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(54) Title: POLYPEPTIDES HAVING PROTEASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

(57) Abstract: The present invention relates to isolated polypeptides having protease activity, and polynucleotides encoding the polypeptides. The invention further relates to the use of such polypeptides in detergent and/or in cleaning processes. The invention also relates to nucleic acid 5 constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing the polypeptides.

POLYPEPTIDES HAVING PROTEASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

Reference to a Sequence Listing

5 This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

Field of the Invention

10 The present invention relates to the use of polypeptides having protease activity and polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods of producing the polypeptides. The present invention particularly relates to the use of polypeptides having protease activity in food application and in detergents.

15 Background of Invention

Enzymes have been used for many decades in the cleaning compositions such as detergents for various purposes such as laundry and dish wash in house hold care and industrial cleaning. A mixture of different enzymes are used each performing its specific activity to specific substances constituting soil from various stains. Proteases are enzymes which degrade proteins and can be used in cleaning processes such as dish wash and laundry to remove the proteinaceous stains. The most commonly used proteases are the serine proteases in particular subtilases. This family has previously been further grouped into 6 different sub-groups by Siezen RJ and Leunissen JAM, 1997, Protein Science, 6, 501-523. One of these sub-groups is the subtilisin family which includes subtilases such as Savinase®, Alcalase® (Novozymes A/S) and BLAP® (Henkel AG). Over the years the subtilisins and other proteases has been genetically engineered to increase their performance. Typically the proteases are designed to fulfil different purposes such as to increase their wash performance e.g. at low temperature conditions and/or increase their capacity to remove certain stains. Commercially known genetically engineered proteases includes Release®, Polarzyme®, Kannase®, Liquanase®, Ovozyme®, Coronase®, 25 Blaze® (Novozymes A/S), Properase®, Purafect Prime®, Purafect Ox®, FN3®, FN4®, Excellase® and Ultimase® (Danisco/DuPont). Despite the availability of many optimized proteases designed for various purposes the compositions of the soiling and stains are very complex and the wash conditions and detergent composition changes to meet different user needs. All factors which makes the availability of different types of proteases for use in cleaning and detergents 35 advantageous.

Summary of the Invention

The present invention relates to isolated bacillus polypeptides having protease activity, selected from the group consisting of:

- 5 (a) a polypeptide having at least 98% sequence identity to the mature polypeptide of SEQ ID NO: 2;
- (b) a polypeptide encoded by a polynucleotide having at least 98% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
- 10 (c) a variant of the mature polypeptide of SEQ ID NO: 2 comprising a substitution, deletion, and/or insertion at one or more (e.g. several) positions; and
- (d) a fragment of the polypeptide of (a), (b) or (c) that has protease activity.

The present invention also relates to isolated polynucleotides encoding the polypeptides of the present invention; nucleic acid constructs; recombinant expression vectors; recombinant host cells comprising the polynucleotides; and methods of producing the polypeptides.

- 15 The present invention also relates to detergent compositions, the use of the proteases of the invention in cleaning and in detergents, the use of detergent compositions comprising a protease of the invention, to methods of doing cleaning and to stains removal processes.

- 20 The present invention also relates to nucleic acid constructs, expression vectors, and recombinant host cells comprising the polynucleotides; and methods of producing a protein of the invention.

Overview of Sequence Listing

SEQ ID NO: 1 is the DNA sequence of *Bacillus sp* protease

SEQ ID NO: 2 is the amino acid sequence as deduced from SEQ ID NO: 1

- 25 SEQ ID NO: 3 is the amino acid sequence of the mature *Bacillus sp* protease

SEQ ID NO: 4 is the amino acid sequence of the TY-145 protease (WO2004/067737, SEQ ID NO: 1)

SEQ ID NO: 5 is the amino acid sequence of *Bacillus lentus* protease

SEQ ID NO: 6 forward primer

- 30 SEQ ID NO: 7 reverse primer

SEQ ID NO: 8 is the amino acid sequence of a *Bacillus clausii* secretion signal

Definitions

- 35 "Polypeptides having protease activity", or proteases, are sometimes also designated peptidases, proteinases, peptide hydrolases, or proteolytic enzymes. Proteases may be of the exo-

type that hydrolyses peptides starting at either end thereof, or of the endo-type that act internally in polypeptide chains (endopeptidases). Endopeptidases show activity on N- and C-terminally blocked peptide substrates that are relevant for the specificity of the protease in question.

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. The term "subtilases" refer 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 or serine peptidases is a subgroup of proteases characterised by having a serine in the active site, which forms a covalent adduct with the substrate. Further the subtilases (and the serine proteases) are characterised by having two active site amino acid residues apart from the serine, namely a histidine and an aspartic acid residue. 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. The term "protease activity" means a proteolytic activity (EC 3.4). Proteases of the invention are endopeptidases (EC 3.4.21). For purposes of the present invention, protease activity is determined according to the procedure described in the Examples.

The term "protease activity" means a proteolytic activity (EC 3.4.21.) that catalyzes the hydrolysis of amide bond or a protein by hydrolysis of the peptide bond that link amino acids together in a polypeptide chain. Several assays for determining protease activity are available in the art. For purposes of the present invention, protease activity may be determined using Suc-AAPF-pNA assay as described in the Examples of the present application.

The term "isolated polypeptide" as used herein refers to a polypeptide that is isolated from a source. In one aspect, the polypeptide is at least 20% pure, more preferably at least 40% pure, more preferably at least 60% pure, even more preferably at least 80% pure, most preferably at least 90% pure and even most preferably at least 95% pure, as determined by SDS-PAGE. The term "pure" as used herein, refers to the degree of purity of polypeptide in a sample, composition or the like. Thus, such as at least 95% pure means that no more than 5% of the sample, composition or the like consists of impurities. It is within the knowledge of the skilled person to determine the purity of an isolated polypeptide.

The term "substantially pure polypeptide" denotes herein a polypeptide preparation that contains at most 10%, preferably at most 8%, more preferably at most 6%, more preferably at most 5%, more preferably at most 4%, more preferably at most 3%, even more preferably at most

2%, most preferably at most 1%, and even most preferably at most 0.5% by weight of other polypeptide material with which it is natively or recombinantly associated. It is, therefore, preferred that the substantially pure polypeptide is at least 92% pure, preferably at least 94% pure, more preferably at least 95% pure, more preferably at least 96% pure, more preferably at least 97% pure, more preferably at least 98% pure, even more preferably at least 99%, most preferably at least 99.5% pure, and even most preferably 100% pure by weight of the total polypeptide material present in the preparation. The polypeptides of the present invention are preferably in a substantially pure form. This can be accomplished, for example, by preparing the polypeptide by well-known recombinant methods or by classical purification methods.

10 The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having protease activity. In one aspect the mature polypeptide is a polypeptide with SEQ ID NO: 3. In another aspect, the mature polypeptide is encoded by nucleotide 319 to 1254 of SEQ ID NO: 1 and amino acid 1 to 312 of SEQ ID NO: 2.

15 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 degree of 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 in Genetics* 16: 276-277; <http://emboss.org>), preferably version 20 3.0.0 or later. Version 6.1.0 was used. The optional 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:

$$\text{(Identical Residues x 100)} / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment}).$$

25 For purposes of the present invention, the degree of 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*; <http://emboss.org>), preferably version 3.0.0 or later. Version 6.1.0 was used. The optional 30 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 labelled "longest identity" (obtained using the `-nobrief` option) is used as the percent identity and is calculated as follows:

$$\text{(Identical Deoxyribonucleotides x 100)} / (\text{Length of Alignment} - \text{Total Number of Gaps in$$

Alignment).

The term “fragment” means a polypeptide having one or more (i.e. several) amino acids deleted from the amino and/or carboxyl terminus of a mature polypeptide, wherein the fragment has protease activity.

5 The term “functional fragment of a polypeptide” or “functional fragment thereof” is used to describe a polypeptide which is derived from a longer polypeptide, e.g., a mature polypeptide, and which has been truncated either in the N-terminal region or the C-terminal region or in both regions to generate a fragment of the parent polypeptide. To be a functional polypeptide the fragment must maintain at least 20%, preferably at least 40%, more preferably at least 50%, more preferably at
10 least 60%, more preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95%, and even most preferably at least 100% of the protease activity of the full-length/mature polypeptide.

The term “subsequence” means a polynucleotide having one or more (several) nucleotides deleted from the 5' and/or 3' end of a mature polypeptide coding sequence, wherein the
15 subsequence encodes a fragment having protease activity.

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
20 of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

The term “variant” means a polypeptide having protease activity comprising an alteration, i.e., a substitution, insertion, and/or deletion of one or more (i.e. several) amino acid residues at one or more (several) positions. A substitution means a replacement of an amino acid occupying a position with a different amino acid; a deletion means removal of an amino acid occupying a
25 position; and an insertion means adding 1-3 amino acids adjacent to an amino acid occupying a position.

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 letters amino acid abbreviations are employed. Amino acid positions are indicated with #₁, #₂, etc.

30 The term “substitution” as used herein refers to an amino acid substitution, wherein the following nomenclature is used: Original amino acid, position, substituted amino acid. Accordingly, the substitution of threonine at position #₁ with alanine is designated as “Thr#₁Ala” or “T#₁A”. Multiple mutations are separated by addition marks (“+”), e.g., “Gly2#₂Arg + Ser#₅Phe” or “G#₂R +

S#₅F”, representing substitutions at positions #₂ and #₅ of glycine (G) with arginine (R) and serine (S) with phenylalanine (F), respectively.

The term “deletion” as used herein, refers to an amino acid deletion, wherein the following nomenclature is used: Original amino acid, position, *. Accordingly, the deletion of glycine at position #₂ is designated as “Gly#₂*” or “G#₂*”. Multiple deletions are separated by addition marks (“+”), e.g., “Gly#₂* + Ser#₃*” or “G#₂* + S#₃*”.

The term “insertion” as used herein, refers to an amino acid insertion, wherein the following nomenclature is used: Original amino acid, position, original amino acid, inserted amino acid. Accordingly the insertion of lysine after glycine at position #₂ is designated “Gly#₂GlyLys” or “G#₂GK”. An insertion of multiple amino acids is designated [Original amino acid, position, original amino acid, inserted amino acid #1, inserted amino acid #2; etc.]. For example, the insertion of lysine and alanine after glycine at position #₂ is indicated as “Gly#₂GlyLysAla” or “G#₂GKA”.

In such cases, the inserted amino acid residue(s) are 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 the above example, the sequence would thus be:

| Parent: | Variant: |
|----------------|--|
| # ₂ | # ₂ # _{2a} # _{2b} |
| G | G - K - A |

If multiple alteration is present, the variants comprising such multiple alterations are separated by addition marks (“+”), e.g., “Arg#₃Tyr+Gly#₄Glu” or “R#₃Y+G#₄E” representing a substitution of arginine and glycine at positions #₃ and #₄ with tyrosine and glutamic acid, respectively.

The terms “cleaning compositions” and “cleaning formulations,” refer to compositions that find use in the removal of undesired compounds from items to be cleaned, such as fabric, carpets, dishware including glassware, contact lenses, hard surfaces such as tiles, zincs, floors, and table surfaces, hair (shampoos), skin (soaps and creams), teeth (mouthwashes, toothpastes), etc. The terms encompasses any materials/compounds selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid, gel, granule, powder, or spray compositions), as long as the composition is compatible with the protease and other enzyme(s) used in the composition. The specific selection of cleaning composition materials is readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use. These terms further refer to any composition that is suited for cleaning, bleaching, disinfecting, and/or sterilizing any object and/or surface. It is intended that

the terms include, but are not limited to detergent composition (e.g., liquid and/or solid laundry detergents and fine fabric detergents; hard surface cleaning formulations, such as for glass, wood, ceramic and metal counter tops and windows; carpet cleaners; oven cleaners; fabric fresheners; fabric softeners; and textile and laundry pre-spotters, as well as dish detergents).

5 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
10 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.

15 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
20 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 protease 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,
25 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.

 The term "fabric" encompasses any textile material. Thus, it is intended that the term encompass garments, as well as fabrics, yarns, fibers, non-woven materials, natural materials,
30 synthetic materials, and any other textile material.

 The term "textile" refers to woven fabrics, as well as staple fibers and filaments suitable for conversion to or use as yarns, woven, knit, and non-woven fabrics. The term encompasses yarns made from natural, as well as synthetic (e.g., manufactured) fibers. The term, "textile materials" is a general term for fibers, yarn intermediates, yarn, fabrics, and products made from fabrics (e.g.,
35 garments and other articles).

