

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(10) International Publication Number  
**WO 2015/193488 A1**

(43) International Publication Date  
23 December 2015 (23.12.2015)

- (51) **International Patent Classification:**  
*C12N 9/52* (2006.01) *C11D 3/386* (2006.01)
- (21) **International Application Number:**  
PCT/EP2015/063847
- (22) **International Filing Date:**  
19 June 2015 (19.06.2015)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
14173280.0 20 June 2014 (20.06.2014) EP
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- (81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))



WO 2015/193488 A1

(54) **Title:** METALLOPROTEASE FROM KRIBBELLA ALUMINOSA AND DETERGENT COMPOSITIONS COMPRISING THE METALLOPROTEASE

(57) **Abstract:** The present invention relates to a new metalloprotease and the use thereof in cleaning processes, such as laundry and dish wash. The invention also relates to detergent compositions and cleaning compositions comprising a metalloprotease.

## METALLOPROTEASE FROM KRIBBELLA ALUMINOSA AND DETERGENT COMPOSITIONS COMPRISING THE METALLOPROTEASE

### Reference to sequence listing

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

### 5 FIELD OF THE INVENTION

The present invention relates to new metalloproteases (E.C 3.4.24) from *Kribbella aluminosa* and the use thereof in cleaning processes, such as dish wash and laundry. The invention further relates to cleaning and/or detergent compositions comprising metalloproteases.

### BACKGROUND OF THE INVENTION

10 The detergent industry has for more than 30 years implemented different enzymes in detergent formulations, most commonly used enzymes includes proteases, amylases and lipases each adapted for removing various types of stains. In addition to the enzymes detergent compositions typically include a complex combination of ingredients. For example, most cleaning products include surfactant system, bleaching agents or builders.

15 The proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. In textile laundry the proteases remove protein stains such as grass, blood, egg and human sweat. These organic stains have a tendency to adhere strongly to textile fibres. Proteases hydrolyse the proteins in the stains and break them down into more soluble polypeptides or free  
20 amino acids.

Alkaline serine proteases are the most important group of protease exploited commercially in the detergent industry. Subtilisins are a prototypical group of bacterial serine proteases used extensively in detergents due to their stability and activity at high temperature and alkaline pH. A commercially important subtilisin used extensively in the detergent industry is Savinase®.

25 New interest in properties such as low-temperature performance and development of new detergent compositions has led to renewed interest for new proteases and other types of detergent enzymes. The search for new proteases is not limited to subtilisins, but is also directed at finding new protease backbones suitable for application in detergent products.

30 Metalloproteases is a group of proteases which has not yet been commercially applied in detergent industry, mainly due to low stability in detergent compositions as well as under the conditions during the wash process. Metalloproteases are proteolytic enzymes having an absolute requirement for metal ion for their activity. Most metalloproteases are zinc-dependent, although some use other transition metals. Metalloproteases have been widely used in different

industries like food and brewing industry. One group of metalloproteases is the M4 family metalloproteases which have been used in various applications. For example, the M4 metalloprotease from *Bacillus amyloliquefaciens*, also known as Neutrase®, has been used for many years as an additive in various food and feed products. This metalloprotease has also been described for use in detergent and cleaning compositions and processes e.g., in WO 2007/044993. Use of storage-stable metalloprotease variants in detergent and a method of production is described in WO 2009/058518. EP 1 288 282, describes a blend of a metalloprotease and a serine protease for use in dish washing. WO 2000/60042 also describes detergent compositions containing a metalloprotease.

However, known metalloproteases are very unstable under conventional wash conditions and in conventional detergent compositions. Thus, the use of metalloproteases in detergents and in wash and cleaning processes has not yet been commercialized.

Therefore there remains a need for metalloproteases having increased stability under wash conditions and/or in detergent compositions. The present invention is directed to providing such enzymes.

A nucleotide sequence encoding a predicted metalloendopeptidase has been identified in a *Streptomyces* sp. The encoded protein UNIPROT:S2YJW6 is 84% identical to the metalloprotease of the present invention.

## SUMMARY OF THE INVENTION

The present invention relates to an isolated polypeptide having protease activity selected from the group consisting of:

- a. a polypeptide having at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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 of SEQ ID NO: 2;
- 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 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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;

- 5
- d. a variant comprising a substitution, deletion, and/or insertion of one or more (e.g. several) amino acids of the mature polypeptide of SEQ ID NO: 2; and
  - e. a fragment of a polypeptide of (a), (b), (c), or (d) that has protease activity.

The isolated polypeptide of the invention is a metalloprotease belonging to the M4 metalloprotease group. Preferably the isolated polypeptide is derived from *Kribbella* sp. Even more preferably the isolated polypeptide is derived from *Kribbella aluminosa*.

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The invention further relates to 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% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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 of SEQ ID NO: 2;
  - b. a polypeptide encoded by a polynucleotide that hybridizes under high 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 the mature polypeptide of SEQ ID NO: 2; and
  - e. a fragment of a polypeptide of (a), (b), (c), or (d) that has protease activity.
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The invention further relates to such compositions which are in particular detergent compositions, and to the use of such composition in a cleaning process such as laundry and hard surface cleaning e.g. automated dish wash.

In a particular embodiment, the invention relates to polynucleotides encoding the polypeptides of the invention, constructs, expression vectors and host cells comprising such polynucleotides and the use thereof for the production of the polypeptide of the invention.

### Overview of sequence listing

SEQ ID NO 1: DNA sequence of metalloprotease from *Kribbella aluminosa*.

