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DESCRIPTION

Description

Background of the Invention

Field of the Invention

[0001] The present invention relates to a detergent composition comprising novel subtilase variants exhibiting increased stability and/or improved wash performance in liquid detergent compositions. Further, the present invention relates to methods of producing said detergent compositions and to the use of said detergent compositions in cleaning applications.

Description of the Related Art

[0002] In the detergent industry, enzymes have for many decades been implemented in washing formulations. Enzymes used in such formulations comprise proteases, lipases, amylases, cellulases, mannosidases as well as other enzymes or mixtures thereof. Commercially the most important enzymes are proteases.

[0003] A wild type subtilase that have been used in laundry is the BLAP protease disclosed in WO 91/02792.

[0004] An increasing number of commercially used proteases are protein engineered variants of naturally occurring wild type proteases Everlase®, Relase®, Ovozyme®, Polarzyme®, Liquanase®, Liquanase Ultra® and Kannase® (Novozymes a/s), Purafast®, Purafect OXP®, FN3®, FN4® and Excellase® (Genencor International, Inc.). Further, a number of variants are described in the art, such as in WO2004/041979 (NOVOZYMES A/S) which describes subtilase variants exhibiting alterations relative to the parent subtilase in e.g. wash performance, thermal stability, stability during wash or catalytic activity. The variants are suitable for use in e.g. cleaning or detergent compositions.

[0005] Variants of the BLAP protease and suitable for use in cleaning or detergent compositions have been disclosed in e.g. EP 701 605. From DE 102009029513 A1, DE 102012215642 A1, EP 2100948 A1, WO 2003/038082 A2, WO 2012/08201 A2, WI 2012/080202 A1, WO 2013/060621 A1, DE 102011118021 A1, WO 2011/072117 A1, WO

2011/140316 A1, WO 95/30011 A2 and WO 2010/056653 A2, CA 2829859 A1, WO 2004/083362 A2, WO 2005/095592 A2, WO 2003/062380 A2, EP 3106508 A1 and WO 92/00345 A1 also proteases suitable for use in cleaning or washing compositions are known.

[0006] WO99/57155 discloses detergent enzymes such as proteases modified by attachment of a cellulose binding domain to the enzymes. It is suggested that binding the detergent enzymes such as protease to textile containing cellulose would enhance wash performance.

[0007] However, various factors make further improvement of the proteases advantageous. In particular liquid detergent compositions remain a challenge for many detergent proteases and loss of activity during storage remains a problem for many good detergent proteases. Thus despite the intensive research in protease development there remains a need for new and improved proteases that have a satisfactory wash performance and increased stability in detergent compositions.

Summary of the Invention

[0008] The invention relates to a detergent composition comprising subtilase variant having improved stability and/or improved wash performance in liquid detergents compared with the parent subtilase.

[0009] The invention relates to a detergent composition comprising subtilase variant having at least 90% sequence identity to SEQ ID NO: 3, preferably at least 95% sequence identity, preferably at least 96% sequence identity, preferably at least 97% sequence identity, preferably at least 98% sequence identity to SEQ ID NO: 3, wherein the variant has a glutamic acid residue (E) in position 101, and where the variant has increased stability in a liquid detergent composition compared to the subtilase having the amino acid sequence of SEQ ID NO: 3, and wherein the subtilase further comprises the substitution P129D (according to numbering of SEQ ID NO:1).

[0010] The invention further relates to the use of the detergent compositions of the invention to laundry or hard surface cleaning, such as dish wash e.g. automated dish wash.

[0011] The present invention further relates to a method for producing a detergent composition comprising the step of adding a variant subtilase obtained by a method for obtaining said variant subtilase according to present invention, and recovering the variant. The invention also relates to detergent compositions obtained by such method.

Definitions

[0012] Allelic variant: The term "allelic variant" means any of two or more alternative forms of

a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

[0013] cDNA: The term "cDNA" means a DNA molecule that can be prepared by reverse transcription from a mature, spliced, mRNA molecule obtained from a eukaryotic or prokaryotic cell.

[0014] cDNA lacks intron sequences that may be present in the corresponding genomic DNA. The initial, primary RNA transcript is a precursor to mRNA that is processed through a series of steps, including splicing, before appearing as mature spliced mRNA.

[0015] Coding sequence: The term "coding sequence" means a polynucleotide, which directly specifies the amino acid sequence of a variant. The boundaries of the coding sequence are generally determined by an open reading frame, which begins with a start codon such as ATG, GTG or TTG and ends with a stop codon such as TAA, TAG, or TGA. The coding sequence may be a genomic DNA, cDNA, synthetic DNA, or a combination thereof.

[0016] Control sequences: The term "control sequences" means nucleic acid sequences necessary for expression of a polynucleotide encoding a variant of the present invention. Each control sequence may be native (*i.e.*, from the same gene) or foreign (*i.e.*, from a different gene) to the polynucleotide encoding the variant or native or foreign to each other. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a variant.

[0017] Detergent composition: The term "detergent composition", includes unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, soap bars, mouthwashes, denture cleaners, car or carpet shampoos, bathroom cleaners; hair shampoos and hair-rinses; shower gels, foam baths; metal cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types. The terms "detergent composition" and "detergent formulation" are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some embodiments, the term is used in reference to laundering fabrics and/or garments (e.g.,

"laundry detergents"). In alternative embodiments, the term refers to other detergents, such as those used to clean dishes, cutlery, etc. (e.g., "dishwashing detergents"). It is not intended that the present invention be limited to any particular detergent formulation or composition. The term "detergent composition" is not intended to be limited to compositions that contain surfactants. It is intended that in addition to the variants according to the invention, the term encompasses detergents that may contain, e.g., surfactants, builders, chelators or chelating agents, bleach system or bleach components, polymers, fabric conditioners, foam boosters, suds suppressors, dyes, perfume, tannish inhibitors, optical brighteners, bactericides, fungicides, soil suspending agents, anticorrosion agents, enzyme inhibitors or stabilizers, enzyme activators, transferase(s), hydrolytic enzymes, oxido reductases, bluing agents and fluorescent dyes, antioxidants, and solubilizers.

[0018] In addition to containing a subtilase variant as disclosed herein, the detergent formulation may contain one or more additional enzymes (such as proteases, amylases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidaes, haloperoxygenases, catalases and mannanases, or any mixture thereof), and/or components such as surfactants, builders, chelators or chelating agents, bleach system or bleach components, polymers, fabric conditioners, foam boosters, suds suppressors, dyes, perfume, tannish inhibitors, optical brighteners, bactericides, fungicides, soil suspending agents, anti-corrosion agents, enzyme inhibitors or stabilizers, enzyme activators, transferase(s), hydrolytic enzymes, oxido reductases, bluing agents and fluorescent dyes, antioxidants, and solubilizers.

[0019] Dish wash: The term "dish wash" refers to all forms of washing dishes, e.g. by hand or automatic dish wash. Washing dishes includes, but is not limited to, the cleaning of all forms of crockery such as plates, cups, glasses, bowls, all forms of cutlery such as spoons, knives, forks and serving utensils as well as ceramics, plastics such as melamine, metals, china, glass and acrylics.

[0020] Dish washing composition: The term "dish washing composition" refers to all forms of compositions for cleaning hard surfaces. The present invention is not restricted to any particular type of dish wash composition or any particular detergent.

[0021] Expression: The term "expression" includes any step involved in the production of a variant including, but not limited to, transcription, post-transcriptional modification, translation, posttranslational modification, and secretion.

[0022] Expression vector: The term "expression vector" means a linear or circular DNA molecule that comprises a polynucleotide encoding a variant and is operably linked to control sequences that provide for its expression.

[0023] Hard surface cleaning: The term "Hard surface cleaning" is defined herein as cleaning of hard surfaces wherein hard surfaces may include floors, tables, walls, roofs etc. as well as surfaces of hard objects such as cars (car wash) and dishes (dish wash). Dish washing

includes but are not limited to cleaning of plates, cups, glasses, bowls, and cutlery such as spoons, knives, forks, serving utensils, ceramics, plastics such as melamine, metals, china, glass and acrylics.

[0024] Host cell: The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, or the like with a nucleic acid construct or expression vector comprising a polynucleotide of the present invention. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

[0025] Improved wash performance: The term "improved wash performance" is defined herein as a subtilase variant displaying an alteration of the wash performance relative to the parent subtilase (i.e. relative to a subtilase having the identical amino acid sequence of said variant but excluding the alterations in said variant), such as relative to the mature polypeptide of SEQ ID NO: 2 e.g. by increased stain removal. The term "wash performance" includes wash performance in dish wash but also in laundry. The wash performance may be determined by calculating the so-called intensity value (Int) as defined in the Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash in the Materials and Methods section herein.

[0026] Isolated: The term "isolated" means a substance in a form or environment which does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated (*e.g.*, multiple copies of a gene encoding the substance; use of a stronger promoter than the promoter naturally associated with the gene encoding the substance). An isolated substance may be present in a fermentation broth sample.

[0027] Laundering: The term "laundering" relates to both household laundering and industrial laundering and means the process of treating textiles and/or fabrics with a solution containing a detergent composition of the present invention. The laundering process can for example be carried out using e.g. a household or an industrial washing machine or can be carried out by hand.

[0028] Mutant: The term "mutant" means a polynucleotide encoding a variant.

[0029] Parent: The term "parent" means a protease to which an alteration is made to produce the enzyme variants of the present invention. Thus the parent is a protease having the identical amino acid sequence of said variant but not having the alterations at one or more e.g. two or more of said specified positions. It will be understood that in the present context the expression "having identical amino acid sequence" relates to 100% sequence identity. The parent may be a naturally occurring (wild-type) polypeptide or a variant thereof. In a particular

embodiment the parent is a protease with at least 60 % identity, such as at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to a polypeptide with the mature polypeptide of SEQ ID NO: 2.

[0030] Protease: The term "protease" is defined herein as an enzyme that hydrolyses peptide bonds. It includes any enzyme belonging to the EC 3.4 enzyme group (including each of the thirteen subclasses thereof). The EC number refers to Enzyme Nomenclature 1992 from NC-IUBMB, Academic Press, San Diego, California, including supplements 1-5 published in Eur. J. Biochem. 1994, 223, 1-5; Eur. J. Biochem. 1995, 232, 1-6; Eur. J. Biochem. 1996, 237, 1-5; Eur. J. Biochem. 1997, 250, 1-6; and Eur. J. Biochem. 1999, 264, 610-650; respectively.

[0031] Protease activity: The term "protease activity" means a proteolytic activity (EC 3.4). Proteases of the invention are endopeptidases (EC 3.4.21). There are several protease activity types: The three main activity types are: trypsin-like where there is cleavage of amide substrates following Arg or Lys at P1, chymotrypsin-like where cleavage occurs following one of the hydrophobic amino acids at P1, and elastase-like with cleavage following an Ala at P1. For purposes of the present invention, protease activity is determined according to the procedure described in "Materials and Methods" below. The subtilase variants of the present invention have at least 20%, *e.g.*, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, and at least 100% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

[0032] Sequence identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity". For purposes of the present invention, the sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment)

[0033] For purposes of the present invention, the sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *supra*) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.,* 2000, *supra*), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief

option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment - Total Number of Gaps in Alignment)

[0034] Stability: The term "stability" includes storage stability and stability during use, e.g. during a wash process (in wash stability) and reflects the stability of the subtilase variant according to the invention as a function of time e.g. how much activity is retained when the protease is kept in solution, in particular in a detergent solution. The stability is influenced by many factors e.g. pH, temperature, detergent composition e.g. amount of builder, surfactants etc. The protease stability may be measured using the 'stability assay' as described in the Materials and Methods section herein. The term "improved stability" or "increased stability" is defined herein as a variant protease displaying an increased stability in solutions, relative to the stability of the parent protease. The terms "improved stability" and "increased stability" includes "improved chemical stability", "detergent stability" or "improved detergent stability.

[0035] The term "improved chemical stability" is defined herein as a variant enzyme displaying retention of enzymatic activity after a period of incubation in the presence of a chemical or chemicals, either naturally occurring or synthetic, which reduces the enzymatic activity of the parent enzyme. Improved chemical stability may also result in variants being more able to catalyze a reaction in the presence of such chemicals. In a particular aspect of the invention the improved chemical stability is an improved stability in a detergent, in particular in a liquid detergent. The term "detergent stability" or "improved detergent stability is in particular an improved stability of the protease activity when a protease variant of the present invention is mixed into a liquid detergent formulation, especially into a liquid detergent formulation according to table 1and then stored at temperatures between 15 and 50 °C, e. g. 20°C, 30°C or 40°C.

[0036] The term "improved thermal activity" means a variant displaying an altered temperature-dependent activity profile at a specific temperature relative to the temperature-dependent activity profile of the parent or relative to a protease with SEQ ID NO: 3.The thermal activity value provides a measure of the variant's efficiency in enhancing catalysis of a hydrolysis reaction over a range of temperatures. A more thermo active variant will lead to an increase in enhancing the rate of hydrolysis of a substrate by an enzyme composition thereby decreasing the time required and/or decreasing the enzyme concentration required for activity. Alternatively, a variant with a reduced thermal activity will enhance an enzymatic reaction at a temperature lower than the temperature optimum of the parent defined by the temperature-dependent activity profile of the parent.

[0037] Stringency conditions: The different stringency conditions are defined as follows.

[0038] The term "very low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200

micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 60°C.

[0039] The term "low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 1X SSC, 0.2% SDS at 60°C.

[0040] The term "medium stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 1x SSC, 0.2% SDS at 65°C.

[0041] The term "medium-high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.5X SSC, 0.2% SDS at 65°C.

[0042] The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.3X SSC, 0.2% SDS at 65°C.

[0043] The term "very high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.15X SSC, 0.2% SDS at 65°C.

[0044] Substantially pure variant: The term "substantially pure variant" means a preparation that contains at most 10%, at most 8%, at most 6%, at most 5%, at most 4%, at most 3%, at most 2%, at most 1%, and at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. Preferably, the variant is at least 92% pure, *e.g.*, at least 94% pure, at least 95% pure, at least 96% pure, at least 97% pure, at least 98% pure, at least 99%, at least 99.5% pure, and 100% pure by weight of the total polypeptide material present in the preparation. The variants of the present invention are preferably in a substantially pure form. This can be accomplished, for example, by preparing the variant by well-known recombinant methods or by classical purification methods.

[0045] Variant: The term "variant" means a polypeptide having protease activity comprising an alteration, *i.e.*, a substitution, insertion, and/or deletion, at one or more (*e.g.*, several) positions. A substitution means replacement of the amino acid occupying a position with a different amino acid; a deletion means removal of the amino acid occupying a position; and an insertion means adding one or more (e.g. several) amino acids, e.g. 1, 2, 3, 4 or 5 amino acids adjacent to and immediately following the amino acid occupying a position.

[0046] Wash performance: The term "wash performance" is used as an enzyme's ability to remove stains present on the object to be cleaned during e.g. wash, such as laundry or hard surface cleaning. The improvement in the wash performance may be quantified by calculating the so-called intensity value (Int) defined in AMSA assay, as described in Materials and Methods section.

[0047] Wild-Type subtilase: The term "wild-type subtilase" means a protease expressed by a naturally occurring organism, such as a bacterium, archaea, yeast, fungus, plant or animal found in nature. An example of a wild-type subtilase is BLAP i.e. the subtilase having the amino acid sequence of SEQ ID NO: 2.

Conventions for Designation of Variants

[0048] For purposes of the present invention, the mature polypeptide BPN' disclosed in SEQ ID NO: 1 is used to determine the corresponding amino acid residue in another protease. The amino acid sequence of another protease is aligned with the mature polypeptide disclosed in SEQ ID NO: 1, and based on the alignment, the amino acid position number corresponding to any amino acid residue in the mature polypeptide disclosed in SEQ ID NO: 1 is determined using the Needleman-Wunsch algorithm as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix.

[0049] Identification of the corresponding amino acid residue in another protease can be determined by an alignment of multiple polypeptide sequences using several computer programs including, but not limited to, MUSCLE (multiple sequence comparison by log-expectation; version 3.5 or later), MAFFT (version 6.857 or later), and EMBOSS EMMA employing ClustalW (1.83 or later), using their respective default parameters.

[0050] When the other enzyme has diverged from the mature polypeptide of SEQ ID NO: 1 such that traditional sequence-based comparison fails to detect their relationship, other pairwise sequence comparison algorithms can be used. Greater sensitivity in sequence-based searching can be attained using search programs that utilize probabilistic representations of polypeptide families (profiles) to search databases. For example, the PSI-BLAST program generates profiles through an iterative database search process and is capable of detecting

remote homologs. Even greater sensitivity can be achieved if the family or superfamily for the polypeptide has one or more representatives in the protein structure databases. Programs such as GenTHREADER utilize information from a variety of sources (PSI-BLAST, secondary structure prediction, structural alignment profiles, and solvation potentials) as input to a neural network that predicts the structural fold for a query sequence. Similarly, the method of Gough et al., 2000, J. Mol. Biol. 313: 903-919, can be used to align a sequence of unknown structure with the superfamily models present in the SCOP database. These alignments can in turn be used to generate homology models for the polypeptide, and such models can be assessed for accuracy using a variety of tools developed for that purpose.

[0051] For proteins of known structure, several tools and resources are available for retrieving and generating structural alignments. For example the SCOP superfamilies of proteins have been structurally aligned, and those alignments are accessible and downloadable. Two or more protein structures can be aligned using a variety of algorithms such as the distance alignment matrix or combinatorial extension, and implementation of these algorithms can additionally be utilized to query structure databases with a structure of interest in order to discover possible structural homologs.

[0052] In describing the variants of the present invention, the nomenclature described below is adapted for ease of reference. The accepted IUPAC single letter or three letter amino acid abbreviation is employed.

[0053] Substitutions. For an amino acid substitution, the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine at position 226 with alanine is designated as "Thr226Ala" or "T226A". Multiple mutations are separated by addition marks ("+"), *e.g.*, "Gly205Arg + Ser411Phe" or "G205R + S411F", representing substitutions at positions 205 and 411 of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively.

[0054] Deletions. For an amino acid deletion, the following nomenclature is used: Original amino acid, position, *. Accordingly, the deletion of glycine at position 195 is designated as "Gly195*" or "G195*". Multiple deletions are separated by addition marks ("+"), *e.g.*, "Gly195* + Ser411*"or "G195* + S411*".

[0055] Insertions: The insertion of an additional amino acid residue such as *e.g.* a lysine after G195 may be indicated by: Gly195GlyLys or G195GK. Alternatively insertion of an additional amino acid residue such as lysine after G195 may be indicated by: *195aL. When more than one amino acid residue is inserted, such as *e.g.* a Lys and Ala after G195 this may be indicated as: Gly195GlyLysAla or G195GKA. In such cases, the inserted amino acid residue(s) may also be numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s), in this example: *195aK *195bA. In the above example, the sequences 194 to 196 would thus be:

	194 195 196
Savinase	A - G - L

	194 195 195a 195b 196
Variant	A - G - K - A - L

[0056] In cases where a substitution and an insertion occur at the same position this may be indicated as S99SD+S99A or in short S99AD. The same modification may also be indicated as S99A + *99aD.

[0057] In cases where an amino acid residue identical to the existing amino acid residue is inserted it is clear that degeneracy in the nomenclature arises. If for example a glycine is inserted after the glycine in the above example this would be indicated by G195GG or *195aGbG. The same actual change could just as well be indicated as A194AG or *194aG for the change from:

	194 195 196
Savinase	A - G - L
To:	
	194 195 195a 196
Variant	A - G - G - L
	194 194a 195 196

[0058] Such instances will be apparent to the skilled person and the indication G195GG and corresponding indications for this type of insertions are thus meant to comprise such equivalent degenerate indications.

[0059] Multiple alterations: Variants comprising multiple alterations are separated by addition marks ("+"), *e.g.*, "Arg170Tyr+Gly195Glu" or "R170Y+G195E" representing a substitution of arginine and glycine at positions 170 and 195 with tyrosine and glutamic acid, respectively. Alternatively, multiple alterations may be separated be space or a comma e.g. A170Y G195E or A170Y, G195E respectively.