The term "non-fabric detergent compositions" include non-textile surface detergent compositions, including but not limited to compositions for hard surface cleaning, such as dishwashing detergent compositions, oral detergent compositions, denture detergent compositions, and personal cleansing compositions.

5 The term "effective amount of enzyme" refers to the quantity of enzyme necessary to achieve the enzymatic activity required in the specific application, e.g., in a defined detergent composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and are based on many factors, such as the particular enzyme used, the cleaning application, the specific composition of the detergent composition, and whether a liquid or dry (e.g., granular, bar)
10 composition is required, and the like. The term "effective amount" of a protease refers to the quantity of protease described hereinbefore that achieves a desired level of enzymatic activity, e.g., in a defined detergent composition.

The term "water hardness" or "degree of hardness" or "dH" or "°dH" as used herein refers to German degrees of hardness. One degree is defined as 10 milligrams of calcium oxide per liter of
15 water.

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

The term "adjunct materials" means any liquid, solid or gaseous material selected for the particular type of detergent composition desired and the form of the product (e.g., liquid, granule,
20 powder, bar, paste, spray, tablet, gel, or foam composition), which materials are also preferably compatible with the protease used in the composition. In some embodiments, granular compositions are in "compact" form, while in other embodiments, the liquid compositions are in a "concentrated" form.

25 The term "stain removing enzyme" as used herein, describes an enzyme that aids the removal of a stain or soil from a fabric or a hard surface. Stain removing enzymes act on specific substrates, e.g., protease on protein, amylase on starch, lipase and cutinase on lipids (fats and oils), pectinase on pectin and hemicellulases on hemicellulose. Stains are often depositions of complex mixtures of different components which either results in a local discolouration of the
30 material by itself or which leaves a sticky surface on the object which may attract soils dissolved in the washing liquor thereby resulting in discolouration of the stained area. When an enzyme acts on its specific substrate present in a stain the enzyme degrades or partially degrades its substrate thereby aiding the removal of soils and stain components associated with the substrate during the washing process. For example, when a protease acts on a grass stain it degrades the protein
35 components in the grass and allows the green/brown colour to be released during washing.

The term "reduced amount" means in this context that the amount of the component is smaller than the amount which would be used in a reference process under otherwise the same conditions. In a preferred embodiment the amount is reduced by, e.g., at least 5%, such as at least 10%, at least 15%, at least 20% or as otherwise herein described.

5 The term "low detergent concentration" system includes detergents where less than about 800 ppm of detergent components is present in the wash water. Asian, e.g., Japanese detergents are typically considered low detergent concentration systems.

The term "medium detergent concentration" system includes detergents wherein between about 800 ppm and about 2000 ppm of detergent components is present in the wash water. North
10 American detergents are generally considered to be medium detergent concentration systems.

The term "high detergent concentration" system includes detergents wherein greater than about 2000 ppm of detergent components is present in the wash water. European detergents are generally considered to be high detergent concentration systems.

15 Detailed Description of the Invention

In one aspect, the present invention relates to an isolated polypeptide having protease activity, selected from the group consisting of: (a) a polypeptide having at least 98% sequence identity to the mature polypeptide of SEQ ID NO: 2; (b) a polypeptide encoded by a polynucleotide having at least 98% sequence identity to the mature polypeptide coding sequence of SEQ ID NO:
20 1; (c) a variant of the mature polypeptide of SEQ ID NO: 2 comprising a substitution, deletion, and/or insertion at one or more (e.g. several) positions; and (d) a fragment of the polypeptide of (a), (b) or (c) that has protease activity.

In an embodiment, the present invention relates to an isolated polypeptide having a sequence identity to SEQ ID NO: 3 of at least 98%, at least 99%, or 100%, which have protease
25 activity. In one aspect, the polypeptide differ by no more than 20 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 from the polypeptide with SEQ ID NO: 3. Thus, in one embodiment, the isolated polypeptide has a substitution in one or more positions corresponding to positions S173 and S175 of SEQ ID NO: 4. In a particular embodiment, the substitution in the position corresponding to position S173 of SEQ ID NO: 3 is S173P or S173Y. In
30 another particular embodiment, the polypeptide comprises two substitutions in the positions corresponding to positions S173 and S175 of SEQ ID NO: 4, wherein the substitutions are S173P+S175P.

In another embodiment, the number of amino acid substitutions, deletions, and/or insertions introduced into the mature polypeptide of SEQ ID NO:3, is not more than 40, e.g. 1, 2, 3, 4, 5, 6, 7,
35 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34,

35, 36, 37, 38, or 39.

A polypeptide of the present invention preferably comprises or consists of the amino acid sequence of SEQ ID NO: 3 or an allelic variant thereof; or is a fragment thereof having protease activity. In another aspect, the polypeptide comprises or consists of the mature polypeptide of SEQ ID NO: 2. In another aspect, the polypeptide comprises or consists of amino acids 1 to 312 of SEQ ID NO: 2

In another embodiment, the present invention relates to a polypeptide having a sequence identity to SEQ ID NO: 3 of at least 98%, at least 99%, or 100%, which have protease activity. In one aspect, the polypeptide differ by no more than 20 amino acids, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or 19 from the polypeptide with SEQ ID NO: 3. In another embodiment, the number of amino acid substitutions, deletions, and/or insertions introduced into the polypeptide with SEQ ID NO:3, is not more than 40, e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39.

In one embodiment, the protease is a variant of a protease with SEQ ID NO 3 or a protease having at least 70% sequence identity to SEQ ID NO 3, wherein the variant comprises one or more of the mutations selected from the list consisting of P8L, P8D, P8K, G25L, G25C, G25W, G25R, K30E, N39D, T40R, L82H, L82G, L82F, L82I, L82V, K123P, S144L, S144I, S144F, S144V, S145R, V163W, V163R, D172N, D172K, D172E, D172W, S173P, S173Y, P174H, S175P, A175S, A175K, P174*+ A175*, D176P, D176P, G180I, G180Y, G180M, D207P, D207P, Y241P, D242G, Q287G, Q287H, Q287T, Y302R, I290C, T294P, Y302I and Y302M.

In another embodiment, the present invention relates to an isolated polypeptide having protease activity encoded by a polynucleotide that hybridizes under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the mature polypeptide coding sequence of SEQ ID NO: 1 or the full-length complement thereof (Sambrook *et al.*, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotides of SEQ ID NO: 1 or a subsequence thereof, as well as the polypeptide with SEQ ID NO: 3 or a fragment thereof may be used to design nucleic acid probes to identify and clone DNA encoding polypeptides having protease activity 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 may 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 ³²P, ³H, ³⁵S, biotin, or avidin). Such probes are encompassed by the present invention.

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 polypeptide having protease activity. 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. In order to identify a clone or DNA that hybridizes with SEQ ID NO: 1 or a subsequence thereof, the carrier material is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding to (i) SEQ ID NO: 1; (ii) the mature polynucleotide coding sequence of SEQ ID NO: 1; (iii) the full-length complement thereof; or (iv) a subsequence thereof; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film or any other detection means known in the art.

Thus, in one aspect, the nucleic acid probe is nucleotides 1 to 1254, nucleotides 200 to 1100, nucleotides 400 to 900, or nucleotides 600 to 800 of SEQ ID NO: 1. In another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2; the mature polypeptide thereof; or a fragment thereof. In another aspect, the nucleic acid probe is SEQ ID NO: 1.

In one embodiment, the present invention relates to an isolated polypeptide having protease activity encoded by a polynucleotide having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least 98%, at least 99%, or 100%.

In one embodiment, the present invention relates to variants of the mature polypeptides of SEQ ID NO: 2 comprising a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions. In an embodiment, the number of amino acid substitutions, deletions and/or insertions introduced into the mature polypeptides of SEQ ID NO: 2 is not more than 20, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or 19. 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.

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.

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.

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 (Cunningham and Wells, 1989, *Science* 244: 1081-1085). 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. See also, Hilton *et al.*, 1996, *J. Biol. Chem.* 271: 4699-4708. 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. See, for example, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *J. Mol. Biol.* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Lett.* 309: 59-64. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide. The identity of essential amino acids can also be inferred from an alignment with a related polypeptide. For the polypeptide of the invention the catalytic triad comprising the amino acids D38, H75 and S254 is essential for protease activity of the enzyme.

In an embodiment, the variant has improved catalytic activity compared to the parent enzyme.

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, such as those disclosed by Reidhaar-Olson and Sauer, 1988, *Science* 241: 53-57; Bowie and Sauer, 1989, *Proc. Natl. Acad. Sci. USA* 86: 2152-2156;

WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone PCR, phage display (e.g., Lowman *et al.*, 1991, *Biochemistry* 30: 10832-10837; U.S. Patent No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire *et al.*, 1986, *Gene* 46: 145; Ner *et al.*, 1988, *DNA* 7: 127).

5 Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness *et al.*, 1999, *Nature Biotechnology* 17: 893-896). 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
10 amino acid residues in a polypeptide.

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.

The polypeptide 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
15 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
20 using intein technology in which fusion polypeptides are created post-translationally (Cooper *et al.*, 1993, *EMBO J.* 12: 2575-2583; Dawson *et al.*, 1994, *Science* 266: 776-779).

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. Examples of cleavage sites include, but are not limited to, the sites disclosed in Martin *et al.*, 2003, *J. Ind.*
25 *Microbiol. Biotechnol.* 3: 568-576; Svetina *et al.*, 2000, *J. Biotechnol.* 76: 245-251; Rasmussen-Wilson *et al.*, 1997, *Appl. Environ. Microbiol.* 63: 3488-3493; Ward *et al.*, 1995, *Biotechnology* 13: 498-503; and Contreras *et al.*, 1991, *Biotechnology* 9: 378-381; Eaton *et al.*, 1986, *Biochemistry* 25: 505-512; Collins-Racie *et al.*, 1995, *Biotechnology* 13: 982-987; Carter *et al.*, 1989, *Proteins: Structure, Function, and Genetics* 6: 240-248; and Stevens, 2003, *Drug Discovery World* 4: 35-48.

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Sources of Polypeptides Having protease Activity

Polypeptides having protease activity of the present invention 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 polypeptide encoded by a
35 polynucleotide is produced by the source or by a strain in which the polynucleotide from the source

has been inserted. In one aspect, the polypeptide obtained from a given source is secreted extracellularly.

The polypeptides may be a bacterial protease. For example, the polypeptides may be a Gram-positive bacterial polypeptide such as a *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*,
5 *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.

In one embodiment, the polypeptide is a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*,
10 *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

In one embodiment, the polypeptide is a *Bacillus* protease, in another embodiment the protease is a *Bacillus sp.* In a specific embodiment the polypeptide is a protease with SEQ ID NO:
15 3 or the mature polypeptide of SEQ ID NO: 2.

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
20 (NRRL).

The parent polypeptide 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
25 the art. A polynucleotide encoding a parent polypeptide 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 polypeptide 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 (see, e.g., Sambrook *et al.*, 1989, *supra*).

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Polynucleotides

The present invention also relates to isolated polynucleotides encoding a polypeptide or a catalytic domain of the present invention, as described herein. Thus, in one aspect, the present invention relates to a polynucleotide having at least 97% sequence identity to the mature
35 polynucleotide coding sequence of SEQ ID NO: 1. In one embodiment, the polynucleotide of the

invention encodes a mature polypeptide having at least 97% sequence identity to the mature polypeptide of SEQ ID NO: 2.

The techniques used to isolate or clone a polynucleotide are known in the art and include isolation from genomic DNA or cDNA, or a combination thereof. The cloning of the polynucleotides from genomic DNA can be effected, *e.g.*, by using the well-known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, *e.g.*, Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligation activated transcription (LAT) and polynucleotide-based amplification (NASBA) may be used. The polynucleotides may be cloned from a strain of *bacillus* or a related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the polynucleotide.

Modification of a polynucleotide encoding a polypeptide of the present invention may be necessary for synthesizing polypeptides substantially similar to the polypeptide. The term “substantially similar” to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, *e.g.*, variants that differ in specific activity, thermostability, pH optimum, or the like. The variants may be constructed on the basis of the polynucleotide presented as the mature polypeptide coding sequence of SEQ ID NO: 1, *e.g.*, a subsequence thereof, and/or by introduction of nucleotide substitutions that do not result in a change in the amino acid sequence of the polypeptide, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions that may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, *e.g.*, Ford *et al.*, 1991, *Protein Expression and Purification 2*: 95-107.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a polynucleotide 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. Thus, in one embodiment, the nucleic acid construct comprises a polynucleotide sequence having at least 97% sequence identity to the mature polynucleotide coding sequence of SEQ ID NO: 1, and wherein the polynucleotide is 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.

A polynucleotide may be manipulated in a variety of ways to provide for expression of the

polypeptide. 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.

