cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and incubated overnight at 37°C. Subsequently, the colonies with halos were inoculated in 120 µl of LB media containing10 µg/mL neomycin.

RCL 6 Combinatorial Libraries

"RCL6" refers to a group of combinatorial libraries created by PCR fusion using several BPN' mutants as parent (template) molecules. A mixture of BPN' mutants were used as templates (parent molecules) in the construction of each of these libraries. The five different mixes of parent molecules used to create these libraries are provided in Table 11-9, and the mutations introduced in each library are listed in Table 11-10.

To create each mutant, two PCR reactions were carried out using either the common 3' gene-flanking primer (P4976, CCTCTCGGTTATGAGTTAGTTC; SEQ ID NO:61) and the mutagenic primer, or the common 5'gene-flanking primer (P4974, GCCTCACATTTGTGCCACCTA; SEQ ID NO:60) and mutagenic primer as shown for each library in Table 11-10. These PCR reactions generated two PCR fragments, one encoding the 5' half of the mutant BPN' gene (5' gene fragment) and the other encoding the 3' half of the mutant BPN' gene (3' gene fragment). Each PCR amplification reaction contained 30 pmol of each primer and 100 ng of the parent molecules listed in Table 11-9. Amplifications were carried out using Vent DNA polymerase (NEB). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 40 sec. Following amplification, the 5' and 3' gene fragments were gel-purified by the QIAGEN® gel-band purification kit, mixed (50 ng of each fragment) and amplified by PCR once again using the primers P4973 (AAAGGATCCTAATCGGCGCTTTTC; SEQ ID NO:62) and P4950 (CTTGTCTCCAAGCTTAAAATAAAA; SEQ ID NO:63) to generate the full-length gene fragment. The PCR conditions were same as described above, except the extension phase, which was carried out at 72°C for 2 min. The full-length DNA fragment was gel-purified by the QIAGEN® gel-band purification kit, digested using *Bam*HI and *Hin*dIII restriction enzymes and ligated with the pHPLT-BPN' partial opt vector that also was digested with the same restriction enzymes. Ligation mixtures were amplified using rolling circle amplification by Illustra Templiphi kit according to the manufacturer's instructions (GE Healthcare) to generate multimeric DNA for transformation into *Bacillus subtilis*. For this purpose, 1 µl of the ligation mixture was mixed with 5 µl of the sample buffer, heated to 95°C for 3 min and cooled on ice. Next, 5 µl of the reaction buffer and 0.2 µl of the enzyme were added to each tube, followed by

incubation at 30°C for 10 hours. Products of the rolling circle amplification were diluted 100 times and used to transform *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and incubated overnight at 37°C. Subsequently, the colonies with halos were inoculated in 120 µl of LB media containing 10µg/mL neomycin.

Table 11-9: Parent Molecules of BPN' Used to Create RCL6 Libraries

Mixes of Parent Molecules				
Mix 1	**Mix 2**	**Mix 3**	**Mix 4**	**Mix 5**
G97A-G128A-Y217Q-S24G-N25G	G97A-G128A-Y217Q-T55P	G97A-G128A-Y217Q-L75S-N76Y	G97A-G128A-Y217Q-P86S-S87G-A88V	G97A-G128A-Y217Q-P129Q-S130G-G131S
G97A-G128A-Y217Q-S24R	G97A-G128A-Y217Q-N61P-S63H		G97A-G128A-Y217Q-S87G-A88V-S89A	
G97A-G128A-Y217Q-G23A-S24G-N25G	G97A-G128A-Y217Q-Q59S-N61P		G97A-G128A-Y217Q-S87T-A88L-S89G	

Table 11-10: Mutations Introduced in the RCL6 Libraries

Mutations Introduced	Gene Fragment	Common 5' & 3' Gene Flanking Primer Names	Mutagenic Primer Name	Mutagenic Primer Sequence	SEQ ID NO:
V68C, A69G	3'	P4976	P5462	CATGGCACACACTGCGGAGGAACGGTTGCGGCGTTAAAC	565
	5'	P4974	P5463	GCAACCGTTCCTCCGCAGTGTGTGCCATGAGAATTGTTA	566
V72I,A73G,delA74,L75S	3'	P4976	P5464	GTCGCAGGAACGATTGGTTCAAACAATTCTATTGGCGTGCTTG	567
	5'	P4974	P5465	CAATAGAATTGTTTGAACCAATCGTTCCTGCGACGTGTGTGCCAT	568
L75H, N76G	3'	P4976	P5466	AACGGTTGCGGCGCATGGAAATTCTATTGGCGTGCTTGGTG	569
	5'	P4974	P5467	CAATAGAATTTCCATGCGCCGCAACCGTTCCTGCGACGTGT	570
L75R,N76G,N77S	3'	P4976	P5468	AACGGTTGCGGCGAGAGGAGGTTCTATTGGCGTGCTTGGTGTA	571

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5469	CACGCCAATAGAACCTCC TCTCGCCGCAACCGTTCCT GCGACGTGT	572
L75G, N76G, N77G	3'	P4976	P5470	AACGGTTGCGGCGGGAGG CGGTTCTATTGGCGTGCTT GGTGTA	573
	5'	P4974	P5471	CACGCCAATAGAACCGCC TCCCGCCGCAACCGTTCCT GCGACGTGT	574
A92G	3'	P4976	P5472	TGCTTCGCTCTACGGCGTT AAAGTTCTTGCAGCAGAC	575
	5'	P4974	P5473	CAAGAACTTTAACGCCGT AGAGCGAAGCAGACGGG GCTA	576
delV93, K94S, V95C,L96S	3'	P4976	P5474	TTCGCTCTACGCCTCATGT TCTGCAGCAGACGGATCA GGCCAA	577
	5'	P4974	P5475	ATCCGTCTGCTGCAGAAC ATGAGGCGTAGAGCGAAG CAGACG	578
V121I_I12 2S_N123C	3'	P4976	P5476	AATAACATGGATATATCT TGCATGAGCCTGGGAGCA CCAAG	579
	5'	P4974	P5477	CAGGCTCATGCAAGATAT ATCCATGTTATTCGCGATG GCCCATT	580
V121L_N1 23C	3'	P4976	P5478	CGAATAACATGGATCTTA TCTGCATGAGCCTGGGAG CACCAAG	581
	5'	P4974	P5479	CCAGGCTCATGCAGATAA GATCCATGTTATTCGCGAT GGCCCATT	582
I122C_N12 3S_M124L	3'	P4976	P5480	TAACATGGATGTATGCTC ATTGAGCCTGGGAGCACC AAGCGGCA	583
	5'	P4974	P5481	TGCTCCCAGGCTCAATGA GCATACATCCATGTTATTC GCGATG	584
N123C	3'	P4976	P5482	ACATGGATGTAATCTGCA TGAGCCTGGGAGCACCAA G	585

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5483	TCCCAGGCTCATGCAGATTACATCCATGTTATTCGCGAT	586
M124I	3'	P4976	P5484	GGATGTAATCAACATCAGCCTGGGAGCACCAAGCGGCA	587
	5'	P4974	P5485	TGCTCCCAGGCTGATGTTGATTACATCCATGTTATTCG	588
M124V	3'	P4976	P5486	GGATGTAATCAACGTAAGCCTGGGAGCACCAAGCGGCA	589
	5'	P4974	P5487	TGCTCCCAGGCTTACGTTGATTACATCCATGTTATTCG	590
M124V-L126A	3'	P4976	P5488	GGATGTAATCAACGTAAGCGCGGGAGCACCAAGCGGCAGTG	591
	5'	P4974	P5489	TTGGTGCTCCCGCGCTTACGTTGATTACATCCATGTTATTCG	592
L126F, delP129	3'	P4976	P5490	AATCAACATGAGCTTCGGAGCAAGCGGCAGTGCGGCACTTAA	593
	5'	P4974	P5491	CACTGCCGCTTGCTCCGAAGCTCATGTTGATTACATCCATGT	594
G127Y	3'	P4976	P5492	AACATGAGCCTGTACGCACCAAGCGGCAGTGCGGCACTTA	595
	5'	P4974	P5493	CACTGCCGCTTGGTGCGTACAGGCTCATGTTGATTACATCC	596
G127S_P129D	3'	P4976	P5494	CAACATGAGCCTGTCAGCAGATAGCGGCAGTGCGGCACTTAAA	597
	5'	P4974	P5495	GCACTGCCGCTATCTGCTGACAGGCTCATGTTGATTACATCC	598
G127N,P129R	3'	P4976	P5496	CAACATGAGCCTGAACGCACGTAGCGGCAGTGCGGCACTTAAA	599
	5'	P4974	P5497	GCACTGCCGCTACGTGCGTTCAGGCTCATGTTGATTACATCC	600

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
G128N, insS, P129S	3'	P4976	P5498	ATGAGCCTGGGAAATTCATCTAGCGGCAGTGCGGCACTTAAA	601
	5'	P4974	P5499	GCACTGCCGCTAGATGAATTTCCCAGGCTCATGTTGATTAC	602
G128S_P129V	3'	P4976	P5500	CATGAGCCTGGGATCAGTTAGCGGCAGTGCGGCACTTAAA	603
	5'	P4974	P5501	GCACTGCCGCTAACTGATCCCAGGCTCATGTTGATTAC	604
G128S, P129D	3'	P4976	P5502	CATGAGCCTGGGATCAGATAGCGGCAGTGCGGCACTTAAA	605
	5'	P4974	P5503	GCACTGCCGCTATCTGATCCCAGGCTCATGTTGATTAC	606
G128S, P129G	3'	P4976	P5504	CATGAGCCTGGGATCAGGTAGCGGCAGTGCGGCACTTAAA	607
	5'	P4974	P5505	GCACTGCCGCTACCTGATCCCAGGCTCATGTTGATTAC	608
H128H, P129Y	3'	P4976	P5506	CATGAGCCTGGGACACTATAGCGGCAGTGCGGCACTTAAA	609
	5'	P4974	P5507	GCACTGCCGCTATAGTGTCCCAGGCTCATGTTGATTAC	610
P129D	3'	P4976	P5508	GAGCCTGGGAGCAGACAGCGGCAGTGCGGCACTTAA	611
	5'	P4974	P5509	TGCCGCACTGCCGCTGTCTGCTCCCAGGCTCATGTTGAT	612
P129E	3'	P4976	P5510	GAGCCTGGGAGCAGAAAGCGGCAGTGCGGCACTTAA	613
	5'	P4974	P5511	TGCCGCACTGCCGCTTTCTGCTCCCAGGCTCATGTTGAT	614
P129V	3'	P4976	P5512	GAGCCTGGGAGCAGTAAGCGGCAGTGCGGCACTTAA	615
	5'	P4974	P5513	TGCCGCACTGCCGCTTACTGCTCCCAGGCTCATGTTGAT	616

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
P129G, delS130	3'	P4976	P5514	GAGCCTGGGAGCAGGAGG CAGTGCGGCACTTAAAGC	617
	5'	P4974	P5515	AGTGCCGCACTGCCTCCT GCTCCCAGGCTCATGTTG AT	618
P129H, delS130, S132N	3'	P4976	P5516	AGCCTGGGAGCACACGGC AATGCGGCACTTAAAGCA GCAGTT	619
	5'	P4974	P5517	TTTAAGTGCCGCATTGCC GTGTGCTCCCAGGCTCAT GTTGAT	620
A134T	3'	P4976	P5518	AAGCGGCAGTGCGACACT TAAAGCAGCAGTTGATAA AG	621
	5'	P4974	P5519	AACTGCTGCTTTAAGTGTC GCACTGCCGCTTGGTGCT C	622
G97R, insG, A98C	3'	P4976	P5520	GTTAAAGTTCTTCGTGGTT GTGACGGATCAGGCCAAT ACTC	623
	5'	P4974	P5521	CTGATCCGTCACAACCAC GAAGAACTTTAACGGCGT AGAGC	624
A98G, D99G	3'	P4976	P5522	TTAAAGTTCTTGCAGGAG GCGGATCAGGCCAATACT CATG	625
	5'	P4974	P5523	TATTGGCCTGATCCGCCTC CTGCAAGAACTTTAACGG CGTAG	626
A98G, insR	3'	P4976	P5524	TTAAAGTTCTTGCAGGAC GTGACGGATCAGGCCAAT ACTCA	627
	5'	P4974	P5525	CTGATCCGTCACGTCCTGC AAGAACTTTAACGGCGTA G	628
A98D, D99G	3'	P4976	P5526	TTAAAGTTCTTGCAGACG GCGGATCAGGCCAATACT CATG	629
	5'	P4974	P5527	TATTGGCCTGATCCGCCGT CTGCAAGAACTTTAACGG CGTAG	630
A98H, D99G, G100D	3'	P4976	P5528	TAAAGTTCTTGCACATGG AGATTCAGGCCAATACTC ATGGATTAT	631

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5529	AGTATTGGCCTGAATCTC CATGTGCAAGAACTTTAA CGGCGTAG	632
D99R, insN	3'	P4976	P5530	AAGTTCTTGCAGCACGTA ACGGATCAGGCCAATACT CATG	633
	5'	P4974	P5531	TATTGGCCTGATCCGTTAC GTGCTGCAAGAACTTTAA CGGCGTA	634
D99V, S101D	3'	P4976	P5532	AGTTCTTGCAGCAGTAGG AGATGGCCAATACTCATG GATTATCAA	635
	5'	P4974	P5533	TGAGTATTGGCCATCTCCT ACTGCTGCAAGAACTTTA ACGGCGTA	636
D99C, insS	3'	P4976	P5534	TTAAAGTTCTTGCAGCAT GTAGCGGATCAGGCCAAT ACTCATG	637
	5'	P4974	P5535	TATTGGCCTGATCCGCTAC ATGCTGCAAGAACTTTAA CGGCGTA	638
G100S	3'	P4976	P5536	AAGTTCTTGCAGCAGACT CTTCAGGCCAATACTCAT GGATTAT	639
	5'	P4974	P5537	ATGAGTATTGGCCTGAAG AGTCTGCTGCAAGAACTT TAACG	640
G100S, S101V	3'	P4976	P5538	TTCTTGCAGCAGACTCTGT AGGCCAATACTCATGGAT TATCA	641
	5'	P4974	P5539	CATGAGTATTGGCCTACA GAGTCTGCTGCAAGAACT TTAACG	642
G100D	3'	P4976	P5540	AAGTTCTTGCAGCAGACG ATTCAGGCCAATACTCAT GGATTAT	643
	5'	P4974	P5541	ATGAGTATTGGCCTGAAT CGTCTGCTGCAAGAACTT TAACG	644
G100N	3'	P4976	P5542	AAGTTCTTGCAGCAGACA ATTCAGGCCAATACTCAT GGATTAT	645
	5'	P4974	P5543	ATGAGTATTGGCCTGAAT TGTCTGCTGCAAGAACTTT AACG	646

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
S100N, S101L	3'	P4976	P5544	TTCTTGCAGCAGACAATC TAGGCCAATACTCATGGA TTATCA	647
	5'	P4974	P5545	CATGAGTATTGGCCTAGA TTGTCTGCTGCAAGAACTT TAACG	648
S101G	3'	P4976	P5546	TTCTTGCAGCAGACGGAG GAGGCCAATACTCATGGA TTATCAA	649
	5'	P4974	P5547	ATGAGTATTGGCCTCCTCC GTCTGCTGCAAGAACTTT A	650
S101D	3'	P4976	P5548	TTCTTGCAGCAGACGGAG ATGGCCAATACTCATGGA TTATCAA	651
	5'	P4974	P5549	ATGAGTATTGGCCATCTC CGTCTGCTGCAAGAACTT TA	652
S101V, Q103N	3'	P4976	P5550	TGCAGCAGACGGAGTAGG CAACTACTCATGGATTAT CAACGGCAT	653
	5'	P4974	P5551	ATAATCCATGAGTAGTTG CCTACTCCGTCTGCTGCAA GAACTTTA	654
S101E	3'	P4976	P5552	TTCTTGCAGCAGACGGAC GTGGCCAATACTCATGGA TTATCAA	655
	5'	P4974	P5553	ATGAGTATTGGCCACGTC CGTCTGCTGCAAGAACTT TA	656
A116S,N117G,N118R	3'	P4976	P5554	AATGGGCCATCTCTGGTA GAATGGATGTAATCAACA TGAGCCT	657
	5'	P4974	P5555	GATTACATCCATTCTACCA GAGATGGCCCATTCGATG CCGTT	658
A116G,N117R	3'	P4976	P5556	AATGGGCCATCGGACGTA ACATGGATGTAATCAACA TGAG	659
	5'	P4974	P5557	GATTACATCCATGTTACGT CCGATGGCCCATTCGATG CCGTT	660
A116N,N117S, N118G	3'	P4976	P5558	AATGGGCCATCAATTCTG GAATGGATGTAATCAACA TGAGCCT	661

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5559	GATTACATCCATTCCAGA ATTGATGGCCCATTCGAT GCCGTT	662
M222Q	3'	P4976	P5560	AAAACGGGACTTCCCAGG CCTCGCCGCATGTAGCTG	663
	5'	P4974	P5561	TACATGCGGCGAGGCCTG GGAAGTCCCGTTTTGCGC AC	664
S24R	3'	P4976	P5562	AAGGCTACACTGGAAGAA ATGTTAAAGTAGCGGTTA TCGA	665
	5'	P4974	P5563	CTACTTTAACATTTCTTCC AGTGTAGCCTTGAGAGTG	666
N25Y	3'	P4976	P5564	CTACACTGGATCATATGTT AAAGTAGCGGTTATCGAC A	667
	5'	P4974	P5565	TAACCGCTACTTTAACAT ATGATCCAGTGTAGCCTT GAGA	668
P52D	3'	P4976	P5566	CTTCTATGGTGGATTCCGA AACAAACCCGTTTCAAG	669
	5'	P4974	P5567	GGTTTGTTTCGGAATCCAC CATAGAAGCCCCTCCAG	670
S63T	3'	P4976	P5568	TTTCAAGATAACAATACA CATGGCACACACGTCGCA GGA	671
	5'	P4974	P5569	TGTGTGCCATGTGTATTGT TATCTTGAAACGGGTTTGT	672
N61E	3'	P4976	P5570	GTTTCAAGATGAAAATTC TCATGGCACACACGTC	673
	5'	P4974	P5571	TGTGCCATGAGAATTTTC ATCTTGAAACGGGTTTGTT TCG	674
N61P	3'	P4976	P5572	AACCCGTTTCAAGATCCA AATTCTCATGGCACACAC GTC	675
	5'	P4974	P5573	TGCCATGAGAATTTGGAT CTTGAAACGGGTTTGTTTC G	676
N62Q	3'	P4976	P5574	GTTTCAAGATAACCAATC TCATGGCACACACGTCGC AGGAA	677

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5575	TGTGTGCCATGAGATTGG TTATCTTGAAACGGGTTTG TTT	678
N62D	3'	P4976	P5576	GTTTCAAGATAACGATTC TCATGGCACACACGTCGC AGGAA	679
	5'	P4974	P5577	TGTGTGCCATGAGAATCG TTATCTTGAAACGGGTTTG TTT	680
S63Q	3'	P4976	P5578	TCAAGATAACAATCAACA TGGCACACACGTCGCAGG	681
	5'	P4974	P5579	ACGTGTGTGCCATGTTGA TTGTTATCTTGAAACGGGT TTG	682
V68A	3'	P4976	P5580	TCATGGCACACACGCAGC AGGAACGGTTGCGGCGTT AA	683
	5'	P4974	P5581	CAACCGTTCCTGCTGCGT GTGTGCCATGAGAATTGT TA	684
S87D	3'	P4976	P5582	TGTAGCCCCGGATGCTTC GCTCTACGCCGTTAA	685
	5'	P4974	P5583	CGTAGAGCGAAGCATCCG GGGCTACACCAAGCACG	686
L96T	3'	P4976	P5584	CGTTAAAGTTACAGCAGC AGACGGATCAGGCCAATA	687
	5'	P4974	P5585	TGATCCGTCTGCTGCTGTA ACTTTAACGGCGTAGAGC GAA	688
L126A	3'	P4976	P5586	TAATCAACATGAGCGCGG GAGCACCAAGCGGCAGTG	689
	5'	P4974	P5587	TTGGTGCTCCCGCGCTCAT GTTGATTACATCCATG	690
L126T	3'	P4976	P5588	TAATCAACATGAGCACGG GAGCACCAAGCGGCAGTG	691
	5'	P4974	P5589	TTGGTGCTCCCGTGCTCAT GTTGATTACATCCATG	692
S125A	3'	P4976	P5590	ATGTAATCAACATGGCAC TGGGAGCACCAAGCGGCA GT	693
	5'	P4974	P5591	TTGGTGCTCCCAGTGCCAT GTTGATTACATCCATGTTA TT	694

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
S130P	3'	P4976	P5592	TGGGAGCACCACCAGGCA GTGCGGCACTTAAAGC	695
	5'	P4974	P5593	GTGCCGCACTGCCTGGTG GTGCTCCCAGGCTCATGT	696
P129L	3'	P4976	P5594	TGAGCCTGGGAGCACTTA GCGGCAGTGCGGCACTTA A	697
	5'	P4974	P5595	TGCCGCACTGCCGCTAAG TGCTCCCAGGCTCATGTTG AT	698
P129E	3'	P4976	P5596	TGAGCCTGGGAGCAGAAA GCGGCAGTGCGGCACTTA A	699
	5'	P4974	P5597	TGCCGCACTGCCGCTTTCT GCTCCCAGGCTCATGTTG AT	700
P129S	3'	P4976	P5598	TGAGCCTGGGAGCATCTA GCGGCAGTGCGGCACTTA A	701
	5'	P4974	P5599	TGCCGCACTGCCGCTAGA TGCTCCCAGGCTCATGTTG AT	702
P40E	3'	P4976	P5600	GACTCGAGCCATGAAGAT CTTAAAGTCGCTGGAGG	703
	5'	P4974	P5601	GACTTTAAGATCTTCATG GCTCGAGTCGATACCGCT	704
Y6Q	3'	P4976	P5602	GCAGTCCGTGCCTCAAGG CGTATCACAAATTAAAGC CCCT	705
	5'	P4974	P5603	ATTTGTGATACGCCTTGA GGCACGGACTGCGCGTAC GCAT	706
G102A	3'	P4976	P5604	CAGACGGATCAGCACAAT ACTCATGGATTATCAACG GCAT	707
	5'	P4974	P5605	TAATCCATGAGTATTGTG CTGATCCGTCTGCTGCAA GAAC	708
S101N	3'	P4976	P5606	GCAGCAGACGGAAACGGC CAATACTCATGGATTATC AA	709
	5'	P4974	P5607	CATGAGTATTGGCCGTTTC CGTCTGCTGCAAGAACTT TA	710

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
G100E	3'	P4976	P5608	TTCTTGCAGCAGACGAATCAGGCCAATACTCATGGATTAT	711
	5'	P4974	P5609	TGAGTATTGGCCTGATTCGTCTGCTGCAAGAACTTTAACG	712
I115V	3'	P4976	P5610	ATCGAATGGGCCGTAGCGAATAACATGGATGTAATCAA	713
	5'	P4974	P5611	CATCCATGTTATTCGCTACGGCCCATTCGATGCCGTTGAT	714
A144K	3'	P4976	P5612	GTTGATAAAGCTGTTAAATCTGGTGTCGTCGTAGTAGC	715
	5'	P4974	P5613	GACGACACCAGATTTAACAGCTTTATCAACTGCTGCTT	716
S145D	3'	P4976	P5614	GATAAAGCTGTTGCAGATGGTGTCGTCGTAGTAGCGGCA	717
	5'	P4974	P5615	TACTACGACGACACCATCTGCAACAGCTTTATCAACTGCT	718
S159K	3'	P4976	P5616	AATGAGGGAACAAAAGGATCATCGAGTACCGTCGGTTA	719
	5'	P4974	P5617	ACGGTACTCGATGATCCTTTTGTTCCCTCATTCCCAGCTG	720
S162K	3'	P4976	P5618	AACATCCGGATCAAAAAGTACCGTCGGTTATCCAGGCAA	721
	5'	P4974	P5619	ATAACCGACGGTACTTTTTGATCCGGATGTTCCCTCATT	722
V147P	3'	P4976	P5620	TGTTGCATCTGGTCCAGTCGTAGTAGCGGCAGCTGGGAAT	723
	5'	P4974	P5621	TGCCGCTACTACGACTGGACCAGATGCAACAGCTTTATCA	724
S161P	3'	P4976	P5622	AGGGAACATCCGGACCATCGAGTACCGTCGGTTATCCA	725

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5623	ACCGACGGTACTCGATGG TCCGGATGTTCCCTCATTC CCA	726
A187D	3'	P4976	P5624	CTTCAAATCAACGTGACT CTTTTTCCTCCGTGGGACC GGA	727
	5'	P4974	P5625	ACGGAGGAAAAAGAGTC ACGTTGATTTGAAGAGTC TACAG	728
F189D	3'	P4976	P5626	TCAACGTGCCTCTGATTCC TCCGTGGGACCGGAGCTG GAT	729
	5'	P4974	P5627	TCCCACGGAGGAATCAGA GGCACGTTGATTTGAAGA G	730
L267V	3'	P4976	P5628	TACTATGGAAAAGGGGTA ATCAACGTACAGGCGGCA GC	731
	5'	P4974	P5629	CTGTACGTTGATTACCCCT TTTCCATAGTAGAAAGAA T	732
Q206E	3'	P4976	P5630	TGGCGTTTCTATTGAATCG ACGCTTCCAGGGAACAA	733
	5'	P4974	P5631	CTGGAAGCGTCGATTCAA TAGAAACGCCAGGGGCCA T	734
K213T	3'	P4976	P5632	CTTCCAGGGAACACATAT GGTGCGCAAAACGGGACT	735
	5'	P4974	P5633	GTTTTGCGCACCATATGTG TTCCCTGGAAGCGTCGAT T	736
K213L	3'	P4976	P5634	CTTCCAGGGAACCTTTAT GGTGCGCAAAACGGGACT	737
	5'	P4974	P5635	GTTTTGCGCACCATAAAG GTTCCCTGGAAGCGTCGA TT	738
K265N	3'	P4976	P5636	TTTCTACTATGGAAACGG GCTGATCAACGTACAGGC GGCA	739
	5'	P4974	P5637	ACGTTGATCAGCCCGTTTC CATAGTAGAAAGAATCAC CAA	740
N240K	3'	P4976	P5638	TTTCTAAGCACCCGAAAT GGACAAACACTCAAGTCC GCA	741

Table 11-10: Mutations Introduced in the RCL6 Libraries					
Mutations Introduced	**Gene Fragment**	**Common 5' & 3' Gene Flanking Primer Names**	**Mutagenic Primer Name**	**Mutagenic Primer Sequence**	**SEQ ID NO:**
	5'	P4974	P5639	GAGTGTTTGTCCATTTCGGGTGCTTAGAAAGAATCAAT	742
P239R	3'	P4976	P5640	TTCTTTCTAAGCACCGTAACTGGACAAACACTCAAGTCC	743
	5'	P4974	P5641	TGTTTGTCCAGTTACGGTGCTTAGAAAGAATCAATGCG	744
T242R	3'	P4976	P5642	CACCCGAACTGGCGTAACACTCAAGTCCGCAGCAGT	745
	5'	P4974	P5643	TGCGGACTTGAGTGTTACGCCAGTTCGGGTGCTTAGAAAG	746
S89Y	3'	P4976	P5644	CGTCTGCTTACCTCTACGCCGTTAAAGTTCTTG	747
	5'	P4974	P5645	ACTTTAACGGCGTAGAGGTAAGCAGACGGGGCTACACCAA	748
P129Q	3'	P4976	P5646	AGCCTGGGAGCACAAAGCGGCAGTGCGGCACTTAAA	749
	5'	P4974	P5647	CACTGCCGCTTTGTGCTCCCAGGCTCATGTTGAT	750
G211T	3'	P4976	P5648	TTCAATCGACGCTTCCAACGAACAAGTATGGTGCGCAAAAC	751
	5'	P4974	P5649	CACCATACTTGTTCGTTGGAAGCGTCGATTGAATAGAAA	752
I111V	3'	P4976	P5650	TGGATTATCAACGGCGTAGAATGGGCCATCGCGAATAAC	753
	5'	P4974	P5651	CGATGGCCCATTCTACGCCGTTGATAATCCATGAGTATT	754

RCL 7 Combinatorial Variants

"RCL7" refers to a set of combinatorial variants created by PCR fusion using several BPN' mutants as parent (template) plasmid. The mutations introduced in each parent plasmid are listed in Table 11-11, and the mutagenic primers used to create the mutants are described in Table 11-10.

To create each mutant, two PCR reactions were carried out using either the common 3' gene-flanking primer (P4976, CCTCTCGGTTATGAGTTAGTTC; SEQ ID NO:61) and the mutagenic primer,

or the common 5'gene-flanking primer (P4974, GCCTCACATTTGTGCCACCTA; SEQ ID NO:60) and mutagenic primer as shown for each library in Table 11-10. These PCR reactions generated two PCR fragments, one encoding the 5' half of the mutant BPN' gene (5' gene fragment) and the other encoding the 3' half of the mutant BPN' gene (3' gene fragment). Each PCR amplification reaction contained 30 pmol of each primer and 100 ng of the parent molecules listed in Table 11-11. Amplifications were carried out using Vent DNA polymerase (NEB). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 40 sec. Following amplification, the 5' and 3' gene fragments were gel-purified using a QIAGEN® gel-band purification kit, mixed (50ng of each fragment) and amplified by PCR once again using the primers P4973 (AAAGGATCCTAATCGGCGCTTTTC; SEQ ID NO:62) and P4950 (CTTGTCTCCAAGCTTAAAATAAAA; SEQ ID NO:63) to generate the full-length gene fragment. The PCR conditions were same as described above, except the extension phase, which was carried out at 72°C for 2 min. The full-length DNA fragment was gel-purified using a QIAGEN® gel-band purification kit, digested using *Bam*HI and *Hin*dIII restriction enzymes, and ligated with the pHPLT-BPN' partial opt that also was digested with the same restriction enzymes. Ligation mixtures were amplified using rolling circle amplification by Illustra Templiphi kit according to the manufacturer's instructions (GE Healthcare) to generate multimeric DNA for transformation into *Bacillus subtilis*. For this purpose, 1µl of the ligation mixture was mixed with 5 µl of the sample buffer, heated to 95°C for 3 min and cooled on ice. Next, 5 µl of the reaction buffer and 0.2 µl of the enzyme were added to each tube, followed by incubation at 30°C for 10 hours. Products of the rolling circle amplification were diluted 100 times and used to transform *B. subtilis* cells (ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and incubated overnight at 37°C. Subsequently, the colonies with halos were inoculated in 120 µl of LB media containing 10 µg/mL neomycin.

Table 11-11: List of Parent Plasmids and Introduced Mutations in the RCL7 variants

Combinatorial Variant #	Parent Plasmid	Mutation(s) Introduced
1	G97A-G128A-Y217Q-T55P	S24R
2	G97A-G128A-Y217Q-N61E	S24R
3	G97A-G128A-Y217Q-N61P-S63H	S24R
4	G97A-G128A-Y217Q-L75S-N76Y	S24R
5	G97A-G128A-Y217Q-S87T-A88L-S89G	S24R
6	G97A-G128A-Y217Q-S89Y	S24R
7	G97A-G128A-Y217Q-I111V	S24R
8	G97A-G128A-Y217Q-I115V	S24R
9	G97A-G128A-Y217Q-P129Q-S130G-G131S	S24R
10	G97A-G128A-Y217Q-P129Q	S24R
11	G97A-G128A-Y217Q-A134T	S24R

Table 11-11: List of Parent Plasmids and Introduced Mutations in the RCL7 variants		
Combinatorial Variant #	**Parent Plasmid**	**Mutation(s) Introduced**
12	G97A-G128A-Y217Q-A144K	S24R
13	G97A-G128A-Y217Q-S145D	S24R
14	G97A-G128A-Y217Q-S159K	S24R
15	G97A-G128A-Y217Q-S162K	S24R
16	G97A-G128A-Y217Q-S161P-S78N	S24R
17	G97A-G128A-Y217Q-V203Y	S24R
18	G97A-G128A-Y217Q-G211T-S78N	S24R
19	G97A-G128A-Y217Q-K213T	S24R
20	G97A-G128A-Y217Q-P239R	S24R
21	G97A-G128A-Y217Q-N240K	S24R
22	G97A-G128A-Y217Q-L267V-S78N	S24R
23	G97A-G128A-Y217Q-A273S-S78N	S24R
24	G97A-G128A-Y217Q-L75S-N76Y	T55P
25	G97A-G128A-Y217Q-S87T-A88L-S89G	T55P
26	G97A-G128A-Y217Q-S89Y	T55P
27	G97A-G128A-Y217Q-I111V	T55P
28	G97A-G128A-Y217Q-I115V	T55P
29	G97A-G128A-Y217Q-P129Q-S130G-G131S	T55P
30	G97A-G128A-Y217Q-P129Q	T55P
31	G97A-G128A-Y217Q-A134T	T55P
32	G97A-G128A-Y217Q-A144K	T55P
33	G97A-G128A-Y217Q-S145D	T55P
34	G97A-G128A-Y217Q-S159K	T55P
35	G97A-G128A-Y217Q-S162K	T55P
36	G97A-G128A-Y217Q-S161P	T55P
37	G97A-G128A-Y217Q-V203Y	T55P
38	G97A-G128A-Y217Q-G211T	T55P
39	G97A-G128A-Y217Q-K213T	T55P
40	G97A-G128A-Y217Q-P239R	T55P
41	G97A-G128A-Y217Q-N240K	T55P
42	G97A-G128A-Y217Q-L267V	T55P
43	G97A-G128A-Y217Q-A273S	T55P
44	G97A-G128A-Y217Q-L75S-N76Y	N61E
45	G97A-G128A-Y217Q-S87T-A88L-S89G	N61E
46	G97A-G128A-Y217Q-S89Y	N61E
47	G97A-G128A-Y217Q-I111V	N61E
48	G97A-G128A-Y217Q-I115V	N61E
49	G97A-G128A-Y217Q-P129Q-S130G-G131S	N61E
50	G97A-G128A-Y217Q-P129Q	N61E
51	G97A-G128A-Y217Q-A134T	N61E
52	G97A-G128A-Y217Q-A144K	N61E
53	G97A-G128A-Y217Q-S145D	N61E
54	G97A-G128A-Y217Q-S159K	N61E

Table 11-11: List of Parent Plasmids and Introduced Mutations in the RCL7 variants		
Combinatorial Variant #	**Parent Plasmid**	**Mutation(s) Introduced**
55	G97A-G128A-Y217Q-S162K	N61E
56	G97A-G128A-Y217Q-S161P	N61E
57	G97A-G128A-Y217Q-V203Y	N61E
58	G97A-G128A-Y217Q-G211T	N61E
59	G97A-G128A-Y217Q-K213T	N61E
60	G97A-G128A-Y217Q-P239R	N61E
61	G97A-G128A-Y217Q-N240K	N61E
62	G97A-G128A-Y217Q-L267V	N61E
63	G97A-G128A-Y217Q-A273S	N61E
64	G97A-G128A-Y217Q-L75S-N76Y	N61P-S63H
65	G97A-G128A-Y217Q-S87T-A88L-S89G	N61P-S63H
66	G97A-G128A-Y217Q-S89Y	N61P-S63H
67	G97A-G128A-Y217Q-I111V	N61P-S63H
68	G97A-G128A-Y217Q-I115V	N61P-S63H
69	G97A-G128A-Y217Q-P129Q-S130G-G131S	N61P-S63H
70	G97A-G128A-Y217Q-P129Q	N61P-S63H
71	G97A-G128A-Y217Q-A134T	N61P-S63H
72	G97A-G128A-Y217Q-A144K	N61P-S63H
73	G97A-G128A-Y217Q-S145D	N61P-S63H
74	G97A-G128A-Y217Q-S159K	N61P-S63H
75	G97A-G128A-Y217Q-S162K	N61P-S63H
76	G97A-G128A-Y217Q-S161P	N61P-S63H
77	G97A-G128A-Y217Q-V203Y	N61P-S63H
78	G97A-G128A-Y217Q-G211T	N61P-S63H
79	G97A-G128A-Y217Q-K213T	N61P-S63H
80	G97A-G128A-Y217Q-P239R	N61P-S63H
81	G97A-G128A-Y217Q-N240K	N61P-S63H
82	G97A-G128A-Y217Q-L267V	N61P-S63H
83	G97A-G128A-Y217Q-A273S	N61P-S63H
84	G97A-G128A-Y217Q-S87T-A88L-S89G	L75S-N76Y
85	G97A-G128A-Y217Q-S89Y	L75S-N76Y
86	G97A-G128A-Y217Q-I111V	L75S-N76Y
87	G97A-G128A-Y217Q-I115V	L75S-N76Y
88	G97A-G128A-Y217Q-P129Q-S130G-G131S	L75S-N76Y
89	G97A-G128A-Y217Q-P129Q	L75S-N76Y
90	G97A-G128A-Y217Q-A134T	L75S-N76Y
91	G97A-G128A-Y217Q-A144K	L75S-N76Y
92	G97A-G128A-Y217Q-S145D	L75S-N76Y
93	G97A-G128A-Y217Q-S159K	L75S-N76Y
94	G97A-G128A-Y217Q-S162K	L75S-N76Y
95	G97A-G128A-Y217Q-S161P	L75S-N76Y
96	G97A-G128A-Y217Q-V203Y	L75S-N76Y
97	G97A-G128A-Y217Q-G211T	L75S-N76Y

Table 11-11: List of Parent Plasmids and Introduced Mutations in the RCL7 variants		
Combinatorial Variant #	**Parent Plasmid**	**Mutation(s) Introduced**
98	G97A-G128A-Y217Q-K213T	L75S-N76Y
99	G97A-G128A-Y217Q-P239R	L75S-N76Y
100	G97A-G128A-Y217Q-N240K	L75S-N76Y
101	G97A-G128A-Y217Q-L267V	L75S-N76Y
102	G97A-G128A-Y217Q-A273S	L75S-N76Y
103	G97A-G128A-Y217Q-I111V	S87T-A88L-S89G
104	G97A-G128A-Y217Q-I115V	S87T-A88L-S89G
105	G97A-G128A-Y217Q-P129Q-S130G-G131S	S87T-A88L-S89G
106	G97A-G128A-Y217Q-P129Q	S87T-A88L-S89G
107	G97A-G128A-Y217Q-A134T	S87T-A88L-S89G
108	G97A-G128A-Y217Q-A144K	S87T-A88L-S89G
109	G97A-G128A-Y217Q-S145D	S87T-A88L-S89G
110	G97A-G128A-Y217Q-S159K	S87T-A88L-S89G
111	G97A-G128A-Y217Q-S162K	S87T-A88L-S89G
112	G97A-G128A-Y217Q-S161P	S87T-A88L-S89G
113	G97A-G128A-Y217Q-V203Y	S87T-A88L-S89G
114	G97A-G128A-Y217Q-G211T	S87T-A88L-S89G
115	G97A-G128A-Y217Q-K213T	S87T-A88L-S89G
116	G97A-G128A-Y217Q-P239R	S87T-A88L-S89G
117	G97A-G128A-Y217Q-N240K	S87T-A88L-S89G
118	G97A-G128A-Y217Q-L267V	S87T-A88L-S89G
119	G97A-G128A-Y217Q-A273S	S87T-A88L-S89G
120	G97A-G128A-Y217Q-I111V	S89Y
121	G97A-G128A-Y217Q-I115V	S89Y
122	G97A-G128A-Y217Q-P129Q-S130G-G131S	S89Y
123	G97A-G128A-Y217Q-P129Q	S89Y
124	G97A-G128A-Y217Q-A134T	S89Y
125	G97A-G128A-Y217Q-A144K	S89Y
126	G97A-G128A-Y217Q-S145D	S89Y
127	G97A-G128A-Y217Q-S159K	S89Y
128	G97A-G128A-Y217Q-S162K	S89Y
129	G97A-G128A-Y217Q-S161P	S89Y
130	G97A-G128A-Y217Q-V203Y	S89Y
131	G97A-G128A-Y217Q-G211T	S89Y
132	G97A-G128A-Y217Q-K213T	S89Y
133	G97A-G128A-Y217Q-P239R	S89Y
134	G97A-G128A-Y217Q-N240K	S89Y
135	G97A-G128A-Y217Q-L267V	S89Y
136	G97A-G128A-Y217Q-A273S	S89Y
137	G97A-G128A-Y217Q-P129Q-S130G-G131S	I111V
138	G97A-G128A-Y217Q-P129Q	I111V
139	G97A-G128A-Y217Q-A134T	I111V