[0060] Different alterations: Where different alterations can be introduced at a position, the different alterations are separated by a comma, *e.g.*, "Arg170Tyr,Glu" represents a substitution of arginine at position 170 with tyrosine or glutamic acid. Thus, "Tyr167Gly,Ala + Arg170Gly,Ala" designates the following variants:
"Tyr167Gly+Arg170Gly", "Tyr167Gly+Arg 170Ala", "Tyr167Ala+Arg170Gly", and "Tyr167 Ala+Arg 170Ala".

[0061] Alternatively different alterations or optional substitutions may be indicated in brackets e.g. Arg170 [Tyr, Glu] or Arg170{Tyr, Glu} or in short R170 [Y,E] or R170 {Y, E}.

Numbering of amino acid positions/residues

[0062] If nothing else is mentioned the amino acid numbering used herein correspond to that of the subtilase BPN' (BASBPN) sequence. For further description of the BPN' sequence, see SEQ ID NO: 1 or Siezen et al., Protein Eng. 4 (1991) 719-737.

Detailed Description of the Invention

[0063] The invention relates to detergent compositions comprising subtilase variants having improved stability and/or improved wash performance in liquid detergents. Preferably the subtilase variant also has good wash performance, more preferred the variant has improved wash performance compared with the parent subtilase, SEQ ID NO: 2 or SEQ ID NO: 3. Whenever the terms "variant(s)" or "subtilase variant(s)" are mentioned further on, they refer to detergent compositions comprising such protease variant(s).

[0064] The parent subtilase may in principle be any natural occurring subtilase or it may be a modified subtilase generated by methods known in the art such as preparation of hybrids or two or more individual subtilases or by substituting, deleting or inserting one or more amino acid residues in a given subtilase.

[0065] Many subtilases have a proven record or good wash performance and there is an abundance of publications describing such subtilases and their use in laundry or cleaning processes, however, for use in detergent it is also important that the subtilases have a satisfactory stability in detergent compositions, such as in liquid detergent. It is preferred to use a parent subtilase having a good wash performance in order to provide variants of such subtilases having improved stability in liquid detergent.

[0066] A preferred parent subtilase comprised in the detergent composition of the invention is the BLAP protease having the amino acid sequence of SEQ ID NO: 2, or a subtilase having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2. The preferred parent subtilase may be a subtilase having the amino acid sequence of SEQ ID NO: 2, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids have been modified compared with SEQ ID NO: 2, and wherein each modification is independently a substitution of one amino acid residue with another amino acid residue, a deletion of an amino acid residue or an insertion of one amino acid residue.

[0067] In another aspect, the parent comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another aspect, the parent comprises or consists of amino acids 1 to 269 of SEQ ID NO: 2. In another embodiment, the parent is an allelic variant of the mature

polypeptide of SEQ ID NO: 2.

[0068] One preferred parent subtilase comprised in the detergent composition of the invention is the subtilase having the amino acid sequence of SEQ ID NO: 2 with a substitution of the arginine residue in position 101 to a glutamic acid residue (R101E). The sequence of this preferred subtilase is shown in SEQ ID NO: 3.

[0069] Other preferred parent subtilase comprised in the detergent composition according to the invention is the protease having the amino acid sequence of SEQ ID NO: 3, or a subtilase having at least 60%, *e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100%sequence identity to SEQ ID NO: 3. In another aspect, the parent comprises or consists of the amino acid sequence of SEQ ID NO: 3. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 3. In another aspect, the parent comprises or consists of amino acids 1 to 269 of SEQ ID NO: 3. In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 3.

[0070] The preferred parent subtilase may be a subtilase having the amino acid sequence of SEQ ID NO: 3, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids have been modified compared with SEQ ID NO: 3, and wherein each modification is independently a substitution of one amino acid residue with another amino acid residue, a deletion of an amino acid residue or an insertion of one amino acid residue.

[0071] Further detergent compositions according to the invention include subtilase variants having at least 90% sequence identity to SEQ ID NO: 3, which variant comprises the substitution P129D, wherein the positions are numbered according to SEQ ID NO: 1.

[0072] In a preferred embodiment the detergent compositions according to the invention comprises subtilase variants having both improved stability in liquid detergent and improved wash performance in comparison with the subtilase having the sequence of SEQ ID NO: 3. Examples of such preferred subtilases of this embodiment includes subtilase variants having at least 90% sequence identity to SEQ ID NO: 3 and comprising the substitution P129D.

[0073] The variants may have other substitutions e.g. substitutions known in the art to impart a particular beneficial property to the subtilase variants. There is an abundance of substitutions in subtilases known in the art and it is contemplated that such known substitutions may be used in the present invention in order to impart such known beneficial effects to the variants comprised in the detergent compositions of the present invention. The subtilase variants of the invention may comprise one or more further substitutions which may be used in the present invention in order to impart additional beneficial effects and/or to improve an existing effect such as stability and wash performance.

[0074] Preferred additional mutations includes one or more of the following substitutions V4I,

N76D, V104T, N128Q, S141H, R145H, A194P, G195E, V205I, N218Q, A228V, N238E, orS265H.

[0075] Further additional mutations might include S256D; L262E; Q137H; S3T; R45E,D; P55N; T58W,Y,L; Q59D,M,N,T; G61D,R; S87E; G97S; A98D,E,R; S106A,W; N117E; H120V,D,K,N; S124M; E136Q; S143W; S161T; S163A,G; Y171L; A172S; N185Q; V199M; Y209W; M222Q; N238H; N261T; L262N,Q,D.

[0076] Particular preferred examples of detergent compositions according to the invention comprise subtilase variants having improved stability and/or improve wash performance in liquid detergent compared with the subtilase having the amino acid sequence of SEQ ID NO: 3 include variants comprising the amino acid sequences:
SEQ ID NO: 3 + P129D.

[0077] The subtilase variants comprised in the detergent compositions of the present invention have improved stability in liquid detergent compared with the parent enzyme, preferably the variants have improved stability in liquid detergent compared with the protease having SEQ ID NO: 2 or SEQ ID NO: 3.

[0078] As examples of preferred variants having improved stability in liquid detergent and improved wash performance compared with the parent enzyme can be mentioned:
SEQ ID NO: 3 + P129D.

[0079] These preferred variants have improved stability such as detergent stability and/or improved or on par wash performance compared with the parent subtilase. In this connection improved wash performance is intended to mean that the wash performance of the variant is higher on at least one stain than the wash performance of the parent subtilase where wash performance is determined using a suitable wash performance assay in a given detergent composition under suitable conditions.

[0080] In one preferred embodiment improved wash performance is measured using AMSA test, e.g. as disclosed in the Methods and Materials section of this application.

[0081] The wash performance of the variant is preferably at least 1 unit higher than the wash performance of the parent subtilase, preferably at least 2 units higher, such as at least 3 units higher, such as at least 4 units higher, such as at least 5 units higher, such as at least 6 units higher, such as at least 7 units higher such as at least 8 units higher, such as at least 9 units higher.

[0082] The subtilase variants may further comprise one or more additional alterations at one or more (e.g., several) other positions, selected from the group consisting of positions: 3, 4, 9, 12, 14, 15, 40, 43, 68, 72, 79, 86, 88, 92, 98, 99, 101, 120, 146, 183, 184, 188, 194, 216, 218, 224, 228, 236, 245, 255, 261, 267 and 270, preferably positions 9, 15, 68 and/or 120 (numbering according to SEQ ID NO: 2). It will be clear to the skilled artisan that if a position

has already been altered once, then it will not be altered a second time. In a preferred embodiment, the alteration at any of the positions selected from the group consisting of 3, 4, 9, 12, 14, 15, 40, 43, 68, 72, 79, 86, 88, 92, 98, 99, 101, 120, 146, 183, 184, 188, 194, 216, 218, 224, 228, 236, 245, 255, 261, 267 and 270 is a substitution. In a more preferred embodiment, the subtilase variant further comprises one or more substitutions selected from the group consisting of 3{D, E, L}, 4I, 9{H, K, R, G}, 12{D, E}, 14T, 15{G, M, S, T}, 40{A, G, M, S, T}, 43{D, E}, 63G, 68{A, G, I, L, M, S, T}, 72{V, L}, N76{D, E}, 79T, 86H, 88V, 92S, 98T, 99{E, T, A, G, M, D}, 101L, 120 {I, N}, 146S, 183{E, D}, 184{E, D}, 188G, 194P, 216{D, E}, 218{E, D}, 224{S, A, T, G, M}, 228T, 236D, 245{H, K, R}, 255{D, E}, 261{D, E}, 267{I, L, V} and/or 270{G, M, S, T} (numbering according to SEQ ID NO: 2). In an even more preferred embodiment, the subtilase variant further comprises one or more substitutions selected from the group consisting of S3{D, E, L}, V4I, S9{H, K, R, G}, Q12{D, E}, P14T, A15{G, M, S, T}, P40{A, G, M, S, T}, N43{D, E}, V68{A, G, I, L, M, S, T}, I72{V, L}, N76{D, E}, I79T, P86H, A88V, A92S, A98T, S99{E, T, A, G, M, D}, S101L, H120{I, N}, G146S, N183{E, D}, N184{E, D}, S188G, A194P, S216{D, E}, N218{E, D}, T224{S, A, T, G, M}, A228T, S236D, Q245{H, K, R}, T255{D, E}, N261{D, E}, L267{I, L, V} and/or A270{G, M, S, T} in the mature polypeptide of SEQ ID NO: 3, or a polypeptide having at least 60 %, preferably at least 70%, preferably at least 80%, preferably at least 90%, preferably at least 95% sequence identity hereto, wherein each position corresponds to the corresponding position of the mature polypeptide of SEQ ID NO: 1.

[0083] The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

[0084] Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, In, The Proteins, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

[0085] Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

[0086] Essential amino acids in a polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis. In the latter

technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for protease activity to identify amino acid residues that are critical to the activity of the molecule. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. For BPN' (SEQ ID NO: 1) the catalytic triad comprising the amino acids S221, H64, and D32 is essential for protease activity of the enzyme.

[0087] The subtilase variants may consist of 150 to 350, e.g., 175 to 330, 200 to 310, 220 to 300, 240 to 290, 260 to 280 or 269, 270, 271, 272, 273, 274 or 275 amino acids.

[0088] In one embodiment, the subtilase variant has improved wash performance. In another embodiment, the subtilase variant has improved stability, preferably improved stability during wash.

[0089] In an embodiment, the subtilase variant has improved stability in liquid detergent compared to the parent enzyme wherein the stability is measured using the 'stability assay' as described in the example 4 of Materials and Methods section herein. In an embodiment, the subtilase variant has improved stability compared to the polypeptide of SEQ ID NO: 2 wherein stability is measured using the 'stability assay' as described in example 4 of the Materials and Methods section herein. In an embodiment, the subtilase variant has improved stability compared to the polypeptide of SEQ ID NO: 3 wherein stability is measured using the 'stability assay' as described in example 4 of the Materials and Methods section herein.

[0090] In an embodiment, the subtilase variant has improved wash performance compared to the parent enzyme wherein wash performance is measured using the Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash as described in example 7 of the Materials and Methods section herein. In an embodiment, the subtilase variant has improved wash performance compared to the polypeptide of SEQ ID NO: 2, wherein wash performance is measured using the Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash as described in example 7 of the Materials and Methods section herein. In an embodiment, the subtilase variant has improved wash performance compared to the polypeptide of SEQ ID NO: 3 wherein wash performance is measured

SEQ ID NO: 3 +T58L+S106A

SEQ ID NO: 3 +T58L+A228V

SEQ ID NO: 3 +S3T+T58L

SEQ ID NO: 3 +T58L+S156D

SEQ ID NO: 3 +T58L+Y91H

SEQ ID NO: 3 +T58L+H120D

SEQ ID NO: 3 +T58L+S163G

SEQ ID NO: 3 +S163G+N261D

SEQ ID NO: 3 +T58L+N261D

SEQ ID NO: 3 +T58L+N76D

SEQ ID NO: 3 +S3T+N76D+H120D

SEQ ID NO: 3 +S3T+N76D+A228V

SEQ ID NO: 3 +S3T+N76D+S156D

SEQ ID NO: 3 +S3T+ N76D+ Y209W

SEQ ID NO: 3 +S3T+ N76D +Y209W +V244T

SEQ ID NO: 3 +N76D H120D

SEQ ID NO: 3 +N76D S156D

SEQ ID NO: 3 +H120D S156D

SEQ ID NO 3 +R45E+L262E

SEQ ID NO 3 +Q59D+G61D

SEQ ID NO 3 +S87E+L262E

SEQ ID NO 3 +G61D+L262E

SEQ ID NO 3 +Q59D+L262E

SEQ ID NO 3 +R45E+Q59D

SEQ ID NO 3 +Q59D+S156D

SEQ ID NO 3 +S156D+L262E

SEQ ID NO 3 +S163G+N238E+L262E

SEQ ID NO 3 +S3T+V4I+S163G+N261D

SEQ ID NO 3 +H120D+S163G+N261D

SEQ ID NO 3 +Y91H+N117H+N238H

SEQ ID NO 3 +T58L+S163G+N261D

SEQ ID NO 3 +S3T+V4I+S163G+N261D

SEQ ID NO 3 +S87E+S163G+L262E

SEQ ID NO 3 +S156D+S163G+L262E

SEQ ID NO 3 +T58LS163G+N261D

SEQ ID NO 3 +S156DS163G+L262E

SEQ ID NO 3 +S3T+N76D+Y209W+N261D+L262E

[0091] The subtilase variants comprised in the detergent compositions of the present invention have improved stability in liquid detergent compared with the parent enzyme, preferably the variants have improved stability in liquid detergent compared with the protease having SEQ ID NO: 2 or SEQ ID NO: 3.

[0092] As examples of preferred variants having improved stability in liquid detergent and improved wash performance compared with the parent enzyme can be mentioned:

SEQ ID NO: 3 + R45E,D,Q

SEQ ID NO: 3 + Q58L

SEQ ID NO: 3 + Q59D,

SEQ ID NO: 3 + G61D,

SEQ ID NO: 3 + S87E,

SEQ ID NO: 3 + G97S,

SEQ ID NO: 3 + A98E,

SEQ ID NO: 3 + N117E,

SEQ ID NO: 3 + H120D,K,V

SEQ ID NO: 3 + P129D,

SEQ ID NO: 3 + E136Q,

SEQ ID NO: 3 + Q137H,

SEQ ID NO: 3 + S156D,

SEQ ID NO: 3 + S160A,

SEQ ID NO: 3 + S163A,G,

SEQ ID NO: 3 + V199M

SEQ ID NO: 3 + M222Q

SEQ ID NO: 3 + N261T

SEQ ID NO: 3 + L262E,Q,N.

[0093] These preferred variants have improved stability such as detergent stability and/or improved or on par wash performance compared with the parent subtilase. In this connection improved wash performance is intended to mean that the wash performance of the variant is higher on at least one stain than the wash performance of the parent subtilase where wash performance is determined using a suitable wash performance assay in a given detergent composition under suitable conditions.

[0094] In one preferred embodiment improved wash performance is measured using AMSA test, e.g. as disclosed in the Methods and Materials section of this application.

[0095] The wash performance of the variant is preferably at least 1 unit higher than the wash performance of the parent subtilase, preferably at least 2 units higher, such as at least 3 units higher, such as at least 4 units higher, such as at least 5 units higher, such as at least 6 units higher, such as at least 7 units higher such as at least 8 units higher, such as at least 9 units higher.

[0096] The subtilase variants may further comprise one or more additional alterations at one or more (*e.g.*, several) other positions, selected from the group consisting of positions: 3, 4, 9, 12, 14, 15, 40, 43, 68, 72, 79, 86, 88, 92, 98, 99, 101, 120, 146, 183, 184, 188, 194, 216, 218, 224, 228, 236, 245, 255, 261, 267 and 270, preferably positions 9, 15, 68 and/or 120 (numbering according to SEQ ID NO: 2). It will be clear to the skilled artisan that if a position has already been altered once, then it will not be altered a second time. In a preferred embodiment, the alteration at any of the positions selected from the group consisting of 3, 4, 9, 12, 14, 15, 40, 43, 68, 72, 79, 86, 88, 92, 98, 99, 101, 120, 146, 183, 184, 188, 194, 216, 218, 224, 228, 236, 245, 255, 261, 267 and 270 is a substitution. In a more preferred embodiment, the subtilase variant further comprises one or more substitutions selected from the group consisting of 3{D, E, L}, 4I, 9{H, K, R, G}, 12{D, E}, 14T, 15{G, M, S, T}, 40{A, G, M, S, T}, 43{D, E}, 63G, 68{A, G, I, L, M, S, T}, 72{V, L}, N76{D, E}, 79T, 86H, 88V, 92S, 98T, 99{E, T, A, G, M, D}, 101L, 120 {I, N}, 146S, 183{E, D}, 184{E, D}, 188G, 194P, 216{D, E}, 218{E, D}, 224{S, A, T, G, M}, 228T, 236D, 245{H, K, R}, 255{D, E}, 261{D, E}, 267{I, L, V} and/or 270{G, M, S, T} (numbering according to SEQ ID NO: 2). In an even more preferred embodiment, the subtilase variant further comprises one or more substitutions selected from the group consisting of S3{D, E, L}, V4I, S9{H, K, R, G}, Q12{D, E}, P14T, A15{G, M, S, T}, P40{A, G, M, S, T}, N43{D, E}, V68{A, G, I, L, M, S, T}, I72{V, L}, N76{D, E}, I79T, P86H, A88V, A92S, A98T, S99{E, T, A, G, M, D}, S101L, H120{I, N}, G146S, N183{E, D}, N184{E, D}, S188G, A194P, S216{D, E}, N218{E, D}, T224{S, A, T, G, M}, A228T, S236D, Q245{H, K, R}, T255{D, E}, N261{D, E}, L267{I, L, V} and/or A270{G, M, S, T} in the mature polypeptide of SEQ ID NO: 3,

or a polypeptide having at least 60 %, preferably at least 70%, preferably at least 80%, preferably at least 90%, preferably at least 95% sequence identity hereto, wherein each position corresponds to the corresponding position of the mature polypeptide of SEQ ID NO: 1.

[0097] The amino acid changes may be of a minor nature, that is conservative amino acid substitutions or insertions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of 1-30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

[0098] Examples of conservative substitutions are within the groups of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do not generally alter specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, In, The Proteins, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

[0099] Alternatively, the amino acid changes are of such a nature that the physico-chemical properties of the polypeptides are altered. For example, amino acid changes may improve the thermal stability of the polypeptide, alter the substrate specificity, change the pH optimum, and the like.

[0100] Essential amino acids in a polypeptide can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis. In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for protease activity to identify amino acid residues that are critical to the activity of the molecule. The active site of the enzyme or other biological interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. For BPN' (SEQ ID NO: 1) the catalytic triad comprising the amino acids S221, H64, and D32 is essential for protease activity of the enzyme.

[0101] The subtilase variants may consist of 150 to 350, e.g., 175 to 330, 200 to 310, 220 to 300, 240 to 290, 260 to 280 or 269, 270, 271, 272, 273, 274 or 275 amino acids.