The control sequence may be a promoter, a polynucleotide that is recognized by a host cell for expression of a polynucleotide encoding a polypeptide of the present invention. The promoter contains transcriptional control sequences that mediate the expression of the polypeptide. 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.

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 (Agaïsse and Lereclus, 1994, *Molecular Microbiology* 13: 97-107), *E. coli lac* operon, *E. coli trc* promoter (Egon *et al.*, 1988, *Gene* 69: 301-315), *Streptomyces coelicolor* agarase gene (*dagA*), and prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proc. Natl. Acad. Sci. USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert *et al.*, 1980, *Scientific American* 242: 74-94; and in Sambrook *et al.*, 1989, *supra*. Examples of tandem promoters are disclosed in WO 99/43835.

Examples of suitable promoters for directing transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus nidulans* acetamidase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Aspergillus oryzae* TAKA amylase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (WO 96/00787), *Fusarium venenatum* amyloglucosidase (WO 00/56900), *Fusarium venenatum* Daria (WO 00/56900), *Fusarium venenatum* Quinn (WO 00/56900), *Rhizomucor miehei* lipase, *Rhizomucor miehei* aspartic proteinase, *Trichoderma reesei* beta-glucosidase, *Trichoderma reesei* cellobiohydrolase I, *Trichoderma reesei* cellobiohydrolase II, *Trichoderma reesei* endoglucanase I, *Trichoderma reesei* endoglucanase II, *Trichoderma reesei* endoglucanase III, *Trichoderma reesei* endoglucanase IV, *Trichoderma reesei* endoglucanase V, *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma reesei* beta-xylosidase, as well as the NA2-tpi promoter (a modified promoter from an *Aspergillus* neutral alpha-amylase gene in which the untranslated leader has

been replaced by an untranslated leader from an *Aspergillus* triose phosphate isomerase gene; non-limiting examples include modified promoters from an *Aspergillus niger* neutral alpha-amylase gene in which the untranslated leader has been replaced by an untranslated leader from an *Aspergillus nidulans* or *Aspergillus oryzae* triose phosphate isomerase gene); and mutant, truncated, and hybrid promoters thereof.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH1, ADH2/GAP), *Saccharomyces cerevisiae* triose phosphate isomerase (TPI), *Saccharomyces cerevisiae* metallothionein (CUP1), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488.

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

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 (*rmB*).

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase, *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

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.

Examples of suitable mRNA stabilizer regions are obtained from a *Bacillus thuringiensis cryIIIA* gene (WO 94/25612) and a *Bacillus subtilis* SP82 gene (Hue *et al.*, 1995, *Journal of Bacteriology* 177: 3465-3471).

The control sequence may also be a leader, a nontranslated region of an mRNA that is important for translation by the host cell. The leader is operably linked to the 5'-terminus of the polynucleotide encoding the polypeptide. Any leader that is functional in the host cell may be used.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, 5 *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence; a sequence operably linked to the 3'-terminus of the polynucleotide and, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence 10 that is functional in the host cell may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

15 Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Mol. Cellular Biol.* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that encodes a signal peptide linked to the N-terminus of a polypeptide and directs the polypeptide into the cell's secretory pathway. The 5'-end of the coding sequence of the polynucleotide may inherently 20 contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the polypeptide. 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 25 coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell may be used.

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* 30 *licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding sequences for filamentous fungal host cells are the signal 35 peptide coding sequences obtained from the genes for *Aspergillus niger* neutral amylase,

Aspergillus niger glucoamylase, *Aspergillus oryzae* TAKA amylase, *Humicola insolens* cellulase, *Humicola insolens* endoglucanase V, *Humicola lanuginosa* lipase, and *Rhizomucor miehei* aspartic proteinase.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding sequences are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding sequence that encodes a propeptide positioned at the N-terminus of a polypeptide. 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 (WO 95/33836), *Rhizomucor miehei* aspartic proteinase, and *Saccharomyces cerevisiae* alpha-factor.

Where both signal peptide and propeptide sequences are present, the propeptide sequence is positioned next to the N-terminus of a polypeptide and the signal peptide sequence is positioned next to the N-terminus of the propeptide sequence.

It may also be desirable to add regulatory sequences that regulate expression of the polypeptide 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. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the *Aspergillus niger* glucoamylase promoter, *Aspergillus oryzae* TAKA alpha-amylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used. Other examples of regulatory sequences are those that allow for gene amplification. In eukaryotic systems, these regulatory sequences include the dihydrofolate reductase gene that is amplified in the presence of methotrexate, and the metallothionein genes that are amplified with heavy metals. In these cases, the polynucleotide encoding the polypeptide would be operably linked with the regulatory sequence.

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Expression Vectors

The present invention also relates to recombinant expression vectors comprising a polynucleotide of the present invention, a promoter, and transcriptional and translational stop signals. Thus, in one embodiment, the recombinant expression vector comprises a polynucleotide sequence having at least 97% sequence identity to the mature polynucleotide coding sequence of

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SEQ ID NO:1, 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 polypeptide at such sites.

5 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.

10 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.

15 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
20 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.

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
25 auxotrophs, and the like.

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. Suitable markers for yeast host cells include, but are not limited to, ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for
30 use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are *Aspergillus nidulans* or *Aspergillus oryzae* *amdS* and
35 *pyrG* genes and a *Streptomyces hygrosopicus* *bar* gene.

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.

For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the polypeptide 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.

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*.

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 pAMB1 permitting replication in *Bacillus*.

Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6.

Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991, *Gene* 98: 61-67; Cullen *et al.*, 1987, *Nucleic Acids Res.* 15: 9163-9175; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

More than one copy of a polynucleotide of the present invention may be inserted into a host cell to increase production of a polypeptide. 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.

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 (see, e.g.,
5 Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant host cells, comprising a polynucleotide of the present invention operably linked to one or more control sequences that direct the production
10 of a polypeptide of the present invention. Thus, in one embodiment, the recombinant host cell comprises a polynucleotide sequence having at least 97% sequence identity to the mature polynucleotide coding sequence of SEQ ID NO:1 linked to one or more control sequences that direct the production of a polypeptide having at least 97% sequence identity to the mature polypeptide of SEQ ID NO:2. A construct or vector comprising a polynucleotide is introduced into a
15 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 polypeptide and its source.

20 The host cell may be any cell useful in the recombinant production of a polypeptide of the present invention, e.g., a prokaryote or a eukaryote.

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*.
25 Gram-negative bacteria include, but are not limited to, *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, and *Ureaplasma*.

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.
30

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.

35 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.

The introduction of DNA into a *Bacillus* cell may be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Mol. Gen. Genet.* 168: 111-115), competent cell transformation (see, e.g., Young and Spizizen, 1961, *J. Bacteriol.* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *J. Mol. Biol.* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *J. Bacteriol.* 169: 5271-5278). The introduction of DNA into an *E. coli* cell may be effected by protoplast transformation (see, e.g., Hanahan, 1983, *J. Mol. Biol.* 166: 557-580) or electroporation (see, e.g., Dower *et al.*, 1988, *Nucleic Acids Res.* 16: 6127-6145). The introduction of DNA into a *Streptomyces* cell may be effected by protoplast transformation, electroporation (see, e.g., Gong *et al.*, 2004, *Folia Microbiol. (Praha)* 49: 399-405), conjugation (see, e.g., Mazodier *et al.*, 1989, *J. Bacteriol.* 171: 3583-3585), or transduction (see, e.g., Burke *et al.*, 2001, *Proc. Natl. Acad. Sci. USA* 98: 6289-6294). The introduction of DNA into a *Pseudomonas* cell may be effected by electroporation (see, e.g., Choi *et al.*, 2006, *J. Microbiol. Methods* 64: 391-397) or conjugation (see, e.g., Pinedo and Smets, 2005, *Appl. Environ. Microbiol.* 71: 51-57). The introduction of DNA into a *Streptococcus* cell may be effected by natural competence (see, e.g., Perry and Kuramitsu, 1981, *Infect. Immun.* 32: 1295-1297), protoplast transformation (see, e.g., Catt and Jollick, 1991, *Microbios* 68: 189-207), electroporation (see, e.g., Buckley *et al.*, 1999, *Appl. Environ. Microbiol.* 65: 3800-3804), or conjugation (see, e.g., Clewell, 1981, *Microbiol. Rev.* 45: 409-436). However, any method known in the art for introducing DNA into a host cell can be used.

The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota as well as the Oomycota and all mitosporic fungi (as defined by Hawksworth *et al.*, In, *Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK).

The fungal host cell may be a yeast cell. "Yeast" as used herein includes ascosporeogenous yeast (Endomycetales), basidiosporeogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, Passmore, and Davenport, editors, *Soc. App. Bacteriol. Symposium Series* No. 9, 1980).

The yeast host cell may be a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell, such as a *Kluyveromyces lactis*, *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces*

oviformis, or *Yarrowia lipolytica* cell.

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

The filamentous fungal host cell may be an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Chrysosporium*, *Coprinus*, *Coriolus*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Phanerochaete*, *Phlebia*, *Piromyces*, *Pleurotus*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes*, or *Trichoderma* cell.

For example, the filamentous fungal host cell may be an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus fumigatus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Bjerkandera adusta*, *Ceriporiopsis aneirina*, *Ceriporiopsis caregiea*, *Ceriporiopsis gilvescens*, *Ceriporiopsis pannocinta*, *Ceriporiopsis rivulosa*, *Ceriporiopsis subrufa*, *Ceriporiopsis subvermispora*, *Chrysosporium inops*, *Chrysosporium keratinophilum*, *Chrysosporium lucknowense*, *Chrysosporium merdarium*, *Chrysosporium pannicola*, *Chrysosporium queenslandicum*, *Chrysosporium tropicum*, *Chrysosporium zonatum*, *Coprinus cinereus*, *Coriolus hirsutus*, *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Phanerochaete chrysosporium*, *Phlebia radiata*, *Pleurotus eryngii*, *Thielavia terrestris*, *Trametes villosa*, *Trametes versicolor*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* and *Trichoderma* host cells are described in EP 238023, Yelton *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1470-1474, and Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be

transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *J. Bacteriol.* 153: 163; and Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75: 1920.

5

Methods of Production

The present invention also relates to methods of producing the polypeptides of the present invention, comprising (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. In a preferred embodiment, the cell is a *Bacillus* cell. In a more preferred embodiment, the cell is a *Bacillus sp.* cell. In a most preferred embodiment, the cell is selected from *Bacillus sp.* producing the polypeptide with SEQ ID NO: 3.

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Thus, one aspect of the invention relates to a method of producing a polypeptide having at least 97 % identity to SEQ ID NO: 3, comprising: (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

15

The present invention also relates to methods of producing a polypeptide of the present invention, comprising (a) cultivating a recombinant host cell of the present invention under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

20

Thus, one aspect of the invention relates to a method of producing the polypeptide having at least 97% identity to SEQ ID NO: 3, comprising:

(a) cultivating a host cell under conditions conducive for production of the polypeptide; and

(b) recovering the polypeptide.

25

The host cell may be a bacterial host cell such a *Bacillus*, *Streptococcus* or *Streptomyces* cell. The host cell may also be a eukaryote, such as a mammalian, insect, plant, or fungal cell. The host cell may be a fungal cell, which may be a yeast cell. Various suitable host cells are described in the "host cells" section of the present application. Thus, in one particular embodiment, the cell is a bacillus.

30

The cell or the host cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, or small-scale 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 polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts,

35

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 polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell
5 lysates.

The polypeptide may be detected using methods known in the art that are specific for the polypeptides. 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 polypeptide.

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

The polypeptide may be purified by a variety of procedures known in the art including, but
15 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 (see, e.g., *Protein Purification*, Janson and Ryden, editors, VCH Publishers, New York, 1989) to obtain substantially pure polypeptides.

20 In an alternative aspect, the polypeptide is not recovered, but rather a host cell of the present invention expressing the polypeptide is used as a source of the polypeptide.

Detergent Compositions

The present invention also relates to compositions comprising a protease of the invention.
25 Preferably, the compositions are enriched with a protease of the invention. The term "enriched" indicates that the protease activity of the composition has been increased.

In one embodiment, the present invention relates to compositions in particular to cleaning compositions and/or detergent compositions comprising a protease of the invention and at least one suitable cleaning/detergent relevant ingredient, such as surfactants, builders, bleaches as described
30 below.

The present invention also relates to compositions, preferably detergent compositions comprising an isolated polypeptide having protease activity selected from the group consisting of: a) a polypeptide having at least 60% sequence identity, at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85%
35 sequence identity, at least 90% sequence identity, at least 92% sequence identity, at least 93%

sequence identity, at least 94% sequence identity, at least 95% sequence identity, at least 96% sequence identity, at least 97% sequence identity, at least 98% sequence identity or at least 99% sequence identity to SEQ ID NO: 3.