Table 11-11: List of Parent Plasmids and Introduced Mutations in the RCL7 variants		
Combinatorial Variant #	**Parent Plasmid**	**Mutation(s) Introduced**
140	G97A-G128A-Y217Q-A144K	I111V
141	G97A-G128A-Y217Q-S145D	I111V
142	G97A-G128A-Y217Q-S159K	I111V
143	G97A-G128A-Y217Q-S162K	I111V
144	G97A-G128A-Y217Q-S161P	I111V
145	G97A-G128A-Y217Q-V203Y	I111V
146	G97A-G128A-Y217Q-G211T	I111V
147	G97A-G128A-Y217Q-K213T	I111V
148	G97A-G128A-Y217Q-P239R	I111V
149	G97A-G128A-Y217Q-N240K	I111V
150	G97A-G128A-Y217Q-L267V	I111V
151	G97A-G128A-Y217Q-A273S	I111V
152	G97A-G128A-Y217Q-P129Q-S130G-G131S	I115V
153	G97A-G128A-Y217Q-P129Q	I115V
154	G97A-G128A-Y217Q-A134T	I115V
155	G97A-G128A-Y217Q-A144K	I115V
156	G97A-G128A-Y217Q-S145D	I115V
157	G97A-G128A-Y217Q-S159K	I115V
158	G97A-G128A-Y217Q-S162K	I115V
159	G97A-G128A-Y217Q-S161P	I115V
160	G97A-G128A-Y217Q-V203Y	I115V
161	G97A-G128A-Y217Q-G211T	I115V
162	G97A-G128A-Y217Q-K213T	I115V
163	G97A-G128A-Y217Q-P239R	I115V
164	G97A-G128A-Y217Q-N240K	I115V
165	G97A-G128A-Y217Q-L267V	I115V
166	G97A-G128A-Y217Q-A273S	I115V
167	G97A-G128A-Y217Q-A144K	P129Q-S130G-G131S
168	G97A-G128A-Y217Q-S145D	P129Q-S130G-G131S
169	G97A-G128A-Y217Q-S159K	P129Q-S130G-G131S
170	G97A-G128A-Y217Q-S162K	P129Q-S130G-G131S
171	G97A-G128A-Y217Q-S161P	P129Q-S130G-G131S
172	G97A-G128A-Y217Q-V203Y	P129Q-S130G-G131S
173	G97A-G128A-Y217Q-G211T	P129Q-S130G-G131S
174	G97A-G128A-Y217Q-K213T	P129Q-S130G-G131S
175	G97A-G128A-Y217Q-P239R	P129Q-S130G-G131S
176	G97A-G128A-Y217Q-N240K	P129Q-S130G-G131S
177	G97A-G128A-Y217Q-L267V	P129Q-S130G-G131S
178	G97A-G128A-Y217Q-A273S	P129Q-S130G-G131S
179	G97A-G128A-Y217Q-A144K	P129Q
180	G97A-G128A-Y217Q-S145D	P129Q
181	G97A-G128A-Y217Q-S159K	P129Q
182	G97A-G128A-Y217Q-S162K	P129Q

Table 11-11: List of Parent Plasmids and Introduced Mutations in the RCL7 variants		
Combinatorial Variant #	**Parent Plasmid**	**Mutation(s) Introduced**
183	G97A-G128A-Y217Q-S161P	P129Q
184	G97A-G128A-Y217Q-V203Y	P129Q
185	G97A-G128A-Y217Q-G211T	P129Q
186	G97A-G128A-Y217Q-K213T	P129Q
187	G97A-G128A-Y217Q-P239R	P129Q
188	G97A-G128A-Y217Q-N240K	P129Q
189	G97A-G128A-Y217Q-L267V	P129Q
190	G97A-G128A-Y217Q-A273S	P129Q
191	G97A-G128A-Y217Q-A144K	A134T
192	G97A-G128A-Y217Q-S145D	A134T
193	G97A-G128A-Y217Q-S159K	A134T
194	G97A-G128A-Y217Q-S162K	A134T
195	G97A-G128A-Y217Q-S161P	A134T
196	G97A-G128A-Y217Q-V203Y	A134T
197	G97A-G128A-Y217Q-G211T	A134T
198	G97A-G128A-Y217Q-K213T	A134T
199	G97A-G128A-Y217Q-P239R	A134T
200	G97A-G128A-Y217Q-N240K	A134T
201	G97A-G128A-Y217Q-L267V	A134T
202	G97A-G128A-Y217Q-A273S	A134T
203	G97A-G128A-Y217Q-S159K	A144K
204	G97A-G128A-Y217Q-S162K	A144K
205	G97A-G128A-Y217Q-S161P	A144K
206	G97A-G128A-Y217Q-V203Y	A144K
207	G97A-G128A-Y217Q-G211T	A144K
208	G97A-G128A-Y217Q-K213T	A144K
209	G97A-G128A-Y217Q-P239R	A144K
210	G97A-G128A-Y217Q-N240K	A144K
211	G97A-G128A-Y217Q-L267V	A144K
212	G97A-G128A-Y217Q-A273S	A144K
213	G97A-G128A-Y217Q-S159K	S145D
214	G97A-G128A-Y217Q-S162K	S145D
215	G97A-G128A-Y217Q-S161P	S145D
216	G97A-G128A-Y217Q-V203Y	S145D
217	G97A-G128A-Y217Q-G211T	S145D
218	G97A-G128A-Y217Q-K213T	S145D
219	G97A-G128A-Y217Q-P239R	S145D
220	G97A-G128A-Y217Q-N240K	S145D
221	G97A-G128A-Y217Q-L267V	S145D
222	G97A-G128A-Y217Q-A273S	S145D
223	G97A-G128A-Y217Q-V203Y	S159K
224	G97A-G128A-Y217Q-G211T	S159K
225	G97A-G128A-Y217Q-K213T	S159K

Table 11-11: List of Parent Plasmids and Introduced Mutations in the RCL7 variants		
Combinatorial Variant #	**Parent Plasmid**	**Mutation(s) Introduced**
226	G97A-G128A-Y217Q-P239R	S159K
227	G97A-G128A-Y217Q-N240K	S159K
228	G97A-G128A-Y217Q-L267V	S159K
229	G97A-G128A-Y217Q-A273S	S159K
230	G97A-G128A-Y217Q-V203Y	S162K
231	G97A-G128A-Y217Q-G211T	S162K
232	G97A-G128A-Y217Q-K213T	S162K
233	G97A-G128A-Y217Q-P239R	S162K
234	G97A-G128A-Y217Q-N240K	S162K
235	G97A-G128A-Y217Q-L267V	S162K
236	G97A-G128A-Y217Q-A273S	S162K
237	G97A-G128A-Y217Q-V203Y	S161P
238	G97A-G128A-Y217Q-G211T	S161P
239	G97A-G128A-Y217Q-K213T	S161P
240	G97A-G128A-Y217Q-239R	S161P
241	G97A-G128A-Y217Q-N240K	S161P
242	G97A-G128A-Y217Q-L267V	S161P
243	G97A-G128A-Y217Q-A273S	S161P
244	G97A-G128A-Y217Q-G211T	V203Y
245	G97A-G128A-Y217Q-K213T	V203Y
246	G97A-G128A-Y217Q-P239R	V203Y
247	G97A-G128A-Y217Q-N240K	V203Y
248	G97A-G128A-Y217Q-L267V	V203Y
249	G97A-G128A-Y217Q-A273S	V203Y
250	G97A-G128A-Y217Q-P239R	G211T
251	G97A-G128A-Y217Q-N240K	G211T
252	G97A-G128A-Y217Q-L267V	G211T
253	G97A-G128A-Y217Q-A273S	G211T
254	G97A-G128A-Y217Q-P239R	K213T
255	G97A-G128A-Y217Q-N240K	K213T
256	G97A-G128A-Y217Q-L267V	K213T
257	G97A-G128A-Y217Q-A273S	K213T
258	G97A-G128A-Y217Q-L267V	P239R
259	G97A-G128A-Y217Q-A273S	P239R
260	G97A-G128A-Y217Q-L267V	N240K
261	G97A-G128A-Y217Q-A273S	N240K

EXAMPLE 12

Table of Detergents

The compositions of the detergents used in the assays for Part I Example 12 are shown in Table 12-1. BPN' variant protein samples were added to the detergent compositions as described in Part I Example 1 to assay for the various properties listed.

Table 12-1: Composition of Detergents Used in the Assays to Test BPN' Variants

Ingredient	Composition (wt% of Composition)		
	1	2	3
C_{12-15} Alkylethoxy(1.8)sulphate	14.7	11.6	
$C_{11.8}$ Alkylbenzene sulfonate	4.3	11.6	8.3
C_{16-17} Branched alkyl sulphate	1.7	1.29	
C_{12-14} Alkyl -9-ethoxylate	0.9	1.07	
C_{12} dimethylamine oxide	0.6	0.64	
Citric acid	3.5	0.65	3
C_{12-18} fatty acid	1.5	2.32	3.6
Sodium Borate (Borax)	2.5	2.46	1.2
Sodium C_{12-14} alkyl ethoxy 3 sulfate			2.9
C_{14-15} alkyl 7-ethoxylate			4.2
C_{12-14} Alkyl -7-ethoxylate			1.7
Ca formate	0.09	0.09	
A compound having the following general structure: bis((C_2H_5O)(C_2H_4O)n)(CH_3)-N^+-C_xH_{2x}-N^+-(CH_3)-bis((C_2H_5O)(C_2H_4O)n), wherein n = from 20 to 30, and x = from 3 to 8, or sulphated or sulphonated variants thereof			1.2
Random graft co-polymer[1]		1.46	0.5
Ethoxylated Polyethylenimine [2]	1.5	1.29	
Diethylene triamine pentaacetic acid	0.34	0.64	
Diethylene triamine penta(methylene phosphonic acid)			0.3

Table 12-1: Composition of Detergents Used in the Assays to Test BPN' Variants			
Ingredient	**Composition (wt% of Composition)**		
	1	**2**	**3**
Tinopal AMS-GX		0.06	
Tinopal CBS-X	0.2	0.17	
Amphiphilic alkoxylated grease cleaning polymer [3]	1.28	1	0.4
Ethanol	2	1.58	1.6
Propylene Glycol	3.9	3.59	1.3
Diethylene glycol	1.05	1.54	
Polyethylene glycol	0.06	0.04	
Monoethanolamine	3.05	2.41	0.4
NaOH	2.44	1.8	
Sodium Cumene Sulphonate			1
Sodium Formate		0.11	
Water, Aesthetics (Dyes, perfumes) and Minors (Enzymes, solvents, structurants)	balance	balance	balance

[1] "Random graft copolymer" is a polyvinyl acetate grafted polyethylene oxide copolymer having a polyethylene oxide backbone and multiple polyvinyl acetate side chains. The molecular weight of the polyethylene oxide backbone is about 6000 and the weight ratio of the polyethylene oxide to polyvinyl acetate is about 40 to 60 and no more than 1 grafting point per 50 ethylene oxide units.

[2] Polyethylenimine (MW = 600) with 20 ethoxylate groups per -NH.

[3] Amphiphilic alkoxylated grease cleaning polymer is a polyethylenimine (MW = 600) with 24 ethoxylate groups per –NH and 16 propoxylate groups per –NH.

Stain Removal Performance of BPN' Combinatorial Variants

Experiments to evaluate the stain removal performance of BPN' combinatorial variants generated as described in Example 11 were performed using BMI stained microswatches. The assay was performed as described in Example 1 (BMI microswatch assay). Table 12-2 provides Performance Index (PI) values of variants generated from RCL4 library using BMI microswatch assay in Detergent Composition 1 at pH 8 and 16°C and Detergent Composition 1 at pH 8 and 32°C and BMI microswatch assay in heat

deactivated commercial TIDE® 2X Cold (Procter & Gamble) detergent at 16°C and pH 8. Heat inactivation of commercial detergent formulas serves to destroy the endogenous enzymatic activity of any protein components while retaining the properties of nonenzymatic components. Heat inactivation of the detergents was performed by placing pre-weighed amounts of liquid detergent (in a glass bottle) in a water bath at 95°C for 2 hours. The TIDE® 2X Cold detergent was purchased from local supermarket stores. Both unheated and heated detergents were assayed within 5 minutes of dissolving the detergent, in order to accurately determine percentage deactivated. Enzyme activity was tested by AAPF assay. Working solutions were made from the heat inactivated stock. Appropriate amounts of water hardness and buffer were added to the detergent solutions to match the desired conditions (Table 12-2). The solutions were mixed by vortexing or inverting the bottles.

Table 12-2: Working Detergent Solutions

Detergent	Temp (°C)	Detergent g/L	pH	Buffer	Hardness Gpg
TIDE® 2X Cold	16, 32	0.98	8	5mM HEPES	6

The sequences of the variants listed in Table 12-3 are relative to BPN'-v3: G97A-G128A-Y217Q. The PI values are calculated relative to BPN'-v3. All mutants in this list have a PI cutoff equal or greater than 0.5 for at least one property tested. "Det. Comp." means Detergent Composition.

Table 12-3: Performance Index Values of Variants Generated from RCL4 Library

Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	TIDE® Detergent pH 8, 16°C, BMI PI	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 1, pH 8, 32°C, BMI PI
S87T-A88L-S89G-G97A-G128A-Y217Q	S87T-A88L-S89G	1.00	1.12	0.99
N61P-S63H-G97A-G128A-Y217Q	N61P-S63H	1.03	1.12	1.01
S87G-A88V-S89A-G97A-G128A-Y217Q	S87G-A88V-S89A	1.02	1.11	0.99
P86S-S87G-A88V-G97A-G128A-Y217Q	P86S-S87G-A88V	1.00	1.10	1.00
Q59S-N61P-G97A-G128A-Y217Q	Q59S-N61P	1.01	1.09	1.00
S24G-N25G-G97A-G128A-Y217Q	S24G-N25G	0.99	1.09	1.02
N61P-N62S-G97A-G128A-Y217Q	N61P-N62S	0.99	1.06	0.98
G97A-G128A-P129Q-S130G-G131S-Y217Q	P129Q-S130G-G131S	0.96	1.06	0.99
L75S-N76Y-G97A-G128A-Y217Q	L75S-N76Y	0.99	1.06	1.00
G97A-G128A-V203Y-Y217Q	V203Y	0.99	1.05	1.01

Table 12-3: Performance Index Values of Variants Generated from RCL4 Library				
Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	TIDE® Detergent pH 8, 16°C, BMI PI	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 1, pH 8, 32°C, BMI PI
T55P-G97A-G128A-Y217Q	T55P	0.98	1.04	0.98
A88V-L90I-G97A-G128A-Y217Q	A88V-L90I	0.99	1.04	1.00
G97A-G128A-G211R-N212S-K213V-Y217Q	G211R-N212S-K213V	0.97	1.04	0.98
G23A-S24G-N25G-G97A-G128A-Y217Q	G23A-S24G-N25G	0.98	1.04	0.98
T22N-S24A-G97A-G128A-Y217Q	T22N-S24A	0.98	1.03	0.97
S24R-G97A-G128A-Y217Q	S24R	0.95	1.02	0.99
G97A-A98S-G128A-Y217Q	A98S	0.95	1.02	0.99
BPN'-v3: G97A-G128A-Y217Q	**BPN'-v3**	**1.00**	**1.00**	**1.00**
G97A-G128A-T158G-S159G-Y217Q	T158G-S159G	0.95	0.99	0.97
Q59E-N61P-G97A-G128A-Y217Q	Q59E-N61P	0.90	0.94	0.90
G97A-A98E-G128A-Y217Q	A98E	0.92	0.91	0.90

The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from those in Table 12-3, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. The proteolytic activity of such protease variant may be greater than that of the BPN' or BPN'-v3 protease. Each such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Table 12-4 provides Performance Index (PI) values of variants generated from RCL 5-7 and FS1-3 using BMI microswatch assay in Detergent Composition 1 at 16°C and pH 8, BMI microswatch assay in Detergent Composition 2 at 16°C and pH 8, and stability measured in Detergent Composition 3. PI values for specific activity by AAPF hydrolysis (Specific AAPF PI) were also determined. All assays were performed as described in Example 1. The sequences of the variants listed are relative to BPN'-v3: G97A-G128A-Y217Q. PI values were calculated relative to BPN'-v3. All mutants in this list have a PI cutoff equal or greater than 0.5 for at least one property tested. PI values less than 0.01 were modified to display 0.01 in bold italics.

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3						
Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
RCL6	G97A-G128A-Y217Q-P86S-S87G-A88V-A116N-N117S-N118G	P86S-S87G-A88V-A116N-N117S-N118G	1.21	1.14	1.17	0.03
FS1	G97A-G128A-Y217Q-S24G-N25G-N61P-S101N	S24G-N25G-N61P-S101N	1.05	1.12	1.96	1.00
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S87T-A88L-S89G-S101N-V203Y	S24G-N25G-S53G-T55P-S87T-A88L-S89G-S101N-V203Y	1.04	1.10	1.71	0.03
FS1	G97A-G128A-Y217Q-N61P-S78N-S101N-V203Y	N61P-S78N-S101N-V203Y	1.03	1.10	1.73	0.33
FS1	G97A-G128A-Y217Q-T55P-N61P-S78N-S101N-V203Y	T55P-N61P-S78N-S101N-V203Y	1.06	1.10	1.97	0.39
FS1	G97A-G128A-Y217Q-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N	S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N	1.03	1.10	2.22	0.33
RCL7	G97A-G128A-Y217Q-V203Y-L267V	V203Y-L267V	1.10	1.09	1.12	0.09
FS1	G97A-G128A-Y217Q-S24G-N25G-T55P-S101N	S24G-N25G-T55P-S101N	1.04	1.09	1.82	1.13
RCL7	G97A-G128A-Y217Q-A134T-L267V	A134T-L267V	1.13	1.08	0.93	0.66
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G	S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G	1.01	1.08	1.10	0.29

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-N61P-S101N-V203Y	S24G-N25G-S53G-N61P-S101N-V203Y	1.07	1.08	2.11	0.09
RCL6	G97A-G128A-Y217Q-N25Y-Q59S-N61P	N25Y-Q59S-N61P	1.07	1.08	0.85	0.66
RCL7	G97A-G128A-Y217Q-I111V-S161P	I111V-S161P	1.10	1.08	0.66	0.98
RCL7	G97A-G128A-Y217Q-I115V-L267V	I115V-L267V	1.10	1.08	1.07	0.63
FS1	G97A-G128A-Y217Q-T55P-S78N-S87T-A88L-S89G-S101N-V203Y	T55P-S78N-S87T-A88L-S89G-S101N-V203Y	0.99	1.08	1.39	0.16
RCL6	G97A-G128A-Y217Q-N25Y-P129Q-S130G-G131S-A137T	N25Y-P129Q-S130G-G131S-A137T	1.07	1.08	0.88	0.67
RCL7	G97A-G128A-Y217Q-N61P-S63H-A128S-P129Q	N61P-S63H-A128S-P129Q	1.04	1.08	1.59	0.78
FS1	G97A-G128A-Y217Q-S53G-N61P-S101N-V203Y	S53G-N61P-S101N-V203Y	1.03	1.08	1.91	0.09
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N	S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N	1.08	1.07	1.44	0.33
FS1	G97A-G128A-Y217Q-N61P-S78N-S87T-A88L-S89G-S101N	N61P-S78N-S87T-A88L-S89G-S101N	1.07	1.07	1.80	0.31
RCL6	G97A-G128A-Y217Q-N25Y-N61P-S63H	N25Y-N61P-S63H	1.06	1.07	0.74	0.60
RCL5	G97A-G128A-Y217Q-Q59S-N61P-V203Y	Q59S-N61P-V203Y	0.99	1.07	0.69	0.09

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
RCL6	G97A-G128A-Y217Q-V8L-N25Y-P129Q-S130G-G131S	V8L-N25Y-P129Q-S130G-G131S	1.08	1.07	1.22	0.03
RCL6	G97A-G128A-Y217Q-P86S-S87G-A88V-P239R	P86S-S87G-A88V-P239R	1.16	1.07	1.26	0.03
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S101N-V203Y	S24G-N25G-S53G-T55P-N61P-S101N-V203Y	1.02	1.07	2.07	0.11
RCL5	G97A-G128A-Y217Q-S24G-N25G-P129Q-S130G-G131S	S24G-N25G-P129Q-S130G-G131S	1.09	1.06	1.25	1.00
FS3	G97A-G128A-Y217Q-N240K	N240K	1.04	1.06	0.90	0.94
RCL5	G97A-G128A-Y217Q-G23A-S24G-N25G-G211R-N212S-K213V	G23A-S24G-N25G-G211R-N212S-K213V	1.06	1.06	1.05	0.06
RCL7	G97A-G128A-Y217Q-N61P-S63H-S78N-I111V-A134T	N61P-S63H-S78N-I111V-A134T	1.03	1.06	0.43	1.14
RCL6	G97A-G128A-Y217Q-S63T-P86S-S87G-A88V	S63T-P86S-S87G-A88V	1.17	1.06	1.46	0.02
RCL6	G97A-G128A-Y217Q-G23A-S24G-N25G-A116N-N117S-N118G	G23A-S24G-N25G-A116N-N117S-N118G	1.08	1.06	0.91	0.21
FS1	G97A-G128A-Y217Q-S78N-S87T-A88L-S89G-S101N	S78N-S87T-A88L-S89G-S101N	1.03	1.06	1.40	0.33
RCL6	G97A-G128A-Y217Q-S24G-N25G-A116N-N117S-N118G	S24G-N25G-A116N-N117S-N118G	1.06	1.06	0.74	1.05
RCL7	G97A-G128A-Y217Q-T55P-N240K	T55P-N240K	1.02	1.06	0.91	0.93

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
RCL6	G97A-G128A-Y217Q-T55P-P129V-P194S	T55P-P129V-P194S	1.07	1.06	0.85	0.86
RCL6	G97A-G128A-Y217Q-N25Y-S87G-A88V-S89A	N25Y-S87G-A88V-S89A	1.07	1.05	1.14	0.08
FS1	G97A-G128A-Y217Q-S24G-N25G-S87T-A88L-S89G-S101N	S24G-N25G-S87T-A88L-S89G-S101N	1.02	1.05	1.19	0.06
RCL7	G97A-G128A-Y217Q-P129Q-S130G-G131S-V203Y	P129Q-S130G-G131S-V203Y	1.04	1.05	0.94	0.05
RCL6	G97A-G128A-Y217Q-Q59S-N61P-N240K	Q59S-N61P-N240K	1.05	1.05	0.80	0.84
FS3	G97A-G128A-Y217Q-S24R-P40E-P129E-S159K-K265R	S24R-P40E-P129E-S159K-K265R	1.13	1.05	1.08	1.31
RCL7	G97A-G128A-Y217Q-P52S-T55P-V203Y	P52S-T55P-V203Y	1.09	1.05	0.53	0.15
RCL6	G97A-G128A-Y217Q-S24R-P129E	S24R-P129E	1.11	1.05	0.88	0.59
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-N61P-S78N	S24G-N25G-S53G-N61P-S78N	0.98	1.05	1.17	1.16
FS1	G97A-G128A-Y217Q-S24G-N25G-T55P-S78N-S101N	S24G-N25G-T55P-S78N-S101N	1.07	1.05	1.71	1.29
RCL6	G97A-G128A-Y217Q-P86S-S87G-A88V-A116S-N117G-N118R	P86S-S87G-A88V-A116S-N117G-N118R	1.10	1.05	0.76	0.05
RCL6	G97A-G128A-Y217Q-N61P-S87T-A88L-S89G	N61P-S87T-A88L-S89G	1.00	1.05	1.12	0.05

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S78N-S87T-A88L-S89G	S24G-N25G-S53G-T55P-S78N-S87T-A88L-S89G	0.96	1.05	0.96	0.29
RCL5	G97A-G128A-Y217Q-G23A-S24G-N25G-N61P-S63H	G23A-S24G-N25G-N61P-S63H	1.06	1.05	0.89	0.13
RCL6	G97A-G128A-Y217Q-S24R-Q59S-N61P	S24R-Q59S-N61P	1.07	1.05	0.94	0.49
RCL6	G97A-G128A-Y217Q-N61P-P129Q-S130G-G131S	N61P-P129Q-S130G-G131S	1.07	1.05	1.13	0.78
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N	S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N	1.05	1.04	1.98	0.32
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S101N-V203Y	S24G-N25G-S53G-T55P-S101N-V203Y	1.05	1.04	1.57	0.15
FS1	G97A-G128A-Y217Q-N61P-S78N-S87T-A88L-S89G-S101N-V203Y	N61P-S78N-S87T-A88L-S89G-S101N-V203Y	1.02	1.04	1.60	0.12
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-S78N-S101N	S24G-N25G-S53G-T55P-S78N-S101N	1.03	1.04	2.01	1.27
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S101N-V203Y	S24G-N25G-S53G-S101N-V203Y	1.01	1.04	1.77	0.07
FS1	G97A-G128A-Y217Q-S24G-N25G-S78N-S101N-V203Y	S24G-N25G-S78N-S101N-V203Y	1.09	1.04	1.54	0.28

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
RCL7	G97A-G128A-Y217Q-P129Q-S130G-G131S-A133V-L267V	P129Q-S130G-G131S-A133V-L267V	1.14	1.04	1.37	0.56
FS1	G97A-G128A-Y217Q-S87T-A88L-S89G-S101N	S87T-A88L-S89G-S101N	1.05	1.04	1.44	0.07
RCL6	G97A-G128A-Y217Q-G23A-S24G-N25G-P239R	G23A-S24G-N25G-P239R	1.07	1.04	0.92	0.16
RCL6	G97A-G128A-Y217Q-S87G-A88V-S89A-A116N-N117S-N118G	S87G-A88V-S89A-A116N-N117S-N118G	1.07	1.04	0.92	0.37
RCL6	G97A-G128A-Y217Q-Q59S-N61P-A116S-N117G-N118R	Q59S-N61P-A116S-N117G-N118R	1.09	1.04	0.68	0.91
RCL5	G97A-G128A-Y217Q-Q59S-N61P-S87T-A88L-S89G	Q59S-N61P-S87T-A88L-S89G	1.03	1.04	0.81	0.08
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S87T-A88L-S89G-V203Y	S24G-N25G-S53G-S87T-A88L-S89G-V203Y	0.96	1.04	0.73	0.04
RCL7	G97A-G128A-Y217Q-A134T-G211T	A134T-G211T	1.11	1.04	0.71	0.35
RCL7	G97A-G128A-Y217Q-T55P-A128S-P129Q	T55P-A128S-P129Q	1.03	1.04	2.71	1.02
FS1	G97A-G128A-Y217Q-T55P-S78N-S87T-A88L-S89G-S101N	T55P-S78N-S87T-A88L-S89G-S101N	1.05	1.04	1.56	0.33
RCL6	G97A-G128A-Y217Q-P86S-S87G-A88V-T242R	P86S-S87G-A88V-T242R	1.09	1.04	0.83	0.04
RCL7	G97A-G128A-Y217Q-S161P-V203Y	S161P-V203Y	1.04	1.04	0.81	0.09

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S24G-N25G-T55P-N61P-S78N-S101N-V203Y	S24G-N25G-T55P-N61P-S78N-S101N-V203Y	1.05	1.04	2.01	0.44
RCL7	G97A-G128A-Y217Q-G211T-L267V	G211T-L267V	1.08	1.03	1.16	0.30
RCL6	G97A-G128A-Y217Q-P40E-T55P-N269K	P40E-T55P-N269K	1.08	1.03	0.71	0.05
RCL6	G97A-G128A-Y217Q-S24R-A128S-P129G	S24R-A128S-P129G	1.08	1.03	1.63	0.64
RCL6	G97A-G128A-Y217Q-S24G-N25G-N61P-N62S-P194L-A232T	S24G-N25G-N61P-N62S-P194L-A232T	1.15	1.03	3.06	0.35
RCL6	G97A-G128A-Y217Q-T55P-A116S-N117G-N118R	T55P-A116S-N117G-N118R	1.07	1.03	0.83	1.06
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S101N-V203Y	S24G-N25G-S53G-S78N-S101N-V203Y	1.03	1.03	1.76	0.32
RCL7	G97A-G128A-Y217Q-P129Q-S130G-G131S-N240K	P129Q-S130G-G131S-N240K	1.08	1.03	0.90	0.83
FS1	G97A-G128A-Y217Q-S53G-T55P-N61P-S78N-S87T-A88L-S89G	S53G-T55P-N61P-S78N-S87T-A88L-S89G	0.93	1.03	1.00	0.33
RCL6	G97A-G128A-Y217Q-N25Y-P129Q-S130G-G131S	N25Y-P129Q-S130G-G131S	1.08	1.03	0.93	0.69
RCL6	G97A-G128A-Y217Q-T55P-I115V	T55P-I115V	1.06	1.03	1.07	1.07
RCL6	G97A-G128A-Y217Q-N25Y-T55P	N25Y-T55P	1.05	1.03	1.00	0.80

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific ΔΔPF PI	Det. Comp. 3, Stability PI
RCL6	G97A-G128A-Y217Q-G23A-S24G-N25G-A128S-P129D	G23A-S24G-N25G-A128S-P129D	0.97	1.03	1.75	0.15
FS1	G97A-G128A-Y217Q-S53G-S78N-S87T-A88L-S89G-S101N-P129S-V203Y	S53G-S78N-S87T-A88L-S89G-S101N-P129S-V203Y	1.00	1.03	1.68	0.13
RCL7	G97A-G128A-Y217Q-T55P-A134T	T55P-A134T	1.06	1.03	0.94	1.05
RCL7	G97A-G128A-Y217Q-N61P-S63H-S78N-I111V	N61P-S63H-S78N-I111V	1.03	1.02	0.49	1.18
FS2	Y217Q-N61P-A97G-G102A-A128G-P129S	N61P-A97G-G102A-A128G-P129S	0.98	1.02	*NA*	*NA*
FS1	G97A-G128A-Y217Q-S53G-N61P-S101N	S53G-N61P-S101N	1.02	1.02	2.08	0.88
RCL5	G97A-G128A-Y217Q-Q59S-N61P-S87G-A88V-S89A	Q59S-N61P-S87G-A88V-S89A	1.01	1.02	0.90	0.20
FS1	G97A-G128A-Y217Q-S53G-S87T-A88L-S89G-S101N-V203Y	S53G-S87T-A88L-S89G-S101N-V203Y	0.96	1.02	1.11	0.04
RCL6	G97A-G128A-Y217Q-S87T-A88L-S89G-P129S	S87T-A88L-S89G-P129S	1.06	1.02	1.21	0.10
FS1	G97A-G128A-Y217Q-S53G-T55P-S78N-S101N-V203Y	S53G-T55P-S78N-S101N-V203Y	1.03	1.02	1.70	0.48
RCL5	G97A-G128A-Y217Q-T55P-P129Q-S130G-G131S	T55P-P129Q-S130G-G131S	1.01	1.02	1.18	0.91
RCL5	G97A-G128A-Y217Q-Q59S-N61P-P129Q-S130G-G131S	Q59S-N61P-P129Q-S130G-G131S	0.98	1.02	0.83	0.80
RCL7	G97A-G128A-Y217Q-A134T-P239R	A134T-P239R	1.03	1.02	0.53	0.98

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific ΔΔPF PI	Det. Comp. 3, Stability PI
RCL5	G97A-G128A-Y217Q-T55P-V203Y	T55P-V203Y	1.01	1.01	1.02	0.23
RCL7	G97A-G128A-Y217Q-T55P-S78N-S89Y	T55P-S78N-S89Y	1.03	1.01	1.05	1.25
RCL5	G97A-G128A-Y217Q-T22N-S24A-N61P-S63H	T22N-S24A-N61P-S63H	1.00	1.01	0.69	0.46
RCL7	G97A-G128A-Y217Q-S161P-L267V	S161P-L267V	1.05	1.01	1.00	0.66
RCL6	G97A-G128A-Y217Q-T55P-L75H-N76G	T55P-L75H-N76G	1.06	1.01	0.69	0.59
RCL7	G97A-G128A-Y217Q-A134T-S161P	A134T-S161P	1.07	1.01	0.73	1.00
RCL7	G97A-G128A-Y217Q-S87T-A88L-S89G-A134T	S87T-A88L-S89G-A134T	1.08	1.01	0.66	0.13
RCL6	G97A-G128A-Y217Q-T55P-A116N-N117S-N118G	T55P-A116N-N117S-N118G	1.06	1.01	1.06	1.11
v12	G97A-G128S-Y217Q	A128S	1.02	1.01	1.65	1.00
RCL7	G97A-G128A-Y217Q-T55P-S78N-I115V	T55P-S78N-I115V	1.07	1.00	1.00	1.32
RCL6	G97A-G128A-Y217Q-Y6Q-P129Q-S130G-G131S	Y6Q-P129Q-S130G-G131S	1.03	1.00	0.98	0.23
RCL7	G97A-G128A-Y217Q-S24R-P129Q-S130G-G131S	S24R-P129Q-S130G-G131S	1.06	1.00	1.11	0.61
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S101N	S24G-N25G-S53G-S78N-S101N	0.99	1.00	1.61	1.21
RCL6	G97A-G128A-Y217Q-T55P-P129V	T55P-P129V	1.07	1.00	0.70	1.00

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
v3	G97A-G128A-Y217Q	BPN'-v3	1.00	1.00	1.00	1.00
FS2	G97A-Y217Q-N61P-N62Q-G100N-A128G	N61P-N62Q-G100N-A128G	0.95	1.00	*NA*	*NA*
RCL6	G97A-G128A-Y217Q-T55P-P129Q	T55P-P129Q	1.10	1.00	1.77	1.15
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y	S24G-N25G-S53G-T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y	1.05	1.00	1.84	0.12
RCL6	G97A-G128A-Y217Q-S87T-A88L-S89G-N240K	S87T-A88L-S89G-N240K	1.04	1.00	0.83	0.10
RCL7	G97A-G128A-Y217Q-A134T-N240K	A134T-N240K	1.07	0.99	0.61	0.96
RCL7	G97A-G128A-Y217Q-S87T-A88L-S89G-P239R	S87T-A88L-S89G-P239R	1.04	0.99	0.68	0.14
RCL7	G97A-G128A-Y217Q-P129Q-S130G-G131S-L267V	P129Q-S130G-G131S-L267V	1.08	0.99	1.35	0.61
RCL7	G97A-G128A-Y217Q-P129Q-N240K	P129Q-N240K	1.06	0.99	1.39	1.00
FS1	G97A-G128A-Y217Q-S78N-S87T-A88L-S89G-V203Y	S78N-S87T-A88L-S89G-V203Y	0.93	0.99	0.74	0.15
RCL7	G97A-G128A-Y217Q-I111V-A273S	I111V-A273S	1.00	0.99	0.48	0.27
FS1	G97A-G128A-Y217Q-S24G-N25G-T55P-S78N-A88V-S101N	S24G-N25G-T55P-S78N-A88V-S101N	1.03	0.99	2.17	0.71
FS1	G97A-G128A-Y217Q-S24G-N25G-T55P-S78N	S24G-N25G-T55P-S78N	0.96	0.98	1.15	1.20

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S101N-V203Y	S24G-N25G-S53G-S78N-S87T-A88L-S101N-V203Y	1.02	0.98	1.27	0.10
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S89G-V203Y	S24G-N25G-S53G-S78N-S87T-A88L-S89G-V203Y	0.97	0.98	0.75	0.14
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N-V203Y	S24G-N25G-S53G-S78N-S87T-A88L-S89G-S101N-V203Y	1.06	0.98	1.21	0.14
RCL5	G97A-G128A-Y217Q-S87G-A88V-S89A-P129Q-S130G-G131S	S87G-A88V-S89A-P129Q-S130G-G131S	1.01	0.98	1.18	0.29
RCL7	G97A-G128A-Y217Q-N61P-S63H-S78N-S161P	N61P-S63H-S78N-S161P	1.03	0.98	0.73	1.16
FS1	G97A-G128A-Y217Q-T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y	T55P-N61P-S78N-S87T-A88L-S89G-S101N-V203Y	1.04	0.98	1.75	0.14
RCL7	G97A-G128A-Y217Q-I111V-P129Q-S130G-G131S	I111V-P129Q-S130G-G131S	1.00	0.98	0.52	0.97
RCL5	G97A-G128A-Y217Q-T22N-S24A-T55P	T22N-S24A-T55P	0.96	0.98	0.94	0.64
RCL7	G97A-G128A-Y217Q-I115V-N240K	I115V-N240K	1.04	0.98	0.66	0.90
RCL6	G97A-G128A-Y217Q-S87G-A88V-S89A-A116N-N117S-N118G-P172H	S87G-A88V-S89A-A116N-N117S-N118G-P172H	0.98	0.98	0.66	0.40

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific ΔΔPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S24G-N25G-S78N-S87T-A88L-S89G-S101N	S24G-N25G-S78N-S87T-A88L-S89G-S101N	1.01	0.98	1.28	0.31
RCL6	G97A-G128A-Y217Q-S24G-N25G-I115V-A134T	S24G-N25G-I115V-A134T	1.03	0.98	0.59	1.13
RCL6	G97A-G128A-Y217Q-T55P-A128S-P129D	T55P-A128S-P129D	0.97	0.97	1.39	1.00
RCL7	G97A-G128A-Y217Q-I111V-S159K	I111V-S159K	0.99	0.97	0.64	1.12
RCL7	G97A-G128A-Y217Q-N240K-A273S	N240K-A273S	1.03	0.96	0.64	0.19
RCL7	G97A-G128A-Y217Q-S159K-L267V	S159K-L267V	1.00	0.96	1.08	0.79
RCL7	G97A-G128A-Y217Q-I111V-P129Q-G211T	I111V-P129Q-G211T	1.01	0.96	0.83	0.42
RCL7	G97A-G128A-Y217Q-I115V-A273S	I115V-A273S	1.05	0.95	0.74	0.20
RCL6	G97A-G128A-Y217Q-S89Y	S89Y	0.99	0.95	0.70	0.88
RCL6	G97A-G128A-Y217Q-S24R-A116N-N117S-N118G	S24R-A116N-N117S-N118G	1.08	0.95	0.91	0.60
RCL7	G97A-G128A-Y217Q-N61E-A144K	N61E-A144K	1.03	0.95	0.97	1.10
RCL6	G97A-G128A-Y217Q-P129Q-S130G-G131S-P239R	P129Q-S130G-G131S-P239R	1.02	0.95	0.85	0.99
RCL7	G97A-G128A-Y217Q-S87T-A88L-S89G-I115V	S87T-A88L-S89G-I115V	0.97	0.95	0.59	0.13
RCL6	G97A-G128A-Y217Q-T55P-A92G	T55P-A92G	0.96	0.94	0.56	0.91