[0102] According to one embodiment and/or according to any of the embodiments above the invention relates to a detergent composition comprising subtilase variant having at least 90% sequence identity to SEQ ID NO: 3, wherein the variant has a glutamic acid residue (E) in position corresponding to position 101 of SEQ ID NO: 1, and where the variant has reduced

cellulose binding compared to the subtilase having the amino acid sequence of SEQ ID NO: 3. One embodiment relates to a detergent composition comprising subtilase variant having at least 90% sequence identity to SEQ ID NO: 3, wherein the variant has a glutamic acid residue (E) in position corresponding to position 101 of SEQ ID NO: 1, and where the variant has reduced cellulose binding compared to the subtilase having the amino acid sequence of SEQ ID NO: 3 and wherein the variant comprises a substitution of a positively charged amino acid residue on the surface of the protease with a neutral or negatively charged residue; or a neutral residue on the surface of the protease is substituted with a negatively charged residue. According to one embodiment or any of the above embodiments the invention relates to a detergent composition comprising subtilase variant having at least 90% sequence identity to SEQ ID NO: 3, wherein the variant has a glutamic acid residue (E) in position corresponding to position 101 of SEQ ID NO: 1, and where the variant has reduced cellulose binding compared to the subtilase having the amino acid sequence of SEQ ID NO: 3 and/or wherein the variant comprises a substitution of a positively charged amino acid residue on the surface of the protease with a neutral or negatively charged residue; or a neutral residue on the surface of the protease is substituted with a negatively charged residue wherein the subtilase variant comprising a substitution selected from the group consisting of V4D,E,I, R10N,Q,D,E,S, H17D, K27S,N,Q,E,D, N43E, I44V, R45E,D,Q,N, G46D, S49N,D, P52E, G53D,E, Q59D, G61D, N62D, L75D, N76D, I79D, S87E, G97D, A98E, *103aE, I104T, N117E, H120D, E136K,Q, S156D, R170E,Q,N,D,S N185D, G195E, N218A, K235L,W,N,Q,E,S, K237N,Q,D,E,S, N238D,E, V244D, R246Q,E,D, R247S,E, Q, D, K251S,D,Q,E,N, N261D, L262D,E and S265H, preferable the substitutions are selected among N117E, S156D, N238E, N261D and L262E and preferably the variant further comprise a substitution selected among: S3T, N128Q, Q137H, S141H, R145H, S163G, A194P, V199M, V205I, N218Q or A228V. According to one embodiment and/or according to any of the above embodiment the invention relates to a detergent composition comprising subtilase variant having at least 90% sequence identity to SEQ ID NO: 3, wherein the variant has a glutamic acid residue (E) in position corresponding to position 101 of SEQ ID NO: 1, where the variant has reduced cellulose binding compared to the subtilase having the amino acid sequence of SEQ ID NO: 3 and wherein the subtilase variant further comprising a substitutions selected among N117E+S3T, S156D+S3T, N238E+S3T, N261D+S3T, L262E+S3T, N117E+N128Q, S156D+N128Q, N238E+N128Q, N261D+N128Q, L262E+N128Q, N117E+Q137H, S156D+Q137H, N238E+Q137H, N261D+Q137H, L262E+Q137H, N117E+S141H, S156D+S141H, N238E+S141H, N261D+S141H, L262E+S141H, N117E+R145H, S156D+R145H, N238E+R145H, N261D+R145H, L262E+R145H, N117E+S163G, S156D+S163G, N238E+S163G, N261D+S163G, L262E+S163G, N117E+A194P, S156D+A194P, N238E+A194P, N261D+A194P, L262E+A194P, N117E+V199M, S156D+V199M, N238E+V199M, N261D+V199M, L262E+V199M, N117E+V205I, S156D+V205I, N238E+V205I, N261D+V205I, L262E+V205I, N117E+N218Q, S156D+N218Q, N238E+N218Q, N261D+N218Q, L262E+N218Q, N117E+A228V, S156D+A228V, N238E+A228V, N261D+A228V, L262E+A228V. According to one embodiment and/or according to any of the above embodiment the invention relates to a detergent composition comprising subtilase variant having at least 90% sequence identity to SEQ ID NO: 3, wherein the variant has a glutamic acid residue (E) in position corresponding to position 101 of SEQ ID NO: 1, where the variant has reduced cellulose binding compared to the subtilase having the amino

acid sequence of SEQ ID NO: 3 and wherein the subtilase variant further comprising a substitutions selected among:

SEQ ID NO: 3 +V4D,E,I

SEQ ID NO: 3 + R10N,Q,D,E,S,

SEQ ID NO: 3 + H17D,

SEQ ID NO: 3 + K27S,N,Q,E,D,

SEQ ID NO: 3 + R45E,D,Q,N,

SEQ ID NO: 3 + G53D,

SEQ ID NO: 3 + Q59D,

SEQ ID NO: 3 + G61D,

SEQ ID NO: 3 + L75D,

SEQ ID NO: 3 + N76D,

SEQ ID NO: 3 + I79D,

SEQ ID NO: 3 + S87E,

SEQ ID NO: 3 + G97D,

SEQ ID NO: 3 + A98E,

SEQ ID NO: 3 + *103aE,

SEQ ID NO:3 + N117E,

SEQ ID NO:3 + H120D,

SEQ ID NO:3 + E136K,Q,

SEQ ID NO.3 + S156D,

SEQ ID NO: 3 + R170E,Q,N,D,

SEQ ID NO: 3 + N185D,

SEQ ID NO: 3 + G195E,

SEQ ID NO: 3 + K235L,W,N,Q,E,S,

SEQ ID NO: 3 + K237N,Q,D,E,S,

SEQ ID NO: 3 + N238D,E,

SEQ ID NO: 3 + V244D

SEQ ID NO: 3 + R246Q,E,D,

SEQ ID NO: 3 + R247S,E,

SEQ ID NO: 3 + K251S,D,Q,E,N,

SEQ ID NO: 3 + N261D,

SEQ ID NO: 3 + L262D,E

SEQ ID NO: 3 + S265H

SEQ ID NO: 3 + A194P + G195E

SEQ ID NO: 3 + G195E + V199M

SEQ ID NO: 3 + N76D+A228V+N261D;

SEQ ID NO: 3 + N76D + S163G + N238E

SEQ ID NO: 3 + S156D + L262E

SEQ ID NO: 3 + N238E + L262E

SEQ ID NO: 3 + S3T + N76D + S156D + Y209W

SEQ ID NO: 3 + K27Q + H120D + S163G + N261D

SEQ ID NO: 3 + V104T + H120D + S156D + L262E

SEQ ID NO: 3 + V104T + S156D + L262E

SEQ ID NO: 3 + Q137H + S141H + R145H + N238E + L262E

SEQ ID NO: 3 + S3T+V4I+A228V;

SEQ ID NO: 3 + H120D S163G N261D

SEQ ID NO: 3 + N76D + S101E + A228V + L262E;

SEQ ID NO: 3 + N76D +Q137H + S141H + R145H + S163G + N238E

SEQ ID NO: 3 + S3T + N76D +Q137H+S141H+R145H+ S156D + Y209W

SEQ ID NO: 3 + H120D +Q137H+S141H+R145H+ S163G + N261D

SEQ ID NO: 3 + A194P+G195E+V199M+V205I;

SEQ ID NO: 3 + S3T+N76D+A194P+G195E+V199M+V205I;

SEQ ID NO: 3 + A228V+N261D;

SEQ ID NO: 3 + N76D+A228V;

SEQ ID NO: 3 + S3T+V4I+N261D;

SEQ ID NO: 3 + H120D+A228V;

SEQ ID NO: 3 + N76D+N261D;

SEQ ID NO: 3 + A194P+G195E+V199M+V205I+A228V+N261D;

SEQ ID NO: 3 + A194P+G195E+V205I+A228V; or

SEQ ID NO: 3 + H120D+N261D.

[0103] Preferably the variants are selected from the group consisting of:

SEQ ID NO: 3 + N238E + L262E

SEQ ID NO: 3 + S156D + L262E

SEQ ID NO: 3 + S3T+V4I+A228V;

SEQ ID NO: 3 + G195E + V199M

SEQ ID NO: 3 + H120D S163G N261D

SEQ ID NO: 3 + N76D+A228V+N261D;

SEQ ID NO: 3 + S3T + N76D + S156D + Y209W

SEQ ID NO: 3 + Q137H + S141H + R145H + N238E + L262E

SEQ ID NO: 3 + Q137H + S141H + R145H + S156D + L262E

SEQ ID NO: 3 + N76D+Q137H+S141H+R145H+ A228V+N261D;

SEQ ID NO: 3 + N76D +Q137H + S141H + R145H + S163G + N238E

SEQ ID NO: 3 + H120D +Q137H+S141H+R145H+ S163G + N261D

SEQ ID NO: 3 + S3T + N76D +Q137H+S141H+R145H+ S156D + Y209W,

wherein the positions correspond to the positions in SEQ ID NO: 1 and wherein the subtilase variant has at least at least 60%, such as at least 70%, such as at least 80%, such as at least 90%, such as at least 95%, such as at least 98% or such as at least 99% sequence identity to SEQ ID NO: 3.

[0104] In one embodiment, the subtilase variant has improved wash performance. In another

embodiment, the subtilase variant has improved stability, preferably improved stability during wash.

[0105] In an embodiment, the subtilase variant has improved stability in liquid detergent compared to the parent enzyme wherein the stability is measured using the 'stability assay' as described in the example 4 of Materials and Methods section herein. In an embodiment, the subtilase variant has improved stability compared to the polypeptide of SEQ ID NO: 2 wherein stability is measured using the 'stability assay' as described in example 4 of the Materials and Methods section herein. In an embodiment, the subtilase variant has improved stability compared to the polypeptide of SEQ ID NO: 3 wherein stability is measured using the 'stability assay' as described in example 4 of the Materials and Methods section herein.

[0106] In an embodiment, the subtilase variant has improved wash performance compared to the parent enzyme wherein wash performance is measured using the Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash as described in example 7 of the Materials and Methods section herein. In an embodiment, the subtilase variant has improved wash performance compared to the polypeptide of SEQ ID NO: 2, wherein wash performance is measured using the Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash as described in example 7 of the Materials and Methods section herein. In an embodiment, the subtilase variant has improved wash performance compared to the polypeptide of SEQ ID NO: 3 wherein wash performance is measured using the Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash as described in example 7 of the Materials and Methods section herein.

Parent protease

[0107] Enzymes cleaving the amide linkages in protein substrates are classified as proteases, or (interchangeably) peptidases.

Serine proteases

[0108] A serine protease is an enzyme, which catalyzes the hydrolysis of peptide bonds, and in which there is an essential serine residue at the active site.

[0109] The bacterial serine proteases have molecular weights in the 20,000 to 45,000 Dalton range. They are inhibited by diisopropylfluorophosphate. They hydrolyze simple terminal esters and are similar in activity to eukaryotic chymotrypsin, also a serine protease. A more narrow term, alkaline protease, covering a sub-group, reflects the high pH optimum of some of the serine proteases, from pH 9.0 to 11.0.

Subtilases

[0110] A sub-group of the serine proteases tentatively designated subtilases has been proposed by Siezen et al. (1991), Protein Eng. 4:719-737 and Siezen et al. (1997), Protein Science 6:501-523. They are defined by homology analysis of more than 170 amino acid sequences of serine proteases previously referred to as subtilisin-like proteases. A subtilisin was previously often defined as a serine protease produced by Gram-positive bacteria or fungi, and according to Siezen et al. now is a subgroup of the subtilases. A wide variety of subtilases have been identified, and the amino acid sequence of a number of subtilases has been determined. For a more detailed description of such subtilases and their amino acid sequences reference is made to Siezen et al. (1997).

Subtilisins

[0111] A subgroup of the subtilases is the subtilisins which are serine proteases from the family S8, in particular from the subfamily S8A, as defined by the MEROPS database (http://merops.sanger.ac.uk/cgi-bin/famsum?family=S8).

[0112] BPN' and Savinase have the MEROPS numbers S08.034 and S08.003, respectively.

Parent subtilase

[0113] The term "parent subtilase" describes a subtilase defined according to Siezen et al. (1997), Protein Science 6:501-523. For further details see description of "Subtilases" above. A parent subtilase may also be a subtilase isolated from a natural source, wherein subsequent modifications (such as replacement(s) of the amino acid side chain(s), substitution(s), deletion(s) and/or insertion(s)) have been made while retaining the characteristic of a subtilase. Furthermore, a parent subtilase may be a subtilase which has been prepared by the DNA shuffling technique.

[0114] Alternatively, the term "parent subtilase" may be termed "wild type subtilase". The parent subtilase is preferably of the subtilisin subgroups. One subgroup of the subtilases, I-S1 or "true" subtilisins, comprises the "classical" subtilisins, such as subtilisin 168 (BSS168), subtilisin BPN', subtilisin Carlsberg (ALCALASE®, NOVOZYMES A/S), and subtilisin DY (BSSDY).

[0115] A further subgroup of the subtilases, I-S2 or high alkaline subtilisins, is recognized by Siezen et al. (supra). Sub-group I-S2 proteases are described as highly alkaline subtilisins and comprises enzymes such as subtilisin PB92 (BAALKP) (MAXACAL®, Genencor International Inc.), subtilisin 309 (SAVINASE®, NOVOZYMES A/S), subtilisin 147 (BLS147) (ESPERASE®, NOVOZYMES A/S), and alkaline elastase YaB (BSEYAB). BPN' is subtilisin BPN' from B.

amyloliquefaciens BPN' has the amino acid sequence SEQ ID NO 1.

[0116] For reference, table 1 below gives a list of some acronyms for various subtilases mentioned herein. For further acronyms, see Siezen et al. (1991 and 1997).

Table 1: Acronyms of various subtilases

Organism	Enzyme	Acronym
Bacillus subtilis 168	subtilisin I168,apr	BSS168
Bacillus amyloliquefaciens	subtilisin BPN' (NOVO)	BASBPN
Bacillus subtilis DY	subtilisin DY	BSSDY
Bacillus licheniformis	subtilisin Carlsberg	BLSCAR
Bacillus lentus	subtilisin 309	BLSAVI
Bacillus lentus	subtilisin 147	BLS147
Bacillus alcalophilus PB92	subtilisin PB92	BAPB92
Bacillus YaB	alkaline elastase YaB	BYSYAB
Bacillus sp. NKS-21	subtilisin ALP I	BSAPRQ
Bacillus sp. G-825-6	subtilisin Sendai	BSAPRS
Thermoactinomyces vulgaris	Thermitase	TVTHER

Homologous subtilase sequences

[0117] The homology between two amino acid sequences is in this context described by the parameter "identity" for purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm as described above. The output from the routine is besides the amino acid alignment the calculation of the "Percent Identity" between the two sequences.

[0118] Based on this description it is routine for a person skilled in the art to identify suitable homologous subtilases, which can be modified according to the invention.

[0119] Substantially homologous parent subtilisin variants may have one or more (several) amino acid substitutions, deletions and/or insertions, in the present context the term "one or more" is used interchangeably with the term "several". These changes are preferably of a minor nature, that is conservative amino acid substitutions as described above and other substitutions that do not significantly affect the three-dimensional folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification (an affinity tag), such as a poly-histidine tract, or protein.

[0120] Although the changes described above preferably are of a minor nature, such changes may also be of a substantive nature such as fusion of larger polypeptides of up to 300 amino acids or more both as amino- or carboxyl-terminal extensions.

[0121] The polypeptide of SEQ ID NO: 3 or a fragment thereof, may be used to design nucleic acid probes to identify and clone DNA encoding a parent from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic DNA or cDNA of a cell of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, e.g., at least 25, at least 35, or at least 70 nucleotides in length. Preferably, the nucleic acid probe is at least 100 nucleotides in length, e.g., at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500 nucleotides, at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P, ^{3}H, ^{35}S, biotin, or avidin). Such probes are encompassed by the present invention.

[0122] A genomic DNA or cDNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above and encodes a parent. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material.

[0123] The polypeptide may be a hybrid polypeptide in which a region of one polypeptide is fused at the N-terminus or the C-terminus of a region of another polypeptide.

[0124] The parent may be a fusion polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the present invention. A fusion polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fusion polypeptide is under control of the same promoter(s) and terminator. Fusion polypeptides may also be constructed using intein technology in which fusion polypeptides are created post-translationally.

[0125] A fusion polypeptide can further comprise a cleavage site between the two polypeptides. Upon secretion of the fusion protein, the site is cleaved releasing the two polypeptides.

[0126] The parent may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the parent encoded by a polynucleotide is produced by the source or by a

strain in which the polynucleotide from the source has been inserted. In one aspect, the parent is secreted extracellularly.

[0127] The parent may be a bacterial protease. For example, the parent may be a Gram-positive bacterial polypeptide such as a *Bacillus, Clostridium, Enterococcus, Geobacillus, Lactobacillus, Lactococcus, Oceanobacillus, Staphylococcus, Streptococcus,* or *Streptomyces* protease, or a Gram-negative bacterial polypeptide such as a *Campylobacter, E. coli, Flavobacterium, Fusobacterium, Helicobacter, Ilyobacter, Neisseria, Pseudomonas, Salmonella,* or *Ureaplasma* protease.

[0128] In one aspect, the parent is a *Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus stearothermophilus, Bacillus subtilis,* or *Bacillus thuringiensis* protease

[0129] In one aspect, the parent is a *Bacillus amyloliquefaciens* protease, e.g., the protease of SEQ ID NO: 1 or the mature polypeptide thereof.

[0130] In another aspect, the parent is a *Bacillus lentus* protease, e.g., the protease of SEQ ID NO: 2 or the mature polypeptide thereof.

[0131] Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

[0132] The parent may be identified and obtained from other sources including microorganisms isolated from nature (*e.g.*, soil, composts, water, etc.) or DNA samples obtained directly from natural materials (*e.g.*, soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms and DNA directly from natural habitats are well known in the art. A polynucleotide encoding a parent may then be obtained by similarly screening a genomic DNA or cDNA library of another microorganism or mixed DNA sample. Once a polynucleotide encoding a parent has been detected with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques that are known to those of ordinary skill in the art.

Preparation of Variants

[0133] The present invention also relates to methods for obtaining a subtilase variant having protease activity

[0134] The variants can be prepared using any mutagenesis procedure known in the art, such

as site-directed mutagenesis, synthetic gene construction, semi-synthetic gene construction, random mutagenesis, shuffling, etc.

[0135] Site-directed mutagenesis is a technique in which one or more (*e.g.*, several) mutations are introduced at one or more defined sites in a polynucleotide encoding the parent.

[0136] Site-directed mutagenesis can be accomplished *in vitro* by PCR involving the use of oligonucleotide primers containing the desired mutation. Site-directed mutagenesis can also be performed *in vitro* by cassette mutagenesis involving the cleavage by a restriction enzyme at a site in the plasmid comprising a polynucleotide encoding the parent and subsequent ligation of an oligonucleotide containing the mutation in the polynucleotide. Usually the restriction enzyme that digests the plasmid and the oligonucleotide is the same, permitting sticky ends of the plasmid and the insert to ligate to one another.

[0137] Site-directed mutagenesis can also be accomplished *in vivo* by methods known in the art.

[0138] Any site-directed mutagenesis procedure can be used in the present invention. There are many commercial kits available that can be used to prepare variants.

[0139] Synthetic gene construction entails *in vitro* synthesis of a designed polynucleotide molecule to encode a polypeptide of interest. Gene synthesis can be performed utilizing a number of techniques, such as the multiplex microchip-based technology and similar technologies wherein oligonucleotides are synthesized and assembled upon photo-programmable microfluidic chips.

[0140] Single or multiple amino acid substitutions, deletions, and/or insertions can be made and tested using known methods of mutagenesis, recombination, and/or shuffling, followed by a relevant screening procedure. Other methods that can be used include error-prone PCR, phage display and region-directed mutagenesis.

[0141] Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells. Mutagenized DNA molecules that encode active polypeptides can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

[0142] Semi-synthetic gene construction is accomplished by combining aspects of synthetic gene construction, and/or site-directed mutagenesis, and/or random mutagenesis, and/or shuffling. Semi-synthetic construction is typified by a process utilizing polynucleotide fragments that are synthesized, in combination with PCR techniques. Defined regions of genes may thus be synthesized *de novo,* while other regions may be amplified using site-specific mutagenic primers, while yet other regions may be subjected to error-prone PCR or non-error prone PCR amplification. Polynucleotide subsequences may then be shuffled.

Polynucleotides

[0143] The present invention also relates to polynucleotides encoding a variant of the present invention.

Nucleic Acid Constructs

[0144] The present invention also relates to nucleic acid constructs comprising a polynucleotide encoding a variant of the present invention operably linked to one or more control sequences that direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences.

[0145] The polynucleotide may be manipulated in a variety of ways to provide for expression of a variant. Manipulation of the polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides utilizing recombinant DNA methods are well known in the art.

[0146] The control sequence may be a promoter, a polynucleotide which is recognized by a host cell for expression of the polynucleotide. The promoter contains transcriptional control sequences that mediate the expression of the variant. The promoter may be any polynucleotide that shows transcriptional activity in the host cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0147] Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a bacterial host cell are the promoters obtained from the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus subtilis xylA* and *xylB* genes, *Bacillus thuringiensis cryIIIA* gene,E. *coli lac* operon, *E. coli trc* promoter, *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene, as well as the *tac* promoter.

[0148] The control sequence may also be a transcription terminator, which is recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3'-terminus of the polynucleotide encoding the variant. Any terminator that is functional in the host cell may be used.