5 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 60% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 65% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 70% sequence identity to SEQ ID NO: 3.

10 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 75% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 80% sequence identity to SEQ ID NO: 3.

15 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 85% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 90% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 91% sequence identity to SEQ ID NO: 3.

20 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 93% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 93% sequence identity to SEQ ID NO: 3.

25 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 94% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 95% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 96% sequence identity to SEQ ID NO: 3.

30 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 97% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 98% sequence identity to SEQ ID NO: 3.

35 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 99% sequence identity to SEQ ID NO: 3.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having 100% sequence identity to SEQ ID NO: 3.

In one embodiment, the composition is a detergent composition which may be adapted for specific uses such as laundry, in particular household laundry, dish washing or hard surface cleaning.

5 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.

10 The detergent composition may be suitable for laundry of textiles or for hard surface cleaning including dish wash including automated dish wash.

In one embodiment of the present invention, the a protease of the present invention may be added to a detergent composition in an amount corresponding to 0.001-200 mg of protein, such as 0.005-100 mg of protein, preferably 0.01-50 mg of protein, more preferably 0.05-20 mg of
15 protein, even more preferably 0.1-10 mg of protein per liter of wash liquid.

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
20 formulated as described in, for example, WO 92/19709 and WO 92/19708 or the protease according to the invention may be stabilized using peptide aldehydes or ketones such as described in WO 2005/105826 and WO 2009/118375. A protease of the present invention may also be incorporated in the detergent formulations disclosed in WO97/07202, which is hereby incorporated by reference.

25 Liquid detergent composition

The proteases of the invention may be formulated in liquid laundry compositions such as a liquid laundry compositions composition comprising:

- a) at least 0.005 mg of active protease protein per litre detergent, wherein the protease has at least 60% sequence identity to SEQ ID NO 3,
- 30 b) 2 wt% to 60 wt% of at least one surfactant, and
- c) 5 wt% to 50 wt% of at least one builder

Powder composition

The detergent composition may also be formulated into a granular detergent for laundry or dish wash. One embodiment of the invention concerns a granular detergent composition comprising

- a) at least 0.005 mg protease per gram of composition, wherein the protease has at least 60% sequence identity to SEQ ID NO: 3,
- b) 5 wt % to 50 wt % anionic surfactant
- c) 1 wt % to 8 wt % nonionic surfactant, and
- 5 d) 5 wt % to 40 wt % builder such as carbonates, zeolites, phosphate builder, calcium sequestering builders or complexing agents

The choice of components may include, for textile care, the consideration of the type of textile 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

10 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 and includes conventional ingredients, including the exemplary non-limiting components set forth below.

15 **Surfactants**

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 non-ionic surfactants and one or more anionic surfactants. The surfactant(s) is typically present at a level of

20 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.

When included therein, the detergent will usually contain from about 1% to about 40% by

25 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 sulphates 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 disulphonate, alkyl sulfates (AS) such as sodium dodecyl sulfate (SDS), fatty alcohol sulfates

30 (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,

35 dodecenylyl/tetradecenylyl succinic acid (DTSA), fatty acid derivatives of amino acids, diesters and

monoesters of sulfo-succinic acid or soap, and combinations thereof.

When included therein, the detergent will usually contain from about 1% to about 40% by weight of a cationic surfactant. Non-limiting examples of cationic surfactants include alkyl dimethylethanolamine quat (ADMEAQ), cetyltrimethylammonium bromide (CTAB),
5 dimethyldistearylammonium chloride (DSDMAC), alkylbenzyl dimethylammonium, alkyl quaternary ammonium compounds, alkoxyated quaternary ammonium (AQA) and combinations thereof.

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
10 about 8% to about 12%. Non-limiting examples of non-ionic surfactants include alcohol ethoxylates (AE or AEO), alcohol propoxylates, propoxylated fatty alcohols (PFA), alkoxyated fatty acid alkyl esters, such as ethoxylated and/or propoxylated fatty acid alkyl esters, alkylphenol ethoxylates (APE), nonylphenol ethoxylates (NPE), alkylpolyglycosides (APG), alkoxyated amines, fatty acid monoethanolamides (FAM), fatty acid diethanolamides (FADA), ethoxylated fatty acid
15 monoethanolamides (EFAM), propoxylated fatty acid monoethanolamide (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.

When included therein, the detergent will usually contain from about 1% to about 40% by weight of a semipolar surfactant. Non-limiting examples of semipolar surfactants include amine
20 oxides (AO) such as alkyl dimethylamineoxide, *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.

When included therein, the detergent will usually contain from about 1% to about 40% by weight of a zwitterionic surfactant. Non-limiting examples of zwitterionic surfactants include
25 betaine, alkyl dimethylbetaine, and sulfobetaine, and combinations thereof.

Hydrotropes

The detergent may contain 0-5% by weight, such as about 0.5 to about 5%, or about 3% to
30 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 sulfonates (STS), sodium xylene sulfonates (SXS), sodium cumene sulfonates (SCS), sodium cymene sulfonate, amine oxides, alcohols and polyglycoethers, sodium hydroxynaphthoate, sodium hydroxynaphthalene sulfonate, sodium ethylhexyl sulfate, and combinations thereof.

35

Builders and Co-Builders

The detergent composition may contain about 0-65% by weight, such as about 5% to about 50% of a detergent builder or co-builder, or a mixture thereof. In a dish wash detergent, 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), iminodiethanol (DEA), triethanolamine (TEA), and carboxymethylinulin (CMI), and combinations thereof.

The detergent composition may also contain 0-65% by weight, such as about 5% to about 50%, of a detergent co-builder, or a mixture thereof. The detergent composition may 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-diylbis(phosphonic acid) (HEDP), ethylenediaminetetrakis(methylene)tetrakis(phosphonic acid) (EDTMPA), diethylenetriaminepentakis(methylene)pentakis(phosphonic acid) (DTPMPA), 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-(hydroxyethyl)-ethylenediaminetriacetate (HEDTA), diethanolglycine (DEG), diethylenetriamine penta (Methylene Phosphonic 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.

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Bleaching Systems

The detergent may contain 0-30% by weight, such as about 1% to about 20%, of a bleaching system. Any bleaching system comprising components known in the art for use in cleaning detergents may be utilized. Suitable bleaching system components include sources of hydrogen peroxide; 5 sources of peracids; and bleach catalysts or boosters.

Sources of hydrogen peroxide: Suitable sources of hydrogen peroxide are inorganic persalts, including alkali metal salts such as sodium percarbonate and sodium perborates (usually mono- or tetrahydrate), and hydrogen peroxide—urea (1/1).

Sources of peracids: Peracids may be (a) incorporated directly as preformed peracids or (b) 10 formed in situ in the wash liquor from hydrogen peroxide and a bleach activator (perhydrolysis) or (c) formed in situ in the wash liquor from hydrogen peroxide and a perhydrolase and a suitable substrate for the latter, e.g., an ester.

a) Suitable preformed peracids include, but are not limited to, peroxydicarboxylic acids such as peroxybenzoic acid and its ring-substituted derivatives, peroxy- α -naphthoic acid, peroxyphthalic acid, 15 peroxyauric acid, peroxysebacic acid, ϵ -phthalimidoperoxyhexanoic acid [phthalimidoperoxyhexanoic acid (PAP)], and *o*-carboxybenzamidoperoxyhexanoic acid; aliphatic and aromatic diperoxydicarboxylic acids such as diperoxydodecanedioic acid, diperoxyazelaic acid, diperoxysebacic acid, diperoxybrassylic acid, 2-decyldiperoxybutanedioic acid, and diperoxyphthalic, -isophthalic and -terephthalic acids; perimidic acids; peroxymonosulfuric acid; peroxydisulfuric acid; peroxyphosphoric 20 acid; peroxydicarboxylic acid; and mixtures of said compounds. It is understood that the peracids mentioned may in some cases be best added as suitable salts, such as alkali metal salts (e.g., Oxone®) or alkaline earth-metal salts.

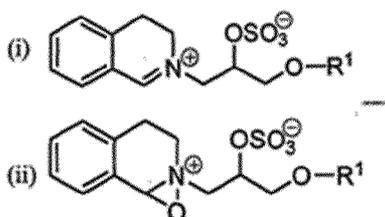
b) Suitable bleach activators include those belonging to the class of esters, amides, imides, nitriles or anhydrides and, where applicable, salts thereof. Suitable examples are 25 tetraacetylenediamine (TAED), sodium 4-[(3,5,5-trimethylhexanoyl)oxy]benzene-1-sulfonate (ISONOBS), sodium 4-(dodecanoyloxy)benzene-1-sulfonate (LOBS), sodium 4-(decanoyloxy)benzene-1-sulfonate, 4-(decanoyloxy)benzoic acid (DOBA), sodium 4-(nonanoyloxy)benzene-1-sulfonate (NOBS), and/or those disclosed in WO98/17767. A particular family of bleach activators of interest was disclosed in EP624154 and particularly preferred in that 30 family is acetyl triethyl citrate (ATC). ATC or a short chain triglyceride like triacetin has the advantage that they are environmentally friendly. Furthermore acetyl triethyl citrate and triacetin have good hydrolytical stability in the product upon storage and are efficient bleach activators. Finally ATC is multifunctional, as the citrate released in the perhydrolysis reaction may function as a builder.

Bleach catalysts and boosters: The bleaching system may also include a bleach catalyst or 35 booster. Some non-limiting examples of bleach catalysts that may be used in the compositions of the

present invention include manganese oxalate, manganese acetate, manganese-collagen, cobalt-amine catalysts and manganese triazacyclononane (MnTACN) catalysts; particularly preferred are complexes of manganese with 1,4,7-trimethyl-1,4,7-triazacyclononane (Me₃-TACN) or 1,2,4,7-tetramethyl-1,4,7-triazacyclononane (Me₄-TACN), in particular Me₃-TACN, such as the dinuclear manganese complex [(Me₃-TACN)Mn(O)₃Mn(Me₃-TACN)](PF₆)₂, and [2,2',2''-nitrilotris(ethane-1,2-diylazanylylidene-κN-methanylylidene)triphenolato-κ3O]manganese(III). The bleach catalysts may also be other metal compounds, such as iron or cobalt complexes.

In some embodiments, where a source of a peracid is included, an organic bleach catalyst or bleach booster may be used having one of the following formulae:

10



(iii) and mixtures thereof; wherein each R₁ is independently a branched alkyl group containing from 9 to 24 carbons or linear alkyl group containing from 11 to 24 carbons, preferably each R₁ 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 R₁ is independently selected from the group consisting of 2-propylheptyl, 2-butyloctyl, 2-pentylnonyl, 2-hexyldecyl, dodecyl, tetradecyl, hexadecyl, octadecyl, isononyl, isodecyl, isotridecyl and isopentadecyl.

Other exemplary bleaching systems are described, e.g. in WO 2007/087258, WO 2007/087244, WO 2007/087259, EP 1867708 (Vitamin K) and WO 2007/087242. Suitable photobleaches may for example be sulfonated zinc or aluminium phthalocyanines.

Polymers

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 polyethylene terephthalate and polyoxyethene terephthalate (PET-POET), PVP, poly(vinylimidazole) (PVI), poly(vinylpyridin-N-oxide) (PVPO or PVPNO) and polyvinylpyrrolidone-vinylimidazole (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.

10 Fabric hueing agents

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 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 WO 2005/03274, WO 2005/03275, WO 2005/03276 and EP 1876226 (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, WO 2007/087243.

Additional enzymes

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

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: 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.

Especially suitable cellulases are the alkaline or neutral cellulases having colour 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 WO 99/001544.

Other cellulases are endo-beta-1, 4-glucanase enzyme having a sequence of at least 97% identity to the amino acid sequence of position 1 to position 773 of SEQ ID NO: 2 of WO 2002/099091 or a family 44 xyloglucanase, which a xyloglucanase enzyme having a sequence of at least 60% identity to positions 40-559 of SEQ ID NO: 2 of WO 2001/062903.

Commercially available cellulases include Celluzyme™, and Carezyme™ (Novozymes A/S) Carezyme Premium™ (Novozymes A/S), Celluclean™ (Novozymes A/S), Celluclean Classic™ (Novozymes A/S), Cellusoff™ (Novozymes A/S), Whitezyme™ (Novozymes A/S), Clazinase™, and Puradax HA™ (Genencor International Inc.), and KAC-500(B)™ (Kao Corporation).

Proteases: Suitable additional proteases to be used with the protease of the invention 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.