10 SEQ ID NO 2: Is the amino acid sequence as deduced from SEQ ID NO: 1.

SEQ ID NO 3: *Bacillus clausii* secretion signal

SEQ ID NO 4: *Bacillus amyloliquefaciens* metalloprotease Uniprot: P06832 (Neutrase®)

SEQ ID NO 5: *Bacillus clausii* serine protease Uniprot: P29600 (Savinase®)

## 15 DETAILED DESCRIPTION OF THE INVENTION

### Definitions

“Polypeptides having protease activity” 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. The present invention provides for the use of polypeptides having protease activity in detergent compositions. It also provides polynucleotides encoding the polypeptides. The proteases of the invention are metalloproteases of the MEOPS family M4. The polypeptides of the present invention have at least 20%, e.g., at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, and at least 100% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

30 “Protease activity” can be measured using any assay, in which a substrate is employed, that includes peptide bonds relevant for the specificity of the protease in question. Assay-pH and assay-temperature are likewise to be adapted to the protease in question. Examples of

assay-pH-values are pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12. Examples of assay-temperatures are 15, 20, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65, 70, 80, 90, or 95°C. Examples of general protease substrates are casein, bovine serum albumin and haemoglobin. In the classical Anson and Mirsky method, denatured haemoglobin is used as substrate and after the assay incubation with the protease in question, the amount of trichloroacetic acid soluble haemoglobin is determined as a measurement of protease activity (Anson, M.L. and Mirsky, A.E., 1932, *J. Gen. Physiol.* 16: 59 and Anson, M.L., 1938, *J. Gen. Physiol.* 22: 79).

For the purpose of the present invention, protease activity was determined using assays which are described in "Materials and Methods", such as the Protazyme OL assay.

The term "metalloprotease" as used herein refers to a protease having one or more metal ions in the binding/active site.

The term "M4 Metalloprotease Family" or "M4 Metalloprotease" or "M4" as used herein means a polypeptide falling into the M4 metalloprotease family according to Rawlings et al., *Biochem. J.*, 290, 205-218 (1993) and as further described in MEROPS - (Rawlings et al., MEROPS: the peptidase database, *Nucl Acids Res*, 34 Database issue, D270-272, 2006). The M4 metalloproteases are neutral metalloproteases containing mainly endopeptidases. All peptidases in the family bind a single, catalytic zinc ion. M4 metalloprotease family members include the common HEXXH motif, where the histidine residues serve as zinc ligands and glutamate is an active site residue. M4 metalloproteases have a pH optimum mainly at neutral pH. The M4 metalloprotease family includes, e.g., Neutrase® (classified as MEROPS subclass M04.014), Thermolysin, Bacillolysin, vibriolysin, pseudolysin, Msp peptidase, coccolysin, aureolysin, vimelysin, lambda toxin neutral peptidase B, PA peptidase (*Aeromonas*-type), griselysin, stearylolysin, MprIII (*Alteromonas* sp. strain O-7), pap6 peptidase, neutral peptidase (*Thermoactinomyces*-type), ZmpA peptidase (*Burkholderia* sp.), zpx peptidase, PrtS peptidase (*Photorhabdus luminescens*), protealysin, ZmpB peptidase (*Burkholderia* sp.). The M4 metalloprotease family of polypeptides has been further characterized and presently includes, according to MEROPS, at least twenty-two subclasses for which a distinct MEROPS ID (i.e., an identifier of the formula M04.xxx) has been assigned, as well as non-peptidase homologues and unassigned peptidases.

The term "isolated" means a substance in a form or environment which does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally

associated (e.g., multiple copies of a gene encoding the substance; use of a stronger promoter than the promoter naturally associated with the gene encoding the substance). An isolated substance may be present in a fermentation broth sample. A fermentation broth resulting from the fermentation of a recombinant host cell expressing the polypeptide of the invention will comprise  
5 the polypeptide in an isolated form.

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  
10 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 variant or polypeptide by well-known recombinant methods or by classical purification methods.

The term "mature polypeptide" means a polypeptide in its final form following translation and any post-translational modifications, such as N-terminal processing, C-terminal truncation, glycosylation, phosphorylation, etc. In one aspect, the mature polypeptide is amino acids 1 to 489 of SEQ ID NO: 2 based on the determination of the N-terminal sequence GTGNSMY. Further, amino acids -191 to -172 of SEQ ID NO: 2 are a signal peptide. Amino acids -171 to -1 of SEQ ID NO 2 are a propeptide, based on the determination of the N-terminal and the signal sequence. Mass spectroscopy showed that a significant part of the polypeptides was C-terminally truncated and consisted of amino acids 1 to 414 of SEQ ID NO: 2. It is well-known in the art that a host cell may produce a mixture of two or more different mature polypeptides (i.e., with a different C-terminal and/or N-terminal amino acid) expressed by the same polynucleotide. In one aspect, the mature polypeptide is amino acids 1 to 414 of SEQ ID NO: 2.  
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The term "mature polypeptide coding sequence" means a polynucleotide that encodes a mature polypeptide having protease activity. In one aspect, the mature polypeptide coding sequence is nucleotides 574 to 2040 of SEQ ID NO: 1 based on the experimental determination of the N-terminal sequence. Nucleotides 1 to 60 of SEQ ID NO: 1 encodes a signal peptide. Nucleotides 61 to 573 of SEQ ID NO: 1 encodes a propeptide.  
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The term "very low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern  
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blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 60°C.

The term “low stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 50°C. The term “medium stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 55°C.