[0149] Preferred terminators for bacterial host cells are obtained from the genes for *Bacillus clausii* alkaline protease (*aprH*), *Bacillus licheniformis* alpha-amylase (*amyL*), and *Escherichia coli* ribosomal RNA (*rrnB*).

[0150] The control sequence may also be an mRNA stabilizer region downstream of a promoter and upstream of the coding sequence of a gene which increases expression of the gene.

[0151] Examples of suitable mRNA stabilizer regions are obtained from a *Bacillus thuringiensis cryIIIA* gene and a *Bacillus subtilis* SP82 gene.

[0152] The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a variant and directs the variant into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the variant. Alternatively, the 5'-end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the variant. However, any signal peptide coding sequence that directs the expressed variant into the secretory pathway of a host cell may be used.

[0153] Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases *(nprT, nprS, nprM),* and *Bacillus subtilis prsA.*

[0154] The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a variant. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to an active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding sequence may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Myceliophthora thermophila* laccase, *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

[0155] Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of the variant and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

[0156] It may also be desirable to add regulatory sequences that regulate expression of the variant relative to the growth of the host cell. Examples of regulatory systems are those that cause expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic

systems include the *lac, tac,* and *trp* operator systems.

Expression Vectors

[0157] The present invention also relates to recombinant expression vectors comprising a polynucleotide encoding a variant of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleotide and control sequences may be joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the polynucleotide encoding the variant at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0158] The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus) that can be conveniently subjected to recombinant DNA procedures and can bring about expression of the polynucleotide. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may be a linear or closed circular plasmid.

[0159] The vector may be an autonomously replicating vector, *i.e.*, a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one that, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

[0160] The vector preferably contains one or more selectable markers that permit easy selection of transformed, transfected, transduced, or the like cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like.

[0161] Examples of bacterial selectable markers are *Bacillus licheniformis* or *Bacillus subtilis dal* genes, or markers that confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, neomycin, spectinomycin or tetracycline resistance.

[0162] The vector preferably contains an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome.

[0163] For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the variant or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional polynucleotides for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, 400 to 10,000 base pairs, and 800 to 10,000 base pairs, which have a high degree of sequence identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding polynucleotides. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

[0164] For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" means a polynucleotide that enables a plasmid or vector to replicate *in vivo.*

[0165] Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli,* and pUB110, pE194, pTA1060, and pAMß1 permitting replication in *Bacillus.*

[0166] More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a variant. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the polynucleotide where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

[0167] The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art.

Host Cells

[0168] The present invention also relates to recombinant host cells, comprising a polynucleotide encoding a variant of the present invention operably linked to one or more control sequences that direct the production of a variant of the present invention. A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal

vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the variant and its source.

[0169] The host cell may be any cell useful in the recombinant production of a variant, *e.g.*, a prokaryote or a eukaryote.

[0170] The prokaryotic host cell may be any Gram-positive or Gram-negative bacterium. Gram-positive bacteria include, but are not limited to, *Bacillus, Clostridium, Enterococcus, Geobacillus, Lactobacillus, Lactococcus, Oceanobacillus, Staphylococcus, Streptococcus,* and *Streptomyces.* Gram-negative bacteria include, but are not limited to, *Campylobacter, E. coli, Flavobacterium, Fusobacterium, Helicobacter, Ilyobacter, Neisseria, Pseudomonas, Salmonella,* and *Ureaplasma.*

[0171] The bacterial host cell may be any *Bacillus* cell including, but not limited to, *Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus stearothermophilus, Bacillus subtilis,* and *Bacillus thuringiensis* cells.

[0172] The bacterial host cell may also be any *Streptococcus* cell including, but not limited to, *Streptococcus equisimilis, Streptococcus pyogenes, Streptococcus uberis,* and *Streptococcus equi* subsp. *Zooepidemicus* cells.

[0173] The bacterial host cell may also be any *Streptomyces* cell, including, but not limited to, *Streptomyces achromogenes, Streptomyces avermitilis, Streptomyces coelicolor, Streptomyces griseus,* and *Streptomyces lividans* cells.

[0174] The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation, competent cell transformation, electroporation, or conjugation . The introduction of DNA into an *E. coli* cell may be effected by protoplast transformation or electroporation. The introduction of DNA into a *Streptomyces* cell may be effected by protoplast transformation, electroporation, conjugation, or transduction. The introduction of DNA into a *Pseudomonas* cell may be effected by electroporation, or conjugation. The introduction of DNA into a *Streptococcus* cell may be effected by natural competence, protoplast transformation, electroporation or conjugation. However, any method known in the art for introducing DNA into a host cell can be used.

Methods of Production

[0175] The present invention also relates to methods of producing a variant, comprising: (a) cultivating a host cell of the present invention under conditions suitable for expression of the variant; and (b) recovering the variant.

[0176] The host cells are cultivated in a nutrient medium suitable for production of the variant using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, or smallscale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the variant to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the variant is secreted into the nutrient medium, the variant can be recovered directly from the medium. If the variant is not secreted, it can be recovered from cell lysates.

[0177] The variant may be detected using methods known in the art that are specific for the variants with protease activity. These detection methods include, but are not limited to, use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the variant.

[0178] The variant may be recovered using methods known in the art. For example, the variant may be recovered from the nutrient medium by conventional procedures including, but not limited to, collection, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

[0179] The variant may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, or extraction to obtain substantially pure variants.

[0180] In an alternative aspect, the variant is not recovered, but rather a host cell of the present invention expressing the variant is used as a source of the variant.

Compositions

[0181] In one certain aspect, the variants according to the invention have improved wash performance compared to the parent enzyme or compared to a protease having the identical amino acid sequence of said variant but not having the alterations at one or more of said specified positions or compared to the mature polypeptide of SEQ ID NO: 2, wherein wash performance is measured using the Automatic Mechanical Stress Assay (AMSA) for Automatic Dish Wash as described in the Materials and Methods section herein.

[0182] In another certain aspect, the variants according to the invention have improved stability, preferably improved stability during wash, compared to the parent enzyme or

compared to a protease having the identical amino acid sequence of said variant but not having the alterations at one or more of said specified positions or compared to the mature polypeptide of SEQ ID NO: 2, wherein stability is measured using the 'in wash stability assay' as described in the Materials and Methods section herein.

[0183] Thus, in a preferred embodiment the composition is a detergent composition, and one aspect of the invention relates to the use of a detergent composition comprising a variant according to the invention in a cleaning process such as laundry or hard surface cleaning.

[0184] The choice of additional components is within the skill of the artisan and includes conventional ingredients, including the exemplary non-limiting components set forth below. The choice of components may include, for fabric care, the consideration of the type of fabric to be cleaned, the type and/or degree of soiling, the temperature at which cleaning is to take place, and the formulation of the detergent product. Although components mentioned below are categorized by general header according to a particular functionality, this is not to be construed as a limitation, as a component may comprise additional functionalities as will be appreciated by the skilled artisan.

Enzyme of the present invention

[0185] In one embodiment of the present invention, the polypeptide may be added to a detergent composition in an amount corresponding to 0.01-200 mg of enzyme protein per liter of wash liqour, preferably 0.05-50 mg of enzyme protein per liter of wash liqour, in particular 0.1-10 mg of enzyme protein per liter of wash liqour.

[0186] A composition for use in automatic dishwash (ADW), for example, may include 0.0001%-50%, such as 0.001%-30%, such as 0.01%-20%, such as 0.5-15% of enzyme protein by weight of the composition.

[0187] A composition for use in laundry granulation, for example, may include 0.0001%-50%, such as 0.001%-20%, such as 0.01%-10%, such as 0.05%-5% of enzyme protein by weight of the composition.

[0188] A composition for use in laundry liquid, for example, may include 0.0001%-10%, such as 0.001-7%, such as 0.1%-5% of enzyme protein by weight of the composition.

[0189] The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, *e.g.*, a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, *e.g.*, an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in, for example, WO 92/19709 and WO 92/19708 or the variants according to the invention may be stabilized using peptide aldehydes or ketones such as described in WO 2005/105826 and WO 2009/118375.

[0190] A variant of the present invention may also be incorporated in the detergent formulations disclosed in WO97/07202, which is hereby incorporated by reference.

Surfactants

[0191] The detergent composition may comprise one or more surfactants, which may be anionic and/or cationic and/or non-ionic and/or semi-polar and/or zwitterionic, or a mixture thereof. In a particular embodiment, the detergent composition includes a mixture of one or more nonionic surfactants and one or more anionic surfactants. The surfactant(s) is typically present at a level of from about 0.1% to 60% by weight, such as about 1% to about 40%, or about 3% to about 20%, or about 3% to about 10%. The surfactant(s) is chosen based on the desired cleaning application, and includes any conventional surfactant(s) known in the art. Any surfactant known in the art for use in detergents may be utilized.

[0192] When included therein the detergent will usually contain from about 1% to about 40% by weight, such as from about 5% to about 30%, including from about 5% to about 15%, or from about 20% to about 25% of an anionic surfactant. Non-limiting examples of anionic surfactants include sulfates and sulfonates, in particular, linear alkylbenzenesulfonates (LAS), isomers of LAS, branched alkylbenzenesulfonates (BABS), phenylalkanesulfonates, alpha-olefinsulfonates (AOS), olefin sulfonates, alkene sulfonates, alkane-2,3-diylbis(sulfates), hydroxyalkanesulfonates and disulfonates, alkyl sulfates (AS) such as sodium dodecyl sulfate (SDS), fatty alcohol sulfates (FAS), primary alcohol sulfates (PAS), alcohol ethersulfates (AES or AEOS or FES, also known as alcohol ethoxysulfates or fatty alcohol ether sulfates), secondary alkanesulfonates (SAS), paraffin sulfonates (PS), ester sulfonates, sulfonated fatty acid glycerol esters, alpha-sulfo fatty acid methyl esters (alpha-SFMe or SES) including methyl ester sulfonate (MES), alkyl- or alkenylsuccinic acid, dodecenyl/tetradecenyl succinic acid (DTSA), fatty acid derivatives of amino acids, diesters and monoesters of sulfo-succinic acid or soap, and combinations thereof.

[0193] When included therein the detergent will usually contain from about 0% to about 10% by weight of a cationic surfactant. Non-limiting examples of cationic surfactants include alklydimethylethanolamine quat (ADMEAQ), cetyltrimethylammonium bromide (CTAB), dimethyldistearylammonium chloride (DSDMAC), and alkylbenzyldimethylammonium, alkyl quaternary ammonium compounds, alkoxylated quaternary ammonium (AQA) compounds, and combinations thereof.

[0194] When included therein the detergent will usually contain from about 0.2% to about 40% by weight of a non-ionic surfactant, for example from about 0.5% to about 30%, in particular from about 1% to about 20%, from about 3% to about 10%, such as from about 3% to about 5%, or from about 8% to about 12%. Non-limiting examples of non-ionic surfactants include alcohol ethoxylates (AE or AEO), alcohol propoxylates, propoxylated fatty alcohols (PFA),

alkoxylated fatty acid alkyl esters, such as ethoxylated and/or propoxylated fatty acid alkyl esters, alkylphenol ethoxylates (APE), nonylphenol ethoxylates (NPE), alkylpolyglycosides (APG), alkoxylated amines, fatty acid monoethanolamides (FAM), fatty acid diethanolamides (FADA), ethoxylated fatty acid monoethanolamides (EFAM), propoxylated fatty acid monoethanolamides (PFAM), polyhydroxy alkyl fatty acid amides, or *N*-acyl *N*-alkyl derivatives of glucosamine (glucamides, GA, or fatty acid glucamide, FAGA), as well as products available under the trade names SPAN and TWEEN, and combinations thereof.

[0195] When included therein the detergent will usually contain from about 0% to about 10% by weight of a semipolar surfactant. Non-limiting examples of semipolar surfactants include amine oxides (AO) such as alkyldimethylamineoxide, *N*-(coco alkyl)-*N,N*-dimethylamine oxide and N-(tallow-alkyl)-*N,N*-bis(2-hydroxyethyl)amine oxide, fatty acid alkanolamides and ethoxylated fatty acid alkanolamides, and combinations thereof.

[0196] When included therein the detergent will usually contain from about 0% to about 10% by weight of a zwitterionic surfactant. Non-limiting examples of zwitterionic surfactants include betaine, alkyldimethylbetaine, sulfobetaine, and combinations thereof.

Hydrotropes

[0197] A hydrotrope is a compound that solubilises hydrophobic compounds in aqueous solutions (or oppositely, polar substances in a non-polar environment). Typically, hydrotropes have both hydrophilic and a hydrophobic character (so-called amphiphilic properties as known from surfactants); however the molecular structure of hydrotropes generally do not favor spontaneous self-aggregation. Hydrotropes do not display a critical concentration above which self-aggregation occurs as found for surfactants and lipids forming miceller, lamellar or other well defined mesophases. Instead, many hydrotropes show a continuous-type aggregation process where the sizes of aggregates grow as concentration increases. However, many hydrotropes alter the phase behavior, stability, and colloidal properties of systems containing substances of polar and non-polar character, including mixtures of water, oil, surfactants, and polymers. Hydrotropes are classically used across industries from pharma, personal care, food, to technical applications. Use of hydrotropes in detergent compositions allow for example more concentrated formulations of surfactants (as in the process of compacting liquid detergents by removing water) without inducing undesired phenomena such as phase separation or high viscosity.

[0198] The detergent may contain 0-5% by weight, such as about 0.5 to about 5%, or about 3% to about 5%, of a hydrotrope. Any hydrotrope known in the art for use in detergents may be utilized. Non-limiting examples of hydrotropes include sodium benzene sulfonate, sodium p-toluene sulfonate (STS), sodium xylene sulfonate (SXS), sodium cumene sulfonate (SCS), sodium cymene sulfonate, amine oxides, alcohols and polyglycolethers, sodium hydroxynaphthoate, sodium hydroxynaphthalene sulfonate, sodium ethylhexyl sulfate, and combinations thereof.

Builders and Co-Builders

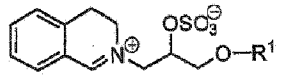
[0199] The detergent composition may contain about 0-65% by weight, such as about 5% to about 45% of a detergent builder or co-builder, or a mixture thereof. In a dish wash deteregent, the level of builder is typically 40-65%, particularly 50-65%. The builder and/or co-builder may particularly be a chelating agent that forms water-soluble complexes with Ca and Mg. Any builder and/or co-builder known in the art for use in laundry detergents may be utilized. Non-limiting examples of builders include zeolites, diphosphates (pyrophosphates), triphosphates such as sodium triphosphate (STP or STPP), carbonates such as sodium carbonate, soluble silicates such as sodium metasilicate, layered silicates (*e.g.*, SKS-6 from Hoechst), ethanolamines such as 2-aminoethan-1-ol (MEA), diethanolamine (DEA, also known as iminodiethanol), triethanolamine (TEA, also known as 2,2',2"-nitrilotriethanol), and carboxymethyl inulin (CMI), and combinations thereof.

[0200] The detergent composition may also contain 0-20% by weight, such as about 5% to about 10%, of a detergent co-builder, or a mixture thereof. The detergent composition may include include a co-builder alone, or in combination with a builder, for example a zeolite builder. Non-limiting examples of co-builders include homopolymers of polyacrylates or copolymers thereof, such as poly(acrylic acid) (PAA) or copoly(acrylic acid/maleic acid) (PAA/PMA). Further non-limiting examples include citrate, chelators such as aminocarboxylates, aminopolycarboxylates and phosphonates, and alkyl- or alkenylsuccinic acid. Additional specific examples include 2,2',2"-nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), iminodisuccinic acid (IDS), ethylenediamine-*N,N'*-disuccinic acid (EDDS), methylglycinediacetic acid (MGDA), glutamic acid-*N,N'*-diacetic acid (GLDA), 1-hydroxyethane-1,1-diphosphonic acid (HEDP), ethylenediaminetetra-(methylenephosphonic acid) (EDTMPA), diethylenetriaminepentakis(methylenephosphonic acid) (DTPMPA or DTMPA), *N*-(2-hydroxyethyl)iminodiacetic acid (EDG), aspartic acid-*N*-monoacetic acid (ASMA), aspartic acid-*N,N*-diacetic acid (ASDA), aspartic acid-*N*-monopropionic acid (ASMP), iminodisuccinic acid (IDA), *N*-(2-sulfomethyl)-aspartic acid (SMAS), *N*-(2-sulfoethyl)-aspartic acid (SEAS), *N*-(2-sulfomethyl)-glutamic acid (SMGL), *N*-(2-sulfoethyl)-glutamic acid (SEGL), *N*-methyliminodiacetic acid (MIDA), α-alanine-*N, N*-diacetic acid (α-ALDA), serine-*N, N*-diacetic acid (SEDA), isoserine-*N, N*-diacetic acid (ISDA), phenylalanine-*N, N*-diacetic acid (PHDA), anthranilic acid-*N, N*-diacetic acid (ANDA), sulfanilic acid-*N, N*-diacetic acid (SLDA) , taurine-*N, N*-diacetic acid (TUDA) and sulfomethyl-*N, N*-diacetic acid (SMDA), *N*-(2-hydroxyethyl)-ethylidenediamine-*N*, *N'*, *N'*-triacetate (HEDTA), diethanolglycine (DEG), diethylenetriamine penta(methylenephosphonic acid) (DTPMP), aminotris(methylenephosphonic acid) (ATMP), and combinations and salts thereof. Further exemplary builders and/or co-builders are described in, e.g., WO 09/102854, US 5977053

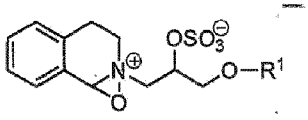
Bleaching Systems

[0201] The detergent may contain 0-50% by weight, such as about 0.1% to about 25%, of a bleaching system. Any bleaching system known in the art for use in laundry detergents may be utilized. Suitable bleaching system components include bleaching catalysts, photobleaches, bleach activators, sources of hydrogen peroxide such as sodium percarbonate and sodium perborates, preformed peracids and mixtures thereof. Suitable preformed peracids include, but are not limited to, peroxycarboxylic acids and salts, percarbonic acids and salts, perimidic acids and salts, peroxymonosulfuric acids and salts, for example, Oxone (R), and mixtures thereof. Non-limiting examples of bleaching systems include peroxide-based bleaching systems, which may comprise, for example, an inorganic salt, including alkali metal salts such as sodium salts of perborate (usually mono- or tetra-hydrate), percarbonate, persulfate, perphosphate, persilicate salts, in combination with a peracid-forming bleach activator. The term bleach activator is meant herein as a compound which reacts with peroxygen bleach like hydrogen peroxide to form a peracid. The peracid thus formed constitutes the activated bleach. Suitable bleach activators to be used herein include those belonging to the class of esters amides, imides or anhydrides. Suitable examples are tetracetylethylene diamine (TAED), sodium 4-[(3,5,5-trimethylhexanoyl)oxy]benzene sulfonate (ISONOBS), diperoxy dodecanoic acid, 4-(dodecanoyloxy)benzenesulfonate (LOBS), 4-(decanoyloxy)benzenesulfonate, 4-(decanoyloxy)benzoate (DOBS), 4-(nonanoyloxy)-benzenesulfonate (NOBS), and/or those disclosed in WO98/17767. A particular family of bleach activators of interest was disclosed in EP624154 and particulary preferred in that family is acetyl triethyl citrate (ATC). ATC or a short chain triglyceride like triacetin has the advantage that it is environmental friendly as it eventually degrades into citric acid and alcohol. Furthermore acetyl triethyl citrate and triacetin has a good hydrolytical stability in the product upon storage and it is an efficient bleach activator. Finally ATC provides a good building capacity to the laundry additive. Alternatively, the bleaching system may comprise peroxyacids of, for example, the amide, imide, or sulfone type. The bleaching system may also comprise peracids such as 6-(phthalimido)peroxyhexanoic acid (PAP). The bleaching system may also include a bleach catalyst. In some embodiments the bleach component may be an organic catalyst selected from the group consisting of organic catalysts having the following formulae:

1. (i)



2. (ii)



3. (iii) and mixtures thereof; wherein each R^1 is independently a branched alkyl group containing from 9 to 24 carbons or linear alkyl group containing from 11 to 24 carbons, preferably each R^1 is independently a branched alkyl group containing from 9 to 18 carbons or linear alkyl group containing from 11 to 18 carbons, more preferably each R^1 is independently selected from the group consisting of 2-propylheptyl, 2-butyloctyl, 2-

pentylnonyl, 2-hexyldecyl, n-dodecyl, n-tetradecyl, n-hexadecyl, n-octadecyl, iso-nonyl, iso-decyl, iso-tridecyl and iso-pentadecyl. Other exemplary bleaching systems are described, e.g. in WO2007/087258, WO2007/087244, WO2007/087259 and WO2007/087242. Suitable photobleaches may for example be sulfonated zinc phthalocyanine

Polymers

[0202] The detergent may contain 0-10% by weight, such as 0.5-5%, 2-5%, 0.5-2% or 0.2-1% of a polymer. Any polymer known in the art for use in detergents may be utilized. The polymer may function as a co-builder as mentioned above, or may provide antiredeposition, fiber protection, soil release, dye transfer inhibition, grease cleaning and/or anti-foaming properties. Some polymers may have more than one of the above-mentioned properties and/or more than one of the below-mentioned motifs. Exemplary polymers include (carboxymethyl)cellulose (CMC), poly(vinyl alcohol) (PVA), poly(vinylpyrrolidone) (PVP), poly(ethyleneglycol) or poly(ethylene oxide) (PEG), ethoxylated poly(ethyleneimine), carboxymethyl inulin (CMI), and polycarboxylates such as PAA, PAA/PMA, poly-aspartic acid, and lauryl methacrylate/acrylic acid copolymers , hydrophobically modified CMC (HM-CMC) and silicones, copolymers of terephthalic acid and oligomeric glycols, copolymers of poly(ethylene terephthalate) and poly(oxyethene terephthalate) (PET-POET), PVP, poly(vinylimidazole) (PVI), poly(vinylpyridine-N-oxide) (PVPO or PVPNO) and polyvinylpyrrolidonevinylimidazole (PVPVI). Further exemplary polymers include sulfonated polycarboxylates, polyethylene oxide and polypropylene oxide (PEO-PPO) and diquaternium ethoxy sulfate. Other exemplary polymers are disclosed in, e.g., WO 2006/130575. Salts of the above-mentioned polymers are also contemplated.