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.

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 WO 89/06279 and protease PD138 described in (WO 93/18140). Other useful proteases may be those described in WO 92/175177, WO 01/016285, WO 02/026024 and WO 02/016547. Examples of

5 trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270, WO 94/25583 and WO 05/040372, and the chymotrypsin proteases derived from *Cellulomonas* described in WO 05/052161 and WO 05/052146.

A further preferred protease is the alkaline protease from *Bacillus lentus* DSM 5483, as described for example in WO 95/23221, and variants thereof which are described in WO 92/21760,

10 WO 95/23221, EP 1921147 and EP 1921148.

Examples of metalloproteases are the neutral metalloprotease as described in WO 07/044993 (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,

15 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, 24, 27, 42, 55, 59, 60, 66, 74, 85, 96, 97, 98, 99, 100, 101, 102, 104, 116, 118, 121, 126, 127, 128, 154, 156, 157, 158, 161, 164, 176, 179, 182, 185, 188, 189, 193, 198, 199, 200, 203, 206, 211, 212, 216, 218, 226, 229, 230, 239, 246, 255, 256, 268 and 269 wherein the positions correspond to the positions of the *Bacillus*

20 *Lentus* protease shown in SEQ ID NO 1 of WO 2016/001449. More preferred the subtilase variants may comprise the mutations: S3T, V4I, S9R, S9E, A15T, S24G, S24R, K27R, N42R, S55P, G59E, G59D, N60D, N60E, V66A, N74D, N85S, N85R, , G96S, G96A, S97G, S97D, S97A, S97SD, S99E, S99D, S99G, S99M, S99N, S99R, S99H, S101A, V102I, V102Y, V102N, S104A, G116V, G116R, H118D, H118N, N120S, S126L, P127Q, S128A, S154D, A156E, G157D, G157P, S158E,

25 Y161A, R164S, Q176E, N179E, S182E, Q185N, A188P, G189E, V193M, N198D, V199I, Y203W, S206G, L211Q, L211D, N212D, N212S, M216S, A226V, K229L, Q230H, Q239R, N246K, N255W, N255D, N255E, L256E, L256D T268A, R269H. The protease variants are preferably variants of the *Bacillus lentus* protease (Savinase®) shown in SEQ ID NO 1 of WO 2016/001449, the *Bacillus amylolichenifaciens* protease (BPN') shown in SEQ ID NO 2 of WO2016/001449. The protease

30 variants preferably have at least 80 % sequence identity to SEQ ID NO 1 or SEQ ID NO 2 of WO 2016/001449.

A protease variant comprising a substitution at one or more positions corresponding to positions 171, 173, 175, 179, or 180 of SEQ ID NO: 1 of WO2004/067737, wherein said protease variant has a sequence identity of at least 75% but less than 100% to SEQ ID NO: 1 of

35 WO2004/067737.

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, Blaze®, Blaze Evity® 100T, Blaze Evity® 125T, Blaze Evity® 150T, Neutrase®, Everlase® and Esperase® (Novozymes A/S), those sold under the tradename Maxatase®, Maxacal®, Maxapem®, , , Purafect Ox®, Purafect OxP®, Puramax®, FN2®, FN3®, FN4®, Excellase®, Excellenz P1000™, Excellenz P1250™, Eraser®, Preferenz P100™, Purafect Prime®, Preferenz P110™, Effectenz P1000™, Purafect®™, Effectenz P1050™, Purafect Ox®™, Effectenz P2000™, Purafast®, Properase®, Opticlean® and Optimase® (Danisco/DuPont), Axapem™ (Gist-Brocades 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: 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 EP 258068 and EP 305216, cutinase from *Humicola*, e.g. *H. insolens* (WO 96/13580), lipase from strains of *Pseudomonas* (some of these now renamed to *Burkholderia*), e.g. *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218272), *P. cepacia* (EP 331376), *P. sp.* strain SD705 (WO 95/06720 & WO 96/27002), *P. wisconsinensis* (WO 96/12012), GDSL-type *Streptomyces* lipases (WO 10/065455), cutinase from *Magnaporthe grisea* (WO 10/107560), cutinase from *Pseudomonas mendocina* (US 5,389,536), lipase from *Thermobifida fusca* (WO 11/084412), *Geobacillus stearothermophilus* lipase (WO 11/084417), lipase from *Bacillus subtilis* (WO 11/084599), and lipase from *Streptomyces griseus* (WO 11/150157) and *S. pristinaespiralis* (WO 12/137147).

Other examples are lipase variants such as those described in EP 407225, WO 92/05249, WO 94/01541, WO 94/25578, WO 95/14783, WO 95/30744, WO 95/35381, WO 95/22615, WO 96/00292, WO 97/04079, WO 97/07202, WO 00/34450, WO 00/60063, WO 01/92502, WO 07/87508 and WO 09/109500.

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

Still other examples are lipases sometimes referred to as acyl transferases or perhydrolases, e.g. acyltransferases with homology to *Candida antarctica* lipase A (WO 10/111143), acyltransferase from *Mycobacterium smegmatis* (WO 05/56782), perhydrolases from the CE 7 family (WO 09/67279), 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 (WO 10/100028).

Amylases: Suitable amylases which can be used together with the protease 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.

5 Suitable amylases include amylases having SEQ ID NO: 2 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,
10 and 444.

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

15 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 or more of the following positions: G48, T49, G107, H156, A181, N190, M197, I201, A209 and Q264. Most
20 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 are those having the substitutions:

M197T;

H156Y+A181T+N190F+A209V+Q264S; or

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

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
30 preferred amylases are those having deletion in positions R181 and G182, or positions H183 and G184.

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
35 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, using SEQ ID 2 of WO 96/023873 for numbering. More preferred variants are those having a deletion in two positions selected from 181, 182, 183 and 184, such as 181 and 182, 182 and 183, 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.

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.

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.

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.

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

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: Suitable peroxidases/oxidases include those of plant, bacterial or
10 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.

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

The detergent enzyme(s) may be included in a detergent composition by adding separate
15 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.

Non-dusting granulates may be produced, e.g. as disclosed in US 4,106,991 and 4,661,452
20 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
25 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.

30

Adjunct materials

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,
anti-soil redeposition agents, anti-wrinkling agents, bactericides, binders, corrosion inhibitors,
35 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.

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.

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, polyvinylloxazolidones 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.

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-hydroxyethylamino)-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-5
s-triazin-6-ylamino) stilbene disulphonate. Tinopal CBS is the disodium salt of 2,2'-bis-(phenylstyryl) 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,
10 from about 0.1 or even from about 0.2 wt % to upper levels of 0.5 or even 0.75 wt%.

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
15 fabrics, in particular the removal of hydrophobic soils from polyester based fabrics. The soil release polymers may for example be nonionic or anionic terephthalate 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 alkoxyated grease cleaning polymers
20 comprising a core structure and a plurality of alkoxyate 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
25 reference). Other soil release polymers are substituted polysaccharide structures especially substituted cellulosic structures such as modified cellulose derivatives 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
30 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.

35 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.

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

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. Detergent formulation forms: Layers (same or different phases), Pouches, versus forms for Machine dosing unit. Pouches may be configured as single or multicompartments. It may 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 may be divided into compartments of the pouch. Preferred films are polymeric materials preferably polymers which are formed into a film or sheet. Preferred polymers, copolymers or derivatives thereof 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 cleaning 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).

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
5 can also give rise to delayed dissolution of selected components in the wash solution.

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
10 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

The enzymes of the invention may be added to laundry soap bars and used for hand
15 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
20 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.

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
25 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
30 organic anion may be, for example, sodium formate.

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, alkoxyated polycarbonates, suds suppressers, structurants, binders, leaching agents,
35 bleaching activators, clay soil removal agents, anti-redeposition agents, polymeric dispersing

agents, brighteners, fabric softeners, perfumes and/or other compounds known in the art.

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

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.

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|----|---------------|---------------|---------------|---------------|---------------|
| | WO2011025615, | WO2011016958, | WO2011005803, | WO2011005623, | WO2011005730, |
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| 25 | WO2011005905, | WO2011005910, | WO2011005813, | WO2010135238, | WO2010120863, |
| | WO2010108002, | WO2010111365, | WO2010108000, | WO2010107635, | WO2010090915, |
| | WO2010033976, | WO2010033746, | WO2010033747, | WO2010033897, | WO2010033979, |
| | WO2010030540, | WO2010030541, | WO2010030539, | WO2010024467, | WO2010024469, |
| | WO2010024470, | WO2010025161, | WO2010014395, | WO2010044905, | WO2010145887, |
| 30 | WO2010142503, | WO2010122051, | WO2010102861, | WO2010099997, | WO2010084039, |
| | WO2010076292, | WO2010069742, | WO2010069718, | WO2010069957, | WO2010057784, |
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| | WO2010142539, | WO2010118959, | WO2010115813, | WO2010105942, | WO2010105961, |
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WO2010066632, WO2010063689, WO2010060821, WO2010049187, WO2010031607, WO2010000636.

Uses

5 The present invention is directed to methods for using the polypeptides having protease activity, or compositions thereof. The invention may be used in compositions thereof in the laundering of textile and fabrics, such as house hold laundry washing and industrial laundry washing. The invention is directed to methods for using the compositions thereof in hard surface cleaning such as automated dish washing (ADW), car wash and cleaning of industrial surfaces.

10

Use of proteases of the invention in detergent compositions and cleaning processes

 The soils and stains that are important for detergent formulators are composed of many different substances, and a range of different enzymes, all with different substrate specificities have been developed for use in detergents both in relation to laundry and hard surface cleaning, such as dishwashing. These enzymes are considered to provide an enzyme detergency benefit, since they specifically improve stain removal in the cleaning process they are applied in as compared to the same process without enzymes. Stain removing enzymes that are known in the art include enzymes such as carbohydrases, amylases, proteases, lipases, cellulases, hemicellulases, xylanases, cutinases, and pectinase.

20 In one aspect, the present invention relates to the use of an isolated polypeptide having a sequence identity to SEQ ID NO: 3 of at least 60%, 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 86%, at least 87%, at least 88%, at least 89%, 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% in detergent compositions and cleaning processes, such as laundry and hard surface cleaning.

 In another aspect, the present invention relates to the use of protease of the invention in detergent compositions and cleaning processes, such as laundry and hard surface cleaning. Thus, in one embodiment, the present invention demonstrates the detergency effect of the protease of the invention on various stains and under various conditions. In a particular embodiment of the invention the detergent composition and the use in cleaning process concerns the use of a protease of the invention together with at least one of the above mentioned stain removal enzymes.

30 In a preferred aspect of the present invention, the protease of the invention may be combined with additional enzymes these additional enzymes are described in details in the section "other enzymes"; preferably the protease of the invention is combined with at least two enzymes,

35

more preferred at least three, four or five enzymes. Preferably, the enzymes have different substrate specificity, e.g., carbolytic activity, proteolytic activity, amylolytic activity, lipolytic activity, hemicellulytic activity or pectolytic activity. In one embodiment of the present invention, a protease of the invention may be combined with one or more metalloproteases, such as a M4
5 Metalloprotease, including Neutrase™ or Thermolysin. Such combinations may further comprise combinations of the other detergent enzymes as outlined above.

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 may for example be household laundering, but it may
10 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 of the invention. The cleaning process or a textile care process may for example be carried out in a machine washing process or in a manual washing process. The washing solution may for example be an aqueous washing
15 solution containing a detergent composition.

The fabrics and/or garments subjected to a washing, cleaning or textile care process of the present invention may be conventional washable laundry, for example household laundry. Preferably, the major part of the laundry is garments and fabrics, including knits, woven, denims, non-woven, felts, yarns, and towelling. The fabrics may be cellulose based such as natural
20 cellulosics, including cotton, flax, linen, jute, ramie, sisal or coir or manmade cellulosics (e.g., originating from wood pulp) including viscose/rayon, ramie, cellulose acetate fibers (tricell), lyocell or blends thereof. The fabrics may also be non-cellulose based such as natural polyamides including wool, camel, cashmere, mohair, rabbit and silk or synthetic polymer such as nylon, aramid, polyester, acrylic, polypropylene and spandex/elastane, or blends thereof as well as blend
25 of cellulose based and non-cellulose based fibers. Examples of blends are blends of cotton and/or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g., polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers, polyvinyl chloride fibers, polyurethane fibers, polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g., rayon/viscose, ramie, flax, linen, jute, cellulose acetate fibers, lyocell).

30 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
35 and amylases is needed to achieve a similar or improved wash performance when compared to

the traditional detergent compositions.

A protease of the invention is usable in 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.