The term “medium-high stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 35% formamide, following standard Southern blotting procedures for 12 to 24 hours.

The term “high stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 65°C.

The term “very high stringency conditions” means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 70°C. 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 3.0.0 or later. 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:

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



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. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the `-nobrief` option) is used as the percent identity and is calculated as follows:

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

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

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 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. An M4 metalloprotease may be truncated such that certain domain is removed to generate a functional fragment, which may be polypeptides where less than 200 amino acids have been removed from the mature M4 Metalloprotease, preferably less than 150 amino acids, more preferably less than 120, 100, 80, 60, 40, 30 amino acids, even more preferably less than 20 amino acids and most preferably less than 10 amino acids, such as 9, 8, 7, 6, 5, 4, 3, 2 or one amino acids have been removed from the 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 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 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 (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 position; and an insertion means adding 1, 2 or even 3 amino acids adjacent to an amino acid occupying a position.

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, tooth-pastes), 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, or spray compositions), as long as the composition is compatible with the metalloprotease 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 dishwash detergents).

The term "detergent composition", includes unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, mouthwashes, denture cleaners, car or carpet shampoos, bathroom cleaners; hair shampoos and hair-rinses; shower gels, foam baths; metal cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types.

The terms "detergent composition" and "detergent formulation" are used in reference to mixtures which are intended for use in a wash medium for the cleaning of soiled objects. In some embodiments, the term is used in reference to laundering fabrics and/or garments (e.g., "laundry detergents"). In alternative embodiments, the term refers to other detergents, such as

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

10           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, 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., garments and other articles).

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

          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) composition is required, and the like. The term "effective amount" of a metalloprotease refers to the quantity of metalloprotease described hereinbefore that achieves a desired level of enzymatic activity, e.g., in a defined detergent composition.

          The term "wash performance" of an enzyme refers to the contribution of an enzyme to washing that provides additional cleaning performance to the detergent without the addition of the enzyme to the composition. Wash performance is compared under relevant washing conditions. Wash performance of enzymes is conveniently measured by their ability to remove certain representative stains under appropriate test conditions. In these test systems, other relevant factors, such as detergent composition, detergent concentration, water hardness, washing me-

chanics, time, pH, and/or temperature, can be controlled in such a way that conditions typical for household application in a certain market segment are imitated.

5 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 litre of 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.

10 The term "improved property" is used to indicate that a better end result is obtained in a property compared to the same process performed without the enzyme. Exemplary properties which are preferably improved in the processes of the present invention include wash performance, enzyme stability, enzyme activity and substrate specificity.

15 The term "improved wash performance" is used to indicate that a better end result is obtained in stain removal from items washed (e.g., fabrics or dishware and/or cutlery) under relevant washing conditions as compared to no enzyme or to a reference enzyme, or that less enzyme, on weight basis, is needed to obtain the same end result relative to no enzyme or to a reference enzyme. Improved wash performance could in this context also be that the same effect, e.g., stain removal effect is obtained in shorter wash time, e.g., the enzymes provide their effect more quickly under the tested conditions.

20 The term "enzyme detergency" or "detergency" or "detergency effect" is defined herein as the advantageous effect an enzyme may add to a detergent compared to the same detergent without the enzyme. Important detergency benefits which can be provided by enzymes are stain removal with no or very little visible soils after washing and/or cleaning, prevention or reduction of redeposition of soils released in the washing process an effect that also is termed anti-  
25 redeposition, restoring fully or partly the whiteness of textiles, which originally were white but after repeated use and wash have obtained a greyish or yellowish appearance an effect that also is termed whitening. Textile care benefits, which are not directly related to catalytic stain removal or prevention of redeposition of soils, are also important for enzyme detergency benefits. Examples of such textile care benefits are prevention or reduction of dye transfer from one  
30 fabric to another fabric or another part of the same fabric an effect that is also termed dye transfer inhibition or anti-back staining, removal of protruding or broken fibers from a fabric surface to decrease pilling tendencies or remove already existing pills or fuzz an effect that also is termed anti-pilling, improvement of the fabric-softness, colour clarification of the fabric and removal of particulate soils which are trapped in the fibers of the fabric or garment. Enzymatic bleaching is

a further enzyme detergency benefit where the catalytic activity generally is used to catalyze the formation of bleaching component such as hydrogen peroxide or other peroxides.

5 The term "anti-redeposition" as used herein describes the reduction or prevention of re-deposition of soils dissolved or suspended in the wash liquor onto the cleaned objects. Redeposition may be seen after one or multiple washing cycles (e.g., as a greying, yellowing or other discolorations).

10 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, powder, bar, paste, spray, tablet, gel, or foam composition), which materials are also preferably compatible with the metalloprotease enzyme 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.

15 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 discoloration of the material by itself or which leaves a sticky surface on the object which may attract soils dissolved in the washing liquor thereby resulting in discoloration 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 components in the grass and allows the green/brown colour to be released during washing.

25 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.

30 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.

35 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 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.

## 5 Polypeptides having protease activity

In an embodiment, the present invention relates to isolated polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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, at least 99% sequence identity or at least 100% sequence identity, which have protease activity. In one aspect, the polypeptides differ by no more than 10 amino acids, *e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, or 9, from the mature polypeptide of SEQ ID NO: 2.