Fabric hueing agents

[0203] The detergent compositions of the present invention may also include fabric hueing agents such as dyes or pigments, which when formulated in detergent compositions can deposit onto a fabric when said fabric is contacted with a wash liquor comprising said detergent compositions and thus altering the tint of said fabric through absorption/reflection of visible light. Fluorescent whitening agents emit at least some visible light. In contrast, fabric hueing agents alter the tint of a surface as they absorb at least a portion of the visible light spectrum. Suitable fabric hueing agents include dyes and dye-clay conjugates, and may also include pigments. Suitable dyes include small molecule dyes and polymeric dyes. Suitable small molecule dyes include small molecule dyes selected from the group consisting of dyes falling into the Colour Index (C.I.) classifications of Direct Blue, Direct Red, Direct Violet, Acid Blue, Acid Red, Acid Violet, Basic Blue, Basic Violet and Basic Red, or mixtures thereof, for example as described in WO2005/03274, WO2005/03275, WO2005/03276 and EP1876226 (hereby incorporated by reference). The detergent composition preferably comprises from about

0.00003 wt% to about 0.2 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. The composition may comprise from 0.0001 wt% to 0.2 wt% fabric hueing agent, this may be especially preferred when the composition is in the form of a unit dose pouch. Suitable hueing agents are also disclosed in, e.g. WO 2007/087257 and WO2007/087243.

Additional Enzymes

[0204] The detergent additive as well as the detergent composition may comprise one or more (additional) enzymes such as a protease, lipase, cutinase, an amylase, carbohydrase, cellulase, pectinase, mannanase, arabinase, galactanase, xylanase, oxidase, *e.g.*, a laccase, and/or peroxidase.

[0205] In general the properties of the selected enzyme(s) should be compatible with the selected detergent, (*i.e.*, pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Cellulases

[0206] Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium, e.g.,* the fungal cellulases produced from *Humicola insolens, Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

[0207] Especially suitable cellulases are the alkaline or neutral cellulases having color care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

[0208] Example of cellulases exhibiting endo-beta-1,4-glucanase activity (EC 3.2.1.4) are those having described in WO02/099091.

[0209] Other examples of cellulases include the family 45 cellulases described in WO96/29397, and especially variants thereof having substitution, insertion and/or deletion at one or more of the positions corresponding to the following positions in SEQ ID NO: 8 of WO 02/099091: 2, 4, 7, 8, 10, 13, 15, 19, 20, 21, 25, 26, 29, 32, 33, 34, 35, 37, 40, 42, 42a, 43, 44, 48, 53, 54, 55, 58, 59, 63, 64, 65, 66, 67, 70, 72, 76, 79, 80, 82, 84, 86, 88, 90, 91, 93, 95, 95d, 95h, 95j, 97, 100, 101, 102, 103, 113, 114, 117, 119, 121, 133, 136, 137, 138, 139, 140a,

141, 143a, 145, 146, 147, 150e, 150j, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160c, 160e, 160k, 161, 162, 164, 165, 168, 170, 171, 172, 173, 175, 176, 178, 181, 183, 184, 185, 186, 188, 191, 192, 195, 196, 200, and/or 20, preferably selected among P19A, G20K, Q44K, N48E, Q119H or Q146 R.

[0210] Commercially available cellulases include Celluzyme™, and Carezyme™ (Novozymes A/S), Clazinase™, and Puradax HA™ (Genencor International Inc.), and KAC-500(B)™ (Kao Corporation).

Proteases

[0211] Suitable proteases include those of bacterial, fungal, plant, viral or animal origin e.g. vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. It may be an alkaline protease, such as a serine protease or a metalloprotease. A serine protease may for example be of the S1 family, such as trypsin, or the S8 family such as subtilisin. A metalloproteases protease may for example be a thermolysin from e.g. family M4 or other metalloprotease such as those from M5, M7 or M8 families.

[0212] The term "subtilases" refers to a sub-group of serine protease according to Siezen et al., Protein Engng. 4 (1991) 719-737 and Siezen et al. Protein Science 6 (1997) 501-523. Serine proteases are a subgroup of proteases characterized by having a serine in the active site, which forms a covalent adduct with the substrate. The subtilases may be divided into 6 sub-divisions, i.e. the Subtilisin family, the Thermitase family, the Proteinase K family, the Lantibiotic peptidase family, the Kexin family and the Pyrolysin family.

[0213] Examples of subtilases are those derived from *Bacillus* such as *Bacillus lentus, B. alkalophilus, B. subtilis, B. amyloliquefaciens, Bacillus pumilus* and *Bacillus gibsonii* described in; US7262042 and WO09/021867, and *subtilisin lentus, subtilisin Novo, subtilisin Carlsberg, Bacillus licheniformis, subtilisin BPN', subtilisin 309, subtilisin* 147 and *subtilisin 168* described in WO89/06279 and protease PD138 described in (WO93/18140). Other useful proteases may be those described in WO92/175177, WO01/016285, WO02/026024 and WO02/016547. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO89/06270, WO94/25583 and WO05/040372, and the chymotrypsin proteases derived from *Cellumonas* described in WO05/052161 and WO05/052146.

[0214] A further preferred protease is the alkaline protease from *Bacillus lentus* DSM 5483, as described for example in WO95/23221, and variants thereof which are described in WO92/21760, WO95/23221, EP1921147 and EP1921148.

[0215] Examples of metalloproteases are the neutral metalloprotease as described in

WO07/044993 (Genencor Int.) such as those derived from *Bacillus amyloliquefaciens.*
Examples of useful proteases are the variants described in: WO92/19729, WO96/034946, WO98/20115, WO98/20116, WO99/011768, WO01/44452, WO03/006602, WO04/03186, WO04/041979, WO07/006305, WO11/036263, WO11/036264, especially the variants with substitutions in one or more of the following positions: 3, 4, 9, 15, 27, 36, 57, 68, 76, 87, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 106, 118, 120, 123, 128, 129, 130, 160, 167, 170, 194, 195, 199, 205, 206, 217, 218, 222, 224, 232, 235, 236, 245, 248, 252 and 274 using the BPN' numbering. More preferred the subtilase variants may comprise the mutations: S3T, V4I, S9R, A15T, K27R, *36D, V68A, N76D, N87S,R, *97E, A98S, S99G,D,A, S99AD, S101G,M,R S103A, V104I,Y,N, S106A, G118V,R, H120D,N, N123S, S128L, P129Q, S130A, G160D, Y167A, R170S, A194P, G195E, V199M, V205I, L217D, N218D, M222S, A232V, K235L, Q236H, Q245R, N252K, T274A (using BPN' numbering).

[0216] Suitable commercially available protease enzymes include those sold under the trade names Alcalase®, Duralase™, Durazym™, Relase®, Relase® Ultra, Savinase®, Savinase® Ultra, Primase®, Polarzyme®, Kannase®, Liquanase®, Liquanase® Ultra, Ovozyme®, Coronase®, Coronase® Ultra, Neutrase®, Everlase® and Esperase® (Novozymes A/S), those sold under the tradename Maxatase®, Maxacal®, Maxapem®, Purafect®, Purafect Prime®, Eraser®, Purafect MA®, Purafect Ox®, Purafect OxP®, Puramax®, Properase®, Ultimase®, FN2®, FN3® , FN4®, Excellase®, , Opticlean® and Optimase® (Danisco/DuPont), Axapem™ (Gist-Brocases N.V.), BLAP (sequence shown in Figure 29 of US5352604) and variants hereof (Henkel AG) and KAP *(Bacillus alkalophilus* subtilisin) from Kao.

Lipases and Cutinases

[0217] Suitable lipases and cutinases include those of bacterial or fungal origin. Chemically modified or protein engineered mutant enzymes are included. Examples include lipase from *Thermomyces,* e.g. from *T. lanuginosus* (previously named *Humicola lanuginosa*) as described in EP258068 and EP305216, cutinase from *Humicola,* e.g. *H. insolens* (WO96/13580), lipase from strains of *Pseudomonas* (some of these now renamed to *Burkholderia*), e.g. *P. alcaligenes* or *P. pseudoalcaligenes* (EP218272), *P. cepacia* (EP331376), *P. sp.* strain SD705 (WO95/06720 & WO96/27002), *P. wisconsinensis* (WO96/12012), GDSL-type *Streptomyces* lipases (WO10/065455), cutinase from *Magnaporthe grisea* (WO10/107560), cutinase from *Pseudomonas mendocina* (US5,389,536), lipase from *Thermobifida fusca* (WO11/084412), *Geobacillus stearothermophilus* lipase (WO11/084417), lipase from *Bacillus subtilis* (WO11/084599), and lipase from *Streptomyces griseus* (WO11/150157) and *S. pristinaespiralis* (WO12/137147).

[0218] Other examples are lipase variants such as those described in EP407225, WO92/05249, WO94/01541, WO94/25578, WO95/14783, WO95/30744, WO95/35381, WO95/22615, WO96/00292, WO97/04079, WO97/07202, WO00/34450, WO00/60063,

WO01/92502, WO07/87508 and WO09/109500.

[0219] Preferred commercial lipase products include include Lipolase™, Lipex™; Lipolex™ and Lipoclean™ (Novozymes A/S), Lumafast (originally from Genencor) and Lipomax (originally from Gist-Brocades).

[0220] Still other examples are lipases sometimes referred to as acyltransferases or perhydrolases, e.g. acyltransferases with homology to *Candida antarctica* lipase A (WO10/111143), acyltransferase from *Mycobacterium smegmatis* (WO05/56782), perhydrolases from the CE 7 family (WO09/67279), and variants of the *M. smegmatis* perhydrolase in particular the S54V variant used in the commercial product Gentle Power Bleach from Huntsman Textile Effects Pte Ltd (WO10/100028).

Amylases

[0221] Suitable amylases which can be used together with subtilase variants of the invention may be an alpha-amylase or a glucoamylase and may be of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, alpha-amylases obtained from *Bacillus, e.g.,* a special strain of *Bacillus licheniformis,* described in more detail in GB 1,296,839.

[0222] Suitable amylases include amylases having SEQ ID NO: 3 in WO 95/10603 or variants having 90% sequence identity to SEQ ID NO: 3 thereof. Preferred variants are described in WO 94/02597, WO 94/18314, WO 97/43424 and SEQ ID NO: 4 of WO 99/019467, such as variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 178, 179, 181, 188, 190, 197, 201, 202, 207, 208, 209, 211, 243, 264, 304, 305, 391, 408, and 444.

[0223] Different suitable amylases include amylases having SEQ ID NO: 6 in WO 02/010355 or variants thereof having 90% sequence identity thereto. Preferred variants are those having a deletion in positions 181 and 182 and a substitution in position 193.

[0224] Other amylases which are suitable are hybrid alpha-amylase comprising residues 1-33 of the alpha-amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 6 of WO 2006/066594 and residues 36-483 of the *B. licheniformis* alpha-amylase shown in SEQ ID NO: 4 of WO 2006/066594 or variants having 90% sequence identity thereof. Preferred variants of this hybrid alpha-amylase are those having a substitution, a deletion or an insertion in one of more of the following positions: G48, T49, G107, H156, A181, N190, M197, I201, A209 and Q264. Most preferred variants of the hybrid alpha-amylase comprising residues 1-33 of the alpha-amylase derived from B. *amyloliquefaciens* shown in SEQ ID NO: 6 of WO 2006/066594 and residues 36-483 of SEQ ID NO: 4 of WO2006/066594 are those having the substitutions:

M197T;

H156Y+A181T+N190F+A209V+Q264S; or

G48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S.

[0225] Further amylases which are suitable are amylases having SEQ ID NO: 6 in WO 99/019467 or variants thereof having 90% sequence identity to SEQ ID NO: 6. Preferred variants of SEQ ID NO: 6 are those having a substitution, a deletion or an insertion in one or more of the following positions: R181, G182, H183, G184, N195, I206, E212, E216 and K269. Particularly preferred amylases are those having deletion in positions R181 and G182, or positions H183 and G184.

[0226] Additional amylases which can be used are those having SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 2 or SEQ ID NO: 7 of WO 96/023873 or variants thereof having 90% sequence identity to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 7. Preferred variants of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 7 are those having a substitution, a deletion or an insertion in one or more of the following positions: 140, 181, 182, 183, 184, 195, 206, 212, 243, 260, 269, 304 and 476. More preferred variants are those having a deletion in positions 181 and 182 or positions 183 and 184. Most preferred amylase variants of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 7 are those having a deletion in positions 183 and 184 and a substitution in one or more of positions 140, 195, 206, 243, 260, 304 and 476.

[0227] Other amylases which can be used are amylases having SEQ ID NO: 2 of WO 08/153815, SEQ ID NO: 10 in WO 01/66712 or variants thereof having 90% sequence identity to SEQ ID NO: 2 of WO 08/153815 or 90% sequence identity to SEQ ID NO: 10 in WO 01/66712. Preferred variants of SEQ ID NO: 10 in WO 01/66712 are those having a substitution, a deletion or an insertion in one of more of the following positions: 176, 177, 178, 179, 190, 201, 207, 211 and 264.

[0228] Further suitable amylases are amylases having SEQ ID NO: 2 of WO 09/061380 or variants having 90% sequence identity to SEQ ID NO: 2 thereof. Preferred variants of SEQ ID NO: 2 are those having a truncation of the C-terminus and/or a substitution, a deletion or an insertion in one of more of the following positions: Q87, Q98, S125, N128, T131, T165, K178, R180, S181, T182, G183, M201, F202, N225, S243, N272, N282, Y305, R309, D319, Q320, Q359, K444 and G475. More preferred variants of SEQ ID NO: 2 are those having the substitution in one of more of the following positions: Q87E,R, Q98R, S125A, N128C, T131I, T165I, K178L, T182G, M201L, F202Y, N225E,R, N272E,R, S243Q,A,E,D, Y305R, R309A, Q320R, Q359E, K444E and G475K and/or deletion in position R180 and/or S181 or of T182 and/or G183. Most preferred amylase variants of SEQ ID NO: 2 are those having the substitutions:

N128C+K178L+T182G+Y305R+G475K;

N128C+K178L+T182G+F202Y+Y305R+D319T+G475K;

S125A+N128C+K178L+T182G+Y305R+G475K; or

S125A+N128C+T131I+T165I+K178L+T182G+Y305R+G475K wherein the variants are C-terminally truncated and optionally further comprises a substitution at position 243 and/or a deletion at position 180 and/or position 181.

[0229] Other suitable amylases are the alpha-amylase having SEQ ID NO: 12 in WO01/66712 or a variant having at least 90% sequence identity to SEQ ID NO: 12. Preferred amylase variants are those having a substitution, a deletion or an insertion in one of more of the following positions of SEQ ID NO: 12 in WO01/66712: R28, R118, N174; R181, G182, D183, G184, G186, W189, N195, M202, Y298, N299, K302, S303, N306, R310, N314; R320, H324, E345, Y396, R400, W439, R444, N445, K446, Q449, R458, N471, N484. Particular preferred amylases include variants having a deletion of D183 and G184 and having the substitutions R118K, N195F, R320K and R458K, and a variant additionally having substitutions in one or more position selected from the group: M9, G149, G182, G186, M202, T257, Y295, N299, M323, E345 and A339, most preferred a variant that additionally has substitutions in all these positions.

[0230] Other examples are amylase variants such as those described in WO2011/098531, WO2013/001078 and WO2013/001087.

[0231] Commercially available amylases are Duramyl™, Termamyl™, Fungamyl™, Stainzyme ™, Stainzyme Plus™, Natalase™, Liquozyme X and BAN™ (from Novozymes A/S), and Rapidase™ , Purastar™/Effectenz™, Powerase and Preferenz S100 (from Genencor International Inc./DuPont).

Peroxidases/Oxidases

[0232] Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus, e.g.,* from *C. cinereus,* and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

[0233] Commercially available peroxidases include Guardzyme™ (Novozymes A/S).

[0234] The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, *i.e.*, a separate additive or a combined additive, can be formulated, for example, as a granulate, liquid, slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates,

liquids, in particular stabilized liquids, or slurries.

[0235] Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

Adjunct materials

[0236] Any detergent components known in the art for use in laundry detergents may also be utilized. Other optional detergent components include anti-corrosion agents, anti-shrink agents, antisoil redeposition agents, anti-wrinkling agents, bactericides, binders, corrosion inhibitors, disintegrants/disintegration agents, dyes, enzyme stabilizers (including boric acid, borates, CMC, and/or polyols such as propylene glycol), fabric conditioners including clays, fillers/processing aids, fluorescent whitening agents/optical brighteners, foam boosters, foam (suds) regulators, perfumes, soil-suspending agents, softeners, suds suppressors, tarnish inhibitors, and wicking agents, either alone or in combination. Any ingredient known in the art for use in laundry detergents may be utilized. The choice of such ingredients is well within the skill of the artisan.

[0237] Dispersants: The detergent compositions of the present invention can also contain dispersants. In particular powdered detergents may comprise dispersants. Suitable water-soluble organic materials include 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. Suitable dispersants are for example described in Powdered Detergents, Surfactant science series volume 71, Marcel Dekker, Inc.

[0238] Dye Transfer Inhibiting Agents: The detergent compositions of the present invention may also 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. When present in a subject composition, the dye transfer inhibiting agents may be present at levels 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 composition.

[0239] Fluorescent whitening agent: The detergent compositions of the present invention will preferably also contain additional components that may tint articles being cleaned, such as fluorescent whitening agent or optical brighteners. Where present the brightener is preferably at a level of about 0,01% to about 0,5%.. Any fluorescent whitening agent suitable for use in a laundry detergent composition may be used in the composition of the present invention. The most commonly used fluorescent whitening agents are those belonging to the classes of diaminostilbene-sulphonic acid derivatives, diarylpyrazoline derivatives and bisphenyl-distyryl derivatives. Examples of the diaminostilbene-sulphonic acid derivative type of fluorescent whitening agents include the sodium salts of: 4,4'-bis-(2-diethanolamino-4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate; 4,4'-bis-(2,4-dianilino-s-triazin-6-ylamino) stilbene-2.2'-disulphonate; 4,4'-bis-(2-anilino-4(N-methyl-N-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-disulphonate, 4,4'-bis-(4-phenyl-2,1,3-triazol-2-yl)stilbene-2,2'-disulphonate; 4,4'-bis-(2-anilino-4(1-methyl-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-disulphonate and 2-(stilbyl-4"-naptho-1.,2':4,5)-1,2,3-trizole-2"-sulphonate. Preferred fluorescent whitening agents are Tinopal DMS and Tinopal CBS available from Ciba-Geigy AG, Basel, Switzerland. Tinopal DMS is the disodium salt of 4,4'-bis-(2-morpholino-4 anilino-s-triazin-6-ylamino) stilbene disulphonate. Tinopal CBS is the disodium salt of 2,2'-bis-(phenyl-styryl) disulphonate. Also preferred are fluorescent whitening agents is the commercially available Parawhite KX, supplied by Paramount Minerals and Chemicals, Mumbai, India. Other fluorescers suitable for use in the invention include the 1-3-diaryl pyrazolines and the 7-alkylaminocoumarins. Suitable fluorescent brightener levels include lower levels of from about 0.01, from 0.05, from about 0.1 or even from about 0.2 wt % to upper levels of 0.5 or even 0.75 wt%.