5 Typical detergent compositions includes 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 removes discolour often by oxidation and many bleaches also have strong bactericidal properties, and are used for disinfecting and
10 sterilizing. Yet other components like builder and chelator softens, e.g., the wash water by removing the metal ions form the liquid.

In a particular embodiment, the invention relates to the use of a composition comprising a protease of the invention, wherein said enzyme composition further comprises at least one or more of the following a surfactant, a builder, a chelator or chelating agent, bleach system or bleach
15 component in laundry or dish wash.

Thus, in one embodiment, the invention relates to the use of a composition comprising a polypeptide having at least 60% sequence identity to SEQ ID NO:3, wherein the composition further comprises a least one or more of the following: a surfactant, a builder, a chelator or chelating agent, bleach system or bleach component in laundry or dish wash.

20 In a preferred embodiment of the invention, the amount of a surfactant, a builder, a chelator or chelating agent, bleach system and/or bleach component are reduced compared to amount of surfactant, builder, chelator or chelating agent, bleach system and/or bleach component used without the added protease of the invention. Preferably the at least one component which is a surfactant, a builder, a chelator or chelating agent, bleach system and/or bleach component is
25 present in an amount that is 1% less, such as 2% less, such as 3% less, such as 4% less, such as 5% less, such as 6% less, such as 7% less, such as 8% less, such as 9% less, such as 10% less, such as 15% less, such as 20% less, such as 25% less, such as 30% less, such as 35% less, such as 40% less, such as 45% less, such as 50% less than the amount of the component in the system without the addition of protease of the invention, such as a conventional amount of such
30 component. In one embodiment, the protease of the invention is used in detergent compositions wherein said composition is free of at least one component which is a surfactant, a builder, a chelator or chelating agent, bleach system or bleach component and/or polymer.

Washing Method

35 The detergent compositions comprising a protease of the present invention are ideally

suited for use in laundry applications. Accordingly, the present invention includes a method for laundering a fabric. The method comprises the steps of contacting a fabric to be laundered with a cleaning laundry solution comprising the detergent composition according to the invention. The fabric may comprise any fabric capable of being laundered in normal consumer use conditions.

5 The solution preferably has a pH of from about 5.5 to about 8. The compositions may be employed at concentrations of 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 90°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 10 90°C. The water to fabric ratio is typically from about 1:1 to about 30:1.

In particular embodiments, the washing method is conducted at a pH of from about 5.0 to about 11.5, or in alternative embodiments, even from about 6 to about 10.5, such as about 5 to about 11, about 5 to about 10, about 5 to about 9, about 5 to about 8, about 5 to about 7, about 5.5 to about 11, about 5.5 to about 10, about 5.5 to about 9, about 5.5 to about 8, about 5.5 to about 7, about 6 to about 11, about 6 to about 10, about 6 to about 9, about 6 to about 8, about 6 to about 7, about 6.5 to about 11, about 6.5 to about 10, about 6.5 to about 9, about 6.5 to about 8, about 6.5 to about 7, about 7 to about 11, about 7 to about 10, about 7 to about 9, or about 7 to about 8, preferably about 5.5 to about 9, and more preferably about 6 to about 8.

In particular embodiments, the washing method is conducted at a degree of hardness of 20 from about 0°dH to about 30°dH, such as about 1°dH, about 2°dH, about 3°dH, about 4°dH, about 5°dH, about 6°dH, about 7°dH, about 8°dH, about 9°dH, about 10°dH, about 11°dH, about 12°dH, about 13°dH, about 14°dH, about 15°dH, about 16°dH, about 17°dH, about 18°dH, about 19°dH, about 20°dH, about 21°dH, about 22°dH, about 23°dH, about 24°dH, about 25°dH, about 26°dH, about 27°dH, about 28°dH, about 29°dH, about 30°dH. Under typical European wash conditions, 25 the degree of hardness is about 15°dH, under typical US wash conditions about 6°dH, and under typical Asian wash conditions, about 3°dH.

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

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

35 Still another embodiment relates to a method for removing stains from fabric which comprises contacting said a fabric with a composition comprising a protease of the invention under conditions suitable for cleaning said object.

In a preferred embodiment, the compositions for use in the methods above further comprises at least one additional enzyme as set forth in the “other enzymes” section above, such as an enzyme selected from the group consisting of carbohydrases, amylases, peptidases, proteases, lipases, cellulase, xylanases or cutinases or a combination hereof. In yet another preferred embodiment the compositions comprises a reduced amount of at least one or more of the following components a surfactant, a builder, a chelator or chelating agent, bleach system or bleach component or a polymer.

Examples

10 Example 1: Expression and Purification:

Isolation, genome sequencing and identification of the encoding gene from *Bacillus sp* (SEQ ID NO 1).

The bacterial strain *Bacillus sp* from the genus *Bacillus* were isolated from an environmental sample and the specie identified by sequencing of the 16S ribosomal subunit genes as listed in Table 1.

Table 1:

| Strain | Source type | Location | Medium | Temperature |
|---------------------|----------------------|----------|---------------|-------------|
| <i>Bacillus sp.</i> | Environmental sample | Turkey | TY medium pH9 | 30°C |

Chromosomal DNA from the bacterial strain was isolated by using the QIAamp DNA Blood Mini Kit” (Qiagen, Hilden, Germany). 2 ug of chromosomal DNA was sent for genome sequencing at FASTERIS SA, Switzerland. The genomes were sequenced by Illumina Sequencing. The resulting genome sequences were analyzed and a S8 protease was identified by comparison to the protease TY-145 (SEQ ID NO: 4) by searching using the BLAST program. The DNA sequence of the identified gene encoding the polypeptide of the invention is included in the sequence listing as SEQ ID NO: 1.

Cloning and expression of a protease from *Bacillus sp.* in *Bacillus subtilis* expression host.

A linear integration vector-system was used for the expression cloning of the protease from *Bacillus sp.* (SEQ ID NO: 3). The linear integration construct was a PCR fusion product made by fusion of the gene between two *Bacillus subtilis* homologous chromosomal regions along with strong promoters and a chloramphenicol resistance marker. The fusion was made by Splicing by Overlap Extension (SOE) PCR (Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R.

(1989) Engineering hybrid genes without the use of restriction enzymes, gene splicing by overlap extension Gene 77: 61-68). The SOE PCR method is also described in patent application WO 2003/095658. The gene was expressed under the control of a triple promoter system (as described in WO 99/43835), consisting of the promoters from *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), and the *Bacillus thuringiensis* cryIIIA promoter including stabilizing sequence. The gene coding for chloramphenicol acetyl-transferase was used as marker (described in e.g. Diderichsen, B.; Poulsen, G.B.; Joergensen, S.T.; A useful cloning vector for *Bacillus subtilis*. Plasmid 30:312 (1993)). The final gene constructs were integrated on the *Bacillus* chromosome by homologous recombination into the pectate lyase locus. The gene fragments were amplified from chromosomal DNA of the corresponding strains with gene specific primers containing overhang to the two flanking vector fragments (primer sequences are listed below). All genes were expressed with a *Bacillus clausii* secretion signal (with the following amino acid sequence: MKKPLGKIVASTALLISVAFSSSIASA (SEQ ID NO: 8) replacing the native secretion signal.

Primers used for PCR amplification:
Forward primer: GTTCATCGATCGCATCGGCTCAGCAGGTTGAGAGCAATGA (SEQ ID NO: 6)
Reverse primer: GCGTTTTTTTATTGATTAACGCGTTTACTTTACACGCGGGAAGC (SEQ ID NO: 7)

The two vector fragments and the gene fragment were subjected to a SOE PCR reaction to assemble the three fragments into one linear vector construct. An aliquot of the PCR product was transformed into *Bacillus subtilis*. Transformants were selected on LB agar plates supplemented with 6 µg of chloramphenicol per ml. The resulting recombinant *Bacillus subtilis* clone containing the integrated expression construct was grown in liquid culture. The enzyme containing supernatants were harvested and the enzymes purified as described below.

Example 2 Purification of a protease from *Bacillus sp.*

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. The 0.2µm filtrate was mixed 1:1 with 3.0M (NH₄)₂SO₄ and the mixture was applied to a Phenyl-sepharose FF (high sub) column (from GE Healthcare) equilibrated in 100mM H₃BO₃, 10mM MES/NaOH, 2mM CaCl₂, 1.5M (NH₄)₂SO₄, pH 6.0. After washing the column with the equilibration buffer, the protease was step-eluted with 100mM H₃BO₃, 10mM MES, 2mM CaCl₂, pH 6.0. The eluted peak (containing the protease activity) was collected and applied to a Bacitracin agarose column (from Upfront chromatography)

equilibrated in 100mM H₃BO₃, 10mM MES, 2mM CaCl₂, pH 6.0. After washing the column extensively with the equilibration buffer, the protease was eluted with 100mM H₃BO₃, 10mM MES, 2mM CaCl₂, 1M NaCl, pH 6.0 with 25%(v/v) 2-propanol. The elution peak (containing the protease activity) was transferred to 20mM MES, 2mM CaCl₂, pH 6.0 on a G25 sephadex column (from GE Healthcare). The G25 transferred peak was the purified preparation and was used for further experiments. The purified protease preparation was analyzed by SDS-PAGE and the gel was stained with coomassie a major band was seen at approx. 36-37kDa and two minor bands were seen at approx. 29Da and 7-8kDa respectively. EDMAN degradation showed that the minor bands represent nicked protease molecules. This is supported by the fact that only one band was seen on a coomassie stained SDS-PAGE gel if this gel was run without reducing agent suggesting an intramolecular sulphur bridge connecting the two parts of the nicked protease molecules.

The purified proteases were tested for activity by a protease activity assay using Suc-AAPF-pNA as substrate. The assay was performed as follows:

pNA substrate : Suc-AAPF-pNA (Bachem L-1400).
15 Temperature : Room temperature (25°C)
Assay buffer : 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS,
1mM CaCl₂, 150mM KCl, 0.01% Triton X-100, pH 9.0.

20µl protease (diluted in 0.01% Triton X-100) was mixed with 100µl assay buffer. The assay was started by adding 100µl pNA substrate (50mg dissolved in 1.0ml DMSO and further diluted 45x with 0.01% Triton X-100). The initial increase in OD₄₀₅ was monitored as a measure of the protease activity.

The skilled person knows of alternative assays that may be used in order to determine the activity of a polypeptide having protease activity, or a protease as such.

25 **Example 3: Construction of protease variants by site-directed mutagenesis**

Site-directed variants were constructed of the bacillus protease (SEQ ID NO: 3) comprising specific substitutions according to the invention. The variants were made by traditional cloning of DNA fragments (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989) using PCR together with properly designed mutagenic oligonucleotides that introduced the desired mutations in the resulting sequence.

Mutagenic oligos were synthesized corresponding to the DNA sequence flanking the desired site(s) of mutation, separated by the DNA base pairs defining the insertions/deletions/substitutions. In this manner, the variants listed in Table 2 below were constructed and produced.

35 In order to test protease variants of the invention, the mutated DNA comprising a variant of

the invention was transformed into a competent *B. subtilis* strain and fermented using standard protocols (TB-glycerol media, 3-4 days, 30°C).