In a particular embodiment, the invention relates to polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of 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%, and wherein the polypeptide has at least at least 70% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

In a particular embodiment, the invention relates to polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of 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%, and wherein the polypeptide has at least at least 75% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

In a particular embodiment, the invention relates to polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of 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%, and wherein the polypeptide has at least at least 80% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

In a particular embodiment, the invention relates to polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of 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%, and wherein the

polypeptide has at least at least 85% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

5 In a particular embodiment, the invention relates to polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of 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%, and wherein the polypeptide has at least at least 90% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

10 In a particular embodiment, the invention relates to polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of 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%, and wherein the polypeptide has at least at least 95% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

15 In a particular embodiment, the invention relates to polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of 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%, and wherein the polypeptide has at least at least 100% of the protease activity of the mature polypeptide of SEQ ID NO: 2.

20 In a preferred embodiment, the present invention relates to isolated polypeptides having protease activity selected from the group consisting of:

(a) a polypeptide having at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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, at least 99% sequence identity or at least 100% sequence identity to the mature polypeptide of SEQ ID NO: 2;

30 (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 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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, at least 99% sequence identity or at least 100% 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 the mature polypeptide of SEQ ID NO: 2; and

(e) a fragment of a polypeptide of (a), (b), (c), or (d) that has protease activity.

The present invention relates to isolated polypeptides having a sequence identity to the mature polypeptide of SEQ ID NO: 2 of at least 85%, at least 86%, at least 87%, at least 88%, at least 89% sequence identity, 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%, which have protease activity. In one aspect, the polypeptides differ by no more than ten amino acids, e.g., by five amino acids, by four amino acids, by three amino acids, by two amino acids, and by one amino acid from the mature polypeptide of SEQ ID NO: 2.

A polypeptide of the present invention preferably comprises or consists of the amino acid sequence of SEQ ID NO: 2 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 or an allelic variant thereof; or is a fragment thereof having protease activity. In another preferred aspect, the polypeptide comprises or consists of amino acids 1 to 489 of SEQ ID NO: 2. In another preferred aspect, the polypeptide comprises or consists of amino acids 1 to 414 of SEQ ID NO: 2.

The present invention also relates to isolated polypeptides having protease activity that are encoded by polynucleotides that hybridize under very low stringency conditions, low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1, (ii) the genomic DNA sequence encoding the mature polypeptide coding sequence of SEQ ID NO: 1, or (iii) the full-length complementary strand of (i) or (ii) (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York).

The polynucleotide of SEQ ID NO: 1 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO: 2 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 or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence,



but should be at least 14, *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 <sup>32</sup>P, <sup>3</sup>H, <sup>35</sup>S, 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 is homologous with SEQ ID NO: 1 or a subsequence thereof, the carrier material is preferably 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 the mature polypeptide coding sequence of SEQ ID NO: 1; the genomic DNA sequence comprising the mature polypeptide coding sequence of SEQ ID NO: 1; its full-length complementary strand; or 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.

In one aspect, the nucleic acid probe is the mature polypeptide coding sequence of SEQ ID NO: 1. In another aspect, the nucleic acid probe is nucleotides 574 to 2040 of SEQ ID NO: 1. In another aspect, the nucleic acid probe is a polynucleotide that encodes the polypeptide of SEQ ID NO: 2 or a fragment thereof. In another preferred aspect, the nucleic acid probe is SEQ ID NO: 1.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), at 50°C (low stringency), at 55°C (medium stringency), at 60°C (medium-high stringency), at 65°C (high stringency), and at 70°C (very high stringency).

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization and hybridization at about 5°C to about 10°C below the calculated  $T_m$  using the calculation according to Bolton and McCarthy (1962, *Proc. Natl. Acad. Sci. USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once in 6X SSC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated  $T_m$ .

The present invention relates to isolated polypeptides having protease activity encoded by polynucleotides having a sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1 of at least at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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, at least 99% sequence identity or at least 100%.

The present invention further relates to an isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids -191 to -172 of SEQ ID NO: 2.

The invention further relates to a polynucleotide comprising a polynucleotide encoding a propeptide comprising or consisting of amino acids -171 to -1 of SEQ ID NO: 2 .

The present invention also relates to variants comprising a substitution, deletion, and/or insertion of one or more (or several) amino acids of the mature polypeptide of SEQ ID NO: 2, or a homologous sequence thereof. Preferably the variant has at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% sequence identity, at least 92% sequence, 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, at least 99% sequence identity %, but less than 100% identity to the mature polypeptide of SEQ ID NO: 2.

Substantially homologous polypeptides of the sequences described above are characterized as having one or more (several) amino acid substitutions, deletions, and/or insertions in the mature polypeptide. Preferably, amino acid changes are 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 one to about nine amino acids, such as one, two, three, four, five, six, seven, eight or nine amino acids; preferably from one to about 15 amino acids, such as 10, 11, 12, 13, 14 or 15 amino acids; and most preferably from one to

about 30 amino acids, such as 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about five to ten residues, preferably from 10 to 15 residues and most preferably from 20 to 25 residues, or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tag, an antigenic epitope, protein A, a carbohydrate binding module or a another binding domain.

Examples of conservative substitutions are within the group 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. The most commonly occurring exchanges 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 parent 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 variant 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 identities of essential amino acids can also be inferred from analysis of identities with polypeptides that are related to the parent polypeptide, such as e.g. the well-known metalloproteinase Thermolysin. The catalytic residues may thus be determined by alignment with known M4 metalloprotease where it has been found that the catalytic residues are conserved in all such proteases. All M4 metalloproteases bind a single, catalytic zinc ion.

All M4 metalloproteases bind a single, catalytic zinc ion. As in many other families of metalloproteases, the present metalloprotease comprises a HEXXH motif, in which H134 and H138 of the HEXXH motif together with E158 are zinc ligands. Residue E135 of the HEXXH motif and H230 are the active site residues (numbering according to SEQ ID NO 2).