[0240] Soil release polymers: The detergent compositions of the present invention may also include one or more soil release polymers which aid the removal of soils from fabrics such as cotton and polyester based fabrics, in particular the removal of hydrophobic soils from polyester based fabrics. The soil release polymers may for example be nonionic or anionic terephthalte based polymers, polyvinyl caprolactam and related copolymers, vinyl graft copolymers, polyester polyamides see for example Chapter 7 in Powdered Detergents, Surfactant science series volume 71, Marcel Dekker, Inc. Another type of soil release polymers are amphiphilic alkoxylated grease cleaning polymers comprising a core structure and a plurality of alkoxylate groups attached to that core structure. The core structure may comprise a polyalkylenimine structure or a polyalkanolamine structure as described in detail in WO 2009/087523 (hereby incorporated by reference). Furthermore random graft co-polymers are suitable soil release polymers Suitable graft co-polymers are described in more detail in WO 2007/138054, WO 2006/108856 and WO 2006/113314 (hereby incorporated by reference). Other soil release polymers are substituted polysaccharide structures especially substituted cellulosic structures such as modified cellulose deriviatives such as those described in EP 1867808 or WO 2003/040279 (both are hereby incorporated by reference). Suitable cellulosic polymers include cellulose, cellulose ethers, cellulose esters, cellulose amides and mixtures thereof. Suitable cellulosic polymers include anionically modified cellulose, nonionically modified cellulose, cationically modified cellulose, zwitterionically modified cellulose, and mixtures thereof. Suitable cellulosic polymers include methyl cellulose, carboxy methyl cellulose, ethyl cellulose, hydroxyl ethyl cellulose, hydroxyl propyl methyl cellulose, ester

carboxy methyl cellulose, and mixtures thereof.

[0241] Anti-redeposition agents: The detergent compositions of the present invention may also include one or more anti-redeposition agents such as carboxymethylcellulose (CMC), polyvinyl alcohol (PVA), polyvinylpyrrolidone (PVP), polyoxyethylene and/or polyethyleneglycol (PEG), homopolymers of acrylic acid, copolymers of acrylic acid and maleic acid, and ethoxylated polyethyleneimines. The cellulose based polymers described under soil release polymers above may also function as anti-redeposition agents.

[0242] Other suitable adjunct materials include, but are not limited to, anti-shrink agents, anti-wrinkling agents, bactericides, binders, carriers, dyes, enzyme stabilizers, fabric softeners, fillers, foam regulators, hydrotropes, perfumes, pigments, sod suppressors, solvents, and structurants for liquid detergents and/or structure elasticizing agents.

Formulation of Detergent Products

[0243] The detergent composition of the invention may be in any convenient form, e.g., a bar, a homogenous tablet, a tablet having two or more layers, a pouch having one or more compartments, a regular or compact powder, a granule, a paste, a gel, or a regular, compact or concentrated liquid. There are a number of detergent formulation forms such as layers (same or different phases), pouches, as well as forms for machine dosing unit.

[0244] Pouches can be configured as single or multicompartments. It can be of any form, shape and material which is suitable for hold the composition, e.g. without allowing the release of the composition from the pouch prior to water contact. The pouch is made from water soluble film which encloses an inner volume. Said inner volume can be devided into compartments of the pouch. Preferred films are polymeric materials preferably polymers which are formed into a film or sheet. Preferred polymers, copolymers or derivates therof are selected polyacrylates, and water soluble acrylate copolymers, methyl cellulose, carboxy methyl cellulose, sodium dextrin, ethyl cellulose, hydroxyethyl cellulose, hydroxypropyl methyl cellulose, malto dextrin, poly methacrylates, most preferably polyvinyl alcohol copolymers and, hydroxyprpyl methyl cellulose (HPMC). Preferably the level of polymer in the film for example PVA is at least about 60%. Preferred average molecular weight will typically be about 20,000 to about 150,000. Films can also be of blend compositions comprising hydrolytically degradable and water soluble polymer blends such as polyactide and polyvinyl alcohol (known under the Trade reference M8630 as sold by Chris Craft In. Prod. Of Gary, Ind., US) plus plasticisers like glycerol, ethylene glycerol, Propylene glycol, sorbitol and mixtures thereof. The pouches can comprise a solid laundry detergent composition or part components and/or a liquid cleaning composition or part components separated by the water soluble film. The compartment for liquid components can be different in composition than compartments containing solids. Ref: (US2009/0011970 A1).

[0245] Detergent ingredients can be separated physically from each other by compartments in

water dissolvable pouches or in different layers of tablets. Thereby negative storage interaction between components can be avoided. Different dissolution profiles of each of the compartments can also give rise to delayed dissolution of selected components in the wash solution.

[0246] A liquid or gel detergent, which is not unit dosed, may be aqueous, typically containing at least 20% by weight and up to 95% water, such as up to about 70% water, up to about 65% water, up to about 55% water, up to about 45% water, up to about 35% water. Other types of liquids, including without limitation, alkanols, amines, diols, ethers and polyols may be included in an aqueous liquid or gel. An aqueous liquid or gel detergent may contain from 0-30% organic solvent. A liquid or gel detergent may be non-aqueous.

Laundry Soap Bars

[0247] The enzymes of the invention may be added to laundry soap bars and used for hand washing laundry, fabrics and/or textiles. The term laundry soap bar includes laundry bars, soap bars, combo bars, syndet bars and detergent bars. The types of bar usually differ in the type of surfactant they contain, and the term laundry soap bar includes those containing soaps from fatty acids and/or synthetic soaps. The laundry soap bar has a physical form which is solid and not a liquid, gel or a powder at room temperature. The term solid is defined as a physical form which does not significantly change over time, i.e. if a solid object (e.g. laundry soap bar) is placed inside a container, the solid object does not change to fill the container it is placed in. The bar is a solid typically in bar form but can be in other solid shapes such as round or oval.

[0248] The laundry soap bar may contain one or more additional enzymes, protease inhibitors such as peptide aldehydes (or hydrosulfite adduct or hemiacetal adduct), boric acid, borate, borax and/or phenylboronic acid derivatives such as 4-formylphenylboronic acid, one or more soaps or synthetic surfactants, polyols such as glycerine, pH controlling compounds such as fatty acids, citric acid, acetic acid and/or formic acid, and/or a salt of a monovalent cation and an organic anion wherein the monovalent cation may be for example Na^+, K^+ or NH_4^+ and the organic anion may be for example formate, acetate, citrate or lactate such that the salt of a monovalent cation and an organic anion may be, for example, sodium formate.

[0249] The laundry soap bar may also contain complexing agents like EDTA and HEDP, perfumes and/or different type of fillers, surfactants e.g. anionic synthetic surfactants, builders, polymeric soil release agents, detergent chelators, stabilizing agents, fillers, dyes, colorants, dye transfer inhibitors, alkoxylated polycarbonates, suds suppressers, structurants, binders, leaching agents, bleaching activators, clay soil removal agents, anti-redeposition agents, polymeric dispersing agents, brighteners, fabric softeners, perfumes and/or other compounds known in the art.

[0250] The laundry soap bar may be processed in conventional laundry soap bar making

equipment such as but not limited to: mixers, plodders, e.g a two stage vacuum plodder, extruders, cutters, logo-stampers, cooling tunnels and wrappers. The invention is not limited to preparing the laundry soap bars by any single method. The premix of the invention may be added to the soap at different stages of the process. For example, the premix containing a soap, an enzyme, optionally one or more additional enzymes, a protease inhibitor, and a salt of a monovalent cation and an organic anion may be prepared and the mixture is then plodded. The enzyme and optional additional enzymes may be added at the same time as the protease inhibitor for example in liquid form. Besides the mixing step and the plodding step, the process may further comprise the steps of milling, extruding, cutting, stamping, cooling and/or wrapping.

Granular detergent formulations

[0251] A granular detergent may be formulated as described in WO09/092699, EP1705241, EP1382668, WO07/001262, US6472364, WO04/074419 or WO09/102854. Other useful detergent formulations are described in WO09/124162, WO09/124163, WO09/117340, WO09/117341, WO09/117342, WO09/072069, WO09/063355, WO09/132870, WO09/121757, WO09/112296, WO09/112298, WO09/103822, WO09/087033, WO09/050026, WO09/047125, WO09/047126, WO09/047127, WO09/047128, WO09/021784, WO09/010375, WO09/000605, WO09/122125, WO09/095645, WO09/040544, WO09/040545, WO09/024780, WO09/004295, WO09/004294, WO09/121725, WO09/115391, WO09/115392, WO09/074398, WO09/074403, WO09/068501, WO09/065770, WO09/021813, WO09/030632, and WO09/015951.

[0252] WO2011025615, WO2011016958, WO2011005803, WO2011005623, WO2011005730, WO2011005844, WO2011005904, WO2011005630, WO2011005830, WO2011005912, WO2011005905, WO2011005910, WO2011005813, WO2010135238, WO2010120863, WO2010108002, WO2010111365, WO2010108000, WO2010107635, WO2010090915, WO2010033976, WO2010033746, WO2010033747, WO2010033897, WO2010033979, WO2010030540, WO2010030541, WO2010030539, WO2010024467, WO2010024469, WO2010024470, WO2010025161, WO2010014395, WO2010044905,

[0253] WO2010145887, WO2010142503, WO2010122051, WO2010102861, WO2010099997, WO2010084039, WO2010076292, WO2010069742, WO2010069718, WO2010069957, WO2010057784, WO2010054986, WO2010018043, WO2010003783, WO2010003792,

[0254] WO2011023716, WO2010142539, WO2010118959, WO2010115813, WO2010105942, WO2010105961, WO2010105962, WO2010094356, WO2010084203, WO2010078979, WO2010072456, WO2010069905, WO2010076165, WO2010072603, WO2010066486, WO2010066631, WO2010066632, WO2010063689, WO2010060821, WO2010049187, WO2010031607, WO2010000636.

Uses

[0255] The present invention is also directed to methods for using the compositions according to the invention in laundering of textile and fabrics, such as house hold laundry washing and industrial laundry washing.

[0256] The invention is also directed to methods for using the compositions according to the invention or in cleaning hard surfaces such as floors, tables, walls, roofs etc. as well as surfaces of hard objects such as cars (car wash) and dishes (dish wash).

[0257] A detergent composition of the present invention may be formulated, for example, as a hand or machine laundry detergent composition including a laundry additive composition suitable for pretreatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

[0258] In a specific aspect, the present invention provides a detergent additive comprising a polypeptide of the present invention as described herein.

[0259] The cleaning process or the textile care process may for example be a laundry process, a dishwashing process or cleaning of hard surfaces such as bathroom tiles, floors, table tops, drains, sinks and washbasins. Laundry processes can for example be household laundering, but it may also be industrial laundering. Furthermore, the invention relates to a process for laundering of fabrics and/or garments where the process comprises treating fabrics with a washing solution containing a detergent composition, and at least one protease variant of the invention. The cleaning process or a textile care process can for example be carried out in a machine washing process or in a manual washing process. The washing solution can for example be an aqueous washing solution containing a detergent composition.

[0260] The last few years there has been an increasing interest in replacing components in detergents, which is derived from petrochemicals with renewable biological components such as enzymes and polypeptides without compromising the wash performance. When the components of detergent compositions change new enzyme activities or new enzymes having alternative and/or improved properties compared to the common used detergent enzymes such as proteases, lipases and amylases is needed to achieve a similar or improved wash performance when compared to the traditional detergent compositions.

[0261] The invention further concerns the use of compositions of the invention comprising the subtilase variants in a proteinaceous stain removing processes. The proteinaceous stains may be stains such as food stains, e.g., baby food, sebum, cocoa, egg, blood, milk, ink, grass, or a combination hereof.

[0262] Typical detergent compositions include various components in addition to the enzymes, these components have different effects, some components like the surfactants lower the

surface tension in the detergent, which allows the stain being cleaned to be lifted and dispersed and then washed away, other components like bleach systems remove discolor often by oxidation and many bleaches also have strong bactericidal properties, and are used for disinfecting and sterilizing. Yet other components like builder and chelator softens, e.g., the wash water by removing the metal ions form the liquid.

[0263] In a particular embodiment, the invention concerns the use of a composition comprising a subtilase variant and one or more detergent components, such as surfactants, hydrotropes, builders, co-builders, chelators or chelating agents, bleaching system or bleach components, polymers, fabric hueing agents, fabric conditioners, foam boosters, suds suppressors, dispersants, dye transfer inhibitors, fluorescent whitening agents, perfume, optical brighteners, bactericides, fungicides, soil suspending agents, soil release polymers, anti-redeposition agents, enzyme inhibitors or stabilizers, enzyme activators, antioxidants, and solubilizers.

[0264] In a particular embodiment, the invention concerns the use of a composition of the invention comprising a subtilase variant as disclosed herein and one or more additional enzymes selected from the group comprising of proteases, amylases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidaes, haloperoxygenases, catalases and mannanases, or any mixture thereof.

[0265] In a particular embodiment, the invention concerns the use of a composition of the invention comprising a subtilase variant as disclosed herein, one or more additional enzymes selected from the group comprising of proteases, amylases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidaes, haloperoxygenases, catalases and mannanases, or any mixture thereof and one or more detergent components, such as surfactants, hydrotropes, builders, co-builders, chelators or chelating agents, bleaching system or bleach components, polymers, fabric hueing agents, fabric conditioners, foam boosters, suds suppressors, dispersants, dye transfer inhibitors, fluorescent whitening agents, perfume, optical brighteners, bactericides, fungicides, soil suspending agents, soil release polymers, anti-redeposition agents, enzyme inhibitors or stabilizers, enzyme activators, antioxidants, and solubilizers.

Washing Method

[0266] The present invention relates to a method of cleaning a fabric, a dishware or hard surface with a detergent composition of the invention.

[0267] A preferred embodiment concerns a method of cleaning, said method comprising the steps of: contacting an object with a detergent composition of the invention under conditions suitable for cleaning said object. In a preferred embodiment the detergent composition is used in a laundry or a dish wash process.

[0268] Still another embodiment relates to a method for removing stains from fabric or

dishware which comprises contacting said fabric or dishware with a composition of the invention under conditions suitable for cleaning said object.

[0269] Also contemplated are compositions and methods of treating fabrics (e.g., to desize a textile) using one or more of the protease of the invention. The protease can be used in any fabric-treating method which is well known in the art (see, e.g., US6,077,316). For example, in one aspect, the feel and appearance of a fabric is improved by a method comprising contacting the fabric with a protease in a solution. In one aspect, the fabric is treated with the solution under pressure.

[0270] The detergent compositions of the present invention are suited for use in laundry and hard surface applications, including dish wash. Accordingly, the present invention includes a method for laundering a fabric or washing dishware. The method comprises the steps of contacting the fabric/dishware to be cleaned with a solution comprising the detergent composition according to the invention. The fabric may comprise any fabric capable of being laundered in normal consumer use conditions. The dishware may comprise any dishware such as crockery, cutlery, ceramics, plastics such as melamine, metals, china, glass and acrylics. The solution preferably has a pH from about 5.5 to about 11.5. The compositions may be employed at concentrations from about 100 ppm, preferably 500 ppm to about 15,000 ppm in solution. The water temperatures typically range from about 5°C to about 95°C, including about 10°C, about 15°C, about 20°C, about 25°C, about 30°C, about 35°C, about 40°C, about 45°C, about 50°C, about 55°C, about 60°C, about 65°C, about 70°C, about 75°C, about 80°C, about 85°C and about 90°C. The water to fabric ratio is typically from about 1:1 to about 30:1.

[0271] The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents and protease inhibitors, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, different salts such as NaCl; KCl; lactic acid, formic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, or a peptide aldehyde such as di-, tri- or tetrapeptide aldehydes or aldehyde analogues (either of the form B1-B0-R wherein, R is H, CH3, CX3, CHX2, or CH2X (X=halogen), B0 is a single amino acid residue (preferably with an optionally substituted aliphatic or aromatic side chain); and B1 consists of one or more amino acid residues (preferably one, two or three), optionally comprising an N-terminal protection group, or as described in WO09118375, WO98/13459) or a protease inhibitor of the protein type such as RASI, BASI, WASI (bifunctional alpha-amylase/subtilisin inhibitors of rice, barley and wheat) or CI2 or SSI. The composition may be formulated as described in e.g. WO 92/19709, WO 92/19708 and US6,472,364. In some embodiments, 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)).

[0272] In some preferred embodiments, the detergent compositions provided herein are typically formulated such that, during use in aqueous cleaning operations, the wash water has

a pH of from about 5.0 to about 11.5, or in alternative embodiments, even from about 6.0 to about 10.5. In some preferred embodiments, granular or liquid laundry products are formulated to have a pH from about 6 to about 8. 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.

[0273] The present invention is further described by the following examples that should not be construed as limiting the scope of the invention.

EXAMPLES

Materials and Methods

Automatic Mechanical Stress Assay (AMSA) for laundry

[0274] In order to assess the wash performance in laundry washing experiments are performed, using the Automatic Mechanical Stress Assay (AMSA). With the AMSA, the wash performance of a large quantity of small volume enzyme-detergent solutions can be examined. The AMSA plate has a number of slots for test solutions and a lid firmly squeezing the laundry sample, the textile to be washed against all the slot openings. During the washing time, the plate, test solutions, textile and lid are vigorously shaken to bring the test solution in contact with the textile and apply mechanical stress in a regular, periodic oscillating manner. For further description see WO02/42740 especially the paragraph "Special method embodiments" at page 23-24.

[0275] The wash performance is measured as the brightness of the colour of the textile washed. Brightness can also be expressed as the intensity of the light reflected from the sample when illuminated with white light. When the sample is stained the intensity of the reflected light is lower, than that of a clean sample. Therefore the intensity of the reflected light can be used to measure wash performance.

[0276] Colour measurements are made with a professional flatbed scanner (Kodak iQsmart, Kodak, Midtager 29, DK-2605 Brøndby, Denmark), which is used to capture an image of the washed textile.

[0277] To extract a value for the light intensity from the scanned images, 24-bit pixel values from the image are converted into values for red, green and blue (RGB). The intensity value (Int) is calculated by adding the RGB values together as vectors and then taking the length of the resulting vector:

$$Int = \sqrt{r^2 + g^2 + b^2}$$

Table 1: Composition of model detergents and test materials

Model detergent and test materials were as follows:	
	0.3 to 0.5% xanthan gum,
	0.2 to 0.4% antifoaming agent,
	6 to 7% glycerol, 0.3 to 0.5% ethanol,
	4 to 7% FAEOS (fatty alcohol ether sulfate),
	24 to 28% nonionic surfactants,
Laundry liquid model detergent	1% boric acid, 1 to 2% sodium citrate (dihydrate),
	2 to 4% soda, 14 to 16% coconut fatty acid,
	0.5% HEDP (1-hydroxyethane-(1,1-diphosphonic acid)),
	0 to 0.4% PVP (polyvinylpyrrolidone),
	0 to 0.05% optical brighteners,
	0 to 0.001% dye, remainder deionized water.
Test material	PC-03 (Chocolate-milk/ink on cotton/polyester)
	C-05 (Blood/milk/ink on cotton)

General molecular biology methods:

[0278] Unless otherwise mentioned the DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989); Ausubel et al. (1995); Harwood and Cutting (1990).