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS3	G97A-G128A-Y217Q-S145D-S159K-N240K-Q275E	S145D-S159K-N240K-Q275E	0.98	0.94	0.76	1.08
RCL7	G97A-G128A-Y217Q-S89Y-P129Q-S130G-G131S	S89Y-P129Q-S130G-G131S	1.04	0.94	0.71	0.70
RCL7	G97A-G128A-Y217Q-P129Q-S130G-G131S-S162K	P129Q-S130G-G131S-S162K	1.01	0.94	1.00	0.93
RCL7	G97A-G128A-Y217Q-I111V-A134T	I111V-A134T	0.98	0.94	0.37	1.06
RCL6	G97A-G128A-Y217Q-P40E-S53Y-S78Y-P86S-S87G-A88V	P40E-S53Y-S78Y-P86S-S87G-A88V	0.99	0.94	0.97	0.35
RCL6	G97A-G128A-Y217Q-S24G-N25G-L75H-N76G	S24G-N25G-L75H-N76G	0.93	0.93	0.58	0.52
FS2	G97A-Y217Q-N61P-A128G-P129S-S130P	N61P-A128G-P129S-S130P	0.85	0.93	0.65	0.92
RCL6	G97A-G128A-Y217Q-S24R-S145D	S24R-S145D	0.99	0.93	0.89	0.59
FS3	G97A-G128A-Y217Q-S24R-S145D-P239R-Q275E	S24R-S145D-P239R-Q275E	0.92	0.92	0.63	0.68
RCL7	G97A-G128A-Y217Q-S24R-S78N-S182P-L267V	S24R-S78N-S182P-L267V	0.95	0.92	1.16	0.55
FS1	G97A-G128A-Y217Q-S53G-N61P-S87T-A88L-S89G-S101N-V203Y	S53G-N61P-S87T-A88L-S89G-S101N-V203Y	1.04	0.92	1.67	0.02
RCL6	G97A-G128A-Y217Q-P5S-S87G-A88V-S89A-A116G-N117R	P5S-S87G-A88V-S89A-A116G-N117R	0.98	0.92	0.64	0.07

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S53G-N61P-S78N-S87T-A88L-S89G-S101N-V203Y	S53G-N61P-S78N-S87T-A88L-S89G-S101N-V203Y	0.99	0.92	1.65	0.11
RCL6	G97A-G128A-Y217Q-Q59S-N61P-A116N-N117S-N118G	Q59S-N61P-A116N-N117S-N118G	0.93	0.92	0.42	1.04
RCL7	G97A-G128A-Y217Q-P239R-A273S	P239R-A273S	0.94	0.91	0.52	0.23
FS1	G97A-G128A-Y217Q-S53G-S78N-S87T-A88L-S89G-S101N-V203Y	S53G-S78N-S87T-A88L-S89G-S101N-V203Y	0.94	0.91	1.16	0.13
RCL6	G97A-G128A-Y217Q-S24R-P129V	S24R-P129V	0.98	0.91	0.52	0.58
RCL7	G97A-G128A-Y217Q-I111V-P239R	I111V-P239R	0.97	0.91	0.38	1.08
FS1	G97A-G128A-Y217Q-S87T-A88L-S89G-S101N-V203Y	S87T-A88L-S89G-S101N-V203Y	0.90	0.91	0.79	0.05
RCL6	G97A-G128A-Y217Q-T55P-P129L	T55P-P129L	0.99	0.91	0.62	0.98
RCL7	G97A-G128A-Y217Q-S87T-A88L-S89G-I111V	S87T-A88L-S89G-I111V	0.92	0.90	0.42	0.13
RCL7	G97A-G128A-Y217Q-S145D-A273S	S145D-A273S	0.91	0.90	0.66	0.17
RCL6	G97A-G128A-Y217Q-P129Q-S130G-G131S-T242R	P129Q-S130G-G131S-T242R	0.94	0.90	0.51	0.89
RCL7	G97A-G128A-Y217Q-S3F-S87T-A88L-S89G-G211T	S3F-S87T-A88L-S89G-G211T	0.93	0.89	0.55	0.07

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
RCL6	G97A-G128A-Y217Q-S87G-A88V-S89A-S162K	S87G-A88V-S89A-S162K	0.97	0.89	1.21	0.37
RCL7	G97A-G128A-Y217Q-S89Y-G211T	S89Y-G211T	0.89	0.88	0.53	0.41
RCL7	G97A-G128A-Y217Q-S87T-A88L-S89G-A144K	S87T-A88L-S89G-A144K	0.93	0.88	0.74	0.12
RCL6	G97A-G128A-Y217Q-P129Q-S130G-G131S-S159K	P129Q-S130G-G131S-S159K	0.95	0.88	1.21	0.96
RCL6	G97A-G128A-Y217Q-A116N-N117S-N118G-P129Q-S130G-G131S	A116N-N117S-N118G-P129Q-S130G-G131S	0.90	0.88	0.54	0.95
RCL6	G97A-G128A-Y217Q-S24G-N25G-P129V	S24G-N25G-P129V	0.86	0.87	0.44	1.02
FS1	G97A-G128A-Y217Q-S24G-N25G-S78N-S87T-A88L-S89G-S101N-V203Y	S24G-N25G-S78N-S87T-A88L-S89G-S101N-V203Y	0.87	0.86	0.83	0.12
FS2	G97A-Y217Q-N123G-A128G	N123G-A128G	0.83	0.86	1.00	0.14
FS2	G97A-G128A-Y217Q-N61P-N62Q-G100N-G102A-M124I	N61P-N62Q-G100N-G102A-M124I	0.82	0.86	5.52	0.40
RCL6	G97A-G128A-Y217Q-S24G-N25G-K141E-T242R	S24G-N25G-K141E-T242R	0.90	0.85	0.65	1.10
RCL6	G97A-G128A-Y217Q-S87G-A88V-S89A-A116N-N117S-N118G-A144T	S87G-A88V-S89A-A116N-N117S-N118G-A144T	0.89	0.85	0.44	0.32
FS1	G97A-G128A-Y217Q-T55P-N61P-S87T-A88L-S89G-G110C-S130P	T55P-N61P-S87T-A88L-S89G-G110C-S130P	0.83	0.85	0.92	0.30

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
RCL6	G97A-G128A-Y217Q-L75S-N76Y-A116S-N117G-N118R	L75S-N76Y-A116S-N117G-N118R	0.84	0.83	0.41	0.13
FS3	G97A-G128A-Y217Q-S145D-S159K-K213L-P239R-N240K	S145D-S159K-K213L-P239R-N240K	0.74	0.81	0.38	0.65
RCL6	G97A-G128A-Y217Q-S24R-S87T-A88L-S89G	S24R-S87T-A88L-S89G	0.86	0.80	0.53	0.08
RCL6	G97A-G128A-Y217Q-G23A-S24G-N25G-P129V	G23A-S24G-N25G-P129V	0.88	0.79	0.53	0.20
RCL7	G97A-G128A-Y217Q-A134T-K213L	A134T-K213L	0.87	0.79	0.51	0.81
RCL7	G97A-G128A-Y217Q-S89Y-A273S	S89Y-A273S	0.81	0.79	0.48	0.19
RCL7	G97A-G128A-Y217Q-S24R-P239R	S24R-P239R	0.90	0.78	0.71	0.69
FS2	G97A-Y217Q-N123G-A128G-P129S	N123G-A128G-P129S	0.76	0.78	0.71	0.12
RCL7	G97A-G128A-Y217Q-S89Y-P239R	S89Y-P239R	0.77	0.76	0.38	0.90
RCL6	G97A-G128A-Y217Q-S24G-N25G-A92G	S24G-N25G-A92G	0.71	0.76	0.25	0.59
RCL6	G97A-G128A-Y217Q-N61P-S63H-I115V-A228V	N61P-S63H-I115V-A228V	0.73	0.74	0.24	0.42
FNA	Y217L	A97G-A128G-Q217L	0.73	0.74	1.81	1.21
RCL6	G97A-G128A-Y217Q-L75S-N76Y-P129V	L75S-N76Y-P129V	0.73	0.73	0.34	0.09
RCL6	G97A-G128A-Y217Q-S24R-P129L	S24R-P129L	0.80	0.73	0.35	0.58

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific ΔΔPF PI	Det. Comp. 3, Stability PI
RCL6	G97A-G128A-Y217Q-S87G-A88V-S89A-P129Q-S182Y-S204Y-P239Q	S87G-A88V-S89A-P129Q-S182Y-S204Y-P239Q	0.69	0.73	0.90	0.36
RCL6	G97A-G128A-Y217Q-S24R-A92G	S24R-A92G	0.75	0.73	0.28	0.44
RCL6	G97A-G128A-Y217Q-S24R-A116S-N117G-N118R	S24R-A116S-N117G-N118R	0.81	0.72	0.48	0.58
RCL6	G97A-G128A-Y217Q-G23A-S24G-N25G-A116G-N117R	G23A-S24G-N25G-A116G-N117R	0.85	0.66	0.63	0.20
RCL6	G97A-G128A-Y217Q-S24G-N25G-P129L	S24G-N25G-P129L	0.72	0.66	0.39	1.03
RCL6	G97A-G128A-Y217Q-S87T-A88L-S89G-S101G	S87T-A88L-S89G-S101G	0.78	0.66	0.65	0.11
RCL6	G97A-G128A-Y217Q-G23A-S24G-N25G-P129L	G23A-S24G-N25G-P129L	0.52	0.59	0.37	0.20
FS1	G97A-G128A-Y217Q-S53G-N61P-G102A-V203Y	S53G-N61P-G102A-V203Y	0.55	0.58	0.29	0.16
RCL6	G97A-G128A-Y217Q-T55P-V147P	T55P-V147P	0.56	0.57	0.22	1.09
RCL6	G97A-G128A-Y217Q-Y6Q-L75S-N76Y	Y6Q-L75S-N76Y	0.55	0.52	0.24	0.17
RCL6	G97A-G128A-Y217Q-N61P-S63H-V147P	N61P-S63H-V147P	0.36	0.37	0.14	0.95
RCL6	G97A-G128A-Y217Q-S24R-V147P	S24R-V147P	0.16	0.22	0.08	0.85
RCL6	G97A-G128A-Y217Q-S24G-N25G-V68C-A69G	S24G-N25G-V68C-A69G	0.21	0.18	0.06	0.71

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3

Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S24G-N25G-N61P-L75S-N76Y-S101N-V203Y	S24G-N25G-N61P-L75S-N76Y-S101N-V203Y	0.19	0.18	0.08	0.38
FS1	G97A-G128A-Y217Q-L75S-N76Y-S78N-S101N-V203Y	L75S-N76Y-S78N-S101N-V203Y	0.13	0.12	0.05	0.72
FS1	G97A-G128A-Y217Q-L75S-N76Y-S78N-S87T-A88L-S89G-S101N-S130P	L75S-N76Y-S78N-S87T-A88L-S89G-S101N-S130P	0.04	0.04	0.07	0.73
FS1	G97A-G128A-Y217Q-S24G-N25G-S53G-S101N-S130P-V203Y	S24G-N25G-S53G-S101N-S130P-V203Y	0.06	0.04	0.06	0.33
RCL6	G97A-G128A-Y217Q-G47E-M50I-L75S-N76Y-S162K	G47E-M50I-L75S-N76Y-S162K	***0.01***	0.03	0.02	1.21
FS1	G97A-G128A-Y217Q-S53G-T55P-S87T-A88L-S89G-S101N-S130P-V203Y	S53G-T55P-S87T-A88L-S89G-S101N-S130P-V203Y	***0.01***	0.02	0.05	0.45
FS1	G97A-G128A-Y217Q-S24G-N25G-L75S-N76Y-A128T-P129T-S130G-G131Q-S132C-A133G-A134T	S24G-N25G-L75S-N76Y-A128T-P129T-S130G-G131Q-S132C-A133G-A134T	***0.01***	***0.01***	0.02	1.21
RCL6	G97A-G128A-Y217Q-P129Q-S130G-G131S-V147P	P129Q-S130G-G131S-V147P	***0.01***	***0.01***	0.03	1.11
FS1	G97A-G128A-Y217Q-S53G-T55P-N61P-L75S-N76Y-S87T-A88L-S89G-G102A-S130P-V203Y	S53G-T55P-N61P-L75S-N76Y-S87T-A88L-S89G-G102A-S130P-V203Y	***0.01***	***0.01***	0.02	1.05

Table 12-4: Performance Index Values of Variants Generated From RCL 5-7 and FS1-3						
Source of Variants	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1, pH 8, 16°C, BMI PI	Det. Comp. 2, pH 8, 16°C, BMI PI	Specific AAPF PI	Det. Comp. 3, Stability PI
FS1	G97A-G128A-Y217Q-S53G-T55P-N61P-L75S-N76Y-S101N-S130P-V203Y	S53G-T55P-N61P-L75S-N76Y-S101N-S130P-V203Y	0.03	***0.01***	0.02	1.03

The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from those listed in Table 12-4, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Each such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Table 12-5 provides the Performance Index (PI) values of BPN' variants (generated as described in "Generation of Variants to Improve BPN' Stability"; see Table 11-3) for stain removal in BMI microswatch assay in Detergent Composition 1 at 16°C and pH 8 (Det. Comp. 1, pH 8, 16°C, BMI PI) and for stability in LAS/EDTA (LAS/EDTA Stability PI). Assays were performed as described in Example 1 (BMI microswatch assay, LAS/EDTA stability assay). The sequences of the variants are shown relative to both BPN' and FNA. That is, each variant sequence is the BPN' or FNA sequence with the specified variant amino acid substitutions. PI values are shown relative to FNA parent, which is BPN'-Y217L.

Table 12-5: Performance Index of Stability-Improved BPN' Variants			
Sequence Relative to FNA: BPN' Y217L	**Sequence Relative to BPN'**	**Det. Comp. 1 pH 8, 16°C, BMI PI**	**LAS/EDTA Stability PI**
P40E-S78N-S87D	P40E-S78N-S87D-Y217L	0.71	11.65
P40E	P40E-Y217L	0.96	8.33
T22V-S78N-Q206E-K213N	T22V-S78N-Q206E-K213N-Y217L	0.75	5.95
T22V-S78N-K213N	T22V-S78N-K213N-Y217L	0.86	5.71
S87D	S87D-Y217L	0.90	4.04
S78N	S78N-Y217L	0.87	3.86
K213N	K213N-Y217L	0.91	1.84
Q206E	Q206E-Y217L	0.86	1.76
T22V	T22V-Y217L	0.97	1.46
FNA	**Y217L**	**1.00**	**1.00**

The invention includes a protease variant having proteolytic activity and/or improved stability relative to FNA, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from those listed in Table 12-5, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Such protease variant may have a proteolytic activity greater than that of BPN' or BPN'-v3. Each such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Table 12-6 provides the Performance Index (PI) values of BPN' variants generated from Library Parent: BPN'-v3: G97A-G128A-Y217Q (as described in "Generation of Variants to Improve BPN' Stability"; see Table 11-3) for stain removal in a BMI microswatch assay in Detergent Composition 1 at 16°C and pH 8 and for stability in LAS/EDTA. Assays were performed as described in Example 1 (BMI microswatch assay and LAS/EDTA stability assay). The Performance Index was calculated relative to BPN'-v3: G97A-G128A-Y217Q. All mutants in this list have a PI cutoff equal or greater than 0.5 for at least one property tested.

Table 12-6: Performance Index of Stability-Improved BPN' Variants			
Sequence Relative to BPN'-v3: BPN' G97A-G128A-Y217Q	Sequence Relative to BPN'	Det. Comp. 1 pH 8, 16°C, BMI PI	LAS/EDTA Stability PI
S87D-N76D-S78N	S87D-N76D-S78N-G97A-G128A-Y217Q	0.62	2.27
P40E-S78N-S87D	P40E-S78N-S87D-G97A-G128A-Y217Q	0.21	2.18
P40E-S87D	P40E-S87D-G97A-G128A-Y217Q	0.18	2.14
S78N-P40E	S78N-P40E-G97A-G128A-Y217Q	0.80	2.03
S87D-N76D	S87D-N76D-G97A-G128A-Y217Q	0.55	1.89
P40E	P40E-G97A-G128A-Y217Q	0.84	1.79
S78N-S87D	S78N-S87D-G97A-G128A-Y217Q	0.79	1.71
S87D	S87D-G97A-G128A-Y217Q	0.78	1.20
S78N	S78N-G97A-G128A-Y217Q	0.93	1.14
BPN'-v3	G97A-G128A-Y217Q	1.00	1.00

The invention includes a protease variant having proteolytic activity and having improved stability relative to BPN'-v3, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from those listed in Table 12-6, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Each such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Table 12-7 provides the Performance Index (PI) values of BPN' variants (generated as described in "Generation of BPN' Variants from Five Different Plasmids"; see Table 11-4) for stain removal in a BMI microswatch assay in Detergent Composition 1at 16°C and pH 8. Assays were performed as described in Example 1 (BMI microswatch assay). The Performance Index of each variant was calculated relative to BPN'-v3: G97A-G128A-Y217Q. All mutants in this list have a PI cutoff equal or greater than 0.5 for at least one property tested.

Table 12-7: Performance Index of BPN' Variants		
Sequence Relative to BPN'-v3 G97A-G128A-Y217Q	Sequence Relative to BPN'	Det. Comp. 1, pH 8, 16°C, BMI PI
S101N	G97A-S101N-G128A-Y217Q	1.12
A137V	G97A-G128A-A137V-Y217Q	1.12
N61P	N61P-G97A-G128A-Y217Q	1.11
S130P	G97A-G128A-S130P-Y217Q	1.09
Q103N	G97A-Q103N-G128A-Y217Q	1.07
S63T	S63T-G97A-G128A-Y217Q	1.03
G102A	G97A-G102A-G128A-Y217Q	1.02
BPN'-v3	**BPN'-v3 (G97A-G128A-Y217Q)**	**1.00**
N109D-S248R	G97A-N109D-G128A-Y217Q-S248R	0.96
S87R	S87R-G97A-G128A-Y217Q	0.95
S188D	G97A-G128A-S188D-Y217Q	0.95
S87D-S248R	S87D-G97A-G128A-Y217Q-S248R	0.94
S188D-S248R	G97A-G128A-S188D-S248R-Y217Q	0.93
S248D	G97A-G128A-S248D-Y217Q	0.86
N109D-S188D-S248R	G97A-N109D-G128A-S188D-S248R-Y217Q	0.83
N109D	G97A-N109D-G128A-Y217Q	0.81
S87R-S248R	S87R-G97A-G128A-Y217Q-S248R	0.79
N109D-S188R	G97A-N109D-G128A-S188R-Y217Q	0.77
N76D	N76D-G97A-G128A-Y217Q	0.75
S87D-N109D-S188D-S248R	S87D-G97A-N109D-G128A-S188D-S248R-Y217Q	0.58
S87R-N109D-S188D-S248R	S87R-G97A-N109D-G128A-S188D-Y217Q-S248R	0.55
S87R-S188R-S248R	S87R-G97A-G128A-S188R-Y217Q-S248R	0.52
A187D	G97A-G128A-A187D-Y217Q	0.48
N109D-S248D	G97A-N109D-G128A-Y217Q-S248D	0.47
S87R-N109R-S188R-S248R	S87R-G97A-N109R-G128A-S188R-Y217Q-S248R	0.39
F189D	G97A-G128A-F189D-Y217Q	0.31
G100N	G97A-G100N-G128A-Y217Q	0.28
S87R-N109D-S188D	S87R-G97A-N109D-G128A-S188D-Y217Q	0.24
S87D-N109D-S188D	S87D-G97A-N109D-G128A-S188D-Y217Q	0.12
S87R-S188D-S248D	S87R-G97A-G128A-S188D-S248D-Y217Q	0.09
N62D	N62D-G97A-G128A-Y217Q	0.09
S87D-N109D-S188D-S248D	S87D-G97A-N109D-G128A-S188D-Y217Q-S248D	0.08

The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from those listed in Table 12-7, wherein positions of the variant are numbered by

correspondence with positions of the SEQ ID NO:2 sequence. Each such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Table 12-8 provides the Performance index (PI) values of BPN' variants (generated as described in "Generation of Combinatorial Libraries and Variants of BPN'-v3 + S78N" as described in Example 3) for stain removal using a BMI microswatch assay in Detergent Composition 1 at 16°C and pH 8. Assays were performed as described in Example 1 (BMI microswatch assay). The Performance Index of each variant was calculated relative to BPN'-S78N-G97A-G128A-Y217Q. PI values less than 0.01 were modified and are indicated as "0.01" in bold italics.

Table 12-8: Performance Index Values of BPN' Variants

Variant	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1 pH 8, 16°C, BMI PI
v3/S78N/I.267V	S78N-G97A-G128A-Y217Q-I.267V	S78N-I.267V	1.12
v3/S78N/S161P	S78N-G97A-G128A-Y217Q-S161P	S78N-S161P	1.05
v3/S78N/I115V	S78N-G97A-G128A-Y217Q-I115V	S78N-I115V	1.04
v3/S78N/A273S	S78N-G97A-G128A-Y217Q-A273S	S78N-A273S	1.03
v3/S78N/G211T	S78N-G97A-G128A-Y217Q-G211T	S78N-G211T	1.00
V3 + S78N	**S78N-G97A-G128A-Y217Q**	**S78N**	**1.00**
v3/S78N/I111V	S78N-G97A-G128A-Y217Q-I111V	S78N-I111V	0.98
v3/S78N/V147L	S78N-G97A-G128A-Y217Q-V147L	S78N-V147L	0.97
v3/S78N/I108V	S78N-G97A-G128A-Y217Q-I108V	S78N-I108V	0.97
v3/S78N/S89Y	S78N-G97A-G128A-Y217Q-S89Y	S78N-S89Y	0.94
v3/S78N/A138T	S78N-G97A-G128A-Y217Q-A138T	S78N-A138T	0.92
v3/S78N/P172V	S78N-G97A-G128A-Y217Q-P172V	S78N-P172V	0.74
v3/S78N/Q59G	S78N-G97A-G128A-Y217Q-Q59G	S78N-Q59G	0.64
GcM96	G97A-G128A-Y217Q-P129T-V147Q-S159D-S161P-S183T-Q185T-G211A-S224A	P129T-V147Q-S159D-S161P-S183T-Q185T-G211A-S224A	0.57

Table 12-8: Performance Index Values of BPN' Variants			
Variant	Sequence Relative to BPN'	Sequence Relative to BPN'-v3: G97A-G128A-Y217Q	Det. Comp. 1 pH 8, 16°C, BMI PI
GcM91	G97A-G128A-Y217Q-Q059V-I108V-V147Q-G211A-N252Q	Q059V-I108V-V147Q-G211A-N252Q	0.55
v3/S78N/Y167A	S78N-G97A-G128A-Y217Q-Y167A	S78N-Y167A	0.53
v3/S78N/A92G	S78N-G97A-G128A-Y217Q-A92G	S78N-A92G	0.49
v3/S78N/P129L	S78N-G97A-G128A-Y217Q-P129L	S78N-P129L	0.48
GcM92	G97A-G128A-Y217Q-N061A-S087E-M124I-S161P-S224A	N061A-S087E-M124I-S161P-S224A	0.36
v3/S78N/N62Q	S78N-G97A-G128A-Y217Q-N62Q	S78N-N62Q	0.27
v3/S78N/V68A	S78N-G97A-G128A-Y217Q-V68A	S78N-V68A	0.24
GcM94	G97A-G128A-Y217Q-S063T-S101A-L126V-S183T-T244N	S063T-S101A-L126V-S183T-T244N	0.12
v3/S78N/M124T	S78N-G97A-G128A-Y217Q-M124T	S78N-M124T	0.05
GcM95	G97A-G128A-Y217Q-P040L-S053G-Q059V-N061A-N062Q-S063T-S087E-G100N	P040L-S053G-Q059V-N061A-N062Q-S063T-S087E-G100N	***0.01***
GcM93	G97A-G128A-Y217Q-N062Q-G100N-S125A-S159D-N240S	N062Q-G100N-S125A-S159D-N240S	***0.01***
GcM100	G97A-G128A-Y217Q-V68A-G102A-G211A-S125A	V68A-G102A-G211A-S125A	***0.01***
GcM90	G97A-G128A-Y217Q-S053G-V068A-G102A-P129T-Q185T	S053G-V068A-G102A-P129T-Q185T	***0.01***

The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one set of amino acid substitutions selected from those listed in Table 12-8, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Each such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Table 12-9 provides Performance index (PI) values of BPN' single variants (constructed using PCR fusion as described in PCT App. No. PCT/US09/46156, filed June 3, 2009, which is incorporated by reference herein for such description) for stain removal in a BMI microswatch assay in Detergent Composition 2 at 16°C and pH 8 and for stability measured in Detergent Composition 3. PI values for specific activity by AAPF hydrolysis (PI specific AAPF) were determined. All assays were performed as described in Example 1. Performance index values were calculated relative to BPN wild type. PI values less than 0.01 are indicated as "0.01" in bold italics. "Det. Comp." means Detergent Composition.

Table 12-9: Performance Index Values for BPN' Single Variants

BPN' Variant	Det. Comp. 2 pH 8, 16°C, BMI PI	PI Specific AAPF	Det. Comp. 3 Stability PI
S182E	1.34	1.05	0.50
N109I	1.28	1.22	0.20
N117H	1.15	0.25	0.20
K237D	1.15	0.60	0.70
L257Q	1.14	0.94	0.80
P225N	1.13	1.02	0.70
S105H	1.11	1.07	***0.01***
S236I	1.10	0.58	0.90
L235H	1.10	0.65	0.70
S249E	1.07	0.72	0.30
N76E	1.07	0.76	0.20
S145N	1.06	1.16	1.10
N243D	1.05	1.03	0.90
R247N	1.04	1.04	0.50
E195N	1.04	1.05	0.40
A98K	1.03	0.75	0.70
S182N	0.99	1.14	0.90
S161H	0.97	1.07	0.90
G83H	0.95	0.72	0.60
G131D	0.95	1.11	1.30
T71C	0.93	1.00	1.30
K136Q	0.93	1.02	0.80
P40D	0.93	1.20	1.20
A187H	0.91	0.95	0.60
L250K	0.90	1.02	0.40
S9I	0.87	0.20	***0.01***
N76M	0.85	0.60	0.60

Table 12-9: Performance Index Values for BPN' Single Variants			
BPN' Variant	**Det. Comp. 2 pH 8, 16°C, BMI PI**	**PI Specific AAPF**	**Det. Comp. 3 Stability PI**
S132D	0.85	0.88	0.70
Q19F	0.83	0.47	0.30
E112H	0.83	1.02	***0.01***
S249P	0.80	1.02	0.50
S53D	0.78	0.29	0.10
V68E	0.78	0.59	1.70
D41I	0.72	1.15	0.90
K43H	0.70	0.28	0.10
V4H	0.66	0.37	0.60
A13Y	0.64	0.48	***0.01***
N62P	0.61	0.60	1.30
L196E	0.56	0.70	0.70
V44D	0.51	0.18	***0.01***

The invention includes a protease variant having proteolytic activity, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 and at least one substitution selected from those listed in Table 12-9, wherein positions of the variant are numbered by correspondence with positions of the SEQ ID NO:2 sequence. Each such protease variant may be an isolated, recombinant, substantially pure, or non-naturally occurring protease variant. Also included are compositions, including cleaning compositions, comprising at least one such protease variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

EXAMPLE 13

Liquid Laundry Detergent Compositions

In this Example, various formulations for liquid laundry detergent compositions are provided. The following liquid laundry detergent compositions of the present invention are prepared as shown below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 13-1: Liquid Laundry Detergent Composition					
Compound	**Formulations**				
	I	**II**	**III**	**IV**	**V**
LAS	24.0	32.0	6.0	3.0	6.0
NaC_{16}-C_{17} HSAS	-	-	-	5.0	-
C_{12}-C_{15} $AE_{1.8}S$	-	-	8.0	7.0	5.0
C_8-C_{10} propyl dimethyl amine	2.0	2.0	2.0	2.0	1.0
C_{12}-C_{14} alkyl dimethyl amine oxide	-	-	-	-	2.0
C_{12}-C_{15} AS	-	-	17.0	-	8.0
CFAA	-	5.0	4.0	4.0	3.0
C_{12}-C_{14} Fatty alcohol ethoxylate	12.0	6.0	1.0	1.0	1.0
C_{12}-C_{18} Fatty acid	3.0	-	4.0	2.0	3.0
Citric acid (anhydrous)	4.5	5.0	3.0	2.0	1.0
DETPMP	-	-	1.0	1.0	0.5
Monoethanolamine	5.0	5.0	5.0	5.0	2.0
Sodium hydroxide	-	-	2.5	1.0	1.5
1 N HCl aqueous solution	#1	#1	-	-	-
Propanediol	12.7	14.5	13.1	10.	8.0
Ethanol	1.8	2.4	4.7	5.4	1.0
DTPA	0.5	0.4	0.3	0.4	0.5
Pectin Lyase	-	-	-	0.005	-
Amylase	0.001	0.002	-		-
Cellulase	-	-	0.0002		0.0001
Lipase	0.1	-	0.1	-	0.1
NprE (optional)	0.05	0.3	-	0.5	0.2
PMN	-	-	0.08	-	-
Protease A (optional)	-	-	-	-	0.1
Aldose Oxidase	-	-	0.3	-	0.003
ZnCl2	0.1	0.05	0.05	0.05	0.02
Ca formate	0.05	0.07	0.05	0.06	0.07
DETBCHD	-	-	0.02	0.01	-
SRP1	0.5	0.5	-	0.3	0.3
Boric acid	-	-	-	-	2.4
Sodium xylene sulfonate	-	-	3.0	-	-
Sodium cumene sulfonate	-	-	-	0.3	0.5
DC 3225C	1.0	1.0	1.0	1.0	1.0
2-butyl-octanol	0.03	0.04	0.04	0.03	0.03
Brightener 1	0.12	0.10	0.18	0.08	0.10
Balance to 100% perfume / dye and/or water					

#1: Add 1N HCl aqueous solution to adjust the neat pH of the formula in the range from about 3 to about 5.

The pH of Formulations (I)-(II) in Table 13-1 is about 5 to about 7 and of Formulations (III)-(V) in Table 13-1 is about 7.5 to about 8.5.

EXAMPLE 14

Hand Dish Liquid Detergent Compositions

In this Example, various hand dish liquid detergent formulations are provided. The following hand dish liquid detergent compositions of the present invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 14-1: Hand Dish Liquid Detergent Compositions

Compound	Formulations					
	I	II	III	IV	V	VI
C_{12}-C_{15} $AE_{1.8}S$	30.0	28.0	25.0	-	15.0	10.0
LAS	-	-	-	5.0	15.0	12.0
Paraffin Sulfonate	-	-	-	20.0	-	-
C_{10}-C_{18} Alkyl Dimethyl Amine Oxide	5.0	3.0	7.0	-	-	-
Betaine	3.0	-	1.0	3.0	1.0	-
C_{12} poly-OH fatty acid amide	-	-	-	3.0	-	1.0
C_{14} poly-OH fatty acid amide	-	1.5	-	-	-	-
$C_{11}E_9$	2.0	-	4.0	-	-	20.0
DTPA	-	-	-	-	0.2	-
Tri-sodium Citrate dehydrate	0.25	-	-	0.7	-	-
Diamine	1.0	5.0	7.0	1.0	5.0	7.0
$MgCl_2$	0.25	-	-	1.0	-	-
nprE (optional)	0.02	0.01	-	0.01	-	0.05
PMN	-	-	0.03	-	0.02	-
Protease A (optional)	-	0.01	-	-	-	-
Amylase	0.001	-	-	0.002	-	0.001
Aldose Oxidase	0.03	-	0.02	-	0.05	-
Sodium Cumene Sulphonate	-	-	-	2.0	1.5	3.0
PAAC	0.01	0.01	0.02	-	-	-
DETBCHD	-	-	-	0.01	0.02	0.01
Balance to 100% perfume / dye and/or water						

The pH of Formulations (I)-(VI) in Table 14-1 is about 8 to about 11.

EXAMPLE 15

Liquid Automatic Dishwashing Detergent Compositions

In this Example, various liquid automatic dishwashing detergent formulations are provided. The following hand dish liquid detergent compositions of the present invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 15-1: Liquid Automatic Dishwashing Detergent Compositions

Compound	Formulations				
	I	II	III	IV	V
STPP	16	16	18	16	16
Potassium Sulfate	-	10	8	-	10
1,2 propanediol	6.0	0.5	2.0	6.0	0.5
Boric Acid	-	-	-	4.0	3.0
$CaCl_2$ dihydrate	0.04	0.04	0.04	0.04	0.04
Nonionic	0.5	0.5	0.5	0.5	0.5
nprE (optional)	0.1	0.03	-	0.03	-
PMN	-	-	0.05	-	0.06
Protease B (optional)	-	-	-	0.01	-
Amylase	0.02	-	0.02	0.02	-
Aldose Oxidase	-	0.15	0.02	-	0.01
Galactose Oxidase	-	-	0.01	-	0.01
PAAC	0.01	-	-	0.01	-
DETBCHD	-	0.01	-	-	0.01
Balance to 100% perfume / dye and/or water					

EXAMPLE 16

Granular and/or Tablet Laundry Compositions

This Example provides various formulations for granular and/or tablet laundry detergents. The following laundry compositions of present invention, which may be in the form of granules or tablet, are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 16-1: Granular and/or Tablet Laundry Compositions					
Compound	**Formulations**				
	I	**II**	**III**	**IV**	**V**
Base Product					
C_{14}-C_{15}AS or TAS	8.0	5.0	3.0	3.0	3.0
LAS	8.0	-	8.0	-	7.0
C_{12}-$C_{15}AE_3S$	0.5	2.0	1.0	-	-
C_{12}-$C_{15}E_5$ or E_3	2.0	-	5.0	2.0	2.0
QAS	-	-	-	1.0	1.0
Zeolite A	20.0	18.0	11.0	-	10.0
SKS-6 (dry add)	-	-	9.0	-	-
MA/AA	2.0	2.0	2.0	-	-
AA	-	-	-	-	4.0
3Na Citrate $\cdot 2H_2O$	-	2.0	-	-	-
Citric Acid (Anhydrous)	2.0	-	1.5	2.0	-
DTPA	0.2	0.2	-	-	-
EDDS	-	-	0.5	0.1	-
HEDP	-	-	0.2	0.1	-
PB1	3.0	4.8	-	-	4.0
Percarbonate	-	-	3.8	5.2	-
NOBS	1.9	-	-	-	-
NACA OBS	-	-	2.0	-	-
TAED	0.5	2.0	2.0	5.0	1.00
BB1	0.06	-	0.34	-	0.14
BB2	-	0.14	-	0.20	-
Anhydrous Na Carbonate	15.0	18.0	-	15.0	15.0
Sulfate	5.0	12.0	5.0	17.0	3.0
Silicate	-	1.0	-	-	8.0
nprE (optional)	0.03	-	0.1	0.06	-
PMN	-	0.05	-	-	0.1
Protease B (optional)	-	0.01	-	-	-
Protease C (optional)	-	-	-	0.01	-
Lipase	-	0.008	-	-	-
Amylase	0.001	-	-	-	0.001
Cellulase	-	0.0014	-	-	-
Pectin Lyase	0.001	0.001	0.001	0.001	0.001
Aldose Oxidase	0.03	-	0.05	-	-
PAAC	-	0.01	-	-	0.05
Balance to 100% Moisture and/or Minors*					

* Perfume, dye, brightener / SRP1 / Na carboxymethylcellulose / photobleach / $MgSO_4$ / PVPVI/ suds suppressor /high molecular PEG/clay.

5

EXAMPLE 17

Liquid Laundry Detergents

This Example provides various formulations for liquid laundry detergents. The following liquid laundry detergent formulations of the present invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 17-1: Liquid Laundry Detergents

Compound	Formulations					
	I	II	III	IV	V	VI
LAS	11.5	11.5	9.0	-	4.0	-
C_{12}-$C_{15}AE_{2.85}S$	-	-	3.0	18.0	-	16.0
C_{14}-$C_{15}E_{2.5}S$	11.5	11.5	3.0	-	16.0	-
C_{12}-$C_{13}E_9$	-	-	3.0	2.0	2.0	1.0
C_{12}-$C_{13}E_7$	3.2	3.2	-	-	-	-
CFAA	-	-	-	5.0	-	3.0
TPKFA	2.0	2.0	-	2.0	0.5	2.0
Citric Acid (Anhydrous)	3.2	3.2	0.5	1.2	2.0	1.2
Ca formate	0.1	0.1	0.06	0.1	-	-
Na formate	0.5	0.5	0.06	0.1	0.05	0.05
ZnCl2	0.1	0.05	0.06	0.03	0.05	0.05
Na Culmene Sulfonate	4.0	4.0	1.0	3.0	1.2	-
Borate	0.6	0.6	1.5	-	-	-
Na Hydroxide	6.0	6.0	2.0	3.5	4.0	3.0
Ethanol	2.0	2.0	1.0	4.0	4.0	3.0
1,2 Propanediol	3.0	3.0	2.0	8.0	8.0	5.0
Monoethanolamine	3.0	3.0	1.5	1.0	2.5	1.0
TEPAE	2.0	2.0	-	1.0	1.0	1.0
nprE (optional)	0.03	0.05	-	0.03	-	0.02
PMN	-	-	0.01	-	0.08	-
Protease A (optional)	-	-	0.01	-	-	-
Lipase	-	-	-	0.002	-	-
Amylase	-	-	-	-	0.002	-
Cellulase	-	-	-	-	-	0.0001
Pectin Lyase	0.005	0.005	-		-	-
Aldose Oxidase	0.05	-	-	0.05	-	0.02
Galactose oxidase	-	0.04				
PAAC	0.03	0.03	0.02	-	-	-
DETBCHD	-	-	-	0.02	0.01	-

Table 17-1: Liquid Laundry Detergents						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
SRP 1	0.2	0.2	-	0.1	-	-
DTPA	-	-	-	0.3	-	-
PVNO	-	-	-	0.3	-	0.2
Brightener 1	0.2	0.2	0.07	0.1	-	-
Silicone antifoam	0.04	0.04	0.02	0.1	0.1	0.1
Balance to 100% perfume/dye and/or water						

EXAMPLE 18

High Density Dishwashing Detergents

This Example provides various formulations for high density dishwashing detergents. The following compact high density dishwashing detergents of the present invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 18-1: High Density Dishwashing Detergents						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
STPP	-	45.0	45.0	-	-	40.0
3Na Citrate·2H_2O	17.0	-	-	50.0	40.2	-
Na Carbonate	17.5	14.0	20.0	-	8.0	33.6
Bicarbonate	-	-	-	26.0	-	-
Silicate	15.0	15.0	8.0	-	25.0	3.6
Metasilicate	2.5	4.5	4.5	-	-	-
PB1	-	-	4.5	-	-	-
PB4	-	-	-	5.0	-	-
Percarbonate	-	-	-	-	-	4.8
BB1	-	0.1	0.1	-	0.5	-
BB2	0.2	0.05	-	0.1	-	0.6
Nonionic	2.0	1.5	1.5	3.0	1.9	5.9
HEDP	1.0	-	-	-	-	-
DETPMP	0.6	-	-	-	-	-
PAAC	0.03	0.05	0.02	-	-	-
Paraffin	0.5	0.4	0.4	0.6	-	-
nprE (optional)	0.072	0.053	-	0.026	-	0.01
PMN	-	-	0.053	-	0.059	-
Protease B (optional)	-	-	-	-	-	0.01
Amylase	0.012	-	0.012	-	0.021	0.006

Table 18-1: High Density Dishwashing Detergents						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
Lipase	-	0.001	-	0.005	-	-
Pectin Lyase	0.001	0.001	0.001	-	-	-
Aldose Oxidase	0.05	0.05	0.03	0.01	0.02	0.01
BTA	0.3	0.2	0.2	0.3	0.3	0.3
Polycarboxylate	6.0	-	-	-	4.0	0.9
Perfume	0.2	0.1	0.1	0.2	0.2	0.2
Balance to 100% Moisture and/or Minors*						

*Brightener / dye / SRP1 / Na carboxymethylcellulose/ photobleach / $MgSO_4$ / PVPVI/ suds suppressor /high molecular PEG/clay.