5 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).

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 amino acid residues in a polypeptide.

20 The total number of amino acid substitutions, deletions and/or insertions of the mature polypeptide of SEQ ID NO: 2 are not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8 or 9.

In addition to the 20 standard amino acids, non-standard amino acids (such as 4-hydroxyproline, 6-N-methyl lysine, 2-aminoisobutyric acid, isovaline, and alpha-methyl serine) may be substituted for amino acid residues of a wild-type polypeptide. A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, and unnatural amino acids may be substituted for amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, and preferably, are commercially available, and include pipercolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

30 The polypeptide may be hybrid polypeptide in which a portion of one polypeptide is fused at the N-terminus or the C-terminus of a portion of another polypeptide.

The polypeptide may be a fused polypeptide or cleavable fusion polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide of the present invention. A fused polypeptide is produced by fusing a polynucleotide encoding another polypeptide to a polynucleotide of the present invention. Techniques for producing fusion

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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 fused polypeptide is under control of the same promoter(s) and terminator. Fusion proteins may also be constructed using intein technology in which fusions 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. 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.

#### 15 Sources of M4 metalloproteases

A M4 Metalloprotease useful in 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 nucleotide sequence is produced by the source in which it is naturally present or by a strain in which the nucleotide sequence from the source has been inserted. In a preferred aspect, the polypeptide obtained from a given source is secreted extracellularly.

A polypeptide of the present invention may be a bacterial polypeptide. For example, the polypeptide may be a gram-positive bacterial polypeptide such as a *Chryseobacterium*, *Bacillus*, *Clostridium*, *Enterococcus*, *Geobacillus*, *Kribbella*, *Lactobacillus*, *Lactococcus*, *Oceanobacillus*, *Staphylococcus*, *Streptococcus*, or *Streptomyces* polypeptide having metalloprotease activity, or a gram-negative bacterial polypeptide such as a *Campylobacter*, *E. coli*, *Flavobacterium*, *Fusobacterium*, *Helicobacter*, *Ilyobacter*, *Neisseria*, *Pseudomonas*, *Salmonella*, and *Ureaplasma* polypeptide.

In one aspect, the polypeptide is a polypeptide derived from *Kribbella*, preferably from *Kribbella aluminosa*, e.g., the polypeptide in 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 (NRRL).

Furthermore, such polypeptides may be identified and obtained from other sources including microorganisms isolated from nature (e.g., soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The polynucleotide may then be obtained by similarly screening a genomic or cDNA library of such a microorganism. Once a polynucleotide sequence encoding a polypeptide has been detected e.g. with the probe(s), the polynucleotide can be isolated or cloned by utilizing techniques which are well known to those of ordinary skill in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

Polypeptides of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleotide sequence (or a portion thereof) encoding another polypeptide to a nucleotide sequence (or a portion thereof) 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 fused polypeptide is under control of the same promoter(s) and terminator.

### **Polynucleotides**

The present invention also relates to isolated polynucleotides encoding a polypeptide of the present invention, as described herein.

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 *Kribbella*, e.g. from a strain of *Kribbella aluminosa*, 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.

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 variant, 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 (Agaisse 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*),  
5 *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*  
10 *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  
15 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 variant, truncated, and hybrid promoters thereof.

20 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.  
25 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  
30 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 (*rrnB*).

Preferred terminators for filamentous fungal host cells are obtained from the genes for  
35 *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*.

5 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).

10 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 15 *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, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

20 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 that is functional in the host cell may be used.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from 25 the genes for *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* glucoamylase, *Aspergillus niger* alpha-glucosidase *Aspergillus oryzae* TAKA amylase, and *Fusarium oxysporum* trypsin-like protease.

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

30 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 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 35 coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. A foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, a foreign

signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of a host cell may be used.

5 Effective signal peptide coding sequences for bacterial host cells are the signal peptide coding sequences obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis* *prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

10 Effective signal peptide coding sequences for filamentous fungal host cells are the signal 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.

15 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  
20 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,  
25 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  
30 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,  
35 *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.

## 5 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. 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  
10 allow for insertion or substitution of the polynucleotide encoding the polypeptide at such sites. Alternatively, the polynucleotide may be expressed by inserting the polynucleotide or a nucleic acid construct comprising the polynucleotide into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

15 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.

20 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  
25 together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids that together contain the total DNA to be introduced into the genome of the host cell, or a transposon, may be used.

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  
30 gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to 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  
35 cells include, but are not limited to, ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for 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 *pyrG* genes and a *Streptomyces hygroscopicus bar* gene.

5 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 pAM $\beta$ 1 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., Sambrook *et al.*, 1989, *supra*).

## 10 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 of a polypeptide of the present invention. A construct or vector comprising a polynucleotide is introduced into a host cell so that the construct or vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

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*. 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.

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.

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 ascosporogenous yeast (Endomycetales), basidiosporogenous 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 The host cell may be a plant cell, comprising a polynucleotide of the present invention so as to express and produce a polypeptide or domain in recoverable quantities. The polypeptide or domain may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the polypeptide or domain may be used as such for improving the quality of a food or feed, *e.g.*, improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

10 The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, *Poa*), forage grass such as *Festuca*, *Lolium*, temperate grass, such as *Agrostis*, and cereals, *e.g.*, wheat, oats, rye, barley, rice, sorghum, and maize (corn).