Protease activity assays:

1) Suc-AAPF-pNA activity assay:

[0279] The proteolytic activity can be determined by a method employing the Suc-AAPF-PNA substrate. Suc-AAPF-PNA is an abbreviation for N-Succinyl-Alanine-Alanine-Proline-Phenylalanine-p-Nitroanilide, and it is a blocked peptide which can be cleaved by endo-proteases. Following cleavage a free PNA molecule is liberated and it has a yellow colour and thus can be measured by visible spectrophotometry at wavelength 405nm. The Suc-AAPF-PNA substrate is manufactured by Bachem (cat. no. L1400, dissolved in DMSO).

[0280] The protease sample to be analyzed was diluted in residual activity buffer (100mM Tris

pH8.6). The assay was performed by transferring 60µl of diluted enzyme samples to 96 well microtiter plate and adding 140µl substrate working solution (0.72mg/ml in 100mM Tris pH8.6). The solution was mixed at room temperature and absorption is measured every 20 sec. over 5 minutes at OD 405 nm.

[0281] The slope (absorbance per minute) of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the protease in question under the given set of conditions. The protease sample should be diluted to a level where the slope is linear.

Tergo-O-Tometer (TOM)

[0282] The Tergo-O-Tometer (TOM) is a medium scale model wash system that can be applied to test 12 different wash conditions simultaneously. A TOM is basically a large temperature controlled water bath with up to 12 open metal beakers submerged into it. Each beaker constitutes one small top loader style washing machine and during an experiment, each of them will contain a solution of a specific detergent/enzyme system and the soiled and unsoiled fabrics its performance is tested on. Mechanical stress is achieved by a rotating stirring arm, which stirs the liquid (1L) within each beaker. Because the TOM beakers have no lid, it is possible to withdraw samples during a TOM experiment and assay for information on-line during wash. In a TOM experiment, factors such as the ballast to soil ratio and the fabric to wash liquor ratio can be varied. Therefore, the TOM provides the link between small scale experiments, such as AMSA and mini-wash, and the more time consuming full scale experiments in full scale washing machines.

[0283] After washing and rinsing the swatches are spread out flat and allowed to air dry at room temperature overnight. All washes are evaluated the day after the wash. Light reflectance evaluations of the swatches are done using a Macbeth Color Eye 7000 reflectance spectrophotometer with large aperture. The measurements are made without UV in the incident light and remission at 460 nm was extracted. Measurements are made on unwashed and washed swatches. The test swatch to be measured is placed on top of another swatch of same type and colour (twin swatch).

[0284] Remission values for individual swatches are calculated by subtracting the remission value of the swatch washed without enzyme (blank) from the swatch washed together with enzyme.

[0285] Calculating the effect of protease variants effect is done by taking the measurements from washed swatches with enzymes and subtracting the measurements from washed without enzyme for each stain.

[0286] The performance of the new protease variants is compared to the performance of Reference (REF) = SEQ ID NO 3 by calculating the relative performance (RP):

$RP = (R_{PROTEASEVARIANT} - R_{BLANK})/(R_{REF} - R_{BLANK})$

Accelerated storage stability assay

[0287] Storage stability of protease variants in liquid detergent was evaluated using an accelerated assay with incubation at elevated temperatures for up to 24 hours.

[0288] All purified protease samples were diluted to concentrations of 0.2 and 0.1 mg/ml based on absorbance at 280 nm and theoretical extinction coefficient using 0.01% Triton X-100. For each variant 2 wells with high protease concentration and 2 wells with low concentration were included. As reference SEQ ID NO 3 was included on each microtiter plate. 30 µl protease sample was mixed with 270 µl detergent (CNS) in the well of a microtiter plate (Nunc U96 PP 0.5 ml) using a magnetic bar (on Zephyr pipetting station (Caliper LifeSciences) for 30 min). 20 µl of this mixture was then transferred to another microtiter plate (Nunc U96 PP 0.5 ml with added magnetic bars) and mixed with 150 µl 100mM Tris pH 8.6 (at least 5 min on Zephyr). 30 µl of this dilution was transferred to a Nunc F 96-MTP, and after addition of 70 µl substrate solution initial activity of unstressed sample was determined by measuring absorbance at 405 nm every 20 see for 5 min (on a SpectraMax Plus). After sealing, the detergent plate was incubated at appropriate temperature (47°C for CNS EDTA pH9) in an Eppendorf Thermomixer (no shaking). After 1-4 and 20-25 hours incubation, 20 µl samples were withdrawn and residual activity of stressed sample was measured as with the initial, unstressed activity.

[0289] Decrease in activity during incubation with detergent was assumed to be exponential. Half lifes (T½) were found from linear regression of Log(Activity) versus incubation time (0, 1-4 and 20-25 hours), and half-life improvement factors (T½ IF) were calculated as half-life of protease variant relative to half-life of SEQ ID NO 3 reference.

Detergent

[0290]

	Alkylbenzenesulfonic acid 8%
	Alcohol ethoxylate 4%
	Sodium lauryl ether sulfate 4%
	Triethanolamine;2,2',2''-nitrilotri(ethan-1-ol) 0.5%
CNS EDTA pH9	Trisodium citrate dihydrate 0.5%
	Sodium hydroxide 1%
	EDTA 0.001%
	pH adjusted to 9
	Up to 100 % demineralised water

Example 1: Preparation and Expression of variants

[0291] Introduction of an expression cassette into Bacillus subtilis was done by transformation of a suitable expression cassette utilizing the natural competence of the organism.

[0292] The following summarizes DNA manipulations such as Tthe introduction of mutations and constructioni ntroduction of an expression cassette into Bacillus subtilis. All DNA manipulations were done by PCR (e.g. Sambrook et al.; Molecular Cloning; Cold Spring Harbor Laboratory Press) and all DNA manipulations and can be repeated by everybody skilled in the art. Recombinant B. *subtilis* constructs encoding subtilase variants were used to inoculate shakeflasks containing a rich media (e.g. PS-1: 100 g/L Sucrose (Danisco cat.no. 109-0429), 40 g/L crust soy (soy bean flour), 10g/L Na2HPO4.12H2O (Merck cat.no. 6579), 0.1ml/L replace- Dowfax63N10 (Dow). Cultivation typically takes 4 days at 30oC shaking with 220rpm.

Example 2: Fermentation of variants

[0293] Fermentation may be performed by methods well known in the art or as follows. A *B. subtilis* strain harboring the relevant expression plasmid was streaked on a LB agar plate, and grown overnight at 37°C. The colonies were transferred to 100 ml PS-1 media in a 500 ml shaking flask.

[0294] Cells and other undissolved material were removed from the fermentation broth by centrifugation at 4500 rpm for 20-25 minutes. Afterwards the supernatant was filtered to obtain a clear solution.

Example 3: Purification of Variants

[0295] The culture broth was centrifuged (26000 x g, 20 min) and the supernatant was carefully decanted from the precipitate. The supernatant was filtered through a Nalgene 0.2µm filtration unit in order to remove the rest of the *Bacillus* host cells. pH in the 0.2µm filtrate was adjusted to pH 8 with 3M Tris base and the pH adjusted filtrate was applied to a MEP Hypercel column (from Pall corporation) equilibrated in 20mM Tris/HCl, 1mM $CaCl_2$, pH 8.0. After washing the column with the equilibration buffer, the column was step-eluted with 20mM CH_3COOH/NaOH, 1mM $CaCl_2$, pH 4.5. Fractions from the column were analysed for protease activity (using the Suc-AAPF-pNA assay at pH 9) and peak-fractions were pooled. The pH of the pool from the MEP Hypercel column was adjusted to pH 6 with 20%(v/v) CH_3COOH or 3M Tris base and the pH adjusted pool was diluted with deionized water to the same conductivity

as 20mM MES/NaOH, 2mM $CaCl_2$, pH 6.0. The diluted pool was applied to a SP-sepharose FF column (from GE Healthcare) equilibrated in 20mM MES/NaOH, 2mM $CaCl_2$, pH 6.0. After washing the column with the equilibration buffer, the protease was eluted with a linear NaCl gradient (0 --> 0.5M) in the same buffer over five column volumes. Fractions from the column were analysed for protease activity (using the Suc-AAPF- pNA assay at pH 9) and active fractions were analysed by SDS-PAGE. The fractions, where only one band was seen on the coomassie stained SDS-PAGE gel, were pooled as the purified preparation and was used for further experiments.

Example 4: Stability of variants of the invention

[0296] Variants of the invention were generated and purified as described in examples 1-3 and tested for stability in liquid detergent at a temperature of 47°C using the stability test disclosed above and the half-life calculated. The reference polypeptide was the subtilase having SEQ ID NO: 3.

Table 2: Stability of variants of SEQ ID NO: 3. First column indicates the substitution in SEQ ID NO: 3. Second column indicates the half-life found in the experiment, and in the third column the data are indicated relative to SEQ ID NO: 3. Last column indicates the standard deviation.

Substitutions (Relative to SEQ ID NO: 3)	**T½(h) (47°C, 24h, 90% detergent)**	**IF (47°C, 24h, 90% detergent)**	**StDev (47°C, 24h, 90% detergent)**
None (=SEQ ID NO: 3)	23	1	0.06
P129D	69	2,6	0,5

[0297] All the variants had significantly improved stability in liquid detergent under these conditions.

Example 5: Wash performance of subtilisin variants

[0298] The wash performance of protease variants that have the same amino acid sequence as SEQ ID NO: 3 except for the substitutions as per the table below on the stains as indicated; reference is the protease according to SEQ ID NO: 3. in laundry was assessed using the Automatic Mechanical Stress Assay (AMSA), where the wash performance of many small volume enzyme-detergent solutions can be examined. The AMSA plate has a number of slots for test solutions and a lid that firmly squeezes the textile to be washed against the slot openings. During the wash, the plate, test solutions, textile and lid were vigorously shaken to bring the test solution in contact with the textile and apply mechanical stress in a regular, periodic, oscillating manner. For further description see WO 02/42740 especially the paragraph "Special method embodiments" at pages 23-24.

[0299] The laundry experiments were conducted under the experimental conditions specified in Table 3.

Table 3:

Detergent dosage	2.0 g/L
Test solution volume	160 µL (20 µL enzyme+140 µL detergent)
pH	8.4
Wash time	20 minutes
Temperature	20°C
Water hardness	12°dH

[0300] Model detergent was used as described in Table 1.

Table 4: Composition of model detergents and test materials

Detergent	Laundry liquid model detergent (Table 1)
Test material	C-05 (Blood/milk/ink on cotton)
	PC-03 (Chocolate milk with carbon black on Polyester/cotton, 65/35)
	CS-37 (Full egg/pigment on cotton)

[0301] Test materials were obtained from Center For Testmaterials BV, 3133 KT Vlaardingen, the Netherlands.

[0302] Water hardness was adjusted to 12°dH by addition of $CaCl_2$, $MgCl_2$, and $NaHCO_3$ (Ca^{2+}:Mg^{2+}= 2:1:4.5) to the test system. After washing, the textiles were flushed in tap water and dried.

[0303] The wash performance was measured as the brightness of the color of the textile washed. Brightness can also be expressed as the intensity of the light reflected from the sample when illuminated with white light. When the sample is stained the intensity of the reflected light is lower than that of a clean sample. Therefore the intensity of the reflected light can be used to measure wash performance.

[0304] Color measurements were made with a Kodak iQsmart flatbed scanner (Kodak, Midtager 29, DK-2605 Brøndby, Denmark), which is used to capture an image of the washed textile.

[0305] To extract a value for the light intensity from the scanned images, 24-bit pixel values from the image were converted into values for red, green and blue (RGB). The intensity value (Int) was calculated by adding the RGB values together as vectors and then taking the length

of the resulting vector:

$$Int=\sqrt{r^2+g^2+b^2}$$

[0306] The results are shown in Table 5. The results are given as relative performance compared to SEQ ID NO: 3 at an enzyme concentration of 30 nM on three different swatches.

Table 5: AMSA relative performance of variants compared to SEQ ID NO 3.

Stain	PC-03	C-05	CS-37
Mutations compared to SEQ ID NO: 3			
P129D	1.1	1.0	0.7

Example 6: Result of Terg-O-tometer (TOM) wash assay

[0307] The TOM wash experiment is conducted under the experimental conditions specified below:

Table 6. TOM wash conditions	
Detergent	Liquid laundry model detergent (Table 1)
Detergent dose	4.66 g/L
pH	pH was measured to be 7.6, but is used "as is" in the current detergent solution and is not adjusted.
Water hardness	16°dH, adjusted by adding $CaCl_2$*$2H_2O$, $MgCl_2$*$6H_2O$ and $NaHCO_3$ (5:1:3) to Milli-Q water.
Enzyme conc.	60 nM
Test solution volume	1000 mL
Test material	2 swatches, each 5x5 cm, of each of the 5 soiled textile types per beaker (i.e. 10 soiled swatches per beaker):
	PC-03 (Chocolate-milk/ink on cotton/polyester)
	C-H010 (Cocoa, cooked up with milk on cotton)
	C-05 (Blood/milk/soot on cotton)
	CS-01 (Aged blood on cotton)
	CS-37 (Full egg/pigment on cotton)
	Cotton ballast swatches (50%:50% WFK10A (Standard Cotton Fabric, Unsoiled):WFK80A (cotton), 5x5 cm (wfk Testgewebe GmbH, Christenfeld 10; D-41379 Brijggen-Bracht; Germany) added to give a total weight of 30 g of soiled textile swatches + ballast per TOM beaker.
Temperature	20°C

Table 6. TOM wash conditions	
Wash time	30 min
Rinse time	5 min
Rotation	120 rpm

Table 7: Relative performance compared to SEQ ID NO: 3 of subtilase variants of having the indicated mutations compared to SEQ ID NO 3.

Delta remission	PC-03	C-H010	C-05	CS-01	CS-37
P129D	1.3	0.8	1.1	0.8	1.3

W: grass on cotton: product no. 164 obtainable from Eidgenössische Material- und Prüfanstalt (EMPA) Testmaterialien AG [Federal materials and testing agency, Testmaterials], St. Gallen, Switzerland.
X: cocoa cooked up with milk: product no. C-H010
Y: blood/milk/ink, product no. EMPA 117 obtainable from Eidgenössische Material- und Prüfanstalt (EMPA) Testmaterialien AG [Federal materials and testing agency, Testmaterials], St. Gallen, Switzerland.
Z: chocolate milk and soot, product no. CFT C03 obtainable from CFT (Center for Testmaterials) BV, Vlaardingen, Netherlands:

[0308] A liquid washing agent with the following composition was used as base formulation (all values in weight percent): 0 to 0.5% xanthan gum, 0.2 to 0.4% antifoaming agent, 0.2 to 8% Triethanolamine, 1 to 7% glycerol, 0.3 to 3% ethanol, 0 to 12% FAEOS (fatty alcohol ether sulfate), 1 to 28% nonionic surfactants, 0.5-4% boric acid, 0.5 to 6% sodium citrate (dihydrate), 1 to 6% soda, 0 to 16% coconut fatty acid, 0.5 to 6% HEDP (1-hydroxyethane-(1,1-diphosphonic acid)), 0 to 0.4% PVP (polyvinylpyrrolidone), 0 to 0.05% optical brighteners, 0 to 0.001% dye, remainder deionized water.

[0309] Based on this base formulation, various detergents were prepared by adding respective proteases as indicated in tables 9. Reference is the protease that has the amino acid sequence of SEQ ID NO.3, the reference protease already showing a good wash performance, especially in liquid detergents. The proteases were added in the same amounts based on total protein content (5 mg/l wash liquor).

[0310] The dosing ratio of the liquid washing agent was 4,7 grams per liter of washing liquor and the washing procedure was performed for 60 minutes at a temperature of 20°C and 40°C, the water having a water hardness between 15.5 and 16.5° (German degrees of hardness).

[0311] The whiteness, i.e. the brightening of the stains, was determined photometrically as an indication of wash performance. A Minolta CM508d spectrometer device was used, which was calibrated beforehand using a white standard provided with the unit.

[0312] The results obtained are the difference values between the remission units obtained with the detergents and the remission units obtained with the detergent containing the

reference protease. A positive value therefore indicates an improved wash performance of the variants in the detergent. It is evident from tables 4a (results at 40°C) and 4b (results at 20°C) that variants according to the invention show improved wash performance.

[0313] Table 4 a and b: Wash performance at 40°C of protease variants that have the same amino acid sequence as SEQ ID NO: 3 except for the substitutions as per the table below on the stains as indicated; reference is the protease according to SEQ ID NO: 3.

Table a)

Protease variant		A	B	C	D	E	F
N76D + A228V + N261D	Diff	1.3	3.0	10.6	nd	nd	nd
	HSD	0.5	2.2	2.4	nd	nd	nd
H120D + S163G + N261D	Diff	1.1	1.5	9.2	nd	nd	nd
	HSD	0.5	2.2	2.4	nd	nd	nd
N76D + A228V + L262E	Diff	1.3	1.3	9.6	nd	nd	nd
	HSD	0.5	2.2	2.4	nd	nd	nd
S156D + L262E	Diff	1.4	3.9	5.8	7.5	3.0	5.6
	HSD	0.7	1.9	2.2	3.7	1.3	3.7
N76D + Q137H + S141H + R145H + A228V + N261D	Diff	1.1	0.8	4.7	0.4	1.2	4.1
	HSD	0.7	1.9	2.2	3.7	1.2	3.7
H120D + S163G + N261D + Q137H + S141H + R145H	Diff	1.0	1.8	5.0	2.2	1.0	4.4
	HSD	0.7	2.0	2.2	3.7	1.2	3.7
N76D + S163G + N238E + Q137H + S141H + R145H	Diff	1.3	3.4	6.2	4.4	1.7	5.9
	HSD	0.7	1.9	2.2	3.7	1.2	3.7
N238E + L262E + Q137H + S141H + R145H	Diff	1.4	3.3	5.6	3.6	1.3	5.3
	HSD	0.7	1.9	2.2	3.7	1.2	3.7
S3T + N76D + S156D + Y209W + Q137H + S141H + R145H	Diff	1.7	3.3	5.6	4.6	1.4	4.0
	HSD	0.7	1.9	2.2	3.7	1.2	3.7

Table b)

Protease Variant	U	G	V	T	W
S163G	0,9	1,5	1,8	nd	0,9
G61D	1,5	1,7	nd	nd	nd
S156D	1,6	2,1	0,5	nd	nd
H120D	0,8	1,6	2,4	nd	nd
G195E + V199M	1,5	0,7	1,1	nd	nd
A228V + N261D	0,5	1,3	1,6	nd	nd
V244T	1,3	1,1	2,7	nd	nd
T58L	nd	2,0	1,1	0,9	0,4

Protease Variant	U	G	V	T	W
S3T + V4I + N261D	1,5	1,7	0,4	nd	nd
A194P + G195E + V199M + V205I	0,8	nd	nd	1,1	nd
H120D + A228V	2,1	1,9	nd	nd	nd
H120D + N261D	nd	nd	0,8	1,2	nd
N76D + A228V + N261D	nd	0,5	nd	1,8	nd

[0314] Tables 4 c-f: Wash performance at 20°C of protease variants that have the same amino acid sequence as SEQ ID NO: 3 except for the substitutions as per the table below on the stains as indicated; reference is the protease according to SEQ ID NO: 3.