Table 2 - Variants of SEQ ID NO: 3

| |
|------------------------------------|
| Mutations compared to SEQ ID NO: 3 |
| D172N |
| V163R |
| G25C |
| G25W |
| G180I |
| D176P |
| P174*,A175* |
| D242G |
| G25L |
| S144I |
| G25R |
| S144V |
| P8K |
| K123P |
| D172K |
| G180Y |
| Y302M |
| S144F |
| P8D |
| P174H D207P |
| T294P |
| P8L G180M |
| D176P |
| Q287G |
| S144L |
| Y302R |
| D172N |
| I290C |

| |
|------------|
| L82V D172W |
| Y241P |
| L82G |
| A175S |
| D172E |
| V163W |
| K30E |
| A175K |
| Q287H |
| Q287T |
| L82H |
| D207P |
| L82F |
| N39D |
| Y302I |
| L82I |
| S145R |
| T40R |

Example 4: TOM wash with the protease from *Bacillus sp.*

The wash performance of the protease from *Bacillus sp.* was tested using laundry liquid model detergent detergent on six different stains using the Tergo-O-Meter (TOM) wash system. The Tergo-O-Meter (TOM) is a medium scale model wash system that can be applied to test up to 16 different wash conditions simultaneously. A TOM is basically a large temperature controlled water bath with up to 16 open metal beakers submerged into it. Each beaker constitutes one small top loader style washing machine and during an experiment, each of them containing a solution of a specific detergent/enzyme system and the soiled and unsoiled fabrics. Using the soiled and unsoiled fabrics the performance of the specific detergent/enzyme system can be determined. Mechanical stress can be achieved by a rotating stirring arm stirring the liquid within each beaker. Because the TOM beakers have no lid, withdrawal of samples during a TOM experiment is possible, and thereby facilitating the option of gathering information on-line during washing. The TOM model wash system may be mainly used in medium scale testing of detergents and enzymes at US or LA (Latin America) or AP (Asian Pacific) wash conditions. In a TOM experiment, factors

such as ‘the ballast to soil’ ratio and ‘the fabric to wash liquid’ 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 top loader washing machines. The TOM experiment was performed by using a water bath with up to 16 steel beakers and one rotating arm per beaker with capacity of 500 or 1200mL of detergent solution. The experiment was performed in the temperature range from 5 to 80°C. The water bath was filled with deionized water, and the rotational speed was set to 70 to 120rpm/min. All beakers were clean and without traces of prior test material. The wash solution was then prepared with the desired amount of detergent, temperature and water hardness in a bucket. Detergent was dissolved during magnet stirring for 10 min. The wash solution was used within 30 to 60 min after preparation. 1000ml wash solution was added to each TOM beaker, and agitation at 120rpm was started. To those beakers used for testing the enzymes of the present invention, the enzymes were added to the beaker. The swatches (also termed “fabrics”) mixed with ballast were sprinkled and loaded into the beaker. Time measurement started when the swatches and ballast were added to the beaker. The washing ran for 30 minutes and was stopped by stopping the agitation of the beakers. The wash load was transferred from the TOM beakers to a sieve in order to rinse with cold tap water. The swatches and ballast were transferred to a European washing machine for a 14 min rinse cycle. The swatches were separated from the ballast and placed on a tray covered with a paper. Another paper was added on top of the swatches. The swatches were left overnight to dry and then measure at the Color Eye as described below. The experimental conditions are summarized in Table 3.

Table 3: Experimental conditions for laundry experiments

| | |
|------------------------|--|
| Detergent dosage | Laundry liquid model detergent B 3.33 g/L (EU detergent) Unilever Small & Mighty 3.33 g/L (EU detergent) Laundry liquid model detergent K 0.8 g/L (US detergent) |
| Test solution volume | 1L |
| pH | 7.5-8.3 |
| Wash time | 30 minutes (EU detergent) and 15 minutes (US detergent) |
| Temperature | 20°C |
| Water hardness | 15°dH (EU conditions) and 9°dH (US conditions) |
| Protease concentration | 30 nM |
| Swatch | CS-37, C-05, PC-03, CS-01, C-H010, EMPA-117, 062KC |

Water hardness was adjusted by addition of CaCl₂, MgCl₂, and NaHCO₃ to the test system.

Table 4: Delta remission value of detergent comprising protease from *Bacillus sp.* compared to detergent without protease at 20°C

| Detergent | Stain | | | | | | |
|----------------------------------|-------|------|-------|-------|--------|-------|----------|
| | CS-37 | C-05 | PC-03 | CS-01 | C-H010 | 062KC | EMPA-117 |
| Laundry liquid model detergent B | 4.8 | 2.7 | 2.2 | 1.0 | 5.0 | 8.5 | NA |
| Unilever Small& Mighty | 9.0 | 3.8 | 3.4 | 0.7 | 4.1 | 0 | NA |
| Laundry liquid model detergent K | 5.8 | 1.0 | 1.1 | 0 | 0 | 1.3 | 6.6 |

5 The results of Table 4 show that detergent comprising *Bacillus sp.* effectively improves wash performance on egg (CS-37), blood/milk/ink (C-05, EMPA117), blood (CS-01), chocolate/milk (C-H010, PC-03) and grass (062KC) stains at 20°C.

Table 5: Relative wash performance of detergent comprising proteases from *Bacillus sp.* compared to detergent TY-145 protease (SEQ ID NO 8) at 20°C

| Detergent | Stain | | | | | | |
|----------------------------------|-------|------|-------|-------|--------|-------|----------|
| | CS-37 | C-05 | PC-03 | CS-01 | C-H010 | 062KC | EMPA-117 |
| Laundry liquid model detergent B | 0.6 | 0.4 | 0.3 | 0.2 | 1.5 | 0.7 | NA |
| Unilever Small& Mighty | 1.1 | 0.4 | 0.3 | 0.2 | 0.4 | 0 | NA |
| Laundry liquid model detergent K | 1.5 | 0.4 | 0.4 | 0 | 0 | 0.7 | 1.4 |

10

Example 5: AMSA wash using the proteases from *Bacillus sp.*

15 The wash performance of the proteases from *Bacillus sp.* was tested using laundry liquid model detergent detergent on five different technical stains using the Automatic Mechanical Stress Assay (AMSA). By AMSA the wash performance in laundry 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 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" on page 20 23-24.

Table 6: Model detergents and test materials were as follows:

| | |
|----------------------------------|--|
| Laundry liquid model detergent | Sodium hydroxide 99 %: 2,95 % Sulfonic acid: 11,52 % Soy soap: 5,5 % Propylenglycole: 5,05 % C13-alkoholethoxylate, 8 EO: 9,45 % Phosphonate, Dequest 2066 type: 1,00 % Triethanolamine: 2,0 % Coco soap: 4,5 % Sodium citrate, dihydrate: 1,0 % IPA-denatured ethanol: 4,63 % Opacifier: 0,12 % Blue dye. Ion exchanged water up to 100% |
| Laundry liquid model detergent B | LAS 7.2% AEOS 4.2% Soy soap 2.75% Coco soap 2.75% AEO 6.6% NaOH 1.2% Ethanol 3% MPG 6% Glycerol 2% TEA 3% Sodium formiate 1% Sodium citrate 2% DTMPA 0.2% PCA 0.2% Ion exchanged water 55.1% |
| Laundry liquid model detergent K | LAS 3% AS 3% AEOS 6% coco fatty acid 1% AEO 3% MEA 0.3% |

| | |
|------------------------------|---|
| | MPG 3% Ethanol 1.5% DTPA (as Na5 salt) 0.1% Sodium citrate 4% Sodium formate 1% KOH 0.6% NaOH 0.4% Ion exchanged water up to 100% |
| Persil Small&Mighty | Commercially available |
| Great value Mandarin Essence | Commercially available |
| Test material | CS-37 Full egg pigment C-05 Blood/milk/ink on cotton PC-03 Chocolate milk/soot CS-01 Aged blood C-H010 Cocoa cooked up milk CS-38 Egg Yolk on cotton C-03 Chocolate milk/soot on cotton EMPA117 Blood/milk/ink on cotton/polyester 062KC Scrubbed Grass on knitted cotton |

Test materials were obtained from EMPA Testmaterials AG, Mövenstrasse 12, CH-9015 St. Gallen, Switzerland, from Center For Testmaterials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands, and WFK Testgewebe GmbH, Christenfeld 10, D-41379 Brüggen, Germany.

5 Water hardness was adjusted to 15°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:CO₃²⁻= 4:1:7.5) to the test system. After washing the textiles were rinsed in tap water and dried.

The wash performance was measured as the brightness of the color of the textile washed. Brightness may also be expressed as the intensity of the light reflected from the sample when
 10 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.

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

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:

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

5 The experiments were conducted using a single cycle wash procedure, with the detergent composition and swatches described in table 1 and the experimental conditions as specified in Table 7 below.

Table 7: Experimental conditions for laundry experiments

| | |
|------------------------|---|
| Detergent dosage | Laundry liquid model detergent B 3.33 g/L |
| Test solution volume | 160 micro L |
| pH | As is |
| Wash time | 20 minutes |
| Temperature | 20°C |
| Water hardness | 15°dH |
| Protease concentration | 0-10-30-60-100nM |
| Swatch | EMPA117EH, PC-03, CS-38, CS-01, C-03 |

10 Water hardness was adjusted to 15°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:CO₃⁻ = 4:1:7.5) to the test system. After washing the textiles were rinsed in tap water and dried.

Table 8: Delta intensity value of detergent comprising proteases from *Bacillus sp.* compared to detergent without protease at 20°C

| Swatch | Enzyme concentration (nM) | EMPA117EH (Blood/Milk/ink on polyester cotton Extra heat treated) | PC-03 | CS-38 (Egg Yolk, with Pigment, Aged by Heating) | CS-01 | C-03 |
|---------------------|---------------------------|---|-------|---|-------|------|
| <i>Bacillus sp.</i> | 10 | 3.7 | 2.0 | 6.8 | 0 | 0.7 |
| | 30 | 9.1 | 2.7 | 9.6 | 0.4 | 2.7 |
| | 60 | 15.5 | 5.4 | 13.5 | 2.2 | 3.3 |
| | 100 | 18.2 | 5.7 | 12.0 | 5.3 | 3.5 |

15 The results of Table 8 show that detergent comprising *Bacillus sp.* effectively improves

wash performance on egg (CS-38), blood/milk/ink (EMPA117EH), blood (CS-01) and chocolate/milk (PC-03, C-03) at 20°C.

Example 6: AMSA dose-response wash of the protease from *Bacillus sp.*

5 The dose-response wash performance of the protease from *Bacillus sp.* was tested using four different detergents on three different stains.

The experiments were conducted as described in the AMSA for laundry method using a single cycle wash procedure, with the detergent composition and swatches described in table 1 and the experimental conditions as specified in Table 9 below.

10 Table 9: Experimental conditions for laundry experiments

| | |
|------------------------|---|
| Detergent dosage | Laundry liquid model detergent B 3.33 g/L (EU detergent) Persil Small&Mighty 2.5 g/L(EU detergent) Great value Mandarin Essence 1.19 g/L(US detergent) Laundry liquid model detergent K 0.8g/L(US detergent) |
| Test solution volume | 160 micro L |
| pH | As is |
| Wash time | 20 minutes |
| Temperature | 20°C |
| Water hardness | 15°dH (EU conditions) or 9°dH (US conditions) |
| Protease concentration | 0 - 2,5nM- 5nM – 10nM - 30 nM |
| Swatch | PC-03, C-05, CS-37 |

Water hardness was adjusted by addition of CaCl₂, MgCl₂, and NaHCO₃ to the test system. After washing the textiles were rinsed in tap water and dried.

15 Table 10: Performance of proteases from *Bacillus sp.* compared to detergent without protease at 20°C

| Detergent | Laundry liquid model detergent B | | Small& Mighty | | Great Value | | Laundry liquid model detergent K | |
|-----------|----------------------------------|-------|---------------|-------|-------------|-------|----------------------------------|-------|
| | 10 nM | 30 nM | 10 nM | 30 nM | 10 nM | 30 nM | 10 nM | 30 nM |
| C-05 | 5.1 | 12.0 | 4.0 | 11.5 | 0 | 1.6 | 3.9 | 5.8 |
| PC-03 | 0.3 | 1.7 | 0.3 | 3.0 | 0.9 | 2.8 | 3.7 | 3.5 |
| CS-37 | 12.2 | 18.9 | 6.3 | 13.9 | 5.2 | 10.4 | 12.5 | 19.0 |

The results of Table 10 show that detergent comprising *Bacillus sp.* effectively improves wash performance on egg, blood/milk and chocolate/milk at 20°C.

5 **Example 7: Mini wash results for the proteases from *Bacillus sp.***

The wash performance of the proteases from *Bacillus sp.* was tested using laundry liquid model detergent on one technical stain using the mini wash system.

The Mini wash assay is a test method where soiled textile is continuously lifted up and down into the test solution and subsequently rinsed.

10

Table 11: The wash experiment is conducted under the experimental conditions specified below:

| | |
|----------------------|--|
| Detergent | Laundry liquid model detergent |
| Detergent dose | 8 g/l |
| pH | As is (i.e. not adjusted) |
| Water hardness | 15°dH, adjusted by adding CaCl ₂ *2H ₂ O, MgCl ₂ *6H ₂ O and NaHCO ₃ (4:1:7.5) to milli-Q water. |
| Enzyme conc. | Example 2.5 nM, 5 nM, 10 nM, 30 nM, 60 nM |
| Test solution volume | 50 ml |
| Test material | PC-03 Chocolate milk/soot |
| Temperature | 30°C |
| Wash time | 20 min |
| Rinse time | 10 min |
| Test system | Soiled textile continuously lifted up and down into the test solutions, 50 times per minute (up-time 0.29 sec, down-time 0.29 sec, lift time 0.17 sec). The test solutions are kept in 125 ml glass beakers. After wash of the textiles are continuously lifted up and down into tap water, 50 times per minute (up-time 0.5 sec, down-time 5 sec, lift time 0.5 sec). |

15 Test materials were obtained from EMPA Testmaterials AG Mövenstrasse 12, CH-9015 St. Gallen, Switzerland, from Center for Testmaterials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands, and WFK Testgewebe GmbH, Christenfeld 10, D-41379 Brüggen, Germany.