15 Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family *Brassicaceae*), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

20 Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers as well as the individual tissues comprising these parts, *e.g.*, epidermis, mesophyll, parenchyme, vascular tissues, meristems. Specific plant cell compartments, such as chloroplasts, apoplasts, mitochondria, vacuoles, peroxisomes and cytoplasm are also considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part. Likewise, plant parts such as specific tissues and cells isolated to facilitate the utilization of the invention are also considered plant parts, *e.g.*, embryos, endosperms, aleurone and seed coats.

Also included within the scope of the present invention are the progeny of such plants, plant parts, and plant cells.

25 The transgenic plant or plant cell expressing the polypeptide or domain may be constructed in accordance with methods known in the art.

### Methods of production

30 The present invention also relates to methods of producing a polypeptide 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 aspect, the cell is a *Kribbella* cell. In a more preferred aspect, the cell is a *Kribbella aluminosa* cell.



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.

One embodiment relates to a method of producing a polypeptide having at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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 of SEQ ID NO: 2; comprising (a) cultivating a recombinant host cell of the present invention under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

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, 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 lysates.

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

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 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.

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.

5

### **Signal peptide and propeptide**

The present invention also relates to an isolated polynucleotide encoding a signal peptide comprising or consisting of amino acids -191 to -172 of SEQ ID NO: 2. The present invention also relates to an isolated polynucleotide encoding a propeptide comprising or  
10 consisting of amino acids -171 to -1 of SEQ ID NO: 2. The present invention also relates to an isolated polynucleotide encoding a signal peptide and a propeptide comprising or consisting of amino acids -191 to -1 of SEQ ID NO: 2. The polynucleotides may further comprise a gene encoding a protein, which is operably linked to the signal peptide and/or propeptide. The protein is preferably foreign to the signal peptide and/or propeptide. In one aspect, the polynucleotide  
15 encoding the signal peptide is nucleotides 1 to 60 of SEQ ID NO: 1. In another aspect, the polynucleotide encoding the propeptide is nucleotides 61 to 573 of SEQ ID NO: 1. In another aspect, the polynucleotide encoding the signal peptide and the propeptide is nucleotides 1 to 573 of SEQ ID NO: 1.

The present invention also relates to nucleic acid constructs, expression vectors and  
20 recombinant host cells comprising such polynucleotides.

The present invention also relates to methods of producing a protein, comprising (a) cultivating a recombinant host cell comprising such polynucleotide; and (b) recovering the protein.

The protein may be native or heterologous to a host cell. The term "protein" is not meant  
25 herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and polypeptides. The term "protein" also encompasses two or more polypeptides combined to form the encoded product. The proteins also include hybrid polypeptides and fused polypeptides.

Preferably, the protein is a hormone, enzyme, receptor or portion thereof, antibody or  
30 portion thereof, or reporter. For example, the protein may be a hydrolase, isomerase, ligase, lyase, oxidoreductase, or transferase, e.g., an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellobiohydrolase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, endoglucanase, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase,  
35 mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, xylanase, or beta-xylosidase.

The gene may be obtained from any prokaryotic, eukaryotic, or other source.

## Compositions

5 The present invention also relates to compositions comprising a metalloprotease of the invention. Preferably, the compositions are enriched in a metalloprotease 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 metalloprotease of the invention and a suitable carrier and/or excipient.

10 The present invention also relates to 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% sequence identity, at least 90% sequence identity, at least 91% sequence identity, at least 92% sequence identity, at least  
15 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, at least 99% sequence identity or even 100% sequence identity to the mature polypeptide of SEQ ID NO: 2; 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%  
20 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 91% 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%  
25 sequence identity, at least 97% sequence identity, at least 98% sequence identity, at least 99% sequence identity or even 100% sequence identity to the mature polypeptide coding sequence of SEQ ID NO: 1; e) a variant comprising a substitution, deletion, and/or insertion of one or more (e.g. several) amino acids of the mature polypeptide of SEQ ID NO: 2; and f) a fragment of a polypeptide of (a), (b), (c), or (d) that has protease activity.

30 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 60% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 65% sequence identity to the mature polypeptide of  
35 SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 70% sequence identity to the mature polypeptide of SEQ ID NO: 2.

5 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 75% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 80% sequence identity to the mature polypeptide of SEQ ID NO: 2.

10 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 85% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 90% sequence identity to the mature polypeptide of  
15 SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 91% sequence identity to the mature polypeptide of SEQ ID NO: 2.

20 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 92% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 93% sequence identity to the mature polypeptide of SEQ ID NO: 2.

25 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 94% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 95% sequence identity to the mature polypeptide of  
30 SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 96% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 97% sequence identity to the mature polypeptide of SEQ ID NO: 2.

5 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 98% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having at least 99% sequence identity to the mature polypeptide of SEQ ID NO: 2.

10 In one embodiment, the compositions of the invention comprise an isolated polypeptide having protease activity and having 100% sequence identity to the mature polypeptide of SEQ ID NO: 2.

In one embodiment, the present invention relates to compositions in particular to cleaning compositions and/or detergent compositions comprising a metalloprotease of the invention and a suitable carrier and/or excipient. In one embodiment, the detergent composition may be adapted for specific uses such as laundry, in particular household laundry, dish washing or hard surface cleaning.

The detergent compositions of the invention may be formulated, for example, as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations. The detergent compositions of the invention may find use in hard surface cleaning, automatic dishwashing applications, as well as cosmetic applications such as dentures, teeth, hair and skin.