Table c)

Protease variant		A	C	D	G	H	I
H120D + N261D	Diff	0.8	7.1	4.3	1.9	2.4	4.8
	HSD	0.7	4.5	2.5	2.6	1.2	1.2
N76D + A228V + N261D	Diff	1.0	9.9	4.9	4.5	2.6	5.4
	HSD	0.7	4.5	2.5	2.6	1.2	1.1

Table d)

Protease variant		A	C	D	J	K	L	M	N	O	P	Q	R	S	T
S156D+ L262E	Diff	0.9	8.0	6.1	5.1	2.3	3.1	4.2	2.6	6.1	1.3	1.9	3.2	2.6	0.6
	HSD	0.9	3.7	4.2	2.0	1.4	2.4	1.5	2.2	3.2	1.1	1.6	2.6	1.9	1.7
N76D+ Q137H+ S141H+ R145H+A 228V+ N261D	Diff	0.2	5.2	3.5	4.3	1.9	1.6	3.7	0.5	5.6	1.5	0.7	2.3	1.3	0.1
	HSD	0.9	3.7	4.2	2.0	1.4	2.4	1.6	2.2	3.3	1.1	1.6	2.7	1.9	1.7
H120D+ S163G+ N261D+ Q137H+ S141H+ R145H	Diff	1.1	7.0	6.0	4.4	2.2	2.9	4.2	3.1	5.9	1.4	2.7	3.5	2.6	1.2
	HSD	0.9	3.7	4.2	2.0	1.4	2.4	1.5	2.2	3.2	1.1	1.6	2.6	1.9	1.7
N76D+ S163G+ N238E+ Q137H+ S141H+ R145H	Diff	1.5	6.3	5.5	4.7	2.1	3.0	3.5	3.5	7.0	1.5	3.4	3.6	3.2	2.6
	HSD	0.9	3.7	4.2	2.0	1.4	2.4	1.5	2.2	3.2	1.1	1.6	2.7	1.9	1.8
	Diff	1.4	5.7	5.9	3.9	1.9	1.5	3.4	1.4	5.8	0.9	1.2	3.6	2.2	0.7
N238E+	HSD	0.9	3.7	4.2	2.0	1.4	2.4	1.5	2.2	3.2	1.1	1.6	2.6	1.9	1.7

Protease variant		A	C	D	J	K	L	M	N	O	P	Q	R	S	T
L262E+ Q137H+ S141H+ R145H															
S3T+ N76D+ S156D+ Y209W+ Q137H+ S141H+ R145H	Diff	2.3	6.7	6.7	4.4	2.6	1.8	3.5	3.0	5.5	1.5	1.9	2.0	2.7	1.8
	HSD	0.9	3.7	4.2	2.0	1.4	2.4	1.5	2.2	3.2	1.1	1.7	2.6	1.9	1.7

Table e)

Protease Variant	U	G	V	T	W
S163G	1,1	1,3	0,5	1,7	nd
G61D	0,2	0,8	0,7	2,1	nd
H120D	nd	0,3	0,6	0,5	1,2
S156D	1,1	1,9	nd	2,1	0,8
H120D	nd	0,8	nd	0,9	1,2
G195E + V199M	1,1	1,0	nd	1,2	1,0
A228V + N261D	0,8	0,8	nd	1,0	0,6
V244T	nd	1,4	0,5	nd	1,5
T58L	1,1	1,2	nd	nd	nd
S3T + V4I + N261D	1,1	1,0	nd	nd	nd
A194P + G195E + V199M + V205I	0,4	1,3	nd	nd	nd
H120D + A228V	1,8	1,4	nd	2,0	nd
N76D + N261D	nd	nd	1,3	2,0	nd
A194P + G195E + V199M + V205I + A228V + N261D	0,6	nd	1,4	1,6	nd
S3T + V4I + A228V	1,7	nd	1,5	0,6	nd
A194P + G195E + V205I + A228V	0,8	nd	2,2	nd	nd
H120D + N261D	nd	1,9	nd	nd	nd
N76D + A228V + N261D	0,7	4,5	0,8	1,3	nd

Table f)

Protease variant		A	C	G	L	X	Y	Z
N238E + L262E	Diff	1,2	4,6	0,6	3,9	5,5	1,2	3,1
	HSD	0,9	2,4	2,0	2,6	2,5	3,3	2,5
S156D + L262E	Diff	1,5	5,7	0,8	3,2	5,8	2,6	4,5

Protease variant		A	C	G	L	X	Y	Z
	HSD	0,9	2,4	2,0	2,5	2,5	3,3	2,3
N76D + S163G + N238E +Q137H + S141H + R145H	Diff	1,1	3,7	0,1	2,9	5,1	2,7	1,6
	HSD	0,9	2,4	2,2	2,5	2,5	3,3	2,3
N238E + L262E + Q137H + S141H + R145H	Diff	1,2	4,4	2,5	1,8	4,2	3,0	4,4
	HSD	0,9	2,4	2,0	2,5	2,5	3,5	2,3
S3T + N76D + S156D + Y209W + Q137H + S141H + R145H	Diff	1,1	3,5	1,9	1,3	3,9	3,5	4,0
	HSD	0,9	2,4	2,0	2,5	2,6	3,3	2,3

Example 7: Wash performance of subtilisin variants

[0315] The wash performance of protease variants that have the same amino acid sequence as SEQ ID NO: 3 except for the substitutions as per the table below on the stains as indicated; reference is the protease according to SEQ ID NO: 3. in laundry was assessed using the Automatic Mechanical Stress Assay (AMSA), where the wash performance of many small volume enzyme-detergent solutions can be examined. The AMSA plate has a number of slots for test solutions and a lid that firmly squeezes the textile to be washed against the slot openings. During the wash, the plate, test solutions, textile and lid were vigorously shaken to bring the test solution in contact with the textile and apply mechanical stress in a regular, periodic, oscillating manner. For further description see WO 02/42740 especially the paragraph "Special method embodiments" at pages 23-24.

[0316] The laundry experiments were conducted under the experimental conditions specified in Table 5.

Table 5:

Detergent dosage	2.0 g/L
Test solution volume	160 µL (20 µL enzyme+140 µL detergent)
pH	8.4
Wash time	20 minutes
Temperature	20°C
Water hardness	12°dH

[0317] Model detergent was used as described in Table 1.

Table 6: Composition of model detergents and test materials

Detergent	Laundry liquid model detergent (Table 1)
Test material	C-05 (Blood/milk/ink on cotton)
	PC-03 (Chocolate milk with carbon black on Polyester/cotton,

	65/35)
	CS-37 (Full egg/pigment on cotton)

[0318] Test materials were obtained from Center For Testmaterials BV, 3133 KT Vlaardingen, the Netherlands.

[0319] Water hardness was adjusted to 12°dH by addition of $CaCl_2$, $MgCl_2$, and $NaHCO_3$ (Ca^{2+}:Mg^{2+}= 2:1:4.5) to the test system. After washing, the textiles were flushed in tap water and dried.

[0320] The wash performance was measured as the brightness of the color of the textile washed. Brightness can also be expressed as the intensity of the light reflected from the sample when illuminated with white light. When the sample is stained the intensity of the reflected light is lower than that of a clean sample. Therefore the intensity of the reflected light can be used to measure wash performance.

[0321] Color measurements were made with a Kodak iQsmart flatbed scanner (Kodak, Midtager 29, DK-2605 Brøndby, Denmark), which is used to capture an image of the washed textile.

[0322] To extract a value for the light intensity from the scanned images, 24-bit pixel values from the image were converted into values for red, green and blue (RGB). The intensity value (Int) was calculated by adding the RGB values together as vectors and then taking the length of the resulting vector:

$$Int=\sqrt{r^2+g^2+b^2}$$

[0323] The results are shown in Table 12. The results are given as relative performance compared to SEQ ID NO: 3 at an enzyme concentration of 30 nM on three different swatches.

Table 7: AMSA relative performance of variants compared to SEQ ID NO 3.

Stain	PC-03	C-05	CS-37
Mutations compared to SEQ ID NO: 3			
R45E	1.0	0.9	0.9
R45D	0.8	0.9	0.9
Q59D	1.0	1.0	1.1
S87E	1.1	1.0	1.2
H120V	0.9	0.9	0.9
L124M	1.0	1.0	1.1
P129D	1.1	1.0	0.7
S156D	0.8	0.9	1.0

Stain Mutations compared to SEQ ID NO: 3	PC-03	C-05	CS-37
S163G	1.1	1.2	1.0
S163A	0.9	1.0	1.0
N185Q	1.1	1.1	1.2
Y209W	0.8	0.9	1.0
M222Q	nd	0.9	3.0
V244T	1.0	1.0	1.0
N261T	1.0	1.1	1.1
L262Q	1.0	1.0	1.1
L262E	1.0	1.0	1.4
Q59D + H120D	1.0	1.0	1.4
G61D + N76D	1.0	0.9	1.6
S3T + N76D	1.1	1.0	1.5
S3T + H120D	1.1	0.9	0.9
G61D + H120D	1.0	0.9	0.9
P55S + H120D	1.0	1.1	1.1
S163G + A228V	1.1	1.1	1.1
S163G + N261D	1.0	1.1	1.0
S3T + S163G	1.1	1.1	1.0
G61D + S163G	1.0	1.1	1.1
S156D + S163G	0.9	1.1	1.3
Q59D S163G	1.0	1.1	1.4
N76D S163G	1.0	1.1	0.9
P55S S163G	1.0	1.1	0.9
H120D S163G	1.0	1.1	0.9
T58L Q59D	1.2	1.0	1.6
P55S T58L	1.0	1.0	0.9
T58L G97D	0.9	1.0	1.3
T58L S106A	1.1	1.0	0.9
T58L A228V	1.1	1.0	0.9
S3T T58L	1.0	1.0	1.1
T58L S156D	1.2	1.1	1.5
T58L Y91H	1.1	1.0	1.0
T58L H120D	1.1	1.0	1.0

Stain Mutations compared to SEQ ID NO: 3	PC-03	C-05	CS-37
T58L S163G	1.2	1.0	0.7
S163G N261D	1.0	0.9	1.3
T58L N261D	0.9	1.0	0.9
T58L N76D	1.0	1.0	1.0
S3T N76D H120D	1.0	0.9	1.3
S3T N76D A228V	1.3	0.9	1.2
S3T N76D S156D	0.9	0.9	2.2
S3T N76D Y209W	1.0	1.0	1.1
S3T N76D Y209W V244T	0.8	0.9	1.0

Table 8: Relative performance compared to SEQ ID NO: 3 at an enzyme concentration of 30 nM on two different swatches.

Stain	PC-03	C-05
Mutations compared to SEQ ID NO 3	(Chocolate milk with carbon black on Polyester/cotton)	(Blood/milk/ink on cotton)
S3T	1.0	1.1
P55N	1.1	1.1
T58W	1.0	1.1
T58Y	1.1	1.0
T58W	1.1	1.1
Q59T	1.2	1.1
Q59N	1.1	1.0
Q59M	1.1	1.0
Q59D	1.2	1.0
G61D	1.2	1.0
G97S	1.1	1.1
A98D	1.1	1.0
S106A	1.1	1.1
H120N	1.1	1.1
H120K	1.0	1.1
H120D	1.3	1.1
S161T	1.0	0.9
S163G	1.4	1.1
N238H	1.0	1.1
Y171L	1.0	1.0

[0324] The results show that the stabilized variants of SEQ ID NO 3 show on par or improved wash performance compared to the wash performance of SEQ ID NO 3.

Example 8: Result of Terg-O-tometer (TOM) wash assay

[0325] The TOM wash experiment is conducted under the experimental conditions specified below:

Table 9. TOM wash conditions	
Detergent	Liquid laundry model detergent (Table 1)
Detergent dose	4.66 g/L
pH	pH was measured to be 7.6, but is used "as is" in the current detergent solution and is not adjusted.
Water hardness	16°dH, adjusted by adding $CaCl_2$*$2H_2O$, $MgCl_2$*$6H_2O$ and $NaHCO_3$ (5:1:3) to Milli-Q water.
Enzyme conc.	60 nM
Test solution volume	1000 mL
Test material	2 swatches, each 5x5 cm, of each of the 5 soiled textile types per beaker (i.e. 10 soiled swatches per beaker):
	PC-03 (Chocolate-milk/ink on cotton/polyester)
	C-H010 (Cocoa, cooked up with milk on cotton)
	C-05 (Blood/milk/soot on cotton)
	CS-01 (Aged blood on cotton)
	CS-37 (Full egg/pigment on cotton)
	Cotton ballast swatches (50%:50% WFK10A (Standard Cotton Fabric, Unsoiled):WFK80A (cotton), 5x5 cm (wfk Testgewebe GmbH, Christenfeld 10; D-41379 Brijggen-Bracht; Germany) added to give a total weight of 30 g of soiled textile swatches + ballast per TOM beaker.
Temperature	20°C
Wash time	30 min
Rinse time	5 min
Rotation	120 rpm

Table 10: Relative performance compared to SEQ ID NO: 3 of subtilase variants of having the indicated mutations compared to SEQ ID NO 3.

Delta remission	PC-03	C-H010	C-05	CS-01	CS-37
R45E	1.4	1.0	1.2	1.9	1.1
R45D	1.2	1.0	1.0	0.7	0.8
N117E	1.2	1.2	1.0	1.2	1.3
M222Q	0.7	0.9	1.1	0.9	1.4
L262E	1.3	1.1	1.1	1.2	1.9
P129D	1.3	0.8	1.1	0.8	1.3
S87E	1.2	1.1	1.2	2.2	1.0
Q59D H120D	1.5	1.0	1.0	0.9	1.1
N76D H120D	1.3	2.1	1.0	1.3	1.6
N76D S156D	1.3	1.0	1.0	2.9	2.1
H120D S156D	1.3	0.9	1.0	2.9	1.6
G61D N76D	1.1	1.0	1.1	1.1	2.3
R45E L262E	1.4	2.2	1.0	1.5	1.2
Q59D G61D	1.5	0.7	1.1	0.8	1.7
S87E L262E	1.2	2.7	1.1	0.7	1.2
G61D L262E	1.4	2.3	1.0	0.9	1.3
Q59D L262E	1.4	1.8	1.0	0.8	1.5
R45E Q59D	1.3	1.1	0.9	1.0	1.4
Q59D S156D	1.9	0.9	1.0	1.1	1.7
S156D L262E	1.4	1.3	1.1	1.1	1.2
S163G N238E L262E	1.5	1.8	0.9	0.9	1.4
S3T V4I S163G N261D	1.6	1.5	1.1	1.3	1.1
H120D S163G N261D	1.5	1.4	1.0	0.9	1.2
Y91H N117H N238H	1.3	1.3	1.0	0.9	1.8
S3T N76D H120D	1.3	0.9	1.0	2.5	1.4
S3T N76D S156D	1.2	1.2	1.0	1.0	1.0
T58L S163G N261D	1.6	1.4	1.1	0.9	1.3
S3T V41 S163G N261D	1.6	1.1	1.0	0.9	1.1
S87E S163G L262E	1.6	1.0	1.1	0.6	1.2
S156D S163G L262E	1.6	2.3	1.2	1.0	1.5
T58LS163G N261D	1.5	2.5	1.2	0.8	1.1
S156D S163G L262E	1.7	1.4	1.1	0.9	1.4
S3T N76D Y209W N261D L262E	1.5	1.4	1.0	1.2	1.5

Example 9: Wash performance of protease variants

[0326] Variants having the mutations as indicated in Table 15 compared to SEQ ID NO: 3 was washed under different conditions, AMSA wash with low concentration of protease (30 nM), AMSA wash with high concentration of protease (300 nM), TOM wash and full scale wash (FSW). The detergent disclosed in Table 1 and the PC-03 (Chocolate-milk/ink on cotton/polyester) test materials were used for the washes. The results were compared with the performance of the reference protease having SEQ ID NO: 3 and the performance shown in the table 15 below where the result for the reference protease is set to 1.00.

Table 11:

Mutations compared to SEQ ID NO: 3	AMSA (30 nM)	AMSA (300 nM)	TOM	FSW
N62D+H120D			1.44	1.39
H120D+N261D			1.52	1.38
N76D+N261D			1.34	
N76D+A228V+N261D			1.46	1.54
A194P+G195E+V205I+N261D			1.41	1.25
R45E	1.06		1.43	1.43
N76D+H120D+N261D			1.13	1.18
H120D+S163G+N261D	1.03		1.63	1.49
S3T+Q59D+N76D			1.15	
S3T+N76D+H120D	1.1		1.31	
S3T+N76D+A194P+G195E+V199M+V205I			1.43	1.53
S3T+N76D+S156D	1.02		1.23	
S3T+N76D+Y209W+N261D			1.33	1.27
S3T+N76D+H120D+Y209W			1.27	
S3T+N76D+S156D+Y209W			1.31	1.38
S3T+V4I+N76D+A228V+N261D			1.34	
S3T+V4I+N76D+H120D			1.32	
H120D+P131F+A194P+N261D			1.45	
N76D+E136H+A228V+N261D			1.12	
N76D+N218S+A228V+N261D	1.01			
N76D+N218Q+A228V+N261D	1.01		1.45	1.46
N76D+N218A+A228V+N261D	1.07		1.47	1.49
K27Q+R45E			1.32	
N76D+A228V+L262E			1.77	1.38

Mutations compared to SEQ ID NO: 3	AMSA (30 nM)	AMSA (300 nM)	TOM	FSW
R45E+A88S	1.06		1.58	1.26
S87E+K237E			1.24	
N261D+L262E			1.41	
S87E+L262E			1.22	
S87E+N238E			1.32	
K27Q+S87E			1.53	
N76D+N117E			1.34	
H120D+N238E			1.08	
Q59D+L262E			1.26	
K27Q+L262E			1.22	
H120D+L262E			1.28	
K27Q+Q59D		1.13		
K27Q+S156D		1.18		
K27Q+G61D		1.21		
Q59D+N261D		1.22	1.49	
Q59D+N117E		1.19		
K237E+N261D		1.11		
Q59D+N238E			1.4	
A15T+H120D+N261D		1.22		
N76D+S163G+N238E		1.18		
H120D+S163G+L262E		1.15		
H120D+ S163G+ N261D	1.08	1.18		

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PATENTKRAV

1. Detergentsammensætning der omfatter en subtilasevariant, hvilken subtilasevariant har mindst 90 % sekvensidentitet til SEQ ID NO: 3, fortrinsvis mindst 95 % sekvensidentitet, fortrinsvis mindst 96 % sekvensidentitet, fortrinsvis mindst 97 % sekvensidentitet, fortrinsvis mindst 98 % sekvensidentitet til SEQ ID NO: 3, hvilken subtilasevariant har en glutaminsyrerest (E) i position 101, og hvor subtilasevarianten yderligere omfatter substitutionen P129D, hvilken subtilasevariant har forøget stabilitet i en flydende detergentsammensætning sammenlignet med subtilasen med aminosyresekvensen i SEQ ID NO: 3, og hvor positionerne svarer til positionerne i SEQ ID NO: 1.

2. Detergentsammensætningen ifølge krav 1, hvor subtilasevarianten yderligere har forbedret vaskeydelse sammenlignet med subtilasen med SEQ ID NO: 3.

3. Detergentsammensætning ifølge krav 1 eller 2, hvor subtilasevarianten omfatter en eller flere substitutioner, som er valgt fra gruppen der består af: S3T; R45E, D, Q; P55N; T58W, Y, L; Q59D, M, N, T; G61D, R; S87E; G97S; A98D, E, R; S160A, W; N117E; H120D, K, V, N; E136Q; Q137H; S143W; S156D; S161T; S163A, G; Y171L; A172S; N185Q; V199M; Y209W; M222Q; N238H; N261T og L262E,Q,N,D, og hvor positionerne svarer til positionerne i SEQ ID NO: 1.

4. Detergentsammensætning ifølge krav 1 til 3, hvor subtilasevarianten er valgt fra gruppen der består af:
SEQ ID NO: 3 + P129D, og hvor positionerne svarer til positionerne i SEQ ID NO: 1.

5. Detergentsammensætning ifølge et hvilket som helst af kravene 1-4, hvilken sammensætning er i form af en bar, en homogen tablet, en tablet med to eller flere lag, en pose med et eller flere rum, et almindeligt eller kompakt pulver, et granulat, en pasta, en gel eller en almindelig, kompakt eller koncentreret væske.

6. Detergentsammensætning ifølge et hvilket som helst af kravene 1-5, hvilken sammensætning yderligere omfatter en eller flere yderligere enzymer, som er valgt blandt: protease, lipase, cutinase, amylase, carbohydrase, cellulase, pectinase, mannanase, arabinase, galactanase, xylanase, oxidase, xanthanase, laccase og/eller peroxidase.

7. Detergentsammensætning ifølge et hvilket som helst af kravene 1-6, hvilken sammensætning er en vaskemiddelsammensætning eller en opvaskemiddelsammensætning, fortrinsvis en sammensætning til maskinopvask.

8. Anvendelse af en detergentsammensætning ifølge et hvilket som helst af kravene 1-7 i en rengøringsproces.

9. Anvendelsen ifølge krav 8, hvor rengøringsprocessen er vask af tøj.

10. Anvendelsen ifølge krav 9, hvor rengøringsprocessen er rengøring af hård overflade, såsom opvask.

11. Fremgangsmåde til fjernelse af en plet fra en overflade, hvilken fremgangsmåde omfatter at bringe overfladen i kontakt med en sammensætning ifølge et hvilket som helst af kravene 1-7.

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

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