The pH of Formulations (I) through (VI) in Table 18-1 is from about 9.6 to about 11.3.

EXAMPLE 19

Tablet Detergent Compositions

This Example provides various tablet detergent formulations. The following tablet detergent compositions of the present invention are prepared by compression of a granular dishwashing detergent composition at a pressure of 13KN/cm^2 using a standard 12 head rotary press. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 19-1: Tablet Detergent Compositions								
Compound	**Formulations**							
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
STPP	-	48.8	44.7	38.2	-	42.4	46.1	46.0
3Na Citrate·2H_2O	20.0	-	-	-	35.9	-	-	-
Na Carbonate	20.0	5.0	14.0	15.4	8.0	23.0	20.0	-
Silicate	15.0	14.8	15.0	12.6	23.4	2.9	4.3	4.2
Lipase	0.001	-	0.01	-	0.02	-	-	-
Protease B (optional)	0.01	-	-	-	-	-	-	-
Protease C (optional)	-	-	-	-	-	0.01	-	-
nprE (optional)	0.01	0.08	-	0.04	-	0.023	-	0.05
PMN	-	-	0.05	-	0.052	-	0.023	-
Amylase	0.012	0.012	0.012	-	0.015	-	0.017	0.002
Pectin Lyase	0.005	-	-	0.002	-	-	-	-
Aldose Oxidase	-	0.03	-	0.02	0.02	-	0.03	-
PB1	-	-	3.8	-	7.8	-	-	4.5

Table 19-1: Tablet Detergent Compositions								
Compound	**Formulations**							
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
Percarbonate	6.0	-	-	6.0	-	5.0	-	-
BB1	0.2	-	0.5	-	0.3	0.2	-	-
BB2	-	0.2	-	0.5	-	-	0.1	0.2
Nonionic	1.5	2.0	2.0	2.2	1.0	4.2	4.0	6.5
PAAC	0.01	0.01	0.02	-	-	-	-	-
DETBCHD	-	-	-	0.02	0.02	-	-	-
TAED	-	-	-	-	-	2.1	-	1.6
HEDP	1.0	-	-	0.9	-	0.4	0.2	-
DETPMP	0.7	-	-	-	-	-	-	-
Paraffin	0.4	0.5	0.5	0.5	-	-	0.5	-
BTA	0.2	0.3	0.3	0.3	0.3	0.3	0.3	-
Polycarboxylate	4.0	-	-	-	4.9	0.6	0.8	-
PEG 400-30,000	-	-	-	-	-	2.0	-	2.0
Glycerol	-	-	-	-	-	0.4	-	0.5
Perfume	-	-	-	0.05	0.2	0.2	0.2	0.2
Balance to 100% Moisture and/or Minors*								

*Brightener / SRP1 / Na carboxymethylcellulose/ photobleach / $MgSO_4$ / PVPVI/ suds suppressor /high molecular PEG/clay.

The pH of Formulations (I) through (VII) in Table 19-1 is from about 10 to about 11.5 and the pH of Formulation (VIII) in Table 19-1 is from 8-10. The tablet weight of Formulations (I) through (VIII) in Table 19-1 is from about 20 grams to about 30 grams.

EXAMPLE 20

Liquid Hard Surface Cleaning Detergents

This Example provides various formulations for liquid hard surface cleaning detergents. The following liquid hard surface cleaning detergent compositions of the present invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 20-1: Liquid Hard Surface Cleaning Detergents							
Compound	**Formulations**						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
C_9-$C_{11}E_5$	2.4	1.9	2.5	2.5	2.5	2.4	2.5
C_{12}-$C_{14}E_5$	3.6	2.9	2.5	2.5	2.5	3.6	2.5
C_7-C_9E_6	-	-	-	-	8.0	-	-
C_{12}-$C_{14}E_{21}$	1.0	0.8	4.0	2.0	2.0	1.0	2.0
LAS	-	-	-	0.8	0.8	-	0.8
Sodium culmene sulfonate	1.5	2.6	-	1.5	1.5	1.5	1.5

Table 20-1: Liquid Hard Surface Cleaning Detergents							
Compound	**Formulations**						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
Isachem® AS	0.6	0.6	-	-	-	0.6	-
Na_2CO_3	0.6	0.13	0.6	0.1	0.2	0.6	0.2
3Na Citrate·$2H_2O$	0.5	0.56	0.5	0.6	0.75	0.5	0.75
NaOH	0.3	0.33	0.3	0.3	0.5	0.3	0.5
Fatty Acid	0.6	0.13	0.6	0.1	0.4	0.6	0.4
2-butyl octanol	0.3	0.3	-	0.3	0.3	0.3	0.3
PEG DME-2000®	0.4	-	0.3	0.35	0.5	-	-
PVP	0.3	0.4	0.6	0.3	0.5	-	-
MME PEG (2000)®	-	-	-	-	-	0.5	0.5
Jeffamine® ED-2001	-	0.4	-	-	0.5	-	-
PAAC	-	-	-	0.03	0.03	0.03	-
DETBCHD	0.03	0.05	0.05	-	-	-	-
nprE (optional)	0.07	-	0.08	0.03	-	0.01	0.04
PMN	-	0.05	-	-	0.06	-	-
Protease B (optional)	-	-	-	-	-	0.01	-
Amylase	0.12	0.01	0.01	-	0.02	-	0.01
Lipase	-	0.001	-	0.005	-	0.005	-
Pectin Lyase	0.001	-	0.001	-	-	-	0.002
ZnCl2	0.02	0.01	0.03	0.05	0.1	0.05	0.02
Calcium Formate	0.03	0.03	0.01	-	-	-	-
PB1	-	4.6	-	3.8	-	-	-
Aldose Oxidase	0.05	-	0.03	-	0.02	0.02	0.05
Balance to 100% perfume / dye and/or water							

The pH of Formulations (I) through (VII) in Table 20-1 is from about 7.4 to about 9.5.

EXAMPLE 21

Cleaning Performance of BPN'-v36 Polypeptide Variants

BPN'-v36 polypeptide variants comprising two amino acid substitutions were constructed by standard PCR fusion using the BPN'-v36 variant as a backbone or parent sequence. For this purpose, two or three partially overlapping fragments were amplified by mutagenic primers prepared such that the primer encoded a desired substitution. PCR amplification reactions were carried out as described in Example 7 of Part I *supra*. The following BPN'-v36 double mutant variants (i.e., BPN'-v36 with the following two amino acid substitution) were constructed: Q019R-N025D, A001Y-Q275R, V004A-S249N, V004E-S260P, V004A-T55A, Y006F-S249C, Y006D-T55A, V008L-Q275R, Q010R-Q275K, L016Q-Q217H, H017R-T158A, S183D-Q206R, P210S-N212D, S018Y-V203A, S018K-V203I, Y021H-D259G, Y021H-D259R, K027R-N269D, K027R-N269T, S037P-S260F, S037T-S260P, D041E-N077D, D041G-N077E, G166V-S183T, N252S-L257H, V044A-Q206H, V044A-Q206K, V044A-Q206R, N076T-N212D, N076P-N212S, N077D-N252D, N077D-N252T, K141I-S248N, T158I-D259N, T158A-

D259P, S161E-Q185H, K237M-H238R, G160A-D259G, G160R-D259V, G215R-D259R, G215D-D259V, N061D-Q206R, N061L-Q206H, S009L-N218S, S161E-S260T, Q019A-N109S, T022S-G166V, Y021H-N252H, P129S-K136R, T022S-T242S, N025K-H238R, N025D-Q185R, S037G-Q275H, K043R-N076S, K043N-Q217R, K043N-S163T, T055A-V147A, N061K-N252K, N062Y-G097D, Y021H-V084E, Y021H-S037E, N062Y-T244A, K027E-Y091F, A074S-P129Q, S249R-Q275R, I079V-Q217H, A098T-T158A, K027R-D120H, Q019R-Q185R, G131S-K265N, A133V-D259N, A144H-T244A, I035V-K043N, G160R-T244A, S161P-T253A, S163T-Q245L, K170R-D259G, S183T-S249R, N184Y-Y262N, V198L-D259G, A200T-H226L, Q206R-S260P, G211V-T244A, Q217R-T244A, L75I-N76D, S260P-Q275L, S260P-Q275R, Y262N-Q275R, V004A-Y006F, H017L-Q019A, N025D-V026A, N118G-V121A, V072F-L075I, S183T-R186K, V203A-Q217R, and S249R-Y262H. The cleaning performance of each of these variants was tested in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C and egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C as described in Example 1 of Part I. Results are provided below.

The following BPN' protease variants were determined to have a PI value equal to or greater than 0.9 and equal or less than 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of S183T-S249R, N61D-Q206R, Y262N-Q275R, K43R-N76S, K170R-D259G, Y6F-S249C, Q19A-N109S, H17L-Q19A, Q19R-Q185R, S18Y-V203A, S161E-S260T, S18K-V203I, V4A-T55A, N252S-L257H, S249R-Y262H, N61L-Q206H, N184Y-Y262N, Q19R-N25D, A74S-P129Q, K27R-D120H, Y21H-N252H, K27R-N269D, A98T-T158A, I79V-Q217H, S9L-N218S, V4A-Y6F, S161P-T253A, V203A-Q217R, T22S-T242S, N76P-N212S, S37T-S260P, T55A-V147A, G160R-T244A, N25D-Q185R, G211V-T244A, A144H-T244A, Y21H-N252H, A1Y-Q275R, V198L-D259G, K141I-S248N, S183T-R186K, S161E-Q185H, P129S-K136R, K43N-S163T, S37G-Q275H, N62Y-T244A, and S260P-Q275R, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to BPN' (SEQ ID NO:2) and a greater PI value than that of BPN' in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), a PI value of equal to or greater than 0.9 and equal to or less than 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity and a PI value of greater than 1.0 to about 5 compared to BPN' in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:6, wherein the variant comprises at least one substitution comprising at least one substitution selected from the group of X1Y, X4A, X6F, X9L, X17L, X18K/Y, X19A/R, X21H, X22S, X25D, X27R, X37G/T, X43N/R, X55A, X61D/L, X62Y, X74S, X76P/S, X79V, X98T, X109S, X120H, X129Q/S, X136R, X141I, X144H, X147A, X158A, X160R, X161E/P, X163T, X170R, X183T, X184Y, X185H/R, X186K, X198L, X203A/I, X206H/R, X211V, X212S, X217H/R, X218S, X242S, X244A, X248N, X249C/R, X252H/S, X253A, X257H, X259G, X260P/T, X262H/N, X269D, and X275H/R, and optionally at least one substitution selected from the group of A1Y, V4A, Y6F, S9L, H17L, S18K/Y, Q19A/R, Y21H, T22S, N25D, K27R, S37G/T, K43N/R, T55A, N61D/L, N62Y, A74S, N76P/S, I79V, A98T, N109S, D120H, P129Q/S, K136R, K141I, A144H, V147A, T158A, G160R, S161E/P, S163T, K170R, S183T, N184Y, Q185H/R, R186K, V198L, V203A/I, Q206H/R, G211V, N212S, Q217H/R, N218S, T242S, T244A, S248N, S249C/R, N252H/S, T253A, L257H, D259G, S260P/T, Y262H/N, N269D, and Q275H/R, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN' (SEQ ID NO:2) and a PI value greater than that of BPN' in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN' subtilisin protease variants were determined to have a PI value equal or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of Y21H-D259G, A133V-D259N, I79V-Q217H, S18K-V203I, T158A-D259P, N61K-N252K, K43N-Q217R, T158A-D259P, Q206R-S260P, A133V-D259N, V198L-D259G, N61K-N252K, S161E-S260T, G160A-D259G, K43N-Q217R, A1Y-Q275R, A200T-H226L, Q217R-T244A, S260P-Q275R, Q206R-S260P, T158I-D259N, Q217R-T244A, L75I-N76D, S161E-Q185H, Y21H-S37E, S249R-Q275R, T158I-D259N, Y21H-S37E, N76T-N212D, S260P-Q275L, G131S-K265N, V4A-S249N, N25D-Q185R, K43R-N76S, S183D-Q206R, Q10R-Q275K, K43N-S163T, Q10R-Q275K, N25D-V26A, G131S-K265N, S260P-Q275L, K141I-S248N, L16Q-Q217H, S249R-Q275R, K27R-N269T, P210S-N212D, L75I-N76D, S183D-Q206R, N118G-V121A, G215D-D259V, N76T-N212D, K27R-N269T, N62Y-G97D, V4E-S260P, G215D-D259V, K27E-Y91F, Y6D-T55A, N77D-N252T, V4E-S260P, Y6D-T55A, N25K-H238R, V44A-Q206H, L16Q-Q217H, V44A-Q206R, V44A-Q206H, and S37P-S260F, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having

proteolytic activity, having enhanced proteolytic activity greater than that of BPN', and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of Y21H-D259G, S183T-S249R, N61D-Q206R, Y262N-Q275R, K043R-N076S, A133V-D259N, and I079V-Q217H, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity and/or a PI value of greater than 1.0 to about 5 compared to BPN' in a BMI microswatch cleaning assay in Detergent Composition 4 at pII 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:6, wherein the variant comprises at least one substitution comprising at least one substitution selected from the group of X021H, X043R, X061D, X076S, X079V, X133V, X183T, X206R, X217H, X249R, X259G/N, X262N, and X275R, and optionally at least one substitution selected from the group of Y021H, K043R, N061D, N076S, I079V, A133V, S183T, Q206R, Q217H, S249R, D259G/N, Y262N,

and Q275R, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN' (SEQ ID NO:2) and a PI value greater than that of BPN' in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than or equal to 1.0 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of K170R-D259G, S18K-V203I, Y6F-S249C, Q19A-N109S, H17L-Q19A, Q19R-Q185R, S18Y-V203A, N61D-Q206R, S161E-S260T, S18K-V203I, V4A-T55A, N252S-L257H, S249R-Y262H, N61L-Q206H, N184Y-Y262N, Q19R-N25D, S249R-Y262H, A74S-P129Q, T158A-D259P, H17L-Q19A, K27R-D120H, V4A-T55A, N61K-N252K, Y21H-N252H, K27R-N269D, K43N-Q217R, T158A-D259P, Q206R-S260P, K27R-N269D, A98T-T158A, I79V-Q217H, S9L-N218S, V4A-Y6F, S161P-T253A, V203A-Q217R, T22S-T242S, N76P-N212S, A133V-D259N, S37T-S260P, T55A-V147A, V198L-D259G, Q19R-Q185R, V4A-Y6F, Q19A-N109S, Y262N-Q275R, G160R-T244A, Q19R-N25D, N25D-Q185R, N61K-N252K, S161E-S260T, A98T-T158A, N61L-Q206H, G211V-T244A, S9L-N218S, A144H-T244A, A144H-T244A, S18Y-V203A, Y21H-N252H, A74S-P129Q, G160A-D259G, K43N-Q217R, A1Y-Q275R, A1Y-Q275R, A200T-H226L, Q217R-T244A, S260P-Q275R, Q206R-S260P, K141I-S248N, S183T-R186K, T158I-D259N, S37T-S260P, K27R-D120H, T22S-T242S, Q217R-T244A, S161E-Q185H, P129S-K136R, G211V-T244A, N76P-N212S, L75I-N76D, S161E-Q185H, Y21H-S37E, S249R-Q275R, G160A-D259G, K43N-S163T, T158I-D259N, Y21H-S37E, S37G-Q275H, S161P-T253A, N76T-N212D, S260P-Q275L, Y6F-S249C, N184Y-Y262N, G131S-K265N, V4A-S249N, N25D-Q185R, N252S-L257H, K43R-N76S, S183D-Q206R, G160R-T244A, Q10R-Q275K, S37G-Q275H, K43N-S163T, Q10R-Q275K, N25D-V26A, P129S-K136R, G131S-K265N, S260P-Q275L, K141I-S248N, T22S-G166V, N62Y-T244A, L16Q-Q217H, S249R-Q275R, S260P-Q275R, K27R-N269T, P210S-N212D, L75I-N76D, S183D-Q206R, N118G-V121A, G215D-D259V, N76T-N212D, V4A-S249N, K27R-N269T, G166V-S183T, N62Y-G97D, V4E-S260P, G215D-D259V, K27E-Y91F, Y21H-D259R, Y6D-T55A, N77D-N252T, V4E-S260P, Y6D-T55A, N77D-N252T, Y21H-D259R, N25K-H238R, N77D-N252D, V44A-Q206H, L16Q-Q217H, V72F-L75I, S37P-S260F, V72F-L75I, N77D-N252D, V44A-Q206R, S163T-Q245L, V44A-Q206H, V44A-Q206R, S37P-S260F, G215R-D259R, V44A-Q206K, and V44A-Q206K, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and equal to or less than 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or

99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

EXAMPLE 22

Cleaning Performance of Additional BPN'-v36 Polypeptide Variants

The following BPN'-v36 variants were synthesized at DNA2.0 (Menlo Park, CA) using the pHPLT-BPN'-v36 plasmid containing the BPN' expression cassette served as template DNA (parent plasmid) for cloning: N109G-A128S-S224A, N109G-A128S-S224A-N243V, A88T-N109G-A116T-A128S-S224A-N243V, N61G-N109G-A128S-S224A, N61G-N109G-A128S-S224A-N243V, N61G-A88T-N109G-A116T-A128S-S224A-N243V, N109Q-A128S-S224A, N109Q-A128S-S224A-N243V, A88T-N109Q-A116T-A128S-S224A-N243V, N109S-A128S-S224A, N109S-A128S-S224A-N243V, A88T-N109S-A116T-A128S-S224A-N243V, N109M-A128S-S224A, N109M-A128S-S224A-N243V, A88T-N109M-A116T-A128S-S224A-N243V, N109G-A114S-A128S, N109G-A114S-A128S-N243V, A88T-N109G-A114S-A116T-A128S-N243V, N109G-A114S-A128S-S224A, N109G-A114S-A128S-S224A-N243V, N109G-A128S-S183V, N109G-A128S-S183L, N109G-A128S-S183L-S224A, N109G-A114S-A128S-S183L-S224A, A88T-N109G-A114S-A116T-A128S-S183L-S224A-N243V, N76D-N109G-A128S-S224A, N101Q-N109Q-A128S-S224A-N243V, N101Q-N109Q-A128S-P129S-S130T-S224A-N243V, N109G-A128S-P129S-S130T-S224A-N243V, S33T-A128S-N218S, S33T-N109G-A128S-N218S-N243V, S33T-N61G-N109G-A128S-N218S-N243V, S33T-N109G-A128S-G169A-N218S-N243V, S33T-S63G-N109G-A128S-N218S-N243V, S33T-N76D-N109G-A128S-N218S-N243V, S33T-S63G-N109G-A128S-G169A-N218S-N243V, I31L-S33T-S63G-N109G-A128S-G169A-N218S-N243V, S33T-N61G-S63G-N109G-A128S-N218S-N243V, S33T-N61G-A88T-N109G-A116T-A128S-N218S-N243V, S33T-N61G-S63G-N109G-A128S-G131H-G169A-N218S-N243V, S33T-N61P-S63G-N109G-A128S-G131H-G169A-N218S-N243V, S33T-N109G-A128S-N218S-S224A-N243V, S63G-N109Q-A128S-S224A-N243V, S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-S224A-N243V, A1G-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, I31L-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-S224A-N243V-S249Q, S33T-T55P-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V-S249Q, A1G-I31L-S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V-S249Q, N61P-S63G-N109Q-A128S-G131H-G169A-S224A-N243V-S249Q, and A1G-I31L-S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-G169A-S224A-N243V-S249Q.

The variants were grown for protein expression as described in Example 11 of Part I. These variants were tested for their performance in the BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C, the egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C, and the AAPF assay as described in Example 1 of Part I. Results are provided below.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of N61P-S63G-N109Q-A128S-S224A-N243V, A88T-N109G-A114S-A116T-A128S-N243V, A88T-N109G-A114S-A116T-A128S-S183L-S224A-N243V, N109G-A128S-S183V, N109G-A128S-N243V-K256R, N109M-A128S-S224A, A88T-N109S-A116T-A128S-S224A-N243V, N109Q-A128S-S224A-N243V, A88T-N109M-A116T-A128S-S224A-N243V, N109S-A128S-S224A-N243V, A88T-N109G-A116T-N243V, N101Q-N109Q-A128S-S224A-N243V, N109G-A116T-N243V-K256R, N109G-A128S-P129S-S130T-S224A-N243V, and A88T-N109Q-A116T-A128S-S224A-N243V, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN'-v36 and/or a PI value of equal to or greater than 1.0 to about 5 compared to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pII 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NON:6, wherein the variant comprises at least one substitution comprising at least one substitution selected from the group of X61G/P/S, X63G, X88T, X101Q, X109G/M/Q/S, X114S, X116T, X128S, X129S, X130T, X158S, X183L/V, X224A, X243V, X248A, and X256R, and optionally at least one substitution selected from the group of N61G/P/S, S63G, A88T,

N101Q, N109G/M/Q/S, A114S, A116T, A128S, P129S, S130T, T158S, S183L/V, S224A, N243V, S248A, and K256R, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN' (SEQ ID NO:2) BPN'-v3, and BPN'-v36 and a PI value greater than that of BPN', BPN'-v3, and BPN'-v36 in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.9 and equal to or less than 1.0 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of G24S-G53S-N78S-G97A-N101S-A128S, G24S-G53S-N78S-G97A-N101S, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V, S33T-N61G-S63G-N109G-A128S-N218S-N243V, S33T-S63G-N109G-A128S-N218S-N243V, S33T-T55P-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-A88T-N109G-A116T-A128S-N218S-N243V, S33T-N109G-A128S-N218S-N243V, S33T-N76D-N109G-A128S-N218S-N243V, S33T-N76D-N109G-A128S-N218S-N243V-S248N-K256R, S33T-N61G-N109G-A128S-N218S-N243V, S33T-A128S-N218S, A1G-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-S224A-N243V-S249Q, N61P-S63G-N109Q-A128S-G131H-S224A-N243V, S63G-N109Q-A128S-G131H-S224A-N243V, N109G-A114S-A128S, N109G-A114S-A128S-S183L-S224A, N109G-A114S-A128S-S224A, N109G-A114S-A128S-S224A-N243V, A88T-N109G-A116T-A128S-S224A-N243V, N61G-A88T-N109G-A116T-A128S-S224A-N243V, N109G-A114S-A128S-N243V, N109G-A128S-S224A-N243V, N109G-A128S-S224A, N109G-A128S-S183L-S224A, N61G-N109G-A128S-S224A, N109G-A128S-S183L, S33T-N76D, N109S-A128S-S224A, N101Q-N109Q-A128S-P129S-S130T-S224A-N243V, S63G-N109Q-A128S-S224A-N243V, N109M-A128S-S224A-N243V, S63G-N109G, N109G-K256R, S63G-N76D, S33T-N109G-A128S-G169A-N218S-N243V, and S33T-N109G-A128S-N218S-S224A-N243V, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity, enhanced proteolytic activity compared to BPN', and/or a PI value equal to or greater than 0.9 and less or equal to 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in a BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of I31L-S33T-S63G-N109G-A128S-G169A-N218S-N243V, A1G-I31L-S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-S63G-N109G-A128S-G131H-G169A-N218S-N243V, S33T-S63G-N109G-A128S-G169A-N218S-N243V, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V-S249Q, S33T-N76D-A128S-N218S, N76D-N109G-A128S-S224A, and S33T-N61P-S63G-N109G-A128S-G131H-G169A-N218S-N243V, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V, S33T-N61G-S63G-N109G-A128S-N218S-N243V, S33T-S63G-N109G-A128S-N218S-N243V, N61P-S63G-N109Q-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-A88T-N109G-A116T-A128S-N218S-N243V, S33T-N109G-A128S-N218S-N243V, S33T-A128S-N218S, A1G-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-S224A-N243V-S249Q, S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-S224A-N243V, A88T-N109G-A114S-A116T-A128S-N243V, A88T-N109G-A114S-A116T-A128S-S183L-S224A-N243V, N109G-A114S-A128S, N109G-A114S-A128S-S183L-S224A, N109G-A114S-A128S-S224A, N109G-A114S-A128S-S224A-N243V, A88T-N109G-A116T-A128S-S224A-N243V, N61G-A88T-N109G-A116T-A128S-S224A-N243V, N109G-A128S-S183V, N109G-A114S-A128S-N243V, N109G-A128S-N243V-S248A, N109G-A128S-S224A-N243V, N109G-A128S-N243V-K256R, N109G-A128S-S224A, N109G-A128S-S183L-S224A, N61G-N109G-A128S-S224A, N109M-A128S-S224A, A88T-N109S-A116T-A128S-S224A-N243V,

N109M-A128S-S224A-N243V, S63G-A128S, A88T-N109G-A116T-N243V, N101Q-N109Q-A128S-S224A-N243V, N109G-A116T-N243V-K256R, N109G-A116T, S63G-N109G, A88T-N109G, N109G-K256R, N61G-N109G-N243V, S33T-N109G-A128S-G169A-N218S-N243V, S33T-N109G-A128S-N218S-S224A-N243V, N109G-A128S-P129S-S130T-S224A-N243V, and A88T-N109Q-A116T-A128S-S224A-N243V, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN'-v36 and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v36 in an egg microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:6, wherein the variant comprises at least one substitution comprising at least one substitution selected from the group of X1G, X33T, X55P, X61G/P/S, X63G, X76D, X88T, X101Q, X109G/M/Q/S, X114S, X116T, X128S, X129S, X130T, X131H, X158S, X169A, X183L/V, X218S, X224A, X243V, X248A/N, X249Q, X256R, and optionally at least one substitution selected from the group of A1G, S33T, T55P, N61G/P/S, S63G, N76D, A88T, N101Q, N109G/M/Q/S, A114S, A116T, A128S, P129S, S130T, G131H, T158S, G169A, S183L/V, N218S, S224A, N243V, S248A/N, S249Q, K256R, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN', BPN'-v3, and BPN'-v36 and a PI value greater than that of BPN', BPN'-v3, and BPN'-v36 in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variant was determined to have a PI value equal to or greater than 0.5 and equal to or less than 1.0 relative to BPN'-v36 in an egg BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising amino acid substitutions selected from the group consisting of

substitutions S33T-N76D-A128S-N218S, N76D-N109G-A128S-S224A and S063G-N76D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and equal to or less than 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising amino acid substitutions selected from the group consisting of S33T-N76D-A128S-N218S, N76D-N109G-A128S-S224A, and S063G-N076D, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variants were determined to have a PI value greater than 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from greater than 1.0 to about 10, from greater than 1.0 to about 8, or from greater than 1.0 to about 5 relative to BPN'-v36 in an AAPF proteolytic assay: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of G24S-G53S-N78S-G97A-N101S-A128S, I31L-S33T-S63G-N109G-A128S-G169A-N218S-N243V, A1G-I31L-S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-S63G-N109G-A128S-G131H-G169A-N218S-N243V, S33T-S63G-N109G-A128S-G169A-N218S-N243V, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V, S33T-T55P-N61P-S63G-A88T-N109G-A116T-A128S-G131H-S224A-N243V-S249Q, S33T-N61G-S63G-N109G-A128S-N218S-N243V, S33T-S63G-N109G-A128S-N218S-N243V, S33T-T55P-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-G169A-S224A-N243V-S249Q, S33T-N61G-A88T-N109G-A116T-A128S-N218S-N243V, S33T-N109G-A128S-N218S-N243V, S33T-N76D-N109G-A128S-N218S-N243V, S33T-N76D-N109G-A128S-N218S-N243V-S248N-K256R, S33T-N61G-N109G-A128S-N218S-N243V, S33T-N76D-A128S-N218S, S33T-A128S-N218S, A1G-N61P-S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-G131H-S224A-N243V-S249Q, N61P-S63G-N109Q-A128S-G131H-S224A-N243V, S63G-N109Q-A128S-G131H-S224A-N243V, N61P-S63G-N109Q-A128S-S224A-N243V, A88T-N109G-A114S-A116T-A128S-N243V, A88T-N109G-A114S-A116T-A128S-S183L-S224A-N243V, N109G-A114S-A128S, N109G-A114S-A128S-S183L-S224A, N109G-A114S-A128S-S224A, N109G-A114S-A128S-S224A-N243V, A88T-N109G-A116T-A128S-S224A-N243V, N61G-A88T-N109G-A116T-A128S-S224A-N243V, N109G-A128S-S183V, N109G-A114S-A128S-N243V, N109G-A128S-N243V-S248A, N109G-A128S-S224A-N243V, N109G-A128S-N243V-K256R, N109G-A128S-S224A, N109G-A128S-S183L-S224A, N61G-N109G-A128S-S224A, N76D-N109G-A128S-S224A, N109M-A128S-S224A, N109G-A128S-S183L, S33T-N76D, A88T-

N109S-A116T-A128S-S224A-N243V, N109Q-A128S-S224A-N243V, N109S-A128S-S224A, A88T-N109M-A116T-A128S-S224A-N243V, N101Q-N109Q-A128S-P129S-S130T-S224A-N243V, S63G-N109Q-A128S-S224A-N243V, N109M-A128S-S224A-N243V, S63G-A128S, N109S-A128S-S224A-N243V, A88T-N109G-A116T-N243V, N61S-N109G-N243V, N101Q-N109Q-A128S-S224A-N243V, N109G-A116T-N243V-K256R, A88T-N109G-A116T-T158S-N243V-K256R, N109G-A116T, S63G-N109G, A88T-N109G, N109G-K256R, N61G-N109G-N243V, S33T-N61P-S63G-N109G-A128S-G131H-G169A-N218S-N243V, S33T-N109G-A128S-G169A-N218S-N243V, S33T-N109G-A128S-N218S-S224A-N243V, N109G-A128S-P129S-S130T-S224A-N243V, and A88T-N109Q-A116T-A128S-S224A-N243V, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN', BPN'-v3, and BPN'-v36, and a greater PI value than that of BPN', BPN'-v3 and BPN'-v36 in this assay. The invention includes a protease variant having enhanced proteolytic activity compared to BPN' (SEQ ID NO:2), enhanced proteolytic activity compared to BPN', BPN'-v3, and BPN'-v36, a PI value of greater than 1.0 to about 5 relative to BPN'-v3, and/or a PI value of greater than 1.0 to about 5 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of amino acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

Also provided is a subtilisin protease variant having enhanced proteolytic activity compared to BPN'-v36 and/or a PI value of greater than 1.0 to about 5 compared to BPN'-v36 in An AAPF proteolytic assay, the variant comprising an amino acid sequence having at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO:2 or SEQ ID NO:6, wherein the variant comprises at least one substitution comprising at least one substitution selected from the group of X1G, X31L, X33T, X55P, X61G/P/S, X63G, X76D, X88T, X101Q, X109G/M/Q/S, X114S, X116T, X128S, X129S, X130T, X131H, X158S, X169A, X183L/V, X218S, X224A, X243V, X248A/N, X249Q, and X256R, and optionally at least one substitution selected from the group of A1G, I31L, S33T, T55P, N61G/P/S, S63G, N76D, A88T, N101Q, N109G/M/Q/S, A114S, A116T, A128S, P129S, S130T, G131H, T158S, G169A, S183L/V, N218S, S224A, N243V, S248A/N, S249Q, and K256R, wherein amino acid positions of the variant are numbered by correspondence with positions of the sequence of SEQ ID NO:2. Such variants have enhanced proteolytic activity compared to the BPN' (SEQ ID NO:2) BPN'-v3, and BPN'-v36 and a PI value greater than that of BPN', BPN'-v3, and BPN'-v36 in this assay. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

The following BPN'-v36 variant was determined to have a PI value equal to or greater than 0.5 and equal to or less than 1.0 relative to BPN'-v36 in an AAPF proteolytic assay: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising amino acid substitutions G24S-G53S-N78S-G97A-N101S or S063G-N76D, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and equal to or less than 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising substitutions G24S-G53S-N78S-G97A-N101S or S063G-N76D, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

EXAMPLE 23

Cleaning Performance of Polypeptide BPN'-v36 Polypeptides Variants from Combinatorial Libraries Based on BPN'-v36 Polypeptide

Two separate combinatorial libraries (AJ1 and AJ2) were synthesized by DNA2.0 (Menlo Park, CA) and were delivered as individual ligation reactions. The pHPLT-BPN'-v36 plasmid (Figure 4) containing the BPN' expression cassette served as template DNA (parent plasmid) for library construction. A list of the possible amino acid positions and substitutions for each library is shown in Table 23-1. The ligation reactions for each library were used to transform *B. subtilis*, and the library variants were grown up for protein expression as described in Example 11. The variants were tested for performance in the BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C as described in Example 1 of Part I.

Table 23-1: Possible Substitutions for Combinatorial Libraries AJ1 and AJ2			
AJ1		AJ2	
Position	Possible Substitutions	Position	Possible Substitutions
S33	G, S	E54	E,Q
D60	D,G	D99	D,N
N62	N,L,S	D120	D,N
S63	S,R,L,N,G	D140	D,N
S125	S,A	E156	E,Q
Q217	Q,R,E,L,G	D197	D,N
M222	M,L,S	K12	K,T
		K27	K,S
		K43	K,T
		K141	K,Y
		K213	K,Q
		K237	K,A
		K256	K,Q

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less or equal to 1.0 relative to BPN'-v36 in the BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16ºC: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of G024S-G053S-N078S-G097A-N101S (i.e., BPN'-v3), G024S-G053S-N078S-G097A-N101S-A128S (i.e., BPN'-v12), N062L, N062L-S063G, N062S, N062S-S063G-Q217L, N062S-S063L-Q217L, N062S-S063N, N062S-S063R, Q217E, S063G, S063G-Q217L, S063G-Q217L-M222S, S063L-Q217L, S063N, S063N-Q217L, D099N-K141Y-K213Q, D099N-K141Y-K256Q, K043T, K043T-K141Y-E156Q, N062L-Q217E, N062L-Q217L, N062L-S063G-Q217E, N062L-S063L, N062L-S063N-Q217L, N062S-Q217L, N062S-S063G, N062S-S063L, N062S-S063N-Q217L, N062S-S063R-Q217E, Q217L, S063G-Q217E, S063N-Q217E, S063R, S063R-Q217E, S063R-Q217L,), D099N-K141Y-K213Q, D099N-K141Y-K256Q, K043T, K043T-K141Y-E156Q, N062L-Q217E, N062L-Q217L, N062L-S063G-Q217E, N062L-S063L, N062S-Q217L, N062S-S063G, N062S-S063L, N062S-S063N-Q217L, Q217L, S063G-Q217E, S063N-Q217E, S063R, S063R-Q217E, and S063R-Q217L, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and equal to or less than 1.0 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%, 85%, 90%,

91%, 92%, 93%, 94%, 95%, 96%, 97%, or 98% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein. Note that a protease variant which is BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising the amino acid substitutions G024S-G053S-N078S-G097A-N101S is BPN'-v3 (SEQ ID NO:4), because the G at position 24, G at position 53, N at position 78, and N at position 101 of SEQ ID NO:6 have been substituted with S at each of positions 24, 53, 78, and 101 of SEQ ID NO:6. In addition, G at position 97 of SEQ ID NO:6 has been substituted with A. Thus, the resultant sequence is SEQ ID NO:4.

The following BPN'-v36 variants were determined to have a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in the BMI microswatch cleaning assay in Detergent Composition 4 at pH 8 and 16°C: BPN'-S024G-S053G-S078N-S101N-G128A-Y217Q amino acid sequence (SEQ ID NO:6) comprising at least one set of amino acid substitutions selected from the group consisting of D099N, K141Y-E156Q, N062L-S063L-Q217L, N062L-S063N, N062L-S063N-Q217E, N062L-S063R, N062L-S063R-Q217L, N062S-Q217E, N062S-S063G-Q217E, N062S-S063G-Q217R, N062S-S063N-Q217R, S063G-S125A, D060G-Q217L, D120N-K141Y-K213Q, K043T-D099N-D120N-K141Y, K043T-D099N-K141Y-K256Q, K043T-K237A, N062L-S063G-Q217R, N062L-S063G-S125A, N062L-S063L-Q217E, N062L-S063N-S125A-Q217L, N062S-Q217R, N062S-S063L-Q217E, N062S-S063R-Q217L, S063G-M222S, S063G-Q217R, D120N-E156Q-K256Q, K141Y-D197N, N062L-Q217R, N062L-S063G-Q217L-M222S, N062L-S063L-Q217R, N062L-S063N-Q217R, N062S-Q217G, N062S-S063G-Q217G, N062S-S063G-Q217L-M222L, N062S-S063G-S125A-Q217L, N062S-S063N-Q217E, Q217G, S033G-N062S-S063G, S063G-Q217G, S063G-Q217L-M222L, S063G-S125A-Q217R, S063L-Q217R, S063N-M222S, S063N-Q217R, S063N-S125A-Q217L, S063R-Q217R, S063R-S125A-Q217L, D099N-E156Q-K256Q, E156Q, K012T-D099N-K213Q, K012T-K256Q, K043T-D099N-K141Y-K213Q, K043T-E156Q, K141Y-K213Q, N062L-Q217G, N062L-Q217L-M222L, N062L-Q217L-M222S, N062L-S063G-M222S, N062L-S063G-Q217L-M222L, N062L-S063G-Q217R-M222S, N062L-S063N-Q217L-M222S, N062L-S063N-S125A, N062L-S063R-S125A, N062L-S125A, N062S-S063G-M222S, N062S-S063G-Q217G-M222S, N062S-S063G-S125A, N062S-S063N-Q217L-M222L, N062S-S063N-S125A-Q217L, N062S-S063R-Q217G, N062S-S063R-Q217L-M222S, Q217G-M222S, Q217L-M222S, Q217R, S033G-S063G-Q217R, S063G-Q217E-M222S, S063G-S125A-Q217G, S063L-Q217E, S063N-Q217G, S063N-Q217G-M222S, S063N-Q217L-M222S, S063R-Q217L-M222S, and S063R-S125A, wherein amino acid positions of the variant are numbered by correspondence with the sequence of SEQ ID NO:2. Such variants have proteolytic activity. The invention includes a protease variant having proteolytic activity and/or a PI value equal to or greater than 0.5 and less than 0.9 relative to BPN'-v36 in this assay, the variant comprising an amino acid sequence having at least 60%, 70%, 80%,

85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 or SEQ ID NO:6 and comprising at least one set of acid substitutions selected from said group above, wherein amino acid positions of the variant are numbered by correspondence with amino acid positions of the SEQ ID NO:2 sequence. Also included are compositions, including, but not limited to, e.g., cleaning compositions, comprising at least one such variant and methods for cleaning utilizing at least one such protease variant as described in greater detail elsewhere herein.

PART II

Series I GG36 Cold Water Protease Variants

The amino acid sequence of wild-type mature *Bacillus lentus* GG36 protease is:

AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQDG
NGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGM
HVANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATD
QNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKN
PSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR (SEQ ID NO:755)

As indicated herein, suitable Series I GG36 cold water protease variants include enzyme variants derived from a parent protease, said parent protease's sequence being at least 60%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to the amino acid sequence of SEQ ID NO:755, said protease variant having one or more of the following characteristics:

a) Test Method 2 performance index of at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from 1.1 to about 10, from 1.1 to about 8, or even from 1.1 to about 5;

b) a Test Method 3 performance index of at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from 1.1 to about 10, from 1.1 to about 8, or even from 1.1 to about 5;

c) a Test Method 4 performance index of at least 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2, from 1.0 to about 10, from 1.0 to about 8, or even from 1.0 to about 5.