25 In one embodiment, the present invention relates to compositions in particular to cleaning compositions and/or detergent compositions comprising a polypeptide having at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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 of SEQ ID NO: 2 and a suitable detergent component such as surfactants, builders, bleaches, polymers as described below.

35 In one embodiment, the present invention relates to compositions in particular to cleaning compositions and/or detergent compositions comprising a polypeptide having at least 85%

sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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 of SEQ ID NO: 2 and an additional enzyme such as additional protease, amylase, lipase, cellulase etc. as described below.

In a preferred embodiment, the detergent compositions comprise one or more conventional carrier(s) and/or excipient(s) such as those exemplified below. The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or non-aqueous.

Unless otherwise noted, all component or composition levels provided herein are made in reference to the active level of that component or composition, and are exclusive of impurities, for example, residual solvents or by-products, which may be present in commercially available sources.

The metalloprotease of the invention is normally incorporated in the detergent composition at a level of from 0.000001% to 2% of enzyme protein by weight of the composition, preferably at a level of from 0.00001% to 1% of enzyme protein by weight of the composition, more preferably at a level of from 0.0001% to 0.75% of enzyme protein by weight of the composition, even more preferably at a level of from 0.001% to 0.5% of enzyme protein by weight of the composition.

Furthermore, the metalloprotease of the invention is normally incorporated in the detergent composition in such amounts that their concentration in the wash water is at a level of from 0.0000001% to 1% enzyme protein, preferably at a level of from 0.000005% to 0.01% of enzyme protein, more preferably at a level of from 0.000001% to 0.005% of enzyme protein, even more preferably at a level of from 0.00001% to 0.001% of enzyme protein in wash water.

As is well known, the amount of enzyme will also vary according to the particular application and/or as a result of the other components included in the compositions.

A composition for use in automatic dishwash (ADW), for example, may include 0.001%-50%, such as 0.01%-25%, such as 0.02%-20%, such as 0.1-15% of enzyme protein by weight of the composition.

A composition for use in laundry granulation, for example, may include 0.0001%-50%, such as 0.001%-20%, such as 0.01%-15%, such as 0.05%-10% of enzyme protein by weight of the composition.

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

In some preferred embodiments, the detergent compositions provided herein are typically formulated such that, during use in aqueous cleaning operations, the wash water has a pH of  
5 from about 5.0 to about 11.5, or in alternative embodiments, even from about 6.0 to about 10.5, such as from about 5 to about 11, from about 5 to about 10, from about 5 to about 9, from about 5 to about 8, from about 5 to about 7, from about 6 to about 11, from about 6 to about 10, from about 6 to about 9, from about 6 to about 8, from about 6 to about 7, from about 7 to about 11, from about 7 to about 10, from about 7 to about 9, or from about 7 to about 8. In some preferred  
10 embodiments, granular or liquid laundry products are formulated such that the wash water has a pH from about 5.5 to about 8. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

The present metalloprotease from *Kribbella aluminosa* is active at alkaline pH as shown in Example 4 and thus may be applied in alkaline detergent systems, such as detergent systems having a pH of 7 to 11, a pH of 8 to 10, e.g., a pH of around 9.5.  
15

Enzyme components weights are based on total protein. All percentages and ratios are calculated by weight unless otherwise indicated. All percentages and ratios are calculated based on the total composition unless otherwise indicated. In the exemplified detergent composition, the enzymes levels are expressed by pure enzyme by weight of the total composition and  
20 unless otherwise specified, the detergent ingredients are expressed by weight of the total composition.

The enzymes of the present invention also find use in detergent additive products. A detergent additive product comprising a metalloprotease of the invention is ideally suited for inclusion in a wash process when, e.g., temperature is low, such as at temperatures about 40°C or  
25 below, the pH is between 6 and 8 and the washing time short, e.g., below 30 min.

The detergent additive product may be a metalloprotease of the invention and preferably an additional enzyme. In one embodiment, the additive is packaged in dosage form for addition to a cleaning process. The single dosage may comprise a pill, tablet, gelcap or other single dosage unit including powders and/or liquids. In some embodiments, filler and/or carrier material(s) are included, suitable filler or carrier materials include, but are not limited to, various salts of sulfate, carbonate and silicate as well as talc, clay and the like. In some embodiments filler and/or carrier materials for liquid compositions include water and/or low molecular weight primary and secondary alcohols including polyols and diols. Examples of such alcohols include, but are not limited to, methanol, ethanol, propanol and isopropanol.  
30

In one particularly preferred embodiment, the metalloprotease according to the invention is employed in a granular composition or liquid, the metalloprotease may be in form of an encapsulated particle. In one embodiment, the encapsulating material is selected from the group consisting of carbohydrates, natural or synthetic gums, chitin and chitosan, cellulose and cellulose derivatives, silicates, phosphates, borates, polyvinyl alcohol, polyethylene glycol, paraffin waxes and combinations thereof.

The compositions according to the invention typically comprise one or more detergent ingredients. The term detergent compositions include articles and cleaning and treatment compositions. The term cleaning composition includes, unless otherwise indicated, tablet, granular or powder- form all-purpose or "heavy-duty" washing agents, especially laundry detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use. The composition can also be in unit dose packages, including those known in the art and those that are water soluble, water insoluble and/or water permeable.

In embodiments in which cleaning and/or detergent components may not be compatible with the metalloprotease of the present invention, suitable methods may be used for keeping the cleaning and/or detergent components and the metalloprotease separated (i.e., not in contact with each other) until combination of the two components is appropriate. Such separation methods include any suitable method known in the art (e.g., gelcaps, encapsulation, tablets, and physical separation e.g., by use of a water dissolvable pouch having one or more compartments).