The textiles were subsequently air-dried and the wash performance was measured as the

brightness of the color of these textiles. Brightness can also be expressed as the Remission (R), which is a measure for the light reflected or emitted from the test material when illuminated with white light. The Remission (R) of the textiles was measured at 460 nm using a Zeiss MCS 521 VIS spectrophotometer. The measurements were done according to the manufacturer's protocol.

5 Calculating the enzyme effect was done by taking the measurements from washed swatches with enzymes and subtract with the measurements from washed without enzyme for each stain, $\Delta\text{Rem}_{\text{enzyme}}$.

10 The experiments were conducted as described in the mini wash assay for laundry method with the detergent composition and swatches described in table 1 and the experimental conditions as specified in Table 12 below.

Table 12: Experimental conditions for mini wash laundry experiments

| | |
|------------------------|--|
| Detergent dosage | Laundry liquid model detergent, 8g/L |
| Test solution volume | 50mL |
| pH | As is |
| Wash time | 20 minutes |
| Temperature | 30°C |
| Water hardness | 15°dH |
| Protease concentration | 0 – 1.2 nM- 2.5 nM – 4.7 nM –9.5 nM – 28.4nM |
| Swatch | PC-03 |

Water hardness was adjusted to 15°dH by addition of CaCl₂, MgCl₂, and NaHCO₃ (Ca²⁺:Mg²⁺:CO₃⁻ = 4:1:7.5) to the test system. After washing the textiles were rinsed in tap water and dried.

15 Table 13: Delta intensity value of detergent comprising proteases from *Bacillus sp.* compared to detergent without protease at 30°C

| Detergent | Enzyme dosage nM | Laundry liquid model detergent |
|---------------------|------------------|--------------------------------|
| <i>Bacillus sp.</i> | 0.0 | 0 |
| | 1.2 | 0 |
| | 2.4 | 0 |
| | 4.7 | 0.3 |
| | 9.5 | 0.4 |
| | 28.4 | 2.3 |

The results in Table 13 show that detergent comprising *Bacillus sp.* improves wash performance on chocolate/milk at 30°C.

Example 8: Full scale wash results for the proteases from *Bacillus sp.*

The wash performance of the protease from *Bacillus sp.* was tested in full scale wash. The wash performance was tested on 14 different stains at 90nM in laundry liquid model detergent

5 B.

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

Calculating the enzyme effect was done by taking the measurements from washed swatches with enzymes and subtract with the measurements from washed without enzyme for each stain, $\Delta\text{Rem}_{\text{enzyme}}$. Wash performance is expressed as a delta remission value (ΔRem).

The experiments were conducted with the detergent composition and swatches described in table 1 and the experimental conditions as specified in Table 14 below.

Table 14: Experimental conditions for full scale wash laundry experiments

| | |
|------------------------|--|
| Detergent dosage | Laundry liquid model detergent B 3.33 g/L |
| Test solution volume | 15 L |
| pH | As is |
| Wash time | 45 min |
| Temperature | 20°C |
| Water hardness | 15°dH |
| Protease concentration | 90nM |
| Swatches | CS-01, WE5DASBWKc, C-05, EMPA116, EMPA 117, C-03, PC-03, C-H010, EMPA 112, CS-37, 10EG, EMPA 164, C-10, 062 KC |

Water hardness was adjusted to 15°dH by addition of CaCl_2 , MgCl_2 , and NaHCO_3 ($\text{Ca}^{2+}:\text{Mg}^{2+}:\text{CO}_3^- = 4:1:7.5$) to the test system.

Table 15: Delta remission value of detergent comprising proteases from *Bacillus sp.* compared to detergent without protease at 20°C

| Stain | 90nM |
|------------|------|
| CS-01 | 2.1 |
| WE5DASBWKc | 2.7 |
| C-05 | 4.6 |
| EMPA 116 | 6.1 |
| EMPA 117 | 7.1 |
| C-03 | 1.2 |
| PC-03 | 6.0 |
| C-H010 | 4.8 |
| EMPA 112 | 5.3 |
| CS-37 | 13.0 |
| 10EG | 7.0 |
| C-10 | 6.2 |

The results of Table 15 show that detergent comprising *Bacillus sp.* effectively improves wash performance on egg (CS-37, WFK10EG), blood (CS-01, WE5DASBWKc), blood/milk/ink (C-05, EMPA116, EMPA117), grass (EMPA164), milk (C-10) and chocolate/milk (C-H010, PC-03, C-03, EMPA112) at 20°C.

Example 9: Storage stability assay of SEQ ID NO: 3 variants

Model B detergent: DC-2011-00146-35

Assay buffer: 100 mM Tris, pH 8.6

10 Substrate solution: 0.72 mg/ml Suc-Ala-Ala-Pro-Phe-pNA (Bachem L-1400) in assay buffer

Storage stability of protease variants in Model B liquid detergent was evaluated by mixing protease with detergent and measuring residual protease activity after 0, 2-2.5 and 23-25 hours incubation at 32 or 35°C. All variants were tested in duplicates and SEQ ID NO 3 reference culture supernatants were included on all plates. 30 µl culture supernatant containing a protease variant (or SEQ ID NO 3, reference) was mixed with 270 µl Model B liquid detergent 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 assay buffer (at least 5 min mixing 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 sec for 5 min (on a SpectraMax Plus). Activity was determined from slope of initial linear increase in absorbance at 405 nm. After sealing, the detergent plate was

incubated at 32 or 35°C in an Eppendorf Thermomixer (no shaking). After 2-2.5 and 23-25 hours incubation, 20 µl samples were withdrawn and residual activity of stressed sample was measured as with the initial, unstressed activity.

Decrease in activity during incubation with detergent was assumed to be exponential. Half
 5 lifes ($T_{1/2}$) were found from linear regression of Log(Activity) versus incubation time, and half-life improvement factors ($T_{1/2}$ IF) were calculated as half-life of protease variant relative to half-life of PTYH004 reference.

10 Table 16: Storage stability of variants in Model B. $T_{1/2}$ IF: Half-life improvement factor relative to SEQ ID NO 3 reference

| Mutation Compared to SEQ ID NO: 3 | $T_{1/2}$ IF relative to SEQ ID NO: 3 |
|--------------------------------------|---------------------------------------|
| D172N | 60 |
| V163R | 14 |
| G25C | 11 |
| G25W | 9 |
| G180I | 8.6 |
| D176P | 8 |
| P174*,A175* | 8 |
| D242G | 7.6 |
| G25L | 7.2 |
| S144I | 7.1 |
| G25R | 6.2 |
| S144V | 5.6 |
| P8K | 5.3 |
| K123P | 5 |
| D172K | 4.9 |
| G180Y | 4.54 |
| Y302M | 4.4 |
| S144F | 4.1 |
| P8D | 3.37 |
| P174H D207P | 3.1 |
| T294P | 2.87 |

| | |
|------------|-------|
| P8L G180M | 2.4 |
| D176P | 1.9 |
| Q287G | 1.9 |
| S144L | 1.6 |
| Y302R | 1.56 |
| D172N | 1.6 |
| I290C | 1.551 |
| L82V D172W | 1.5 |
| Y241P | 1.48 |
| L82G | 1.5 |
| A175S | 1.46 |
| D172E | 1.4 |
| V163W | 1.41 |
| K30E | 1.38 |
| A175K | 1.34 |
| Q287H | 1.32 |
| Q287T | 1.3 |
| L82H | 1.26 |
| D207P | 1.25 |
| L82F | 1.23 |
| N39D | 1.16 |
| Y302I | 1.14 |
| L82I | 1.11 |
| S145R | 1.1 |
| T40R | 1.1 |

CLAIMS

1. An isolated polypeptide having protease activity, selected from the group consisting of:
 - (a) a polypeptide having at least 98% sequence identity to the mature polypeptide of SEQ ID NO: 2;
 - (b) a polypeptide encoded by a polynucleotide having at least 98% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
 - (c) a variant of the mature polypeptide of SEQ ID NO: 2 comprising a substitution, deletion, and/or insertion at one or more (e.g. several) positions; and
 - (d) a fragment of the polypeptide of (a), (b) or (c) that has protease activity.
2. The polypeptide according to claim 1 comprising or consisting of SEQ ID NO: 3, or the mature polypeptide of SEQ ID NO: 2.
3. The polypeptide according to claim 2, wherein the mature polypeptide is amino acids 1 to 312 of SEQ ID NO: 2 or the polypeptide with SEQ ID NO: 3.
4. The polypeptide according to any one of claims 1 to 3, which is a variant of SEQ ID NO: 3 comprising a substitution, deletion, and/or insertion at one or more positions.
5. The polypeptide according to any one of claims 1 to 4, wherein said polypeptide compared to SEQ ID NO: 3 comprises one or more alteration selected from the list consisting of P8L, P8D, P8K, G25L, G25C, G25W, G25R, K30E, N39D, T40R, L82H, L82G, L82F, L82I, L82V, K123P, S144L, S144I, S144F, S144V, S145R, V163W, V163R, D172N, D172K, D172E, D172W, S173P, S173Y, P174H, S175P, A175S, A175K, P174*+ A175*, D176P, D176P, G180I, G180Y, G180M, D207P, D207P, Y241P, D242G, Q287G, Q287H, Q287T, Y302R, I290C, T294P, Y302I and Y302M.
6. A composition comprising an isolated polypeptide having protease activity selected from the group consisting of:
 - (a) at least 60% sequence identity, at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 92% sequence identity, at least 93% sequence identity, at least 94% sequence identity, at least 95% sequence identity, at least 96% sequence identity, at least 97% sequence identity, at least 98% sequence identity or at least 99% sequence identity to SEQ ID NO: 3;

- (b) a polypeptide encoded by a polynucleotide that hybridizes under medium stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1 or (ii) the full-length complementary strand of (i);
- (c) a polypeptide encoded by a polynucleotide having at least 60% sequence identity, at least 65% sequence identity, at least 70% sequence identity, at least 75% sequence identity, at least 80% sequence identity, at least 85% sequence identity, at least 90% sequence identity, at least 92% sequence identity, at least 93% sequence identity, at least 94% sequence identity, at least 95% sequence identity, at least 96% sequence identity, at least 97% sequence identity, at least 98% sequence identity or at least 99% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1;
- (d) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g. several) amino acids of SEQ ID NO: 3; and
- (e) a fragment of a polypeptide of (a), (b), (c), or (d) that has protease activity.
7. The composition according to claim 6 being a detergent composition such as a composition for laundry or automatic dish washing.
8. The composition according to claim 7 further comprising one of more additional enzymes selected among proteases, amylases, lipases, cutinases, cellulases, endoglucanases, xyloglucanases, pectinases, pectin lyases, xanthanases, peroxidases, haloperoxygenases, catalases, mannanases, or any mixture thereof.
9. The composition according to any one of claims 7 or 8 comprising one or more components selected among: surfactants, builders, bleaching systems, polymers and hydrotropes.
10. The detergent composition according to any one of the claims 5 to 9 in form of 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.
11. Use of a composition according to any one of the claims 5 to 10 in a cleaning process, such as laundry, hard surface cleaning, dish wash or automated dish wash.
12. Use of a polypeptide having protease activity in a cleaning process, wherein the polypeptide having protease activity is: a polypeptide having at least 60 % sequence identity to SEQ ID NO: 3.

13. A method for removing a stain from a surface which comprises contacting the surface with a composition according to any one of the claims 5 to 10.
- 5 14. An isolated polynucleotide encoding the polypeptide according to any one of claims 1 to 5.
15. A nucleic acid construct or expression vector comprising the polynucleotide according to claim 14 operably linked to one or more control sequences that direct the production of the polypeptide in an expression host.
- 10 16. A recombinant host cell comprising the polynucleotide according to claim 14 operably linked to one or more control sequences that direct the production of the polypeptide.
17. A method of producing the polypeptide according to any one of claims 1 to 5 comprising:
- 15 (a) cultivating a cell, which in its wild-type form produces the polypeptide, under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.
18. A method according to claim 17, wherein the cell is a *Bacillus*.
- 20 19. A method of producing a polypeptide having protease activity comprising:
- (a) cultivating the host cell according to claim 16 under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.
- 25

INTERNATIONAL SEARCH REPORT

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|---|
| International application No PCT/EP2016/074714 |
|---|

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|--|--|-----------------------|
| A. CLASSIFICATION OF SUBJECT MATTER INV. C12N9/54 C11D3/386 C12N15/75 ADD. | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED | | |
| Minimum documentation searched (classification system followed by classification symbols) C12N C11D | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, BIOSIS, EMBASE | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
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