Test Method 2, Test Method 3, and Test Method 4 are explicitly described in the "Test Methods" section of Part II Example 1. All mutations referenced herein utilize the BPN' numbering scheme as shown in Figure 5. In some aspects, the variants referenced herein refer to variants having amino acid sequences compared to the amino acid sequence of SEQ ID NO:755, using the BPN' numbering scheme.

Suitable Series I GG36 cold water proteases can be variants of subtilisins or derived from subtilisins, particularly those derived from subtilisin *Bacillus lentus* GG36 of SEQ ID NO:755 and in some aspects, comprise one or more of the following mutations: A1R, Q2S, V4R, V4S, S9A, R10S, P14K, A16S, H17R, N18R, G20R, T22A, T22R, S24R, S24W, G25R, G25V, V26F, L42I, N43R, N43A, G46R, P52F, P52E, P52N, T57R, Q59A, N62E, N62Q, V68A, V68C, T71G, I72C, A74C. L75A, L75F,

L75R, N76D, S78R, L82R, P86W, E89P, E89T, E89G, E89H, E89I, E89V, E89W, Y91N, K94N, G100S, S101A, S101N, S101G, S101D, S103G, S103N, V104L, V104I, S106V, S106G, A108I, L111V, E112V, G115K, G115R, N117F, G118I, V121F, S128D, S128F, S128L, S128N, P129E, S144R, L148I, A158E. G159E, S160D, S166D, N185E, N185I, R186H, S188E, S188D, D197F, V203E, Y209S, Y209N, Y209F, Y209T, Y209E, Y209H, Y209G, P210R, S212I, S212F, Y214F, A215N, A215D, A215E, L217E, L217N, T224A, A230E, A231I, Q236F, N238R, N238K, P239K, P239G, P239R, P239S, W241R, S242R, S242L, N243R, V244R, N248I, N248V, II249R, L250I, N252R, T253R, L262D, Y263F, S265F, L267V, L267N. N269I, N269R, E271F, E271I, E271H, E271P, E271T, E271V, E271L and/or A272F, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 (BPN') as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2.

In one aspect, suitable Series I GG36 cold water protease variants include subtilisins, particularly those derived from *Bacillus lentus* GG36 of SEQ ID NO:755, comprising one or more of the following sets of substitutions: T022R-S024R, S009A-E271L, N018R-W241R, N018R-G115R, N043R-H249R, G020R-H249R, V004R-H249R, G020R-S024R, N018R-H249R, S009A-G020R, G020R-W241R, S009A-S078R, G020R-G115R, N018R-S024R, S024R-S242R, T022R-G115R, N018R-N043R, G020R-N043R, N018R-S242R, S242R-N269R, N018R-V244R, S024R-N269R, G020R-E271L, S024R-E271L, V004R-S009A, G020R-N269R, A001R-S024R, V244R-E271L, S009A-N018R, W241R-E271L, V004R-S024R, S009A-H249R, S009A-T022R, N062E-P129E, N062E-G159E, A016S-L148I, A158E-H249R, A016S-N062E, L111V-S188D, T022A-N062E, N062E-L148I, T022A-P129E, N062E-E271F, N062E-A158E, A016S-G159E, N062E-R186H, S128N-G159E, N062E-S188D, N062E-S128N, L148I-G159E, S103G-A158E, L111V-G159E, A158E-E271F, A016S-S188D, T022A-L111V, S128N-A158E, A016S-A158E, V104L-A158E, S128N-R186H, G159E-Y209E, N062E-S101A, L111V-Y209E, L148I-S188D, S101A-Y209E, T022A-S188D, A016S-T022A, S128N-P129E, A016S-Y209E, A016S-S128N, T022A-E089P, S128N-Y209E, E089P-A158E, N062E-S103G, R186H-E271F, A016S-P129E, E089P-G159E, L111V-H249R, S101A-P129E, L148I-Y209E, T022A-G159E, P129E-H249R, P129E-Y209E, V104L-P129E, S128N-S188D, L111V-A158E, T022A-A158E, N062E-Y209E, N062E-H249R, S101A-R186H, E089P-P129E, P129E-E271, T22A-L111V-G159E, S101A-S103G-V104L-Y209E, S101A-S103G-V104L-G159E, S101A-S103G-V104L-S188D, S101G-S103A-V104I-G159D, T22A-S103G-G159E, T22A-S128N-E271F-Y209E, T22A-Y209E-E271F, T22A-S101A-Y209E, S101A-Y209E-E271F, T22A-L111V-S128N, T22A-S101A-G159E, S101A-S103G-V104L, T22A-S101A-S103G-V104L, S101A-S103G-V104L, S101G-S103A-V104I, S101A-S103G-V104L-S128N, S103A-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K, S101G-S103A-V104L-

G159D-A232V-Q236H-Q245R-N248D, N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-S24R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-T253R, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159E-A232V-Q245R-N248D-H249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N238R, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N76D, and/or S101G-S103A-V104I-G159E-A232V-Q245R-N248D-E271F, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 (BPN') as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2.

In another aspect, suitable Series I GG36 cold water protease variants include variants of subtilisins, particularly those derived from *Bacillus lentus* GG36 of SEQ ID NO:755, wherein the variants comprise three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14 15, 16, 17, 18, 19,20, 21, 22, 23, 24, or even 25 mutations within the group of positions comprising positions 1, 2, 4, 9, 10, 14, 16, 17, 18, 20, 22, 24, 25, 26, 42, 43, 46, 52, 57, 59, 62, 68, 71, 72, 74, 75, 76, 78, 82, 86, 89, 91, 94, 100, 101, 103, 104, 106, 108, 111, 112, 115, 117, 118, 121, 128, 129, 144, 148, 158, 159, 160, 166, 185, 186, 188, 197, 203, 209, 210, 212, 214, 215, 217, 224, 230, 231, 236, 238, 239, 241, 242, 243, 244, 248, 249, 250, 252, 253, 262, 263, 265, 267, 269, 271 and 272.

In another aspect, suitable Series I GG36 cold water protease variants include variants of subtilisins, particularly those derived from *Bacillus lentus* GG36 of SEQ ID NO:755, wherein the variants comprise a total of three, four, five, six, seven, eight, nine, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or even 25 mutations selected from: A1R, Q2S, V4R, V4S, S9A, R10S, P14K, A16S, H17R, N18R, G20R, T22A, T22R, S24R, S24W, G25R, G25V, V26F, L42I, N43R, N43A, G46R, P52F, P52E, P52N, T57R, Q59A, N62E, N62Q, V68A, V68C, T71G, I72C, A74C. L75A, L75F, L75R, N76D, S78R, L82R, P86W, E89P, E89T, E89G, E89H, E89I, E89V, E89W, Y91N, K94N, G100S, S101A, S101N, S101G, S101D, S103G, S103N, V104L, V104I, S106V, S106G, A108I, L111V, E112V, G115K, G115R, N117F, G118I, V121F, S128D, S128F, S128L, S128N, P129E, S144R, L148I, A158E. G159E, S160D, S166D, N185E, N185I, R186H, S188E, S188D, D197F, V203E, Y209S, Y209N, Y209F, Y209T, Y209E, Y209H, Y209G, P210R, S212I, S212F, Y214F, A215N, A215D, A215E, L217E, L217N, T224A, A230E, A231I, Q236F, N238R, N238K, P239K, P239G, P239R, P239S, W241R, S242R, S242L, N243R, V244R, N248I, N248V, H249R, L250I, N252R, T253R, L262D, Y263F, S265F, L267V, L267N, N269I, N269R, E271F, E271I, E271H, E271P, E271T, E271V, E271L and A272F; and optionally one or more of the following mutations: S103A, G159D, Q236H, Q245R, N248D and N252K, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by

correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 (BPN') as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2.

In some aspects, the Series I GG36 cold water protease variant comprises one or more mutations, and having a total net charge of -5, -4, -3, -2, -1 or 0 relative to *B. lentus* subtilisin GG36 wild-type (SEQ ID NO:755).

In another aspect, the Series I GG36 cold water protease variants are low ionic strength Series I GG36 cold water protease variants. Such low ionic strength Series I GG36 cold water protease variants comprising one or more mutations, and having a total net charge of -5, -4, -3, -2, -1 or 0 relative to *B. lentus* subtilisin GG36 protease wild-type (SEQ ID NO:755). These mutations can be selected from: (a) two or more of the following mutations: A1R, Q2S, V4R, V4S, S9A, R10S, P14K, A16S, T22A, T22R, S24R, G25V, V26F, L42I, P52F, P52E, P52N, N62E, N62Q, V68A, V68C, T71G, I72C, A74C. L75A, L75F, S78R, E89P, E89T, E89G, E89H, E89W, Y91N, K94N, G100S, S101A, S101N, S101G, S101D, S103G, S103N, V104L, V104I, A108I, L111V, E112V, G115K, N117F, V121F, S128D, S128F, S128L, S128N, P129E, L148I, A158E. G159E, S160D, S166D, N185E, R186H, S188E, S188D, V203E, Y209S, Y209N, Y209F, Y209T, Y209E, Y209H, Y209G, P210R, S212I, S212F, Y214F, A215N, A215D, A215E, L217E, L217N, T224A, A230E, A231I, Q236F, N238R, N238K, P239K, P239G, P239R, N248V, H249R, L250I, L262D, Y263F, S265F, L267V, L267N. N269I, N269R, E271F, E271I, E271H and A272F; and/or (b) one or more of the following sets of mutations: N062E-P129E, N062E-G159E, A016S-L148I, A158E-H249R, A016S-N062E, L111V-S188D, T022A-N062E, N062E-L148I, T022A-P129E, N062E-E271F, N062E-A158E, A016S-G159E, N062E-R186H, S128N-G159E, N062E-S188D, N062E-S128N, L148I-G159E, S103G-A158E, L111V-G159E, A158E-E271F, A016S-S188D, T022A-L111V, S128N-A158E, A016S-A158E, V104L-A158E, S128N-R186H, G159E-Y209E, N062E-S101A, L111V-Y209E, L148I-S188D, S101A-Y209E, T022A-S188D, A016S-T022A, S128N-P129E, A016S-Y209E, A016S-S128N, T022A-E089P, S128N-Y209E, E089P-A158E, N062E-S103G, R186H-E271F, A016S-P129E, E089P-G159E, L111V-H249R, S101A-P129E, L148I-Y209E, T022A-G159E, P129E-H249R, P129E-Y209E, V104L-P129E, S128N-S188D, L111V-A158E, T022A-A158E, N062E-Y209E, N062E-H249R, S101A-R186H, E089P-P129E, P129E-E271F, T22A-L111V-G159E, S101A-S103G-V104L-Y209E, S101A-S103G-V104L-G159E, S101A-S103G-V104L-S188D, S101G-S103A-V104I-G159D, T22A-S103G-G159E, T22A-S128N-E271F-Y209E, T22A-Y209E-E271F, T22A-S101A-Y209E, S101A-Y209E-E271F, T22A-L111V-S128N, T22A-S101A-G159E, S101A-S103G-V104L, T22A-S101A-S103G-V104L, S101A-S103G-V104L, S101G-S103A-V104I, S101A-S103G-V104L-S128N, S103A-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-G159D-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-A232V-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-Q236H-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q245R-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-N248D-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N248D, N62E-S101G-S103A-V104I-G159D-

A232V-Q245R-N248D-E271F, N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-II249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-S24R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-T253R, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-H249R, T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159E-A232V-Q245R-N248D-H249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N238R, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N76D and S101G-S103A-V104I-G159E-A232V-Q245R-N248D-E271F, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 (BPN').

In another aspect, the above low ionic strength Series I GG36 cold water protease variants form part of a detergent composition that is diluted in water, typically within a laundry washing machine, to form a laundry detergent wash liquor, whose conductivity is from about 0.1 mS/cm to about 3 mS/cm, from about 0.3 mS/cm to about 2.5 mS/cm, or even from about 0.5 mS/cm to about 2 mS/cm.

In another aspect, the Series I GG36 cold water protease variants are high ionic strength Series I GG36 cold water protease variants. Such high ionic strength Series I GG36 cold water protease variants comprise two or more mutations, and have a total net charge of +5, +4, +3, +2, +1 or 0 relative to *B. lentus* subtilisin GG36 protease wild-type (SEQ ID NO:755). These mutations can be selected from: (a) two or more of the following mutations V4R, H17R, N18R, G20R, T22R, S24R, S24W, G25R, N43R, N43A, G46R, P52F, P52N, T57R, Q59A, N62Q, T71G, L75R, N76D, S78R, L82R, P86W, E89P, E89W, E89T, E89I, E89II, E89V, V104L, S106V, S106G, G115R, G118I, V121F, S144R, N185I, D197F, Y209N, Y209S, L217E, A231I, P239R, P239S, W241R, S242R, S242L, N243R, V244R, N248I, H249R, N252R, T253R, E271T, E271V, E271L, E271H, E271F, E271P, A1R, S9A, S212F and N269R; and/or (b) one or more of the following sets of mutations T022R-S024R, S009A-E271L, N018R-W241R, N018R-G115R, N043R-H249R, G020R-H249R, V004R-H249R, G020R-S024R, N018R-H249R, S009A-G020R, G020R-W241R, S009A-S078R, G020R-G115R, N018R-S024R, S024R-S242R, T022R-G115R, N018R-N043R, G020R-N043R, N018R-S242R, S242R-N269R, N018R-V244R, S024R-N269R, G020R-E271L, S024R-E271L, V004R-S009A, G020R-N269R, A001R-S024R, V244R-E271L, S009A-N018R, W241R-E271L, V004R-S024R, S009A-H249R, S009A-T022R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-E271F, S101G-S103A-V104I-A158E-A232V-Q245R-N248D-II249R, S101G-S103A-V104I-G159D-A232V-Q245R-N248D-S24R, S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K, S101G-S103A-V104L-A232V-Q236H-Q245R-N248D-N252K, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 (BPN').

In another aspect, the above high ionic strength Series I GG36 cold water protease variants form part of a detergent composition that is diluted in water, typically within a laundry washing machine, to form a laundry detergent wash liquor, whose conductivity is from about 3 mS/cm to about 30 mS/cm, from about 3.5 mS/cm to about 20 mS/cm, or even from about 4mS/cm to about 10 mS/cm.

The charge of the Series I GG36 cold water protease variants is expressed relative to *B. lentus* subtilisin GG36 protease wild-type having the amino acid sequence of SEQ ID NO:755. The amino acids that impart a single negative charge are D and E and those that impart a single positive charge are R, H and K. Any amino acid change versus SEQ ID NO:755 that changes a charge is used to calculate the charge of the Series I GG36 cold water protease variant. For example, introducing a negative charge mutation from a wild-type neutral position will add a net charge of -1 to the Series I GG36 cold water protease variant, whereas introducing a negative charge mutation (D or E) from a wild-type positive amino acid residue (R, H or K) will add a net charge of -2. Summing the charge changes from all the amino acid residues that are different for the Series I GG36 cold water protease variant versus *B. lentus* subtilisin GG36 protease wild-type having the amino acid sequence of SEQ ID NO:755 gives the charge change of the Series I GG36 cold water protease variant. Without wishing to be bound by theory, it is believed that: (a) the preferred charge range for Series I GG36 cold water protease variants to be used in low conductivity laundry detergent solutions is -5, -4, -3, -2, -1, 0, particularly -2, -1; and (b) the preferred charge range for Series I GG36 cold water protease variants to be used in high conductivity laundry detergent solutions is +5, +4, +3, +2, +1, 0, particularly +2, +1. By correctly selecting the charge unexpectedly improved levels of cold water cleaning performance can be obtained. "Low conductivity laundry detergent solutions" are defined as having a conductivity of from about 0.1 mS/cm to about 3 mS/cm, from about 0.3 mS/cm to about 2.5 mS/cm, or even from about 0.5 mS/cm to about 2 mS/cm. "High conductivity laundry detergent solutions" are defined as having a conductivity of from about 3 mS/cm to about 30 mS/cm, from about 3.5 mS/cm to about 20 mS/cm, or even from about 4 mS/cm to about 10 mS/cm. It is intended that the above examples be non-limiting. Once mutations are combined to optimize cold water performance, the enzyme charge can also be balanced by mutations in further positions.

Methods for Making GG36 Cold Water Protease Variants

A variety of methods are known in the art that are suitable for generating modified polynucleotides of the invention that encode GG36 cold water protease variants, including, but not limited to, for example, site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, deletion mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches. Non-limiting exemplary methods of making the Series I GG36 cold water protease variants are provided in the section above entitled "Vectors, Cells, and Methods for Making Protease Variant Polypeptides of the Invention."

For testing of enzyme activity in heat-inactivated detergents, working solutions of detergents are made from the heat inactivated stocks. Appropriate amounts of water hardness (e.g., 6 gpg or 12 gpg) and buffer are added to the detergent solutions to match the desired conditions. The solutions are mixed by vortexing or inverting the bottles. The following Table provides information regarding some of the commercially-available detergents and test conditions used herein. In some experiments, additional and/or other commercially available detergents find use in the following Examples.

Table A. Laundry and Dish Washing Conditions							
Region	**Form**	**Dose**	**Detergent***	**Buffer**	**Gpg**	**pH**	**T (°C)**
Laundry (Heavy Duty Liquid and Granular)							
NA	HDL	0.78 g/l	P&G TIDE® 2X	5 mM HEPES	6	8.0	20
WE	HDL	5.0 g/L	Henkel PERSIL™	5 mM HEPES	12	8.2	40
WE	HDG	8.0 g/L	P&G ARIEL®	2 mM Na_2CO_3	12	10.5	40
JPN	HDG	0.7 g/L	P&G TIDE®	2 mM Na_2CO_3	6	10.0	20
NA	HDG	1.0 g/L	P&G TIDE®	2 mM Na_2CO_3	6	10.0	20
Automatic Dish Washing							
WE	ADW	3.0 g/L	RB CALGONIT™	2 mM Na_2CO_3	21	10.0	40
NA	ADW	3.0 g/L	P&G CASCADE®	2 mM Na_2CO_3	9	10.0	40

In some additional Examples, the following solutions find use:

Table B Working Detergent Solutions					
Detergent	**Temp (C)**	**Detergent g/L**	**pH**	**Buffer**	**Gpg**
TIDE® 2X Cold	16	0.98	8	5mM HEPES	6
TIDE® 2X Cold	32	0.98	8	5mM HEPES	6
TIDE® 2X Cold	16	0.98	7	5mM MOPS	6

Table C provides granular laundry detergent compositions produced in accordance with the invention suitable for laundering fabrics.

Table C. Granular Laundry Detergent Compositions and Their Components						
	Detergent Compositions					
Component	**1**	**2**	**3**	**4**	**5**	**6**
Linear alkylbenzenesulfonate with aliphatic carbon chain length C_{11}-C_{12}	15	12	20	10	12	13
Other surfactants	1.6	1.2	1.9	3.2	0.5	1.2
Phosphate builder(s)	2	3	4			
Zeolite		1		1	4	1
Silicate	4	5	2	3	3	5

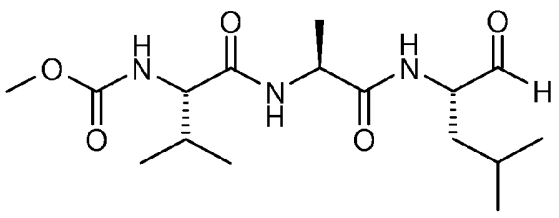
Table C. Granular Laundry Detergent Compositions and Their Components						
	Detergent Compositions					
Component	**1**	**2**	**3**	**4**	**5**	**6**
Sodium Carbonate	2	5	5	4	0	3
Polyacrylate (MW 4500)	1	0.6	1	1	1.5	1
Carboxymethyl cellulose (Finnfix BDA ex CPKelco)	1	-	0.3	-	1.1	-
Celluclean® (15.6mg/g)	0.23	0.17	0.5	0.2	0.2	0.6
Cold Water Protease variant*	0.23	0.17	0.05	0.2	0.03	0.1
Stainzyme Plus® (14mg/g)	0.23	0.17	0.5	0.2	0.2	0.6
Mannaway 4.0T (4mg/g)	0.1			0.1		0.1
Lipex 100T (18.6mg/g)	0.2		0.1		0.3	
Fluorescent Brightener(s)	0.16	0.06	0.16	0.18	0.16	0.16
Diethylenetriamine pentaacetic acid or Ethylene diamine tetraacetic acid	0.6		0.6	0.25	0.6	0.6
$MgSO_4$	1	1	1	0.5	1	1
Bleach(es) and Bleach activator(s)	6.88		6.12	2.09	1.17	4.66
Ethoxylated thiophene Hueing Dye[5]	0.002	0.001	0.003	0.003	-	-
Direct Violet 9 ex Ciba Specialty Chemicals				0.0006	0.0004	0.0006
Sulfate/Citric Acid/ Sodium Bicarbonate/ Moisture/perfume	Balance to 100%					

[1] Random graft copolymer is a polyvinyl acetate grafted polyethylene oxide copolymer having a polyethylene oxide backbone and multiple polyvinyl acetate side chains. The molecular weight of the polyethylene oxide backbone is about 6000 and the weight ratio of the polyethylene oxide to polyvinyl acetate is about 40 to 60 and no more than 1 grafting point per 50 ethylene oxide units.

[2] Polyethylenimine (MW = 600) with 20 ethoxylate groups per -NH.

[3] Amphiphilic alkoxylated grease cleaning polymer is a polyethylenimine (MW = 600) with 24 ethoxylate groups per –NH and 16 propoxylate groups per -NH

[4] Reversible protease inhibitor of structure:



[5]Ethoxylated thiophene Hueing Dye is as described in US 7,208,459 B2.

In Table C, all enzyme levels expressed as % enzyme raw material, except for cold water protease variant (of this invention) which is expressed as % of active protein added to the product. Table D provides granular laundry detergent compositions suitable for top-loading automatic washing machines (detergent compositions 7-9) and front loading washing machines (detergent compositions 10-11). The GG36 protease variant tested and/or cold water protease variant of the present invention is added separately to these formulations.

Table D. Granular Laundry Detergent Compositions and Their Components

	Detergent Composition				
Component	**7**	**8**	**9**	**10**	**11**
Surfactants					
C_{16-17} Branched alkyl sulfate	3.55	15.8			
C_{12-14} alkyl sulphate			1.5		
Sodium linear alkylbenzenesulfonate with aliphatic chain length C_{11}-C_{12}	9.6		10.6	7.5	9
Sodium $C_{14/15}$ alcohol ethoxy – 3 - sulfate	1.15			2.88	
Sodium $C_{14/15}$ alkyl sulphate	2.37				
$C_{14/15}$ alcohol ethoxylate with average 7 moles of ethoxylation				1.17	1
mono-C_{8-10} alkyl mono-hydroxyethyl di-methyl quaternary ammonium chloride					0.45
Di methyl hydroxyl ethyl lauryl ammonium chloride			0.18		
Zeolite A	13.9	4.7	0.01	2.9	1.8
Sodium Silicate 1.6.ratio	4	0.2		4	4
Sodium Silicate 2.35.ratio			8		
Citric Acid				2.5	1.4
Sodium tripolyphosphate			5		
Sodium Carbonate	24.1	30	16.9	24.4	21
Nonanoyloxybenzenesuplhonate	5.78	2.81	0.96		
Oxaziridinium-based bleach booster				0.03	0.017
Tetrasodium S,S,- ethylenediaminedisuccinate				0.2	
Diethylenetriamine penta (methylene phosphonic acid), heptasodium salt	0.61				0.33
Hydroxyethane dimethylene phosphonic acid				0.29	0.45
Ethylene diamine tetraacetate			0.27		
MgSO4			0.47	0.5994	0.782

Table D. Granular Laundry Detergent Compositions and Their Components					
	Detergent Composition				
Component	7	8	9	10	11
Sodium Percarbonate	7	4.4		15.9	19.1
Tetra Acetyl Ethylene Diamine				3.3	4.6
Sodium Perborate Monohydrate			1.2		
Carboxymethyl cellulose (e.g., Finnfix BDA ex CPKelco)	0.1		0.17	1.69	0.23
Sodium Acrylic acid/maleic acid co-polymer (70/30)	0.0236	3.8		2	2.5
Sodium polyacrylate (Sokalan PA30 CL)	4		0.84		
Terephthalate polymer				0.23	
Polyethylene glycol/vinyl acetate random graft co polymer			0.89	0.89	0.91
Photobleach- zinc phthalocyanine tetrasulfonate			0.005	0.001	0.002
C.I. Fluorescent Brightener 260	0.11	0.15	0.04	0.23	0.15
C.I. Fluorescent Brightener 351 (Tinopal® CBS)			0.1		
Suds suppressor granule		0.25		0.07	0.04
Hydrophobically modified carboxy methyl cellulose (Finnifix® SH-1)			0.019	0.028	
Bentonite			8.35		
Miscellaneous (Dyes, perfumes, process aids, moisture and sodium sulphate)	Balance	Balance	Balance	Balance	Balance

In Table D, surfactant ingredients can be obtained from any suitable supplier, including but not limited to BASF (e.g., LUTENSOL®), Shell Chemicals, Stepan, Huntsman, and Clariant (e.g., PRAEPAGEN®). Zeolite can be obtained from sources such as Industrial Zeolite. Citric acid and sodium citrate can be obtained from sources such as Jungbunzlauer. Sodium percarbonate, sodium carbonate, sodium bicarbonate and sodium sesquicarbonate can be obtained from sources such as Solvay. Acrylate/maleate copolymers can be obtained from sources such as BASF. Carboxymethylcellulose and hydrophobically modified carboxymethyl cellulose can be obtained from sources such as CPKelco. C.I. Fluorescent Brightener 260 can be obtained from 3V Sigma (e.g., OPTIBLANC®, OPTIBLANC® 2M/G, OPTIBLANC® 2MG/LT Extra, or OPTIBLANC® Ecobright. Tetrasodium S,S-ethylenediamine disuccinate can be obtained from sources such as Innospec. Terephthalate co-polymer can be obtained from Clariant (e.g., REPELOTEX SF 2). In addition, 1-Hydroxyethane -1,1-diphosphonic acid can be obtained from Thermphos. Oxaziridinium-based bleach booster has the following structure, where R1 = 2-butyloctyl, and was produced according to US 2006/0089284A1.



The enzymes NATALASE®, TERMAMYL®, STAINZYME PLUS®, CELLUCLEAN® and MANNAWAY® can be obtained from Novozymes. Zinc phthalocyanine tetrasulfonate can be obtained from Ciba Specialty Chemicals (e.g., TINOLUX® BMC). Suds suppressor granule can be obtained from Dow Corning. In these detergent compositions, random graft copolymer is a polyvinyl acetate grafted polyethylene oxide copolymer having a polyethylene oxide backbone and multiple polyvinyl acetate side chains. The molecular weight of the polyethylene oxide backbone is about 6000 and the weight ratio of the polyethylene oxide to polyvinyl acetate is about 40 to 60 and no more than 1 grafting point per 50 ethylene oxide units.

PART II EXAMPLES

EXAMPLE 1

Assays and Test Methods

This Example describes the various Test Methods and assays used in the development of the variants described in these Examples. Any deviations from the protocols provided are indicated in the pertinent Examples. The assays were performed using a Biomek FX Robot (Beckman Coulter) or a multichannel pipettor (e.g., Rainin PipetLite, Mettler-Toledo) and a SpectraMAX MTP Reader (type 340; Molecular Devices).

A. TEST METHODS

Test Method 1

A protocol to define whether a dye or pigment material is a fabric hueing agent for the purpose of the invention is provided below:

1) Fill two tergotometer pots with 800ml of Newcastle upon Tyne, UK, City Water (~12 grains per US gallon total hardness, supplied by Northumbrian Water, Pity Me, Durham, Co. Durham, UK).

2) Insert pots into tergotometer, with water temperature controlled at 30°C and agitation set at 40rpm for the duration of the experiment.

3) Add 4.8g of IEC-B detergent (IEC 60456 Washing Machine Reference Base Detergent Type B), supplied by wfk, Brüggen-Bracht, Germany, to each pot.

4) After two minutes, add 2.0mg active colorant to the first pot.

5) After one minute, add 50g of flat cotton vest (supplied by Warwick Equest, Consett, County Durham, UK), cut into 5cm x 5cm swatches, to each pot.

6) After 10 minutes, drain the pots and re-fill with cold Water (16°C) having a water hardness of 14.4 English Clark Degrees Hardness with a 3:1 Calcium to Magnesium molar ratio.

7) After 2 minutes rinsing, remove fabrics.

8) Repeat steps 3-7 for a further three cycles using the same treatments.

9) Collect and line dry the fabrics indoors for 12 hours.

10) Analyze the swatches using a Hunter Miniscan spectrometer fitted with D65 illuminant and UVA cutting filter, to obtain Hunter a (red-green axis) and Hunter b (yellow-blue axis) values.

11) Average the Hunter a and Hunter b values for each set of fabrics. If the fabrics treated with colorant under assessment show an average difference in hue of greater than 0.2 units on either the a axis or b axis, it is deemed to be a fabric hueing agent for the purpose of the invention.

Test Method 2

For Test Method 2, the BMI microswatch assay provided below is run using the granular detergent composition 10 (see Table D above). The laundry detergent is dissolved in water that has a hardness of 12 gpg and adjusted to a temperature of 16°C, and the protease variant enzyme of interest is added. Performance of the protease variant enzymes is then determined as per the BMI microswatch assay described. The performance index is determined by comparing the performance of the protease variant enzyme with that of the *B. lentus* GG36 subtilisin enzyme having the amino acid sequence of SEQ ID NO:755, with in all cases the enzyme dosage being 1.6 ppm. Protease variant enzymes having a performance index of 1.1 or greater are viewed to be cold water protease variants.

Test Method 3

For Test Method 3, the BMI microswatch assay provided below is run using the granular laundry detergent composition 7 (see Table D above). The laundry detergent is dissolved in water that has a hardness of 6 gpg and adjusted to a temperature of 16°C, the GG36 protease variant enzyme of interest is added. Performance of the GG36 protease variant enzymes is then determined as per the BMI microswatch assay described. The performance index is determined by comparing the performance of the GG36 protease variant enzyme with that of the *B. lentus* GG36 subtilisin enzyme having the amino acid sequence of SEQ ID NO:755, with in all cases the enzyme dosage being 4 ppm. GG36 protease variant enzymes having a performance index of 1.1 or greater are viewed to be cold water protease variants.

Test Method 4

For Test Method 4, the BMI microswatch assay provided below is run using the granular laundry detergent composition 7 (see Table D above). The laundry detergent is dissolved in water that has a hardness of 6 gpg and adjusted to a temperature of 16°C, and the GG36 protease variant enzyme of

interest is added. Performance of the GG36 protease variant enzymes is then determined as per the BMI microswatch assay described. The performance index is determined by comparing the performance of the GG36 protease variant enzyme with that of a reference enzyme GG36-A158E, said GG36-A158E reference enzyme consisting of the *B. lentus* subtilisin GG36 protease amino acid sequence of SEQ ID NO:755 with a single substitution of glutamic acid for alanine at position 158 (i.e., the A158E mutation), with in all cases the enzyme dosage being 4 ppm. GG36 protease variant enzymes having a performance index of 1.0 or greater are viewed to be cold water protease variants.

Test Method 5

Electrical conductivity of an aqueous solution is assayed according to the standard method ASTM D1125 and reported in units of milliSiemens/cm, abbreviated to mS/cm herein.

B. ASSAYS

TCA Assay for Protein Content Determination in 96-well Microtiter Plates

For GG36 and GG36 variants, this assay was started using filtered *B. subtilis* culture supernatants from microtiter plates grown 2-3 days at 37 °C with shaking at 250 rpm and humidified aeration. A fresh 96-well flat bottom microtiter plate (MTP; Costar 9017 medium binding clear polystyrene plate) was used for the assay. First, 100 µL/well of 0.25 N HCl was placed in each well. Then, 20-25 µL of filtered culture supernatant were added and the solution was mixed on a table top mixer (e.g., Lab line Instruments, Titer plate shaker, model 4825) for 5-10 seconds. The light scattering/absorbance at 405 nm was then determined, in order to provide the "blank" reading. For the "test" reading, 100 µL/well of 30% (w/v) trichloroacetic acid (TCA) was added to each well containing the mixture of HCl and culture supernatant, and the plate was incubated for 10 minutes at room temperature. After briefly mixing the solution on a table top mixer for no more than 2-3 sec, the light scattering/absorbance at 405 nm was determined. The turbidity/light scatter increase in the samples correlates to the total amount of precipitable protein in the culture supernatant. The calculations were performed by subtracting the "blank" reading (obtained after addition of HCl only, no TCA) from the "test" reading (obtained after addition of TCA, as described above) to provide a relative measure of the protein content in the samples. If desired, a standard curve can be created by calibrating the TCA readings with AAPF assays of clones with known conversion factors. However, the TCA results are linear with respect to protein concentration from 50 to 500 parts per million (ppm) of protein (where 1 ppm corresponds to 1 mg/L) and can thus be plotted directly against enzyme performance for the purpose of choosing variants with desired performance.

AAPF Protease Assay in 96-well Microtiter Plates

In order to determine the protease activity of the serine protease variants, the hydrolysis of N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-p-nitroanilide (suc-AAPF-pNA) was measured. The

reagent solutions used were: 100 mM Tris/HCl, pH 8.6, containing 0.005% TWEEN®-80 (Tris dilution buffer); 100 mM Tris buffer, pH 8.6, containing 1 mM $CaCl_2$ and 0.005% TWEEN®-80 (Tris/Ca buffer); and 160 mM suc-AAPF-pNA in DMSO (suc-AAPF-pNA stock solution) (Sigma: S-7388). To prepare a suc-AAPF-pNA working solution, 1 ml suc-AAPF-pNA stock solution was added to 100 ml Tris/Ca buffer and mixed well for at least 10 seconds. The assay was performed by adding 10 µl of diluted protease solution to each well of a 96-well MTP, immediately followed by the addition of 190 µl of 1 mg/ml suc-AAPF-pNA working solution. The solutions were mixed for 5 sec, and the absorbance change in kinetic mode (25 readings in 5 minutes) was read at 405 nm in an MTP reader, at 25°C. The protease activity was expressed as AU (activity = $\Delta OD \cdot min^{-1} ml^{-1}$).

Eglin C Inhibition Assay

As described herein, serine protease concentration and specific activity was determined by titration with an inhibitor called eglin c. Eglin c from the leech *Hirudo medicinalis* is a tight-binding protein inhibitor of subtilisins and ASP protease (Heinz et al., Biochemistry, 31: 8755-66 [1992]), and can therefore be used to measure protease enzyme concentration, which in turn permits specific activity to be calculated. The gene for eglin c was synthesized and expressed in *E. coli* by standard methods. Its properties and inhibitory potency were the same as eglin c purchased from Sigma.

(i) Concentration Determination of an Eglin C Stock Solution

A sample of *Bacillus lentus* subtilisin of known specific activity was diluted in 100 mM Tris buffer, pH 8.6, containing 1 mM $CaCl_2$ and 0.005% TWEEN®-80 (Tris/Ca buffer), to a concentration appropriate for AAPF protease assay described above. Several dilutions of the eglin c stock solution were also made in the Tris/Ca buffer. An aliquot of each diluted eglin c solution was mixed with an equal volume of the diluted *Bacillus lentus* subtilisin solution. An aliquot of the Tris/Ca buffer only, without eglin c, was also mixed with an equal volume of the diluted *Bacillus lentus* subtilisin solution, in order to measure uninhibited subtilisin activity in the absence of eglin c. The mixed solutions were incubated at room temperature for 15-30 minutes and the protease activity of each sample was then measured by AAPF assay described above. Using the known specific activity of *Bacillus lentus* subtilisin, the concentration of active protease in each sample was determined. The concentration of eglin c in each sample was then calculated based on the decrease of the observed protease activity as compared to the uninhibited subtilisin sample that was mixed with Tris/Ca buffer only (without eglin c). Thus, using the known dilutions and volumes of the eglin c solutions, the concentration of eglin c in the stock solution was determined.

(ii) Concentration and Specific Activity Determination of Subtilisin Variants

Samples of subtilisin variants were diluted in 100 mM Tris buffer, pH 8.6, containing 1 mM $CaCl_2$ and 0.005% TWEEN®-80 (Tris/Ca buffer). Several dilutions of the eglin c stock solution of known concentration were also made in the Tris/Ca buffer. An aliquot of each diluted eglin c solution was mixed with an equal volume of a subtilisin variant solution. The mixed solutions were incubated at room temperature for 15-30 minutes and the protease activity of each sample was then measured by AAPF assay. Using the observed decrease of the protease activity upon addition of each eglin c sample and the known concentration of the eglin c, the concentration of the eglin c necessary for the complete inhibition of each subtilisin enzyme variant was calculated. This concentration is equivalent to the enzyme concentration in the sample. An aliquot of the Tris/Ca buffer only, without eglin c, was also mixed with each subtilisin variant sample and the protease activity in the absence of eglin c was measured by AAPF assay. The specific activity of the subtilisin variants was then calculated using the enzyme concentrations as determined above.

BMI Microswatch Assay

Blood milk and ink (BMI) stained microswatches (EMPA116) of 5.5 millimeter circular diameter were obtained from CFT. In one method, the EMPA116 BMI fabric is pre-rinsed in water prior to cutting them into a 96well microtiter plate (Corning 3641), one microswatch per well. In the second method the EMPA 116 cloth is cut directly into a 96 well microtiter plate (Corning 3641) where the swatches are then rinsed with two water washes. The rinses are carried out by adding 200 µl of Milli Q water to each well/swatch and mixing them on a table top mixer (Lab line instruments, Titer plate shaker, model 4825) for 15 minutes at a setting of 7. The wash liquor is removed and 200 µl of water is added again to the swatch for another 15 minute rinse. The wash water is removed and the swatches are then air dried in the microtiter plate.

Detergent compositions 7-11 were diluted in Milli-Q (deionized) water to final working concentrations described in Table 1-1. These detergents were buffered with 2 mM sodium carbonate, pH 10.3. Additionally, a water hardness composition (3:1 Ca:Mg. - $CaCl_2$: $MgCl_2{\cdot}6H_2O$) was added to each detergent solution to the final concentration described in Table 1-1. The detergent solutions were mixed at room temperature for 0.5 to 2 hours, centrifuged in 50 mL polypropylene conical tubes at 3000xg for 5-10 minutes and were kept at room temperature for the 32°C assays or pre-equilibrated in an ice-water bath for the 16°C assays. Then, 190 µl of the desired detergent solution was added to each well of the MTP containing BMI microswatches. To this mixture, 5 -15 µl of the diluted enzyme master dilution solution were added, making the approximate concentration of enzyme in the reaction 0.25-2 µg/ml. The enzyme master dilution solution was prepared from the filtered culture supernatants (see TCA assay described above) at ~2.5 - 20 µg/mL. The MTP was sealed with tape and placed in the iEMS incubator/shaker (Thermo/Labsystems) pre-set at 16°C in a refrigerator for 30 minutes or at 32°C on the benchtop for 30 minutes, with agitation at 1400 rpm. Following incubation under the appropriate conditions, 120-125 µl of the solution from each well was transferred into a fresh MTP (Corning 9017).

The new MTP containing 125 µl of solution/well was read at 600 nm (with 5 sec mixing mode in the plate reader) using the MTP SpectraMax reader. Blank controls containing a microswatch and detergent without any enzyme were also included. The absorbance value obtained was corrected for the blank value (substrate without enzyme), providing a measure of hydrolytic activity. For each sample (variant) the performance index was calculated. The performance index compares the performance of the variant (measured value) and the standard enzyme (theoretical value) at the same protein concentration. In addition, the theoretical values can be calculated, using the parameters of a performance dose response curve of the standard protease. A performance index (PI) that is greater than 1 (PI>1) identifies a better variant as compared to the standard (e.g., wild-type), while a PI of 1 (PI=1) identifies a variant that performs the same as the standard, and a PI that is less than 1 (PI<1) identifies a variant that performs worse than the standard. Thus, the PI identifies winners as well as variants that are less desirable for use under certain circumstances.

TABLE 1-1: Final Detergent, Water Hardness, and Buffer Concentrations Used for BMI Microswatch Assays

Detergent Composition	Final Detergent Concentration (g/L)	Final Water Hardness* (gpg)	Final Sodium Carbonate Buffer Concentration (mM)
7	0.808	6	2
8	1	3	2
9	2.3	12	2
10	5.9	12	2
11	8.3	12	2

LAS/EDTA Stability Assay

The stability of protease variants in the presence of a representative anionic surfactant (LAS=linear alkylbene sulfonate, sodium dodecylbenzenesulfonate-DOBS) and di-sodium EDTA is measured after incubation under defined conditions and the residual activity is determined using the AAPF assay described above. The reagents used were dodecyllbenzene sulfonate, sodium salt (DOBS; Sigma No. D-2525), TWEEN®-80 (Sigma No. P-8074), di-sodium EDTA (Siegfried Handel No. 164599-02), HEPES (Sigma No. H-7523), unstressed buffer: 50 mM HEPES (11.9 g/l) + 0.005% TWEEN®-80, pH 8.0, Stress buffer: 50 mM HEPES (11.9 g/l), 0.1% (w/v) DOBS (1 g/l), 10 mM EDTA (3.36 g/l), pH 8.0, reference protease and protease variant culture supernatants, containing 200 - 400 µg/ml protein. The equipment used is V- or U-bottom MTP as dilution plates (Greiner 651101 and 650161 respectively), F-bottom MTP (Corning 9017) for unstress and LAS/EDTA buffer as well as for suc-AAPF-pNA plates, Biomek FX (Beckman Coulter), Spectramax Plus 384 MTP Reader (Molecular Devices), and iEMS Incubator/Shaker (Thermo/Labsystems).

The iEMS incubator/shaker (Thermo/Labsystems) is set at 29°C. Culture supernatants were diluted into plates containing unstress buffer to a concentration of ~ 25 ppm (master dilution plate). For the assay, 20 µl of sample from the master dilution plate is added to plates containing 180 µl unstress

buffer to give a final incubation concentration of 2.5 ppm. The contents were mixed and kept at room temperature and the AAPF assay is performed on this plate. In addition, 20 µl of sample from the master dilution plate is also added to plates containing 180 µl stress buffer (50 mM HEPES (11.9 g/l), 0.1% (w/v) DOBS (1 g/l), 10 mM EDTA (3.36 g/l), pH 8.0). The solutions were mixed and immediately placed in 29°C iEMS shaker for 30 min at 400 rpm. Following 30 minutes of incubation, the AAPF assay is performed on the stress plate. The stability of the samples is determined by calculating the ratio of the residual and initial AAPF activity as follows: Residual Activity (%) = [mOD.min-1 stressed]*100 / [mOD. min-1 unstressed].

The final detergent, water hardness and buffer concentrations are determined based on the assay system to be used (e.g., North American, Japanese, Western European, or Central European conditions). In some aspects, the stain removal performance of the protease variants is determined in commercially available detergents. Heat inactivation of commercial detergent formulas serves to destroy the enzymatic activity of any protein components while retaining the properties of non-enzymatic components. Thus, this method is suitable for preparing commercially purchased detergents for use in testing the enzyme variants of the present invention.

Baked Egg Microtiter Assay

For this assay, 96-well baked egg yolk substrate plates are prepared from chicken egg yolks. Chicken egg yolks are separated from the whites, released from the membrane sac, and diluted 20% (vol/weight) with Milli-Q water. The diluted yolk is stirred for 15 min at room temperature using a magnetic stirrer. Five µL are carefully pipetted into the center of each well of a 96-well V-bottom plate (Costar #3894) using an 8-channel pipette. The plates are baked at 90°C for 1 hour and cooled at room temperature. The baked egg yolk substrate plates are stored at room temperature and used within one week of preparation. Automatic dish detergents are prepared as described herein and pre-heated to 50°C. A 190 µL aliquot of detergent is added to each well of the 96-well plate using an 8-channel pipette. Ten µL of diluted enzyme is added to each well using a 96-channel pipetting device. The plate is carefully sealed with an adhesive foil sealer and incubated at 50°C with shaking for 30 min. 120 µL of the reaction mixture is transferred to a new 96-well flat-bottom plate, and the absorbance/light scattering is determined at 405 nm. The absorbance/light scattering at 405 nm is proportional to egg yolk removal.

Egg Yolk Microswatch Assay ("CS-38 Microswatch Assay"; or "EGG" or "Dish")

Automatic dish detergents are prepared as described herein. The equipment used included a New Brunswick Innova 4230 shaker/incubator and a SpectraMAX (type 340) MTP reader. The MTPs are obtained from Costar (type 9017). Aged egg yolk with pigment swatches (CS-38) are obtained from Center for Test Materials (Vlaardingen, Netherlands). Before cutting 0.25-inch circular microswatches, the fabric is washed with water. One microswatch is placed in each well of a 96-well microtiter plate. The test detergent is equilibrated at 50°C. 190 µl of detergent solution is added to each well of the MTP,

containing microswatches. To this mixture, 10 µl of the diluted enzyme solution is added. The MTP is sealed with adhesive foil and placed in the incubator for 30 minutes, with agitation. Following incubation, 100 µl of the solution from each well is transferred into a fresh MTP. This MTP is read at 405 nm using a SpectraMax MTP reader. Blank controls, as well as controls containing microswatches and detergent but no enzyme are also included.

In some aspects, pre-washed microswatches find use. This type of microswatch is pre-washed in deionised water for 20 minutes at ambient temperature. After the pre-washing step, the swatches are put on top of paper towels to dry. The air-dried swatches are then punched using a ¼" circular die on an expulsion press. Finally two microswatches are put into each well of a 96-well MTP vertically to expose the whole surface area (i.e. not flat on the bottom of the well).

Samples of protease variants to be tested are obtained from filtered culture broth of cultures grown in MTP plates. The equipment used is a Biomek FX Robot (Beckman Coulter), a SpectraMAX MTP Reader (type 340; Molecular Devices), an iEMS incubator/shaker (Thermo/Labsystems); F-bottom MTPs (Costar type 9017 used for reading reaction plates after incubation); and V-bottom MTPs (Greiner 651101 used for pre-dilution of supernatant). In this assay, the proteases hydrolyze the substrate and liberate pigment and insoluble particles from the substrate. Thus the rate of turbidity is a measure of enzyme activity.

The stain removal performance of reference serine proteases and variants therefrom on microswatches is determined on a MTP scale in commercially available detergent (Calgonit 5 in1). CS-38 microswatches (egg-yolk with pigment, aged by heating), obtained from CFT Vlaardingen are used as substrate. Two swatches are used per well. ADW tablets from Calgonit 5in1 are used to prepare the detergent solution. To inactivate the protease activity present in the tablets, a 21g tablet is dissolved in Milli-Q water heated in a water bath to a temperature of 60°C. The solution is cooled to room temperature and the volume of water adjusted to 700 mL. The solution is further diluted with water to achieve a final concentration of 3 g/l. Water hardness is adjusted to 21°GH by adding 1.46 ml of the Ca/Mg-mixture (Ca/Mg mixture [(3:1), 1.92 M $CaCl_2$ =282.3 g/L $CaCl_2.2H_20$; 0.64 M $MgCl_2$ = 130.1 g/L $MgCl_2 \cdot 6H_2O$), 15000gpg]. The enzyme samples are prediluted in 10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN®-80 solution and tested at appropriate concentrations.

The incubator is set at the desired temperature of 50°C. 72 µl of dilution buffer is added to the empty V-bottom plate (i.e., a "dilution plate") followed by 8 µl supernatant. 9 µl from the dilution plate is added to plates containing the microswatches incubated in 171 µl detergent solution. 9 µl from the dilution plate is added to plates containing the microswatches to give a total dilution of supernatant of 200X. The microswatch plate (with detergent and enzyme) is covered with tape and placed in the incubator/shaker for 30 minutes at 1400 rpm. Following incubation, 75 µl of the reaction mixture is transferred to an empty F-bottom plate and the absorbance is read in a MTP Reader at 405 nm after de-bubbling with a hair dryer. Blank controls, containing one or two microswatches and detergent without the addition of reference protease containing samples are also included in the test.

The absorbance value obtained is corrected for the blank value (substrate without enzyme), providing a measure of hydrolytic activity. For each sample (variant) the performance index is calculated. The performance index compares the performance of the variant (actual value) and the standard enzyme (theoretical value) at the same protein concentration. In addition, the theoretical values can be calculated, using the parameters of the Langmuir equation of the standard enzyme.

Egg Yolk Stains on Stainless Steel

The stainless steel sheets (10 x 15 cm; brushed on one side) used in these experiments are thoroughly washed at 95°C in a laboratory dishwasher with a high-alkalinity commercial detergent (e.g., ECOLAB® detergent; Henkel) to provide sheets that are clean and grease-free. These sheets are deburred prior to their first use. The sheets are dried for 30 minutes at 80°C in a thermal cabinet before being soiled with egg yolk. The surfaces to be brushed are not touched prior to soiling. Also, no water stains or fluff on the surfaces are permitted. The cooled sheets are weighed before soiling.

The egg yolks are prepared by separating the yolks of approximately 10-11 eggs (200 g of egg yolk) from the whites. The yolks are stirred with a fork in a glass beaker to homogenize the yolk suspension. The yolks are then strained (approx. 0.5 mm mesh) to remove coarse particles and any egg shell fragments. A flat brush (2.5") is used to apply 1.0 ± 0.1 g egg yolk suspension as uniformly as possible over an area of 140 cm^2 on the brushed sides of each of the stainless steel sheets, leaving an approx. 1 cm wide unsoiled rim (adhesive tape is used if needed). The soiled sheets are dried horizontally (to prevent formation of droplets on the edges of the sheets), at room temperature for 4 hours (max. 24 h).

For denaturation, the sheets are immersed for 30 seconds in boiling, demineralized water (using a holding device if necessary). Then, the sheets are dried again for 30 min at 80°C. After drying and cooling, the sheets are weighed. After weighing, the sheets are left for at least 24 hours (20°C, 40-60% relatively humidity) before submitting them to the wash test. In order to meet the testing requirements, only sheets with 500 ± 100 mg/140 cm^2 (egg yolk after denaturation), are used in the testing. After the wash tests are conducted, the sheets are dried for 30 min at 80°C, in the thermal cabinet, and weighed again after cooling. The percent cleaning performance is determined by dividing the (mg of egg yolk released by washing x 100) by the (mg of denatured egg yolk applied).

Minced Meat on Porcelain Plates

For these experiments, dessert plates (Arzberg, white, glazed porcelain) conforming to EN 50242, form 1495, No. 0219, diameter 19 cm are used. A total of 225 g lean pork and beef (half and half) is finely chopped and cooled, after removing visible fat. The mixture is twice run through a mincer. Temperatures above 35°C are avoided. Then, 225 g of the minced meat is mixed with 75 g of egg (white and yolk mixed together). The preparation is then frozen up to three months at -18°C, prior to use. If pork is not available, beef is used.

The minced meat and egg mixture (300 g) is brought up to room temperature and mixed with 80 ml synthetic water. The mixture is then homogenized using a kitchen hand blender for 2 min. Then, a fork is used to spread 3 g of the minced meat/egg/water mixture on each white porcelain plate, leaving an approx. 2 cm wide unsoiled margin around the rim. The amount applied is 11.8 ± 0.5 mg/cm². The plates are dried for 2 hours at 120°C in a preheated thermal cabinet. As soon as the plates are cooled, they are ready for use. The plates are stacked with paper towels between each of the plates.

After washing, the plates are sprayed with ninhydrin solution (1% ethanol) for better identification of the minced meat residues. To promote the color reaction, the plates are heated for 10 min at 80°C in the thermal cabinet. Evaluation of the washing performance is done by visually inspecting the color reactions of the minced meat residues with reference to the IKW photographic catalogue (IKW).

Egg/Milk Stains on Stainless Steel

The stainless steel sheets (10 x 15 cm; brushed on one side) used in these experiments are thoroughly washed at 95°C in a laboratory dishwasher with a high-alkalinity commercial detergent to remove grease and clean the sheets. The sheets are polished dry with a cellulose cloth. The surfaces to be brushed are not touched prior to soiling. Also, no water stains or fluff on the surfaces are permitted. Before soiling, the sheets are placed in a thermal cabinet at 80°C, for 30 min. The cooled sheets are weighed before soiling.

The egg yolks and whites of whole raw eggs (3-4 eggs; 160 g/egg) are placed in a bowl and beaten with an egg whisk. Then, 50 ml semi-skimmed UHT (1.5% fat, ultra-high temperature, homogenized) milk are added to the mixture. The milk and egg are mixed without generating froth. A flat brush is used to uniformly distribute 1.0 ± 0.1 g of the egg/milk mixture on the brushed side of the stainless steel sheets, using a balance to check the distribution. A margin of approximately 1.0 cm is left around the short sides of the sheets. The soiled sheets are dried horizontally (to prevent formation of droplets on the edges of the sheets), at room temperature for 4 hours (max. 24 h).

The sheets are then immersed for 30 seconds in boiling, demineralized water (using a holding device if necessary). Then, the sheets are dried again for 30 min at 80°C. After drying and cooling, the sheets are weighed. After weighing, the sheets are left for at least 24 hours (20°C, 40-60% relatively humidity), before submitting them to the wash test. In order to meet the testing requirements, only sheets with 190 ± 10 mg egg yolk are used.

After the wash tests are conducted, the sheets are dried for 30 min at 80°C, in the thermal cabinet, and weighed again after cooling. The percentage cleaning performance is determined by dividing the (mg of egg/milk released by washing x 100) by the (mg of egg/milk applied).

Preparation of the Spaghetti Mix Stain on Porcelain Plates

Pasta sauce (390 g) is mixed with 150 g of boiled spaghetti pasta, 25 g of minced meat (improved IKW composition-a combination of 225 gram fat free minced meat and 75 gram egg yolk) and 50 g of

Grozette Formaggio cheese. A spoon is used to spread 3 g of this mixture on each white porcelain plate (Arzberg, 19cm diameter, white, glazed porcelain, conforming to EN 50242, form 1495, No. 0219) leaving an approximately 2 cm wide unsoiled margin around the rim. The plates are dried by baking them for 2 hours at 120°C in an oven. As soon as the plates are cooled, they are ready for use. The plates are stacked with paper towels between each of the plates for storage. After washing, the plates are sprayed with iodine solution (0.05N) for better identification of the carbohydrate residues. Evaluation of the washing performance is done by visually inspecting the color reactions of the carbohydrate residues with reference to the IKW photographic catalogue (IKW) and rated on a scale of 0-10 (10 being clean).

Performance index

The performance index compares the performance of the variant (measured value) and the standard enzyme (theoretical value) at the same protein concentration. In addition, the theoretical values can be calculated, using the parameters of a performance dose response curve of the standard protease. Various terms set forth below are used to describe the variant: non-deleterious variants have a PI > 0.05; deleterious variants have a PI = 0.05; combinable variants are those for which the variant has performance index values greater than or equal to 0.2 for at least one property, and >0.05 for all properties. Combinable variants are those that can be combined to deliver proteins with appropriate performance indices for one or more desired properties. These data find use in engineering any subtilisin/subtilase. Even if the subtilase to be engineered has an amino acid different from that of subtilisin GG36 at particular positions, these data find use in finding substitutions that will alter the desired properties by identifying the best choices for substitutions, including substitutions to the GG36 wild type amino acid.

EXAMPLE 2

Generation of GG36 Single Mutants Using Site Evaluation Libraries (SELs)

The construction of GG36 SELs described in this example was performed by GENEART using their proprietary methods and technology platform for gene optimization, gene synthesis, library generation and analysis (WO 2004/059556A3, European Patent Nos. 0 200 362 and 0 201 184; and US Patent Nos. 4,683,195, 4,683,202 and 6,472,184). The GG36 SELs were produced at positions pre-selected by the inventors using the pHPLT-GG36 *B. subtilis* expression plasmid (see Figure 6). This *B. subtilis* expression plasmid contains the GG36 expression cassette shown below, the *B. licheniformis* LAT promoter (Plat), and additional elements from pUB110 (McKenzie et al., Plasmid, 15:93-103, 1986) including a replicase gene (reppUB), a neomycin/kanamycin resistance gene (neo) and a bleomycin resistance marker (bleo) (Figure 4 in U.S. Patent No. 6,566,112). The pHPLT-GG36 plasmid map is provided at Figure 6. The GG36 expression cassette sequence is provided below.

The DNA sequence of GG36 (the signal sequence is shown in lower case letters, propeptide in lower case, underlined text, and GG36 mature sequence in uppercase letters) is provided below:

Gtgagaagcaaaaaattgtggatcgtcgcgtcgaccgcactactcatttctgttgctttcagttcatcgatcgcatcggctgctgaagaagcaaaagaaaa
atatttaattggctttaatgagcaggaagctgtcagtgagtttgtagaacaagtagaggcaaatgacgaggtcgccattctctctgaggaagaggaagtcg
aaattgaattgcttcatgaatttgaaacgattcctgttttatccgttgagttaagcccagaagatgtggacgcgcttgagctcgatccagcgatttcttatattg
aagaggatgcagaagtaacgacaatgGCGCAATCAGTGCCATGGGGAATTAGCCGTGTGCAAGCCCCAGC
TGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCTGTCCTCGATACAGGTATTT
CCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTTGTACCAGGGGAACCATCCACT
CAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACGATTGCTGCTTTAAACAATTCGA
TTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACTATACGCTGTTAAAGTATTAGGGGCGAG
CGGTTCAGGTTCGGTCAGCTCGATTGCCCAAGGATTGGAATGGGCAGGGAACAATGGCATG
CACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCAAGTGCCACACTTGAGCAAGCTGTTAA
TAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCGGCATCTGGAAATTCAGGTGCAGGCTCAA
TCAGCTATCCGGCCCGTTATGCGAACGCAATGGCAGTCGGAGCTACTGACCAAAACAACAA
CCGCGCCAGCTTTTCACAGTATGGCGCAGGGCTTGACATTGTCGCACCAGGTGTAAACGTGC
AGAGCACATACCCAGGTTCAACGTATGCCAGCTTAAACGGTACATCGATGGCTACTCCTCAT
GTTGCAGGTGCAGCAGCCCTTGTTAAACAAAAGAACCCATCTTGGTCCAATGTACAAATCCG
CAATCATCTAAAGAATACGGCAACGAGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTT
GTCAATGCAGAAGCTGCAACTCGTTAA (SEQ ID NO:756)

The protein sequence of GG36 (the signal sequence is shown in lower case letters, propeptide in lower case, underlined text, and GG36 mature protease sequence in uppercase letters) is provided below:

vrskklwivastallisvafsssiasaaeeakekyligfneqeavsefveqveandevailseeeeveiellhefetipvlsvelspedvdaleldpaisy
ieedaevttmAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQD
GNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMHVA
NLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSISYPARYANAMAVGATDQNNNRASFS
QYGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKN
TATSLGSTNLYGSGLVNAEAATR (SEQ ID NO:757).

The method of mutagenesis was based on the codon-specific mutation approach in which all possible amino acid substitutions are simultaneously created at a specific codon of interest using forward and reverse mutagenesis primers that contain a degenerate codon, NNS ((A,C,T or G), (A,C,T or G), (C or G)) at the site of interest. To construct each of the GG36 SELs, three PCR reactions were performed: two mutagenesis reactions (primary PCR1 and PCR2) to introduce the mutated codon of interest in the mature GG36 DNA sequence using the NNS forward and reverse mutagenesis primers (25-45 nucleotides long), and a third reaction to fuse the two mutagenesis PCR products together to construct the pHPLT-GG36 expression vector having the desired mutated codons in the mature GG36 sequence.

The primer sequences used in this Example are provided below:

Table 2-1. Primers	
Sequence	**Primer Name**
CGCGCTT**GAGCTC**GATCCAGCGATTTC (SEQ ID NO:758)	SacI-Fw
GTCTCC**AAGCTT**TAACGAGTTGCAG (SEQ ID NO:759)	HindIII-Rv
GCAATTC**AGATCT**TCCTTCAGGTTATGACC (SEQ ID NO:760)	pHPLT-BglII-Fw
GCATCGA**AGATCT**GATTGCTTAACTGCTTC (SEQ ID NO:761)	pHPLT-BglII-Rv

The Phusion High-Fidelity DNA Polymerase (Finnzymes catalog no. F-530L) was used for all PCRs, and the reactions were executed according to manufacturer's protocols that were supplied with the polymerase. In particular, for primary PCR 1, 1 μL (10 μM) of each of the pHPLT-BglII-Fw primer and a NNS reverse mutagenesis primer were used, and for primary PCR 2, 1 μL (10 μM) of the pHPLT-BglII-Rv primer and a NNS forward mutagenesis primer were used. Each reaction also included 1 μL of the pHPLT-GG36 plasmid template DNA (0.1 – 1 ng/ μL). An MJ Research PTC-200 Peltier thermal cycler was used for the PCRs. The reactions yielded two fragments of approximately 2 to 3 kb having approximately 30 nucleotide overlap surrounding the GG36 codon of interest. The fragments obtained were fused in a third PCR similar to the ones described above using 1 μL of primary PCR 1 reaction mix, 1 μL of primary PCR 2 reaction mix and 1 μL (10 μM) of each of the forward and reverse SacI-Fw and HindIII-Rv primers. The amplified linear 859 bp fragment encoding the GG36 variant gene was purified (using QIAGEN® Qiaquick PCR purification kit) and digested with the *Sac*I and *Hin*dIII restriction enzymes to create cohesive ends on both sides of the fusion fragment. About 50 ng of plasmid pHPLT-GG36 was also purified after digestion with *Sac*I and *Hin*dIII, resulting in a 3.9kb vector backbone fragment. The digested vector fragment was ligated with 50 ng of the digested 859 bp fragment encoding the variant enzyme using the T4 DNA ligase (Invitrogen) following the manufacturer's protocol for cloning of cohesive ends. Subsequently, the ligation mixture was used to transform *B. subtilis* cells (*ΔaprE, ΔnprE, oppA, ΔspoIIE, degUHy32, ΔamyE::[xylR,pxylA-comK]*) as described (WO 2002/014490).

To express the variant proteins for further biochemical analyses, the *B. subtilis* strains carrying the GG36 variant plasmids were inoculated into microtiter plates containing 150μl Luria broth medium supplemented with 10μg/ml neomycin. Plates were grown overnight at 37°C with 300 rpm shaking and 80% humidity using Enzyscreen lids for microtiter plates (Enzyscreen). Ten microliters from the overnight culture plate were used to inoculate a new microtiter plate containing 190μl of MBD medium (a MOPS based defined medium) with 10ug/ml neomycin. MBD medium was prepared essentially as known in the art (see Neidhardt et al., J. Bacteriol. 119:736-747 [1974]), except that NH_4Cl_2, $FeSO_4$, and $CaCl_2$ were omitted from the base medium, 3 mM K_2HPO_4 was used, and the base medium was supplemented with 60 mM urea, and 100ml of a solution made of 210 g/L glucose, and 350 g/L maltodextrin. The micronutrients were made up as a 100X stock solution containing in one liter, 400 mg

$FeSO_4 \cdot 7H_2O$, 100 mg $MnSO_4 \cdot H_2O$, 100 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $CuCl_2 \cdot 2H_2O$, 100 mg $CoCl_2 \cdot 6H_2O$, 100 mg $NaMoO_4 \cdot 2H_2O$, 100 mg $Na_2B_4O_7 \cdot 10H_2O$, 10 ml of 1M $CaCl_2$, and 10 ml of 0.5 M sodium citrate. The MBD medium containing microtiter plates were grown for 68 hours at 37°C, 300 rpm, and 80% humidity using Enzyscreen lids (Enzyscreen) for determining protein expression. The next day, cultures were filtered through a micro-filter plate (0.22µm; Millipore) and the resulting filtrate was used for biochemical analysis. The TCA and BMI microswatch assays for the detergent compositions 7-11 were carried out as described in Example 1. Performance indices were also calculated as described under the BMI assay description in Example 1, and they are shown in Table 2-2 relative to GG36. In the following Tables, the detergent compositions ("Det.") correspond to those shown in Table D, above. Also, as indicated, the amino acid position is listed according to BPN' numbering.

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
GG36 Amino Acid Position (BPN' Numbering)	**WT Residue**	**Mutant Residue**
1	A	R
2	Q	A
2	Q	R
2	Q	S
2	Q	M
2	Q	W
3	S	R
4	V	R
4	V	S
4	V	C
8	I	A
9	S	W
9	S	F
9	S	A
10	R	A
10	R	M
10	R	S
10	R	H
12	Q	F
12	Q	R
14	P	F
14	P	K
14	P	Q
15	A	R
15	A	F
16	A	S
17	H	R
17	H	F
17	H	M
18	N	R
18	N	K
20	G	R
20	G	K
20	G	F
22	T	R
22	T	Q

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
22	T	L
22	T	V
22	T	W
22	T	Y
22	T	A
23	G	A
23	G	S
23	G	F
24	S	R
24	S	W
24	S	H
24	S	L
24	S	Q
24	S	F
25	G	R
25	G	F
25	G	V
26	V	F
27	K	R
27	K	L
27	K	V
27	K	F
28	V	A
28	V	E
28	V	N
29	A	T
30	V	E
31	L	F
33	T	S
33	T	G
33	T	D
34	G	P
35	I	M
36	S	T
36	S	F
36	S	R
38	T	R
38	T	F
38	T	L
40	P	H
40	P	W

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
40	P	R
40	P	N
40	P	T
40	P	L
42	L	I
43	N	R
43	N	A
43	N	S
43	N	W
43	N	F
43	N	I
43	N	D
43	N	M
45	R	T
46	G	R
48	A	R
50	F	C
51	V	W
51	V	F
51	V	H
52	P	F
52	P	N
52	P	E
55	P	Y
57	T	R
59	Q	A
59	Q	F
59	Q	R
60	D	P
60	D	A
60	D	Q
62	N	Q
62	N	E
63	G	S
63	G	A
63	G	M
63	G	V
63	G	T
63	G	H
63	G	Q
63	G	I

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
63	G	D
63	G	E
63	G	P
64	H	F
64	H	T
68	V	A
68	V	C
69	A	N
69	A	T
69	A	W
69	A	P
71	T	G
72	I	C
74	A	C
75	L	R
75	L	A
75	L	E
75	L	F
78	S	R
78	S	I
78	S	N
79	I	Q
79	I	W
81	V	R
82	L	R
82	L	T
82	L	M
82	L	F
82	L	V
85	A	M
86	P	W
86	P	I
86	P	L
89	E	P
89	E	W
89	E	T
89	E	I
89	E	H
89	E	V
89	E	F
89	E	L

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
89	E	W
89	E	G
91	Y	F
91	Y	N
92	A	F
94	K	N
99	S	F
99	S	T
99	S	M
99	S	G
99	S	P
100	G	I
100	G	S
100	G	N
100	G	Q
101	S	N
101	S	G
101	S	T
101	S	A
101	S	D
101	S	F
101	S	D
101	S	E
101	S	P
102	G	A
102	G	N
102	G	T
102	G	E
102	G	H
103	S	N
103	S	G
103	S	D
104	V	L
104	V	I
104	V	E
104	V	D
105	S	T
105	S	Q
105	S	E
106	S	V
106	S	G

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
106	S	T
106	S	A
106	S	E
106	S	D
106	S	F
107	I	F
107	I	M
108	A	I
108	A	G
109	Q	M
111	L	V
111	L	I
112	E	V
112	E	L
112	E	Q
114	A	G
115	G	R
115	G	K
116	N	L
116	N	A
116	N	K
117	N	F
118	G	I
118	G	R
119	M	C
120	H	A
120	H	F
120	H	R
121	V	E
121	V	F
123	N	G
123	N	E
124	L	S
128	S	N
128	S	M
128	S	H
128	S	Q
128	S	I
128	S	F
128	S	L
128	S	D

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
129	P	E
132	S	A
132	S	E
138	A	G
144	S	R
147	V	L
148	L	I
158	A	E
159	G	C
159	G	E
160	S	D
166	S	E
166	S	D
167	Y	W
175	M	V
177	V	C
181	D	A
182	Q	R
183	N	D
183	N	R
183	N	I
183	N	F
183	N	M
185	N	I
185	N	E
185	N	V
186	R	H
186	R	K
188	S	R
188	S	E
188	S	D
192	Y	W
192	Y	H
194	A	V
194	A	F
194	A	E
197	D	F
198	I	L
198	I	F
203	V	E
203	V	C

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
208	T	S
209	Y	N
209	Y	S
209	Y	F
209	Y	T
209	Y	H
209	Y	L
209	Y	G
209	Y	E
210	P	V
210	P	R
210	P	L
211	G	R
211	G	Q
212	S	I
212	S	F
212	S	M
213	T	A
214	Y	F
215	A	F
215	A	N
215	A	H
215	A	E
215	A	D
216	S	F
216	S	A
217	L	E
217	L	N
217	L	D
218	N	P
218	N	E
218	N	D
224	T	A
224	T	G
227	V	I
230	A	E
231	A	I
231	A	C
233	L	C
234	V	F
235	K	F

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
236	Q	N
236	Q	F
238	N	R
238	N	K
238	N	L
239	P	R
239	P	S
239	P	R
239	P	H
239	P	N
239	P	K
239	P	T
239	P	F
239	P	G
240	S	R
241	W	R
242	S	R
242	S	L
243	N	R
243	N	F
244	V	R
246	I	S
248	N	I
248	N	V
248	N	R
249	H	R
249	H	T
250	L	I
251	K	R
251	K	S
252	N	R
252	N	F
252	N	H
252	N	I
253	T	R
253	T	F
253	T	I
254	A	C
256	S	N
258	G	R
260	T	V

Table 2-2. Single Variants of GG36 with Performance Indices of at Least 0.2 Relative to GG36 in Either TCA or BMI Microswatch Cleaning at 16°C in Detergents 7-11.		
260	T	I
262	L	H
262	L	D
263	Y	F
265	S	F
267	L	N
267	L	M
267	L	V
269	N	R
269	N	I
270	A	C
271	E	T
271	E	V
271	E	L
271	E	H
271	E	F
271	E	P
271	E	A
271	E	M
271	E	I
272	A	F
272	A	R
273	A	I
273	A	F
274	T	G

Table 2-3. GG36 Single Variants with Performance Indices of at Least 1.5 Relative to GG36 BMI Microswatch Cleaning at 32°C in Detergent 7.
GG36 Variant
N62E
A158E
G159E

Table 2-4. GG36 Single Variants with Performance Indices of at least 1.2 Relative to GG36 BMI Microswatch Cleaning at 32°C in Detergent 10.
GG36 Variant
A1R
S78R
V244R
N269R
E271L

EXAMPLE 3

Construction and Cleaning Performance of the NHJ1 and WCE1 Sets of GG36 Variants

The NHJ1 and WCE1 set of GG36 variants described herein were constructed at DNA 2.0, Inc., using the pHPLT-GG36 *B. subtilis* expression plasmid described above (Figure 6). The variants were expressed in *B. subtilis* cells (genotype: ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo) as described in Example 2, and were further characterized using the TCA assay for protein content determination, LAS/EDTA stability assay, and BMI microswatch cleaning assay as described in Example 1. These results are shown in Tables 3-1 and 3-2. In the following Tables, the detergent compositions ("Det.") correspond to those shown in Table D, above. Also, as indicated, the amino acid position is listed according to BPN' numbering.

TABLE 3-1. NHJ1 Variants with Performance Indices of at least 0.25 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 7, 8 or 9.
GG36 Variant (BPN' Numbering)
N062E-A158E
S103G-A158E
S128N-A158E
A016S-A158E
V104L-A158E
E089P-A158E
L111V-A158E
T022A-A158E
S101A-A158E
L148I-A158E
P129E-A158E
T022A-E089P
A016S-E089P
N062E-E089P
N062E-E271F
A158E-E271F
R186H-E271F
P129E-E271F
L111V-E271F
Y209E-E271F
A016S-E271F
S188D-E271F
T022A-E271F
G159E-E271F
V104L-E271F
S101A-E271F
E089P-E271F
S128N-E271F
S103G-E271F
L148I-E271F
H249R-E271F
N062E-G159E
A016S-G159E
S128N-G159E
L148I-G159E
L111V-G159E
E089P-G159E
T022A-G159E

TABLE 3-1. NHJ1 Variants with Performance Indices of at least 0.25 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 7, 8 or 9.
GG36 Variant (BPN' Numbering)
P129E-G159E
S103G-G159E
V104L-G159E
A158E-G159E
S101A-G159E
A158E-H249R
L111V-H249R
P129E-H249R
N062E-H249R
A016S-H249R
R186H-H249R
L148I-H249R
G159E-H249R
S101A-H249R
S188D-H249R
V104L-H249R
Y209E-H249R
T022A-H249R
S128N-H249R
S103G-H249R
E089P-H249R
T022A-L111V
S101A-L111V
A016S-L111V
V104L-L111V
N062E-L111V
S103G-L111V
E089P-L111V
A016S-L148I
N062E-L148I
T022A-L148I
P129E-L148I
V104L-L148I
S103G-L148I
S128N-L148I
S101A-L148I
E089P-L148I
L111V-L148I

TABLE 3-1. NHJ1 Variants with Performance Indices of at least 0.25 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 7, 8 or 9.
GG36 Variant (BPN' Numbering)
A016S-N062E
T022A-N062E
N062E-P129E
T022A-P129E
S128N-P129E
A016S-P129E
S101A-P129E
V104L-P129E
E089P-P129E
S103G-P129E
L111V-P129E
N062E-R186H
S128N-R186H
S101A-R186H
T022A-R186H
A016S-R186H
A158E-R186H
E089P-R186H
P129E-R186H
G159E-R186H
S103G-R186H
V104L-R186H
L111V-R186H
L148I-R186H
N062E-S101A
T022A-S101A
A016S-S101A
E089P-S101A
N062E-S103G
T022A-S103G
A016S-S103G
S101A-S103G
E089P-S103G
N062E-S128N
A016S-S128N
T022A-S128N
S101A-S128N
V104L-S128N

TABLE 3-1. NHJ1 Variants with Performance Indices of at least 0.25 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 7, 8 or 9.
GG36 Variant (BPN' Numbering)
E089P-S128N
S103G-S128N
L111V-S128N
L111V-S188D
N062E-S188D
A016S-S188D
L148I-S188D
T022A-S188D
S128N-S188D
S101A-S188D
V104L-S188D
E089P-S188D
P129E-S188D
G159E-S188D
R186H-S188D
S103G-S188D
A158E-S188D
A016S-T022A
A016S-V104L
T022A-V104L
S101A-V104L
N062E-V104L
S103G-V104L
E089P-V104L
G159E-Y209E
L111V-Y209E
S101A-Y209E
A016S-Y209E
S128N-Y209E
L148I-Y209E
P129E-Y209E
N062E-Y209E
T022A-Y209E
S103G-Y209E
A158E-Y209E
S188D-Y209E
V104L-Y209E
E089P-Y209E

TABLE 3-1. NHJ1 Variants with Performance Indices of at least 0.25 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 7, 8 or 9.
GG36 Variant (BPN' Numbering)
R186H-Y209E
GG36

TABLE 3-2. WCE1 Variants with Performance Indices of at least 0.2 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 10 or 11.
GG36 Variant (BPN' Numbering)
N018R-W241R
G020R-W241R
S024R-W241R
S009A-W241R
G020R-W241R
V004R-W241R
N043R-W241R
S078R-W241R
T022R-W241R
G115R-W241R
A001R-W241R
S212F-W241R
L082R-W241R
N018R-V244R
S024R-V244R
S078R-V244R
G020R-V244R
S212F-V244R
S009A-V244R
L082R-V244R
A001R-V244R
N043R-V244R
T022R-V244R
V004R-V244R
G115R-V244R
W241R-V244R
S242R-V244R
A001R-V004R
S009A-T022R
N018R-T022R
G020R-T022R
V004R-T022R
A001R-T022R
S024R-S242R
N018R-S242R
V004R-S242R
G020R-S242R
S212F-S242R

TABLE 3-2. WCE1 Variants with Performance Indices of at least 0.2 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 10 or 11.
GG36 Variant (BPN' Numbering)
L082R-S242R
S078R-S242R
A001R-S242R
S009A-S242R
T022R-S242R
G115R-S242R
N043R-S242R
W241R-S242R
N018R-S212F
T022R-S212F
V004R-S212F
S024R-S212F
A001R-S212F
G115R-S212F
G020R-S212F
S009A-S212F
N043R-S212F
S078R-S212F
L082R-S212F
S009A-S078R
G020R-S078R
S024R-S078R
T022R-S078R
N018R-S078R
V004R-S078R
A001R-S078R
N043R-S078R
T022R-S024R
G020R-S024R
N018R-S024R
A001R-S024R
V004R-S024R
S009A-S024R
V004R-S009A
A001R-S009A
S242R-N269R
S024R-N269R
G020R-N269R

TABLE 3-2. WCE1 Variants with Performance Indices of at least 0.2 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 10 or 11.
GG36 Variant (BPN' Numbering)
T022R-N269R
H249R-N269R
S212F-N269R
N043R-N269R
V244R-N269R
A001R-N269R
N018R-N269R
S078R-N269R
S009A-N269R
G115R-N269R
W241R-N269R
V004R-N269R
L082R-N269R
N018R-N043R
G020R-N043R
V004R-N043R
T022R-N043R
S009A-N043R
A001R-N043R
S024R-N043R
S009A-N018R
V004R-N018R
A001R-N018R
S024R-L082R
S009A-L082R
N018R-L082R
A001R-L082R
S078R-L082R
G020R-L082R
T022R-L082R
V004R-L082R
N043R-L082R
N043R-H249R
G020R-H249R
V004R-H249R
N018R-II249R
S009A-H249R
S212F-H249R

TABLE 3-2. WCE1 Variants with Performance Indices of at least 0.2 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 10 or 11.
GG36 Variant (BPN' Numbering)
T022R-H249R
S024R-H249R
G115R-H249R
A001R-H249R
L082R-H249R
S242R-H249R
W241R-H249R
V244R-H249R
S078R-II249R
N018R-G115R
G020R-G115R
T022R-G115R
S078R-G115R
S009A-G115R
V004R-G115R
A001R-G115R
L082R-G115R
N043R-G115R
S024R-G115R
S009A-G020R
N018R-G020R
V004R-G020R
A001R-G020R
S009A-E271L
G020R-E271L
S024R-E271L
V244R-E271L
W241R-E271L
N043R-E271L
T022R-E271L
H249R-E271L
S212F-E271L
G115R-E271L
S242R-E271L
S078R-E271L
V004R-E271L
N269R-E271L
A001R-E271L

TABLE 3-2. WCE1 Variants with Performance Indices of at least 0.2 Relative to GG36 in Any One of TCA , LAS/EDTA Stability, or BMI Microswatch Cleaning at 16°C in Detergents 10 or 11.
GG36 Variant (BPN' Numbering)
N018R-E271L
L082R-E271L
GG36

EXAMPLE 4

Construction and Cleaning Performance of NHJ4 Set of GG36 Variants

The NHJ4 set of GG36 variants described in Table 4-4 below were constructed using the pHPLT-GG36 *B.* subtilis expression plasmid (Figure 6) using PCR fusion or the QUIKCHANGE® Multi Site-directed mutagenesis kit ("QCMS kit"; Stratagene) as described below.

a) Construction of NHJ4 Variants by QUIKCHANGE® Multi Site-Directed Mutagenesis

Variants created using the QUIKCHANGE® Multi Site-Directed Mutagenesis are shown in Table 4-1. The parent plasmid pHPLT-GG36 (template DNA) was methylated using two micrograms of DNA and Dam methylase (NEB), according to the manufacturer's instructions. Site-directed mutants were made by a QuikChange® Multi Site-Directed Mutagenesis Kit ("QCMS kit"; Stratagene) following the manufacturer's protocol. The following mutations were introduced in the parent plasmid S101A-S103G-V104L, G159E, T22A, Y209E, E271F, S101A, S103G, L111V, S128N, N62E, and S188D, For efficient transformation of *B. subtilis*, DNA from the QCMS reaction mixtures was amplified by rolling circle amplification (RCA) using the Illustra Templiphi kit (GE Healthcare) and the reaction was performed according to the manufacturer's protocol. One microliter of ten-fold diluted amplified DNA was used to transform 50 µL of competent *B. subtilis* cells (genotype: ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). The transformation mixture was shaken at 37°C for 1 hour. Ten microliter aliquots of the transformation mixture were plated on skim milk (1.6%) Luria agar plates supplemented with 10 µg/ml of neomycin (Teknova). Subsequently, the colonies with halos were inoculated in 120 µl of LB media containing 10 µg/mL neomycin for plasmid DNA extraction (QIAprep Spin Miniprep kit, Qiagen). The extracted plasmids were sequenced to confirm the presence of the desired mutations.

b) Construction of NHJ4 Variants by Extension PCR

Ten combinatorial mutants of GG36 were created by extension PCR. The list of mutations introduced in the GG36 gene contained in the pHPLT plasmid were T22A, N62E, S103G, S103G-L111V, S101G-S103A-V104I, S101A-S103G-V104L, S101A, S128N, G159D, G159E, Y209E, and

L111V. To create each mutant, PCR fragments containing the desired mutations were amplified using mutagenic primers as well as forward and reverse primers to amplify the entire GG36 variant. Each PCR amplification reaction contained 30 pmol of each mutagenic primer and 100 ng of the DNA template, pHPLT-GG36 plasmid. Amplifications were carried out using Vent DNA polymerase (NEB). The PCR reaction (20 µL) was initially heated at 95°C for 2.5 min followed by 30 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 15 sec. and extension at 72°C for 1 min. Following amplification, 2 to 4 PCR fragments for each variant were gel-purified, using a QIAGEN® gel-band purification kit and mixed (50 ng of each fragment). These mixtures served as DNA templates for the extension PCR to generate the full-length gene fragments. The PCR conditions were same as described above, except the extension phase, which was carried out at 72°C for 2 min. The full-length DNA fragment was gel-purified using a QIAGEN® gel-band purification kit, digested with the *Bam*HI and *Hin*dIII restriction enzymes and ligated with the pHPLT-GG36, which was digested with the same restriction enzymes. One microliter of the ligation mixtures was amplified using rolling circle amplification by Illustra Templiphi kit according to the manufacturer's instructions (GE Healthcare) to generate multimeric DNA for transformation into *Bacillus subtilis*. Products of the rolling circle amplification were diluted 100-times and used to transform *B. subtilis* cells (genotype: ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). An aliquot of the transformation mix was plated on LB plates containing 1.6% skim milk and 10 µg/mL neomycin and incubated overnight at 37°C. Subsequently, the colonies with halos were inoculated in 120 µl of Luria broth medium containing 10 µg/mL neomycin for plasmid DNA extraction (QIAprep Spin Miniprep kit, Qiagen). The extracted plasmids were sequenced to confirm the presence of the desired mutations. Variants created by the extension PCR are shown in Table 4-1.

To express the NHJ4 set of variant proteins for further biochemical analyses, the *B. subtilis* strains carrying the variant plasmids were inoculated into microtiter plates containing 150µl Luria broth medium supplemented with 10µg/ml neomycin. The cultures were grown up for protein expression as described in Example 2, and they were filtered through a micro-filter plate (0.22µm; Millipore) also as described in Example 2. The resulting filtrate was used for biochemical analysis. The eglin c inhibition assay for protein content determination and BMI microswatch assays tested in various detergents were carried out as described in Example 1. Performance indices are also calculated as described under the BMI assay description in Example 1. Table 4-1 provides information regarding these multiple mutation variants and the results obtained for them. The PI values are relative to GG36. In the following Tables, the detergent compositions ("Det.") correspond to those shown in Table D, above. Also, as indicated, the amino acid position is listed according to BPN' numbering.

TABLE 4-1. NHJ4 Multiple Mutation Variants with BMI Cleaning Performance Indices of at Least 0.2 Relative to GG36 in Detergents 7, 8 or 9 at 16°C.		
Variant Name	**Created by**	**Mutations , (BPN' Numbering)**
		GG36
NHJ4-1	Extension PCR	S101G S103A V104I
NHJ4-10	Extension PCR	T22A S101A Y209E
NHJ4-11	Extension PCR	S103G L111V G159E
NHJ4-12	Extension PCR	T22A S103G G159E
NHJ4-13	QCMS	T22A L111V G159E
NHJ4-14	QCMS	T22A S128N E271F Y209E
NHJ4-15	QCMS	T22A S103G L111V
NHJ4-16	QCMS	N62E L111V S128N
NHJ4-17	QCMS	T22A L111V S128N
NHJ4-18	Extension PCR	T22A N62E L111V
NHJ4-19	QCMS	S101A S103G V104L S188D
NHJ4-2	Extension PCR	S101G S103A V104I G159D
NHJ4-20	Extension PCR	S101A S103G V104L S128N
NHJ4-24	QCMS	T22A S101A G159E
NHJ4-3	Extension PCR	S101A S103G V104L
NHJ4-4	Extension PCR	S101A S103G V104L G159E
NHJ4-5	Extension PCR	T22A S101A S103G V104L
NHJ4-6	QCMS	S101A S103G V104L Y209E
NHJ4-7	QCMS	T22A Y209E E271F
NHJ4-8	QCMS	T22A S101A E271F
NHJ4-9	QCMS	S101A Y209E E271F

EXAMPLE 5

Construction and Cleaning Performance of NHJ3 Set of GG36 Variants

The NHJ3 set of variants described herein are based on a variant of GG36 (referred to as GG36-9) containing the following mutations: S101G, S103A, V104I, G159D, A232V, Q236H, Q245R, N248D, and N252K (BPN' numbering). These variants were created using the QUIKCHANGE® Lightning Site-Directed Mutagenesis Kit (QCLDS kit; Stratagene), with the pRA68 plasmid (see Figure 7) as the DNA template. Plasmid pRA68 was derived from the pBN3 vector (see Babé et al., Biotech. Appl. Biochem. 27:117-124 [1998]).

The DNA sequence of GG36-9 variant (the signal sequence is shown in lower case letters, propeptide in lower case, underlined text, and GG36-9 mature sequence in uppercase letters) is provided below:

Gtgagaagcaaaaaattgtggatcgtcgcgtcgaccgcactactcatttctgttgcttttagttcatcgatcgcatcggctgctgaagaagcaaaagaaaa
atatttaattggctttaatgagcaggaagctgtcagtgagtttgtagaacaagtagaggcaaatgacgaggtcgccattctctctgaggaagaggaagtcg
aaattgaattgcttcatgaatttgaaacgattcctgttttatccgttgagttaagcccagaagatgtggacgcgcttgaactcgatccagcgatttcttatattga
agaggatgcagaagtaacgacaatgGCGCAATCAGTGCCATGGGGAATTAGCCGTGTGCAAGCCCCGGCT
GCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCTGTCCTCGATACAGGTATTTC
CACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTTGTACCAGGGGAACCATCCACTC
AAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACGATTGCTGCTCTAAACAATTCGAT
TGGCGTACTTGGCGTAGCGCCGAGCGCGGAACTATACGCTGTTAAAGTATTAGGGGCGAGC
GGTGGGGGGCGCCATCAGCTCGATTGCCCAAGGATTGGAATGGGCAGGGAACAATGGCATGC
ACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCAAGTGCCACACTTGAGCAAGCTGTTAAT
AGCGCGACTTCTAGGGGCGTTCTTGTTGTAGCGGCATCTGGAAATTCGGGTGCAGACTCAAT
CAGCTATCCGGCCCGTTATGCGAACGCAATGGCAGTCGGAGCTACTGACCAAAACAACAAC
CGCGCCAGCTTTTCACAGTATGGCGCAGGGCTTGACATCGTCGCACCAGGTGTAAACGTGCA
GAGCACATACCCAGGTTCAACGTATGCCAGCTTAAACGGTACATCGATGGCTACTCCTCATG
TTGCAGGTGCAGCAGTCCTTGTTAAACATAAGAACCCATCTTGGTCCAATGTACGAATCCGC
GATCATCTAAAGAAAACGGCAACGAGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTG
TCAATGCCGAAGCTGCAACTCGTTAA (SEQ ID NO:762)

The protein sequence of the GG36-9 variant (the signal sequence is shown in lower case letters, propeptide in lower case, underlined text, and GG36-9 mature protease sequence in uppercase letters) is provided below:

vrskklwivastallisvafsssiasaaeeakekyligfneqeavsefveqveandevailseeeeveiellhefetipvlsvelspedvdaleldpaisy
ieedaevttmAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQD
GNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGGGAISSIAQGLEWAGNNGMHVAN
LSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGADSISYPARYANAMAVGATDQNNNRASFSQ
YGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHVAGAAVLVKQKNPSWSNVRIRDHLKKTA
TSLGSTNLYGSGLVNAEAATR (SEQ ID NO:763)

To create the NHJ3 variants using the QCLSD kit, mutagenic primers were designed for each of the variants according to the manufacturer's protocol. The mutagenesis reaction for each variant consisted of 0.5 µl of 10X Buffer, 0.5 µL of pRA68 plasmid DNA (168ng/µL), 0.5µl forward mutagenic primer (25 µM), 0.5 µl reverse mutagenesis primer (25 µM), 1 µl dNTPs (supplied in the QCLSD kit), 1.5 µl Quik solution (supplied in the QCLMS kit), 1µl Enzyme blend (supplied in the QCLSD kit), and

40 µl of distilled, deionized water to make up a 50 µL reaction volume as per the manufacturer's instructions. The cycling program was 1 cycle at 95°C for 2 minutes, 18 cycles of 95°C for 20 seconds, 60°C for 10 seconds and 68°C for 3 minutes, 22 seconds, and a final cycle of 68°C for 5 minutes. Next, 1 µL of *Dpn*I restriction enzyme supplied in the kit was used to digest the plasmid DNA in the reaction, and then 2 µL of the reaction was used to transform TOP 10 *E.coli* competent cells (Invitrogen). The *E.coli* transformants were selected on Luria broth medium plates containing 50 µg/mL(ppm) carbenicillin after overnight growth at 37°C. Plasmid DNA was extracted from 4-8 *E. coli* colonies grown in LA medium containing 50 µg/mL(ppm) carbenicillin using the QIAprep spin miniprep kit (Qiagen). The plasmids were sequenced to confirm the presence of the desired mutations. The variant plasmids were then transformed into *B. subtilis* cells as described in Example 2. The *B. subtilis* variant strains were grown up as described in Example 2 for further biochemical analysis, such as protein content determination using the eglin c inhibition assay (Example 1) and a BMI microswatch cleaning assay (Example 1). The results are provided below in Tables 5-1 and 5-2. The PIs are relative to GG36. In the following Tables, the detergent compositions ("Det.") correspond to those shown in Table D, above. Also, as indicated, the amino acid position is listed according to BPN' numbering.

TABLE 5-1. NHJ3 Multiple Mutation Variants with BMI Cleaning Performance Indices of at Least 1.1 Relative to GG36 in Detergents 7, 8, or 9 at 16°C.

Variant	Variant Sequence Relative to GG36 (Using BPN' Numbering)
	GG36
NHJ3-1	S103A-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K
NHJ3-2	S101G-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K
NHJ3-3	S101G-S103A-G159D-A232V-Q236H-Q245R-N248D-N252K
NHJ3-4	S101G-S103A-V104L-A232V-Q236H-Q245R-N248D-N252K
NHJ3-5	S101G-S103A-V104L-G159D-Q236H-Q245R-N248D-N252K
NHJ3-6	S101G-S103A-V104L-G159D-A232V-Q245R-N248D-N252K
NHJ3-7	S101G-S103A-V104L-G159D-A232V-Q236H-N248D-N252K
NHJ3-8	S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K
NHJ3-9	S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N248D

TABLE 5-2. NHJ3 Multiple Mutation Variants with BMI Cleaning Performance Indices of at Least 0.3 Relative to GG36 in Detergents 10 or 11 at 16°C	
Variant	**Variant Sequence Relative to GG36 (Using BPN' Numbering)**
	GG36
NHJ3-1	S103A-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K
NHJ3-2	S101G-V104I-G159D-A232V-Q236H-Q245R-N248D-N252K
NHJ3-3	S101G-S103A-G159D-A232V-Q236H-Q245R-N248D-N252K
NHJ3-4	S101G-S103A-V104L-A232V-Q236H-Q245R-N248D-N252K
NHJ3-5	S101G-S103A-V104L-G159D-Q236H-Q245R-N248D-N252K
NHJ3-6	S101G-S103A-V104L-G159D-A232V-Q245R-N248D-N252K
NHJ3-7	S101G-S103A-V104L-G159D-A232V-Q236H-N248D-N252K
NHJ3-8	S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N252K
NHJ3-9	S101G-S103A-V104L-G159D-A232V-Q236H-Q245R-N248D

EXAMPLE 6

Construction and Cleaning Performance of NHJ5 Set of GG36 Variants

The NHJ5 set of variants described herein are based on a variant of GG36 (referred to as GG36-7) containing the following mutations: S101G, S103A, V104I, G159D, A232V, Q245R, N248D, and (BPN' numbering). These variants were created using the QUIKCHANGE® Lightning Multi Site-Directed Mutagenesis Kit ("QCLMS kit") with the pRA96 plasmid as the DNA template (see Figure 8). The mutations incorporated included: H243R (H249R), E265F (E271F), D157E (D159E), A156E (A158E), A156E-D157G (A158E- D159E), T22A, N60E (N62E), N232R (N238R), D242R (D248R), T247R (T253R), S24R, N74D (N76D) {GG36 Numbering and BPN' Numbering Shown in Parentheses.

The variants were generated using the methods described in Example 5.T he *B. subtilis* variant strains were grown up as described in Example 2 for further biochemical analysis, such as protein content determination using the eglin c inhibition assay (Example 1) and the BMI microswatch cleaning assay (Example 1). The results are provided below in Table 6-1. The PI values are relative to GG36. In the following Tables, the detergent compositions ("Det.") correspond to those shown in Table D, above. Also, as indicated, the amino acid position is listed according to BPN' numbering.

The DNA sequence of GG36-7 variant (the signal sequence is shown in lower case letters, propeptide in lower case, underlined text, and GG36-7 mature protease sequence in uppercase letters) is provided below:

gtgagaagcaaaaaattgtggatcgtcgcgtcgaccgcactactcatttctgttgctttagttcatcgatcgcatcggctgctgaagaagcaaaagaaaaa
tatttaattggctttaatgagcaggaagctgtcagtgagtttgtagaacaagtagaggcaaatgacgaggtcgccattctctctgaggaagaggaagtcga

aattgaattgcttcatgaatttgaaacgattcctgttttatccgttgagttaagcccagaagatgtggacgcgcttgaactcgatccagcgatttcttatattgaa
gaggatgcagaagtaacgacaatgGCGCAATCAGTGCCATGGGGAATTAGCCGTGTGCAAGCCCCGGCTG
CCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCTGTCCTCGATACAGGTATTTCC
ACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTTGTACCAGGGGAACCATCCACTCA
AGATGGGAATGGGCATGGCACGCATGTGGCCGGGACGATTGCTGCTCTAAACAATTCGATT
GGCGTACTTGGCGTAGCGCCGAGCGCGGAACTATACGCTGTTAAAGTATTAGGGGCGAGCG
GTGGGGGCGCCATCAGCTCGATTGCCCAAGGATTGGAATGGGCAGGGAACAATGGCATGCA
CGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCAAGTGCCACACTTGAGCAAGCTGTTAATA
GCGCGACTTCTAGGGGCGTTCTTGTTGTAGCGGCATCTGGAAATTCGGGTGCAGACTCAATC
AGCTATCCGGCCCGTTATGCGAACGCAATGGCAGTCGGAGCTACTGACCAAAACAACAACC
GCGCCAGCTTTTCACAGTATGGCGCAGGGCTTGACATCGTCGCACCAGGTGTAAACGTGCA
GAGCACATACCCAGGTTCAACGTATGCCAGCTTAAACGGTACATCGATGGCTACTCCTCATG
TTGCAGGTGCAGCAGTCCTTGTTAAACAAAAGAACCCATCTTGGTCCAATGTACGAATCCGC
GATCATCTAAAGAATACGGCAACGAGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTG
TCAATGCCGAAGCTGCAACTCGT (SEQ ID NO:764)

The protein sequence of GG36-7 variant (signal sequence is shown in lower case letters, propeptide in lower case, underlined text, and GG36-7 mature protease sequence in uppercase letters) is provided below:

vrskklwivastallisvafsssiasaaeeakekyligfneqeavsefveqveandevailseeeeveiellhefetipvlsvelspedvdaleldpaisy
icedacvttmAQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGISTHPDLNIRGGASFVPGEPSTQD
GNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGGGAISSIAQGLEWAGNNGMHVAN
LSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGADSISYPARYANAMAVGATDQNNNRASFSQ
YGAGLDIVAPGVNVQSTYPGSTYASLNGTSMATPHIVAGAAVLVKQKNPSWSNVRIRDHLKNTA
TSLGSTNLYGSGLVNAEAATR (SEQ ID NO:765)

TABLE 6-1: NHJ5 Multiple Mutation Variants with BMI Cleaning Performance Indices of at Least 0.6 Relative to GG36 in Detergents 7-11 at 16°C	
Variant	**Variant Sequence Relative to GG36 (BPN' Numbering)**
	GG36
GG36-7	S101G-S103A-V104I-G159D-A232V-Q245R-N248D
NHJ5-1	S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F
NHJ5-2	S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N238R
NHJ5-3	S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N248R
NHJ5-4	S101G-S103A-V104I-G159D-A232V-Q245R-N248D-T253R

NHJ5-5	S101G-S103A-V104I-G159D-A232V-Q245R-N248D-S24R
NHJ5-6	S101G-S103A-V104I-G159D-A232V-Q245R-N248D-N76D
NHJ5-7	S101G-S103A-V104I-G159E-A232V-Q245R-N248D-H249R
NHJ5-8	S101G-S103A-V104I-G159E-A232V-Q245R-N248D-E271F
NHJ5-9	S101G-S103A-V104I-A158E-A232V-Q245R-N248D-H249R
NHJ5-10	S101G-S103A-V104I-A158E-A232V-Q245R-N248D-E271F
NHJ5-11	T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R
NHJ5-12	T22A-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F
NHJ5-13	N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-H249R
NHJ5-14	N62E-S101G-S103A-V104I-G159D-A232V-Q245R-N248D-E271F

EXAMPLE 7

Construction of NHJ2 Combinatorial Library

This Example describes the construction of a GG36 combinatorial library involving one or more of the following mutations: A16S, T22A, S101A, S103G, V104L, L111V, S128N, and L148I (BPN' numbering). The pHPLT-GG36 *B. subtilis* expression plasmid was provided to DNA 2.0 Inc. for the generation of NHJ2 combinatorial library. A ligation reaction of the constructed NHJ2 library was provided by DNA 2.0, Inc. for transformation in the *B. subtilis* strain (genotype: ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). The variants generated containing one or several of the mutations described herein are tested for cold water cleaning applications using methods and detergent compositions described herein.

EXAMPLE 8

Construction of Additional Libraries and GG36 Variants

Additional libraries and variants are constructed using the following set of mutations: A1R, A230E, E271L, G115R, G20R, H249R, K235F, K27V/F/L, L75E, L82R, N18R, N269R, N43D, N43R, N76D, R45T, S212F, S242R, S24R, S78R, S9A, T22R, V121E, V244R, V28E, V30E, V4R, W241R (BPN' numbering). The variants generated containing one or more of these mutations are tested for cold water cleaning applications using methods and detergent compositions described herein.

Additional sets of GG36 variants are constructed and tested for cold water cleaning applications using methods and detergent compositions described herein include: G20R-N43R-H249R, G20R-T22R-N43R, G20R-N43R-S242R, G20R-N43R-E271L, G20R-N43R-V244R, G20R-S24R-N43R-S242R, S9A-T22R-S78R-S212F-W241R, S9A-G20R-N43R-S212F, S9A-N43R-S212F, G20R-N43R-S212F, G20R-T22R-N43R-S212F, S24R-S78R-S212F, S9A-N43R-S78R, S9A-N43R-S78R-S242R, S9A-G20R-N43R-S78R, G20R-S24R-N43R-S78R-S242R, T22R-S24R-S78R-S212F, S9A-G20R-N43R-

S78R-S242R, G20R-N43R-S78R-H249R, G20R-N43R-S78R, S9A-S78R-S212F, S9A-T22R-N43R-S78R, S9A-G20R-S24R-N43R, S9A-T22R-S78R-S212F, V4R-S9A-T22R-S78R-S212F, G20R-S24R-N43R, A1R-S9A-N43R, G20R-S24R-N43R-G115R, S9A-S24R-N43R, G20R-T22R-S24R-N43R, A1R-S24R-N43R, S9A-G20R-S24R-N43R-S242R, S9A-G20R-T22R-S78R-S212F, S9A-S24R-N43R-V244R, S9A-S24R-N43R-S242R, V4R-S9A-T22R-S24R-S212F, and T22R-S24R-N43R (BPN' numbering).

EXAMPLE 9

Construction and Cleaning Performance of the GG36 Library WCE2

The WCE2 combinatorial library was generated by DNA 2.0, Inc. using the pHPLT-GG36 *B. subtilis* expression plasmid. A ligation reaction of the constructed WCE2 library was provided by DNA 2.0, Inc. for transformation in the *B. subtilis* strain (genotype: ΔaprE, ΔnprE, amyE::xylRPxylAcomK-phleo). The set of mutations used to generate the WCE2 library are A230E, G20R, H249R, N18R, N43R/D, N76D, R45T, S242R, and S24R (BPN' numbering). The variants generated containing one or more of these mutations are tested for cold water cleaning applications using methods and detergent compositions described herein.

EXAMPLE 10

Construction and Cleaning Performance of the WCE3 Set of GG36 Variants

This Example describes the WCE3 set of mutants based on the GG36 variants, GG36-7 (Example 5) and GG36-9 (Example 4). These variants are: S101G-S103A-V104I-A232V-Q245R-N248D, S101G-S103A-V104I-G159D-A232V-Q245R, S101G-S103A-V104I-G159R-A232V-Q245R-N248D, S101G-S103A-V104I-G159D-A232V-Q245R-N248R, S101G-S103A-V104I-A232V-Q245R, S101G-S103A-V104I-A232V-Q245R-N248R, S101G-S103A-V104I-G159R-A232V-Q245R-N248R, and S101G, S103A, V104I, A232V, Q236H, Q245R, and N252K. They were created using the QuikChange® Lightning Multi Site-Directed Mutagenesis Kit (QCLMS kit; Stratagene) with the pRA96 plasmid as the DNA template described in Example 5. The variants generated will be tested for cold water cleaning applications using methods and detergent compositions described in this application.

EXAMPLE 11

Construction of Additional Libraries and Variants of GG36

This Example describes the construction of GG36 variants and libraries using one or more of the following mutations: A16S, T22A, S24R, N62E, N76D, E89P, S101A/G, S103G/A, V104L/I, L111V, S128N, P129E, A232V, L148I, A158E, G159D/E, S166D, R186H, S188D, Y209E, Q236H, N238R, Q245R, N248D/R, H249R, N252K/R, T253R, E271F (BPN' numbering) using a *B. subtilis* expression plasmid (e.g., pHPLT-GG36; Figure 6). The variants generated containing one or more of these

mutations are tested for cold water cleaning applications using methods and detergent compositions described herein.

EXAMPLE 12

Construction of Additional Libraries and Variants of GG36

This Example describes the construction of GG36 variants and libraries in *B. subtilis* using one or more of the following mutations (BPN' numbering): A1R, Q2S, Q2M, Q2A, Q2R, Q2W, S3R, V4R, V4S, V4C, I8A, S9A, S9F, S9W, R10S, R10A, R10H, R10M, Q12F, Q12R, P14K, P14F, P14Q, A15R, A15T, A16S, H17R, H17M, H17F, N18R, N18K, G20F, G20K, G20R, T22A, T22R, T22Y, T22V, T22Q, T22L, T22W, G23A, G23S, G23F, S24R, S24F, S24W, S24Q, S24H, S24L, G25V, G25F, G25R, V26F, K27L, K27F, K27R, K27V, V28A, V28N, V28E, A29T, V30E, L31F, T33S, T33G, T33D, G34P, I35M, S36T, S36F, S36R, T38L, T38F, T38R, P40N, P40L, P40T, P40W, P40H, P40R, L42I, N43A, N43F, N43I, N43S, N43R, N43M, N43W, N43D, R45T, G46R, A48R, F50C, V51W, V51F, V51H, P52F, P52E, P52N, P55Y, T57R, Q59A, Q59F, Q59R, D60P, D60Q, D60A, N62E, N62Q, G63V, G63M, G63T, G63I, G63A, G63S, G63H, G63Q, G63D, G63E, G63P, H64F, H64T, V68A, V68C, A69N, A69T, A69P, A69W, T71G, I72C, A74C, L75A, L75F, L75E, L75R, N76D, S78R, S78N, S78I, I79W, I79Q, V81R, L82F, L82T, L82V, L82R, L82M, A85M, P86W, P86L, P86I, E89P, E89T, E89G, E89H, E89L, E89V, E89W, E89F, E89I, Y91N, Y91F, A92F, K94N, S99F, S99T, S99P, S99G, S99M, G100S, G100N, G100Q, G100I, S101A, S101N, S101G, S101T, S101D, S101E, S101P, S101F, G102A, G102T, G102N, G102H, G102E, S103G, S103N, S103D, S103A, V104L, V104I, V104E, V104D, S105T, S105E, S105Q, S106G, S106T, S106E, S106D, S106A, S106V, S106F, I107M, I107F, A108I, A108G, Q109M, L111V, L111I, E112V, E112L, E112Q, A114G, G115K, G115R, N116K, N116A, N116L, N117F, G118R, G118I, M119C, H120A, H120F, H120R, V121F, V121E, N123G, N123E, L124S, S128D, S128F, S128L, S128N, S128H, S128M, S128I, S128Q, P129E, S132A, S132E, A138G, S144R, V147L, L148I, A158E, G159D, G159E, G159C, S160D, S166D, S166E, Y167W, M175V, V177C, D181A, Q182R, N183I, N183D, N183M, N183F, N183R, N185E, N185V, N185I, R186H, R186K, S188E, S188D, S188R, Y192H, Y192W, A194E, A194V, A194F, D197F, I198L, I198F, V203E, V203C, T208S, Y209S, Y209N, Y209F, Y209T, Y209E, Y209H, Y209G, Y209L, P210R, P210V, P210L, G211Q, G211R, S212I, S212M, S212F, T213A, Y214F, A215N, A215D, A215E, A215H, A215F, S216F, S216A, L217E, L217N, L217D, N218D, N218P, N218E, T224A, T224G, V227I, A230E, A231I, A231C, A232V, L233C, V234F, K235F, Q236F, Q236N, Q236H, N238R, N238K, N238L, P239K, P239G, P239R, P239H, P239T, P239N, P239S, P239F, S240R, W241R, S242L, S242R, N243F, N243R, V244R, Q245R, I246S, N248D, N248V, N248I, N248R, H249R, H249T, L250I, K251R, K251S, N252I, N252F, N252R, N252K, N252H, T253I, T253R, T253F, A254C, S256N, G258R, T260V, T260I, L262D, L262H, Y263F, S265F, L267V, L267N, L267M, N269I, N269R, A270C, E271I, E271V, E271H, E271M, E271L, E271P, E271A, E271F, E271T, A272F, A272F, A272R,

A273F, A273I, and T274G. The variants generated containing one or more of these mutations are tested for cold water cleaning applications using methods and detergent compositions described herein.

EXAMPLE 13

Automatic Dishwash Performance Tests

In this Example, methods used to determine the wash performance of protease variants using some commercially available dish detergents are described. These protease variants are tested under various conditions. These detergents are commercially available from WFK and are referred to by the designations provided below. The protocols for each of the stain types (minced meat, egg yolk, and egg yolk with milk) are provided below. Before the individual soil types can be applied to the test dishes, the dishes must be thoroughly washed. This is particularly necessary, as residues of certain persistent stains may still be present on the dishes from previous tests. New dishes were also subjected to three thorough washes before being used for the first time in a test.

The washing tests are typically performed in an automatic dishwasher (e.g., Miele: G690SC), equipped with soiled dishes and stainless steel sheets, as described above. A defined amount of the detergent is used, as indicated in the tables of results below. In some experiments, the temperatures tested are 45°C, 55°C and 65°C. In some experiments, the water hardness is 9° or 21° GH (German hardness) (374 ppm Ca).

As indicated above, after washing, the plates soiled with minced meat or pasta/sauce/meat/cheese are visually assessed using a photo rating scale of from 0 to 10, wherein "0" designated a completely dirty plate and "10" designated a clean plate. These values correspond to the stain or soil removal (SR) capability of the enzyme-containing detergent.

The washed stainless steel plates soiled with egg yolk and/or egg yolk milk (are analyzed gravimetrically to determine the amount of residual stain after washing.

Some exemplary detergents are provided below.

Phosphate-Free Detergent, IEC-60436 WFK Type B (pH=10.4 in 3g/l)	
Component	**Wt %**
Sodium citrate dehydrate,	30.0
Maleic acid/ acrylic acid copolymer sodium Salt (SOKALAN® CP5; BASF)	12.0
Sodium perborate monohydrate,	5.0
TAED,	2.0
Sodium disilicate: Protil A (Cognis),	25.0
Linear fatty alcohol ethoxylate,	2.0
Sodium carbonate anhydrous,	add to 100

Phosphate-Containing Detergent:, IEC-60436 WFK Type C (pH=10.5 in 3 g/l))	
Component	**Wt %**
Sodium tripolyphosphate	23.0
Sodium citrate dehydrate	22.3
Maleic acid/ Acrylic Acid Copolymer Sodium Salt	4.0
Sodium perborate monohydrate	6.0
TAED	2.0
Sodium disilicate: Protil A (Cognis)	5.0
Linear Fatty Alcohol Ethoxylate	2.0
Sodium Carbonate anhydrous,	add to 100,

EXAMPLE 14

Granular and/or Tablet Laundry Compositions

This Example provides various formulations for granular and/or tablet laundry detergents. The following laundry compositions of present invention, which may be in the form of granules or tablet, are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 14-1: Granular and/or Tablet Laundry Compositions

Compound	**Formulations**				
	I	**II**	**III**	**IV**	**V**
Base Product					
C_{14}-C_{15}AS or TAS	8.0	5.0	3.0	3.0	3.0
LAS	8.0	-	8.0	-	7.0
C_{12}-$C_{15}AE_3S$	0.5	2.0	1.0	-	-
C_{12}-$C_{15}E_5$ or E_3	2.0	-	5.0	2.0	2.0
QAS	-	-	-	1.0	1.0
Zeolite A	20.0	18.0	11.0	-	10.0
SKS-6 (dry add)	-	-	9.0	-	-
MA/AA	2.0	2.0	2.0	-	-
AA	-	-	-	-	4.0
3Na Citrate $2H_2O$	-	2.0	-	-	-

Table 14-1: Granular and/or Tablet Laundry Compositions					
Compound	**Formulations**				
	I	**II**	**III**	**IV**	**V**
Citric Acid (Anhydrous)	2.0	-	1.5	2.0	-
DTPA	0.2	0.2	-	-	-
EDDS	-	-	0.5	0.1	-
HEDP	-	-	0.2	0.1	-
PB1	3.0	4.8	-	-	4.0
Percarbonate	-	-	3.8	5.2	-
NOBS	1.9	-	-	-	-
NACA OBS	-	-	2.0	-	-
TAED	0.5	2.0	2.0	5.0	1.00
BB1	0.06	-	0.34	-	0.14
BB2	-	0.14	-	0.20	-
Anhydrous Na Carbonate	15.0	18.0	-	15.0	15.0
Sulfate	5.0	12.0	5.0	17.0	3.0
Silicate	-	1.0	-	-	8.0
nprE (optional)	0.03	-	0.1	0.06	-
PMN	-	0.05	-	-	0.1
Protease B (optional)	-	0.01	-	-	-
Protease C (optional)	-	-	-	0.01	-
Lipase	-	0.008	-	-	-
Amylase	0.001	-	-	-	0.001
Cellulase	-	0.0014	-	-	-
Pectin Lyase	0.001	0.001	0.001	0.001	0.001
Aldose Oxidase	0.03	-	0.05	-	-
PAAC	-	0.01	-	-	0.05
Balance to 100% Moisture and/or Minors*					

* Perfume, dye, brightener / SRP1 / Na carboxymethylcellulose/ photobleach / $MgSO_4$ / PVPVI/ suds suppressor /high molecular PEG/clay.

EXAMPLE 15

Tablet Detergent Compositions

This Example provides various tablet detergent formulations. The following tablet detergent compositions of the present invention are prepared by compression of a granular dishwashing detergent

composition at a pressure of 13KN/cm^2 using a standard 12 head rotary press. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

5

Table 15-1: Tablet Detergent Compositions								
Compound	**Formulations**							
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
STPP	-	48.8	44.7	38.2	-	42.4	46.1	46.0
3Na Citrate $2H_2O$	20.0	-	-	-	35.9	-	-	-
Na Carbonate	20.0	5.0	14.0	15.4	8.0	23.0	20.0	-
Silicate	15.0	14.8	15.0	12.6	23.4	2.9	4.3	4.2
Lipase	0.001	-	0.01	-	0.02	-	-	-
Protease B (optional)	0.01	-	-	-	-	-	-	-
Protease C (optional)	-	-	-	-	-	0.01	-	-
nprE (optional)	0.01	0.08	-	0.04	-	0.023	-	0.05
PMN	-	-	0.05	-	0.052	-	0.023	-
Amylase	0.012	0.012	0.012	-	0.015	-	0.017	0.002
Pectin Lyase	0.005	-	-	0.002	-	-	-	-
Aldose Oxidase	-	0.03	-	0.02	0.02	-	0.03	-
PB1	-	-	3.8	-	7.8	-	-	4.5
Percarbonate	6.0	-	-	6.0	-	5.0	-	-
BB1	0.2	-	0.5	-	0.3	0.2	-	-
BB2	-	0.2	-	0.5	-	-	0.1	0.2
Nonionic	1.5	2.0	2.0	2.2	1.0	4.2	4.0	6.5
PAAC	0.01	0.01	0.02	-	-	-	-	-
DETBCHD	-	-	-	0.02	0.02	-	-	-
TAED	-	-	-	-	-	2.1	-	1.6
HEDP	1.0	-	-	0.9	-	0.4	0.2	-
DETPMP	0.7	-	-	-	-	-	-	-
Paraffin	0.4	0.5	0.5	0.5	-	-	0.5	-
BTA	0.2	0.3	0.3	0.3	0.3	0.3	0.3	-
Polycarboxylate	4.0	-	-	-	4.9	0.6	0.8	-
PEG 400-30,000	-	-	-	-	-	2.0	-	2.0
Glycerol	-	-	-	-	-	0.4	-	0.5
Perfume	-	-	-	0.05	0.2	0.2	0.2	0.2
Balance to 100% Moisture and/or Minors*								

*Brightener / SRP1 / Na carboxymethylcellulose/ photobleach / $MgSO_4$ / PVPVI/ suds suppressor /high molecular PEG/clay.

The pH of Examples 14(I) through 14(VII) is from about 10 to about 11.5; pH of 14(VIII) is from 8-10. The tablet weight of Examples 14(I) through 14(VIII) is from about 20 grams to about 30 grams.

EXAMPLE 16

Liquid Laundry Detergent Compositions

In this Example, various formulations for liquid laundry detergent compositions are provided. The following liquid laundry detergent compositions of the present invention are prepared as shown below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 16-1: Liquid Laundry Detergent Compositions

Compound	Formulations				
	I	II	III	IV	V
LAS	24.0	32.0	6.0	3.0	6.0
NaC_{16}-C_{17} HSAS	-	-	-	5.0	-
C_{12}-C_{15} $AE_{1.8}S$	-	-	8.0	7.0	5.0
C_8-C_{10} propyl dimethyl amine	2.0	2.0	2.0	2.0	1.0
C_{12}-C_{14} alkyl dimethyl amine oxide	-	-	-	-	2.0
C_{12}-C_{15} AS	-	-	17.0	-	8.0
CFAA	-	5.0	4.0	4.0	3.0
C_{12}-C_{14} Fatty alcohol ethoxylate	12.0	6.0	1.0	1.0	1.0
C_{12}-C_{18} Fatty acid	3.0	-	4.0	2.0	3.0
Citric acid (anhydrous)	4.5	5.0	3.0	2.0	1.0
DETPMP	-	-	1.0	1.0	0.5
Monoethanolamine	5.0	5.0	5.0	5.0	2.0
Sodium hydroxide	-	-	2.5	1.0	1.5
1 N HCl aqueous solution	#1	#1	-	-	-
Propanediol	12.7	14.5	13.1	10.	8.0
Ethanol	1.8	2.4	4.7	5.4	1.0
DTPA	0.5	0.4	0.3	0.4	0.5
Pectin Lyase	-	-	-	0.005	-

Table 16-1: Liquid Laundry Detergent Compositions					
Compound	Formulations				
	I	II	III	IV	V
Amylase	0.001	0.002	-		-
Cellulase	-	-	0.0002		0.0001
Lipase	0.1	-	0.1	-	0.1
NprE (optional)	0.05	0.3	-	0.5	0.2
PMN	-	-	0.08	-	-
Protease A (optional)	-	-	-	-	0.1
Aldose Oxidase	-	-	0.3	-	0.003
ZnCl2	0.1	0.05	0.05	0.05	0.02
Ca formate	0.05	0.07	0.05	0.06	0.07
DETBCHD	-	-	0.02	0.01	-
SRP1	0.5	0.5	-	0.3	0.3
Boric acid	-	-	-	-	2.4
Sodium xylene sulfonate	-	-	3.0	-	-
Sodium cumene sulfonate	-	-	-	0.3	0.5
DC 3225C	1.0	1.0	1.0	1.0	1.0
2-butyl-octanol	0.03	0.04	0.04	0.03	0.03
Brightener 1	0.12	0.10	0.18	0.08	0.10
Balance to 100% perfume / dye and/or water					

#1: Add 1N HCl aqueous solution to adjust the neat pH of the formula in the range from about 3 to about 5.

The pH of Examples above 15(I)-(II) is about 5 to about 7, and of 15(III)-(V) is about 7.5 to about 8.5.

Table 16-2: Liquid Laundry Detergents						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
LAS	11.5	11.5	9.0	-	4.0	-
C_{12}-$C_{15}AE_{2.85}S$	-	-	3.0	18.0	-	16.0
C_{14}-$C_{15}E_{2.5}S$	11.5	11.5	3.0	-	16.0	-
C_{12}-$C_{13}E_9$	-	-	3.0	2.0	2.0	1.0
C_{12}-$C_{13}E_7$	3.2	3.2	-	-	-	-

Table 16-2: Liquid Laundry Detergents						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
CFAA	-	-	-	5.0	-	3.0
TPKFA	2.0	2.0	-	2.0	0.5	2.0
Citric Acid (Anhydrous)	3.2	3.2	0.5	1.2	2.0	1.2
Ca formate	0.1	0.1	0.06	0.1	-	-
Na formate	0.5	0.5	0.06	0.1	0.05	0.05
ZnCl2	0.1	0.05	0.06	0.03	0.05	0.05
Na Culmene Sulfonate	4.0	4.0	1.0	3.0	1.2	-
Borate	0.6	0.6	1.5	-	-	-
Na Hydroxide	6.0	6.0	2.0	3.5	4.0	3.0
Ethanol	2.0	2.0	1.0	4.0	4.0	3.0
1,2 Propanediol	3.0	3.0	2.0	8.0	8.0	5.0
Monoethanolamine	3.0	3.0	1.5	1.0	2.5	1.0
TEPAE	2.0	2.0	-	1.0	1.0	1.0
nprE (optional)	0.03	0.05	-	0.03	-	0.02
PMN	-	-	0.01	-	0.08	-
Protease A (optional)	-	-	0.01	-	-	-
Lipase	-	-	-	0.002	-	-
Amylase	-	-	-	-	0.002	-
Cellulase	-	-	-	-	-	0.0001
Pectin Lyase	0.005	0.005	-		-	-
Aldose Oxidase	0.05	-	-	0.05	-	0.02
Galactose oxidase	-	0.04				
PAAC	0.03	0.03	0.02	-	-	-
DETBCHD	-	-	-	0.02	0.01	-
SRP 1	0.2	0.2	-	0.1	-	-
DTPA	-	-	-	0.3	-	-
PVNO	-	-	-	0.3	-	0.2
Brightener 1	0.2	0.2	0.07	0.1	-	-
Silicone antifoam	0.04	0.04	0.02	0.1	0.1	0.1

Table 16-2: Liquid Laundry Detergents						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
Balance to 100% perfume/dye and/or water						

EXAMPLE 17

Hand Dish Liquid Detergent Compositions

In this Example, various hand dish liquid detergent formulations are provided. The following hand dish liquid detergent compositions of the invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some alternative aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 17-1: Hand Dish Liquid Detergent Compositions						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
C_{12}-C_{15} $AE_{1.8}S$	30.0	28.0	25.0	-	15.0	10.0
LAS	-	-	-	5.0	15.0	12.0
Paraffin Sulfonate	-	-	-	20.0	-	-
C_{10}-C_{18} Alkyl Dimethyl Amine Oxide	5.0	3.0	7.0	-	-	-
Betaine	3.0	-	1.0	3.0	1.0	-
C_{12} poly-OH fatty acid amide	-	-	-	3.0	-	1.0
C_{14} poly-OH fatty acid amide	-	1.5	-	-	-	-
$C_{11}E_9$	2.0	-	4.0	-	-	20.0
DTPA	-	-	-	-	0.2	-
Tri-sodium Citrate dehydrate	0.25	-	-	0.7	-	-
Diamine	1.0	5.0	7.0	1.0	5.0	7.0
$MgCl_2$	0.25	-	-	1.0	-	-
nprE (optional)	0.02	0.01	-	0.01	-	0.05
PMN	-	-	0.03	-	0.02	-
Protease A (optional)	-	0.01	-	-	-	-
Amylase	0.001	-	-	0.002	-	0.001
Aldose Oxidase	0.03	-	0.02	-	0.05	-
Sodium Cumene Sulphonate	-	-	-	2.0	1.5	3.0

Table 17-1: Hand Dish Liquid Detergent Compositions						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
PAAC	0.01	0.01	0.02	-	-	-
DETBCHD	-	-	-	0.01	0.02	0.01
Balance to 100% perfume / dye and/or water						

The pH of Examples 16(I)-(VI) is about 8 to about 11.

EXAMPLE 18

Liquid Automatic Dishwashing Detergent Compositions

In this Example, various liquid automatic dishwashing detergent formulations are provided. The following hand dish liquid detergent compositions of the invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 18-1: Liquid Automatic Dishwashing Detergent Compositions					
Compound	**Formulations**				
	I	**II**	**III**	**IV**	**V**
STPP	16	16	18	16	16
Potassium Sulfate	-	10	8	-	10
1,2 propanediol	6.0	0.5	2.0	6.0	0.5
Boric Acid	-	-	-	4.0	3.0
$CaCl_2$ dihydrate	0.04	0.04	0.04	0.04	0.04
Nonionic	0.5	0.5	0.5	0.5	0.5
nprE (optional)	0.1	0.03	-	0.03	-
PMN	-	-	0.05	-	0.06
Protease B (optional)	-	-	-	0.01	-
Amylase	0.02	-	0.02	0.02	-
Aldose Oxidase	-	0.15	0.02	-	0.01
Galactose Oxidase	-	-	0.01	-	0.01
PAAC	0.01	-	-	0.01	-
DETBCHD	-	0.01	-	-	0.01
Balance to 100% perfume / dye and/or water					

EXAMPLE 19

High Density Dishwashing Detergents

This Example provides various formulations for high density dishwashing detergents. The following compact high density dishwashing detergents of the invention are provided below. In each formulation, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 19-1: High Density Dishwashing Detergents

Compound	Formulations					
	I	II	III	IV	V	VI
STPP	-	45.0	45.0	-	-	40.0
3Na Citrate $2H_2O$	17.0	-	-	50.0	40.2	-
Na Carbonate	17.5	14.0	20.0	-	8.0	33.6
Bicarbonate	-	-	-	26.0	-	-
Silicate	15.0	15.0	8.0	-	25.0	3.6
Metasilicate	2.5	4.5	4.5	-	-	-
PB1	-	-	4.5	-	-	-
PB4	-	-	-	5.0	-	-
Percarbonate	-	-	-	-	-	4.8
BB1	-	0.1	0.1	-	0.5	-
BB2	0.2	0.05	-	0.1	-	0.6
Nonionic	2.0	1.5	1.5	3.0	1.9	5.9
HEDP	1.0	-	-	-	-	-
DETPMP	0.6	-	-	-	-	-
PAAC	0.03	0.05	0.02	-	-	-
Paraffin	0.5	0.4	0.4	0.6	-	-
nprE (optional)	0.072	0.053	-	0.026	-	0.01
PMN	-	-	0.053	-	0.059	-
Protease B (optional)	-	-	-	-	-	0.01
Amylase	0.012	-	0.012	-	0.021	0.006
Lipase	-	0.001	-	0.005	-	-
Pectin Lyase	0.001	0.001	0.001	-	-	-
Aldose Oxidase	0.05	0.05	0.03	0.01	0.02	0.01

Table 19-1: High Density Dishwashing Detergents						
Compound	**Formulations**					
	I	**II**	**III**	**IV**	**V**	**VI**
BTA	0.3	0.2	0.2	0.3	0.3	0.3
Polycarboxylate	6.0	-	-	-	4.0	0.9
Perfume	0.2	0.1	0.1	0.2	0.2	0.2
Balance to 100% Moisture and/or Minors*						

*Brightener / dye / SRP1 / Na carboxymethylcellulose/ photobleach / $MgSO_4$ / PVPVI/ suds suppressor /high molecular PEG/clay.

The pH of Examples 18(I) through (VI) is from about 9.6 to about 11.3.

EXAMPLE 20

Liquid Hard Surface Cleaning Detergents

This Example provides various formulations for liquid hard surface cleaning detergents. The following liquid hard surface cleaning detergent compositions of the invention are provided below. In each of these formulations, at least one protease variant provided herein is included at a concentration of from about 0.0001 to about 10 weight percent. In some aspects, other concentrations will find use, as determined by the formulator, based on their needs.

Table 20-1: Liquid Hard Surface Cleaning Detergents							
Compound	**Formulations**						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
C_9-$C_{11}E_5$	2.4	1.9	2.5	2.5	2.5	2.4	2.5
C_{12}-$C_{14}E_5$	3.6	2.9	2.5	2.5	2.5	3.6	2.5
C_7-C_9E_6	-	-	-	-	8.0	-	-
C_{12}-$C_{14}E_{21}$	1.0	0.8	4.0	2.0	2.0	1.0	2.0
LAS	-	-	-	0.8	0.8	-	0.8
Sodium culmene sulfonate	1.5	2.6	-	1.5	1.5	1.5	1.5
Isachem® AS	0.6	0.6	-	-	-	0.6	-
Na_2CO_3	0.6	0.13	0.6	0.1	0.2	0.6	0.2
3Na Citrate·$2H_2O$	0.5	0.56	0.5	0.6	0.75	0.5	0.75
NaOH	0.3	0.33	0.3	0.3	0.5	0.3	0.5
Fatty Acid	0.6	0.13	0.6	0.1	0.4	0.6	0.4
2-butyl octanol	0.3	0.3	-	0.3	0.3	0.3	0.3
PEG DME-2000®	0.4	-	0.3	0.35	0.5	-	-

Table 20-1: Liquid Hard Surface Cleaning Detergents							
Compound	**Formulations**						
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**
PVP	0.3	0.4	0.6	0.3	0.5	-	-
MME PEG (2000)®	-	-	-	-	-	0.5	0.5
Jeffamine® ED-2001	-	0.4	-	-	0.5	-	-
PAAC	-	-	-	0.03	0.03	0.03	-
DETBCHD	0.03	0.05	0.05	-	-	-	-
nprE (optional)	0.07	-	0.08	0.03	-	0.01	0.04
PMN	-	0.05	-	-	0.06	-	-
Protease B (optional)	-	-	-	-	-	0.01	-
Amylase	0.12	0.01	0.01	-	0.02	-	0.01
Lipase	-	0.001	-	0.005	-	0.005	-
Pectin Lyase	0.001	-	0.001	-	-	-	0.002
ZnCl2	0.02	0.01	0.03	0.05	0.1	0.05	0.02
Calcium Formate	0.03	0.03	0.01	-	-	-	-
PB1	-	4.6	-	3.8	-	-	-
Aldose Oxidase	0.05	-	0.03	-	0.02	0.02	0.05
Balance to 100% perfume / dye and/or water							

The pH of Examples 19(I) through (VII) is from about 7.4 to about 9.5.

All publications, patent applications, and patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or patent were specifically and individually indicated to be incorporated by reference. In particular, all publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated protease variant of a parent protease, the variant comprising an amino acid sequence comprising three amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q, wherein the variant has proteolytic activity and each amino acid position of the variant is numbered by correspondence to an amino acid position in the amino acid sequence of SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2.

2. The variant of claim 1, wherein the variant amino acid sequence comprises four amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q.

3. The variant of claim 1, wherein the variant amino acid sequence comprises five amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q.

4. The variant of claim 1, wherein the variant amino acid sequence comprises six amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X101N, X128A/S, and X217L/Q.

5. The variant of any preceding claim, wherein the variant amino acid sequence further comprises amino acid substitution X097A.

6. The variant of any preceding claim, wherein the variant amino acid sequence comprises amino acid substitutions X024G/R+X053G+X078N+X101N+X128A/S+X217L/Q or X097A+X128A/S+X217L/Q.

7. The variant of any preceding claim, wherein the variant amino acid sequence comprises amino acid substitutions S024G/R+S053G+S078N+S101N+G128A/S+Y217L/Q or G097A+G128A/S+Y217L/Q.

8. The variant of claim 7, wherein the variant amino acid sequence comprises amino acid substitutions S024G+S053G+S078N+S101N+G128S+Y217Q or S024G+S053G+S078N+S101N+G128A+Y217Q, and optionally further comprises a substitution selected from the group consisting of N109G, N076D, S033T, N243V, S248A, A088T, and S063G.

9. The variant of claim 7, wherein the variant amino acid sequence further comprises a set of amino acid substitutions selected from the group consisting of:

A088T+N109G+A116T+G131H+N243V+L257G,

S033T+N076D,

S009T+N109G+K141R+N243V,

S162G+K256R,

N109G+A116T,

N109G+L257G,

S162G+L257G,

N061G+N109G+N243V,

N109G+N243V+S248A,

S033T+N076D+N109G+N218S+N243V+S248N+K256R,

N109G+A116T+N243V+K256R,

A088T+N109G+A116T+G131H+N243V,

A088T+N109G,

N109G+N243V,

T158S+L257G,

N061S+N109G+N243V,

P040A+N109G+N243V+S248N+K256R,

S009T+S018T+Y021N+N109G+K141R,

A088T+N109G+A116T+T158S+N243V+K256R,

A088T+N109G+A116T+T158S+N218S+L257G,

N109G+K256R,

N109G+N243V+K256R,

S063G+K256R,

S063G+N109G,

S063G,

S063G+N076D,

S033T+N076D+N218S, and

N076D+N218S.

10. The variant of claim 7, wherein the variant amino acid sequence comprises amino acid substitutions S024G+S053G+S078N+S101N+G128A+Y217Q and further comprises a set of amino acid substitutions selected from the group consisting of:

A088T+N109G+A116T+G131H+N243V+L257G,

S033T+N076D,
S009T+N109G+A128S+K141R+N243V,
S162G+K256R,
N109G+A116T,
N109G+L257G,
S162G+L257G,
N061G+N109G+N243V,
N109G+A128S+N243V+S248A,
S033T+N076D+N109G+A128S+N218S+N243V+S248N+K256R,
N109G+A116T+N243V+K256R,
A088T+N109G+A116T+G131H+N243V,
A088T+N109G,
N109G+N243V,
T158S+L257G,
N061S+N109G+N243V,
P040A+N109G+A128S+N243V+S248N+K256R,
S009T+S018T+Y021N+N109G+A128S+K141R,
A088T+N109G+A116T+T158S+N243V+K256R,
A088T+N109G+A116T+T158S+N218S+L257G,
N109G+K256R,
N109G+A128S+N243V+K256R,
S063G+K256R,
S063G+N109G,
S063G+A128S,
S063G+N076D,
S033T+N076D+A128S+N218S, and
N076D+N218S.

11. The variant of any preceding claim, wherein the variant amino acid sequence has at least 80% sequence identity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:6.

12. The variant of any preceding claim, wherein the variant amino acid sequence has at least 85% sequence identity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:6.

13. The variant of any of claims 1-12, wherein the variant has enhanced proteolytic activity and/or cleaning activity compared to the parent protease or enhanced proteolytic activity and/or cleaning activity compared to the proteolytic activity of the BPN' protease having the sequence of SEQ ID NO:2.

14. The variant of any of claims 1-12, wherein the variant has enhanced proteolytic activity compared to the proteolytic activity of the protease having the sequence of SEQ ID NO:4.

15. The variant of any of claims 1-12, wherein the variant has enhanced proteolytic activity compared to the proteolytic activity of the protease having the sequence of SEQ ID NO:6.

16. The variant of any preceding claim, wherein the parent protease is a subtilisin protease.

17. The variant of claim 16, wherein the parent protease has at least 80% sequence identity to the *B. amyloliquefaciens* subtilisin protease BPN' having the amino acid sequence of SEQ ID NO:2.

18. The variant of claim 16, wherein the parent protease has at least 85% sequence identity to the amino acid sequence of SEQ ID NO:2.

19. The variant of claim 1, wherein the variant comprises the amino acid sequence of SEQ ID NO:6.

20. An isolated protease variant of a parent protease, the protease variant having proteolytic activity and comprising an amino acid sequence which comprises an alteration at one or more amino acid positions corresponding to amino acid positions of SEQ ID NO:2 selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217, wherein the at least one alteration is independently

(i) an insertion of one or more amino acid residues upstream or downstream of the amino acid residue which occupies the position,

(ii) a deletion of the amino acid residue which occupies the position, or

(iii) a substitution of the amino acid residue which occupies the position with a different amino acid residue,

wherein each amino acid position is numbered by correspondence with an amino acid position in the amino acid sequence of *Bacillus amyloliquefaciens* subtilisin protease BPN' set forth in SEQ ID NO:2 as determined by alignment of the amino acid sequence of the variant with SEQ ID NO:2.

21. The variant of claim 20, wherein the variant comprises an amino acid sequence comprising an alteration at two amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

22. The variant of claim 21, wherein the variant comprises an amino acid sequence comprising an alteration at three amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

23. The variant of claim 22, wherein the variant comprises an amino acid sequence comprising an alteration at four amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

24. The variant of claim 23, wherein the variant comprises an amino acid sequence comprising an alteration at five amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

25. The variant of claim 24, wherein the variant comprises an amino acid sequence comprising an alteration at six amino acid positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

26. The variant of claim 25, wherein the variant comprises an amino acid sequence comprising an alteration at each of the amino acid positions corresponding to positions of SEQ ID NO:2 selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

27. The variant of any of claims 20-26, wherein the variant comprises an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at a position selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

28. The variant of claim 27, wherein the variant comprises an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of two positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

29. The variant of claim 28, wherein the variant comprises an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of four positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

30. The variant of claim 29, wherein the variant comprises an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of five positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

31. The variant of claim 30, wherein the variant comprises an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of six positions selected from the group consisting of positions 24, 53, 78, 97, 101, 128, and 217.

32. The variant of claim 31, wherein the variant comprises an amino acid sequence comprising a substitution of an amino acid residue with a different amino acid residue at each of positions 24, 53, 78, 101, 128, and 217, wherein each position is numbered by correspondence with a position in SEQ ID NO:2.

33. The variant of any of claims 20-32, wherein the variant comprises an amino acid sequence comprising at least one amino acid substitution selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L.

34. The variant of claim 33, wherein the variant comprises an amino acid sequence comprising at least two amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L.

35. The variant of claim 34, wherein the variant comprises an amino acid sequence comprising at least three amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L.

36. The variant of claim 35, wherein the variant comprises an amino acid sequence comprising at least four amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L.

37. The variant of claim 36, wherein the variant comprises an amino acid sequence comprising at least five amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L.

38. The variant of claim 37, wherein the variant comprises an amino acid sequence comprising at least six amino acid substitutions selected from the group consisting of X024G/R, X053G, X078N, X097A, X101N, X128A/S, and X217Q/L.

39. The variant of any of claims 20-38, wherein the variant comprises an amino acid sequence comprising a set of substitutions selected from the group consisting of: (a) X128A/S and/or X217L/Q, (b) G128A/S and/or Y217L/Q, and (c) G097A, G128A/S, and Y217L/Q.

40. The variant of any of claims 20-39, wherein the variant comprises an amino acid sequence comprising amino acid substitutions X024G/R+X053G+X078N+X101N+X128A/S+X217Q/L.

41. The variant of any of claims 20-40, wherein the variant comprises an amino acid sequence having at least 80% sequence identity to the amino acid sequence of SEQ ID NO:2.

42. The variant of claim 41, wherein the variant comprises an amino acid sequence having at least 85% sequence identity to SEQ ID NO:2.

43. The variant of any of claims 20-42, wherein the variant amino acid sequence comprises at least one amino acid substitution selected from the group of S024G, S053G, S078N, S101N, G128A/S, and Y217Q.

44. The variant of any of claims 20-43, wherein the variant amino acid sequence comprises amino acid substitutions S024G+S053G+S078N+S101N+G128A+Y217Q.

45. The variant of any of claims 20-44, wherein the variant has enhanced proteolytic activity has enhanced proteolytic activity and/or cleaning activity compared to the parent protease or enhanced proteolytic activity and/or cleaning activity compared to the proteolytic activity of the protease set forth in SEQ ID NO:2.

46. The variant of any of claims 20-44, wherein the variant has enhanced proteolytic activity compared to the proteolytic activity of the protease set forth in SEQ ID NO:4.

47. The variant of any of claims 20-44, wherein the variant has enhanced proteolytic activity compared to the proteolytic activity of the protease set forth in SEQ ID NO:6.

48. The variant of any of claims 20-47, wherein the parent protease is a subtilisin protease.

49. The variant of claim 48, wherein the parent protease has at least 80% sequence identity to the *B. amyloliquefaciens* subtilisin protease BPN' having the amino acid sequence of SEQ ID NO:2.

50. The variant of claim 49, wherein the parent protease has at least 85% sequence identity to the amino acid sequence of SEQ ID NO:2.

51. An isolated protease variant of a parent protease, wherein (a) the protease variant comprises an amino acid sequence (i) having at least 90% identity to the sequence of SEQ ID NO:2 and (ii) comprising a substitution of glycine at positions 24 and 53, a substitution of asparagine at positions 78 and 101, a substitution of alanine or serine at position 128, and a substitution of glutamine at position 217; (b) the parent protease has at least 90% sequence identity to SEQ ID NO:2; (c) each amino acid position of the variant is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2; and (d) the protease variant has increased proteolytic activity and/or cleaning activity relative to the parent protease.

52. The variant according to any of the preceding claims, wherein the variant is a mature form.

53. An isolated polypeptide having protease activity, said polypeptide comprising an amino acid sequence having at least 85% sequence identity to a polypeptide sequence selected from the group consisting of:

a) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+G131H+N243V+L257G;

b) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S033T+N076D;

c) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S009T+N109G+K141R+N243V;

d) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S162G+K256R;

e) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+A116T;

f) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+L257G;

g) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S162G+L257G;

h) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N061G+N109G+N243V;

i) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+N109G+N243V+S248A;

j) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S033T+N076D+N109G+N218S+N243V+S248N+K256R;

k) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+A116T+N243V+

K256R;

l) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+ G131H+N243V;

m) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G;

n) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+N243V;

o) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+T158S+L257G;

p) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N061S+N109G+N243V;

q) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+P040A+N109G+N243V+ S248N+K256R;

r) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S009T+S018T+Y021N+ N109G+K141R;

s) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+ T158S+N243V+K256R;

t) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+A088T+N109G+A116T+ T158S+N218S+L257G;

u) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N109G+K256R;

v) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+N109G+N243V+K256R;

w) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+K256R;

x) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+N109G;

y) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S063G;

z) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+S063G+N076D;

aa) BPN'-S024G+S053G+S078N+S101N+G128S+Y217Q+S033T+N076D+N218S;

bb) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q+N076D+N218S; and

cc) BPN'-S024G+S053G+S078N+S101N+G128A+Y217Q, wherein each amino acid position of the variant is numbered by correspondence with an amino acid position of the sequence of SEQ ID NO:2.

54. An isolated nucleic acid comprising a polynucleotide sequence encoding a variant of any of claims 1-52 or a polypeptide of claim 53, or a complementary polynucleotide sequence thereof.

55. An isolated nucleic acid comprising a polynucleotide sequence having at least 80% sequence identity to the polynucleotide sequence set forth in SEQ ID NO:3 or SEQ ID NO:5, or a complementary polynucleotide sequence thereof.

56. An expression vector comprising at least one nucleic acid of claim 54 or 55.

57. The expression vector of claim 56, wherein the at least one nucleic acid is operably linked to a promoter.

58. A recombinant host cell comprising: (a) a nucleic acid of claim 64 or 65, or (b) an expression vector of claim 56 or 57.

59. The recombinant host cell of claim 58, wherein the host cell is a bacterial cell.

60. The recombinant host cell of claim 59, wherein the host cell is a *Bacillus* cell.

61. The recombinant host cell of claim 70, wherein the host cell is a *Bacillus* subtilis cell.

62. A cell culture comprising: (a) a nucleic acid of claim 54 or 55, or (b) an expression vector of claim 56 or 57.

63. A method of producing a protease variant, the method comprising cultivating a recombinant host cell of any of claims 58-61 under conditions conducive to produce the variant.

64. The method of claim 63, further comprising recovering the variant from the cell culture.

65. A method of producing a protease variant, the method comprising:

(a) introducing the recombinant expression vector of claim 56 or 57 into a population of cells; and

(b) culturing the cells in a culture medium under conditions conducive to produce the protease variant encoded by the expression vector.

66. The method of claim 65, further comprising:

(c) isolating or recovering the variant from the cells or from the culture medium.

67. A composition comprising a variant of any of claims 1-52 or a polypeptide of claim 53, which composition is not a fabric and home care product.

68. The composition of claim 67, comprising at least one adjunct ingredient or carrier.

69. The composition of claim 67 or 68, wherein the composition comprises an additional enzyme.

70. The composition according to claim 69, wherein the additional enzyme is selected from the group consisting of a hemicellulase, cellulase, amylase, peroxidase, protease, xylanase, lipase, phospholipase, esterase, cutinase, pectinase, pectate lyase, mannanase, keratinase, reductase, oxidase, phenoloxidase, lipoxygenase, ligninase, pullulanase, tannase, pentosanase, malanase, ß-glucanase, arabinosidase, hyaluronidase, chondroitinase, and laccase.

71. The composition of any of claims 67-70, wherein the composition is a detergent composition for cleaning a contact lens.

72. The composition of any of claims 67-70, wherein the composition is a cleaning composition useful in a personal care application.

73. The composition of any of claims 67-72, further comprising at least one builder and/or at least one surfactant.

74. A method for cleaning an item or surface in need of cleaning, the method comprising contacting the item or surface with a variant of any of claims 1-52, a polypeptide of claim 53, or a composition of any of claims 67-73.

75. The method of claim 74, further comprising rinsing the item or surface with water.

76. A method for cleaning a surface or object, the method comprising contacting at least a portion of the item or surface to be cleaned with a variant of any of claims 1-52, a polypeptide of claim 53, or a composition of any of claims 67-73 for a sufficient time and/or under conditions sufficient or effective to clean or wash the item or surface to a desired degree, and optionally comprising rinsing the item or surface with water.

77. A method of treating and/or cleaning a surface or fabric comprising the steps of optionally washing and/or rinsing said surface or fabric, contacting said surface or fabric with a variant of any of claims 1-52, a polypeptide of claim 52, or a composition of any of claims 67-73, then optionally washing and/or rinsing said surface or fabric.

78. The variant of any of claims 1-52, wherein the parent protease is a subtilisin protease, and the variant is a subtilisin protease variant having improved wash performance or improved cleaning performance in a detergent as compared to that of the parent subtilisin protease, or the polypeptide of claim 53, wherein the polypeptide has improved wash performance or improved cleaning performance in a detergent as compared to that of the protease of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

FIGURE 1

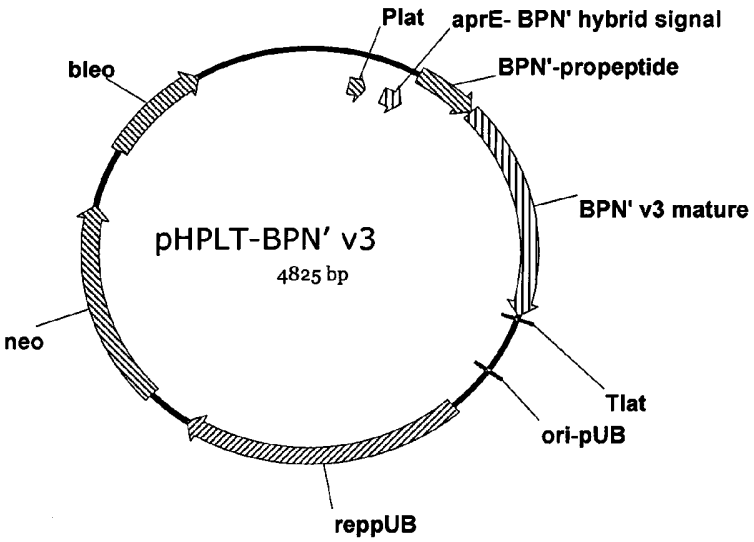

Plat
aprE- BPN' hybrid signal
BPN'-propeptide
bleo
BPN' v3 mature
pHPLT-BPN' v3
4825 bp
neo
Tlat
ori-pUB
reppUB

FIGURE 2

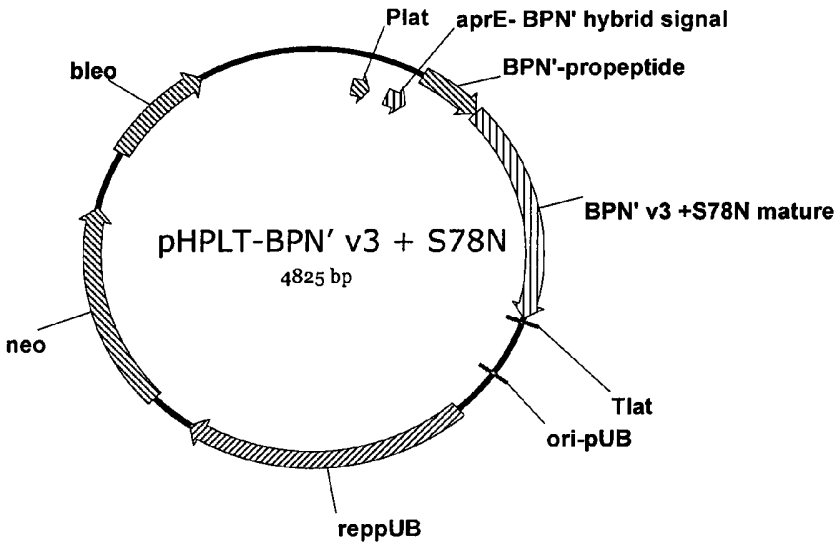

Plat
aprE- BPN' hybrid signal
BPN'-propeptide
bleo
BPN' v3 +S78N mature
pHPLT-BPN' v3 + S78N
4825 bp
neo
Tlat
ori-pUB
reppUB

FIGURE 3

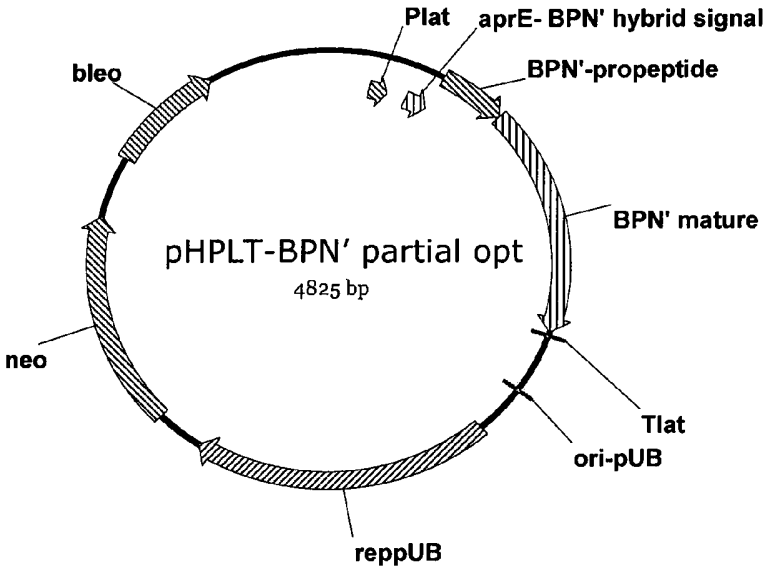

Plat
aprE- BPN' hybrid signal
BPN'-propeptide
bleo
BPN' mature
pHPLT-BPN' partial opt
4825 bp
neo
Tlat
ori-pUB
reppUB

FIGURE 4

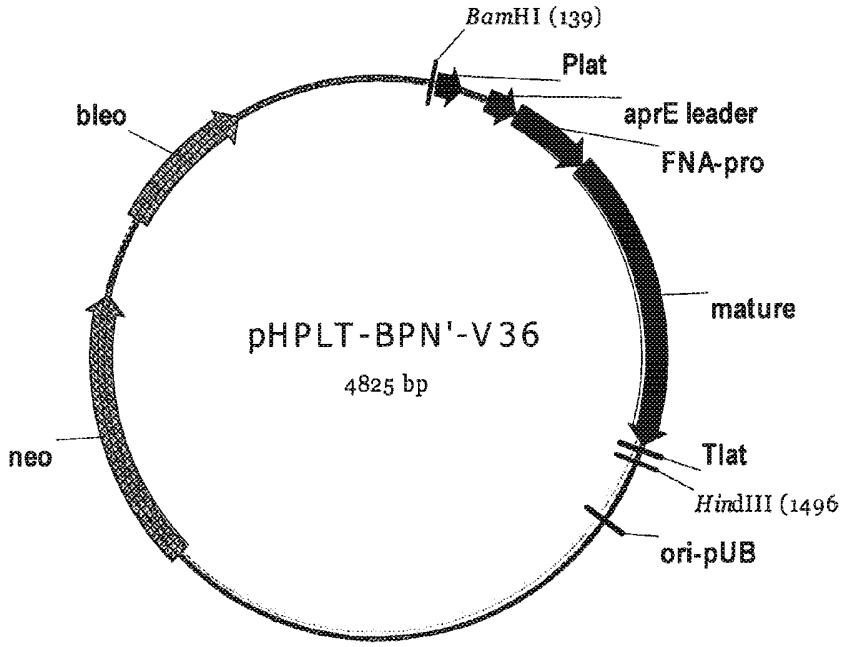

BamHI (139)
Plat
aprE leader
FNA-pro
mature
Tlat
HindIII (1496
ori-pUB
neo
bleo
pHPLT-BPN'-V36
4825 bp

FIGURE 5

```
BPN'          1 AQSVPYGVSQ IKAPALHSQG YTGSNVKVAV IDSGIDSSHP DLKVAGGASM VPSETNPFQD
GG36          1 AQSVPWGISR VQAPAAHNRG LTGSGVKVAV LDTGIS-THP DLNIRGGASF VPGEPST-QD

BPN'         61 NNSHGTHVAG TVAALNNSIG VLGVAPSASL YAVKVLGADG SGQYSWIING IEWAIANNMD
GG36         59 GNGHGTHVAG TIAALNNSIG VLGVAPSAEL YAVKVLGASG SGSVSSIAQG LEWAGNNGMH

                                                          161
BPN'        121 VINMSLGGPS GSAALKAAVD KAVASGVVVV AAAGNEGTSG SSSTVGYPGK YPSVIAVGAV
GG36        119 VANLSLGSPS PSATLEQAVN SATSRGVLVV AASGNSGAGS ----ISYPAR YANAMAVGAT

BPN'        181 DSSNQRASFS QYGPELDVMA PGVSIQSTLP GNKYGAYNGT SMASPHVAGA AALILSKHPN
GG36        175 DQNNNRASFS QYGAGLDIVA PGVNVQSTYP GSTYASLNGT SMATPHVAGA AALVKQKNPS

BPN'        241 WTNTQVRSSL ENTTTKLGDS FYYGKGLINV QAAAQ
GG36        235 WSNVQIRNHL KNTATSLGST NLYGSGLVNA EAATR
```

FIGURE 6

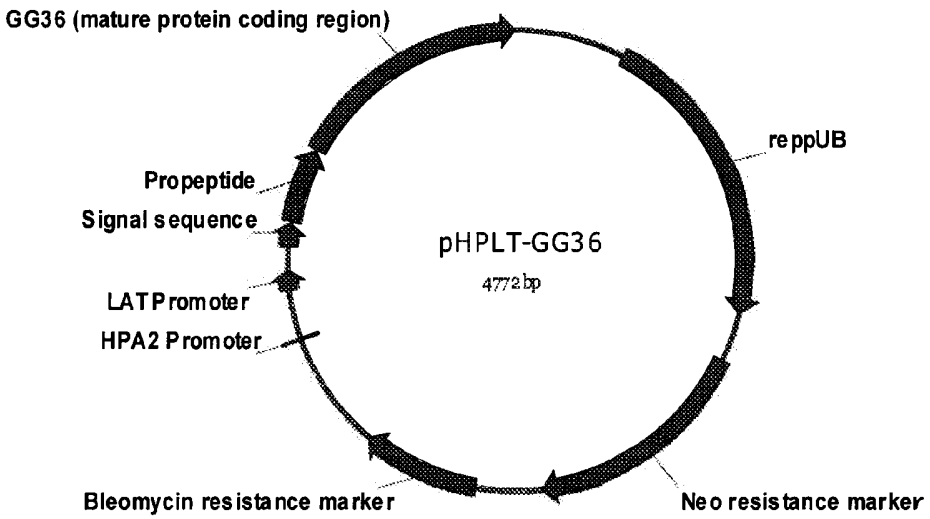

GG36 (mature protein coding region)
reppUB
Propeptide
Signal sequence
pHPLT-GG36
4772 bp
LATPromoter
HPA2 Promoter
Bleomycin resistance marker
Neo resistance marker

FIGURE 7

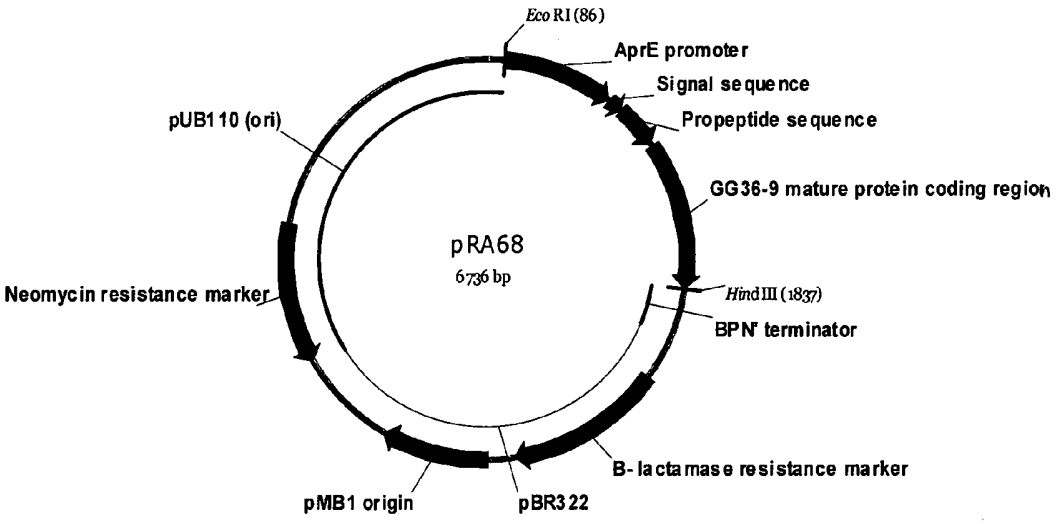
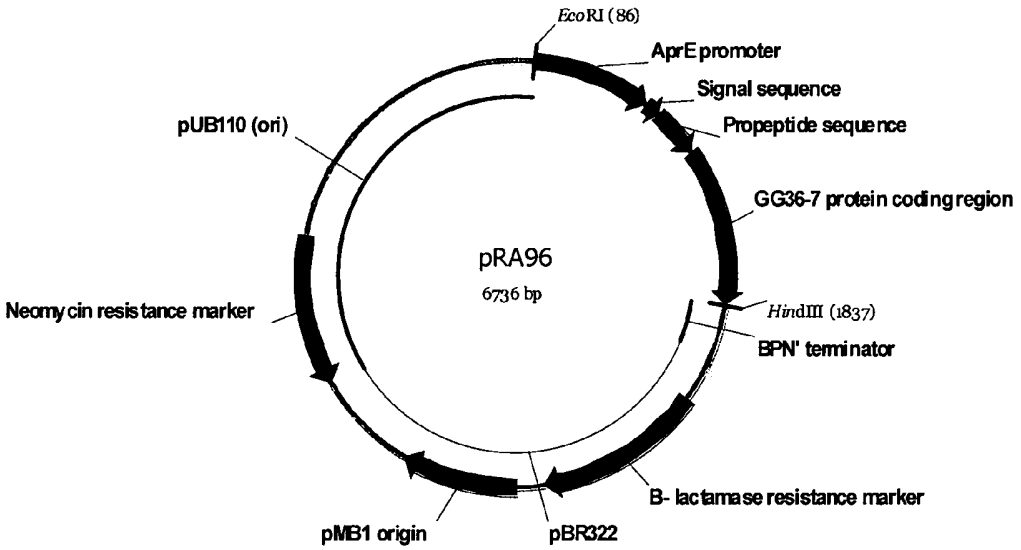

EcoRI (86)
AprE promoter
Signal sequence
Propeptide sequence
GG36-9 mature protein coding region
HindIII (1837)
BPN' terminator
B-lactamase resistance marker
pBR322
pMB1 origin
Neomycin resistance marker
pUB110 (ori)
pRA68
6736 bp

FIGURE 8


EcoRI (86)
AprE promoter
Signal sequence
Propeptide sequence
GG36-7 protein coding region
HindIII (1837)
BPN' terminator
B- lactamase resistance marker
pBR322
pMB1 origin
Neomycin resistance marker
pUB110 (ori)
pRA96
6736 bp