As mentioned when the metalloprotease of the invention is employed as a component of a detergent composition (e.g., a laundry washing detergent composition, or a dishwashing detergent composition), it may, for example, be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are polyethyleneglycol (PEG) products with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591.



In some embodiments, the enzymes employed herein are stabilized by the presence of water-soluble sources of zinc (II), calcium (II) and/or magnesium (II) ions in the finished compositions that provide such ions to the enzymes, as well as other metal ions (e.g., barium (II), scandium (II), iron (II), manganese (II), aluminum (III), tin (II), cobalt (II), copper (II), nickel (II), and oxovanadium (IV)). The enzymes of the detergent compositions of the invention may also be stabilized using conventional stabilizing agents such as polyol, e.g., propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708. The enzymes of the invention may also be stabilized by adding reversible enzyme inhibitors, e.g., of the protein type (as described in EP 544 777) or the boronic acid type. Other enzyme stabilizers are well known in the art, such as peptide aldehydes and protein hydrolysate, e.g. the metalloproteases according to the invention may be stabilized using peptide aldehydes or ketones such as described in WO2005/105826 and WO2009/118375.

Protected enzymes for inclusion in a detergent composition of the invention may be prepared, as mentioned above, according to the method disclosed in EP 238 216.

The composition may be augmented with one or more agents for preventing or removing the formation of the biofilm. These agents may include, but are not limited to, dispersants, surfactants, detergents, other enzymes, anti-microbials, and biocides.

The compositions of the invention may be applied in dosing elements to be used in an auto-dosing device. The dosing elements comprising the composition of the present invention can be placed into a delivery cartridge as that described in WO 2007/052004 and WO 2007/0833141. The dosing elements can have an elongated shape and set into an array forming a delivery cartridge which is the refill for an auto-dosing dispensing device as described in case WO 2007/051989. The delivery cartridge is to be placed in an auto-dosing delivery device, such as that described in WO 2008/053191.

Suitable disclosure of auto-dosing devices can be found in WO 2007/083139, WO 2007/051989, WO 2007/083141, WO 2007/083142 and EP2361964,

#### Other enzymes

In one embodiment, a metalloprotease of the invention is combined with one or more enzymes, such as at least two enzymes, more preferred at least three, four or five enzymes. Preferably, the enzymes have different substrate specificity, e.g., proteolytic activity, amylolytic activity, lipolytic activity, hemicellulytic activity or pectolytic activity.

The detergent additive as well as the detergent composition may comprise one or more enzymes such as a 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 animal, vegetable or microbial origin.

5 Particularly suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered variants 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.

10 Especially suitable cellulases are the alkaline or neutral cellulases having color care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and WO 1999/001544.

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

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

25 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.

30 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  
35 WO89/06279 and protease PD138 described in (WO93/18140). Other useful proteases may be

those described in WO92/175177, WO01/016285, WO02/026024 and WO02/016547. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO89/06270, WO94/25583 and WO05/040372, and the chymotrypsin proteases derived from *Cellulomonas* described in WO05/052161 and WO05/052146.

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

Examples of metalloproteases are the neutral metalloprotease as described in WO07/044993 (Genencor Int.) such as those derived from *Bacillus amyloliquefaciens*.

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

Suitable commercially available protease enzymes include those sold under the trade names Alcalase®, Duralase™, Durazym™, Relase®, Relase® Ultra, Savinase®, Savinase® Ultra, Primase®, Polarzyme®, Blaze®, Blaze Evity®, Kannase®, Liquanase®, Liquanase® Ultra, Ovozyme®, Coronase®, Coronase® Ultra, Neutrase®, Everlase® and Esperase® (Novozymes A/S), those sold under the tradename Maxatase®, Maxacal®, Maxapem®, Purafect®, Purafect Prime®, Preferenz™, Purafect MA®, Purafect Ox®, Purafect OxP®, Puramax®, Properase®, Effectenz™, FN2®, FN3®, FN4®, Excellase®, 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  
30 Kao.

Lipases: Suitable lipases include those of animal, vegetable or microbial origin. Particularly suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered variants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g., from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068  
35 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas* lipase, e.g., from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri*

(GB 1,372,034), *P. fluorescens*, *Pseudomonas* sp. strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g., from *B. subtilis* (Dartois et al., 1993, *Biochemica et Biophysica Acta*, 1131: 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422).

5 Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include Lipolase™, Lipolase Ultra™, and Lipex™ (Novozymes A/S).

10 Amylases: Suitable amylases which can be used together with metalloprotease of the invention may be an alpha-amylase or a glucoamylase and may be of bacterial or fungal origin. Chemically modified or protein engineered variants 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. Suitable amylases include amylases having SEQ ID  
15 NO: 3 in WO 95/10603 or variants having 90% sequence identity to SEQ ID NO: 3 thereof. Preferred variants are described in WO 94/02597, WO 94/18314, WO 97/43424 and SEQ ID NO: 4 of WO 99/019467, such as variants with substitutions in one or more of the following positions: 15, 23, 105, 106, 124, 128, 133, 154, 156, 178, 179, 181, 188, 190, 197, 201, 202, 207, 208, 209, 211, 243, 264, 304, 305, 391, 408, and 444. Different suitable amylases include  
20 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. 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  
25 90% sequence identity thereof. Preferred variants of this hybrid alpha-amylase are those having a substitution, a deletion or an insertion in one of more of the following positions: G48, T49, G107, H156, A181, N190, M197, I201, A209 and Q264. Most preferred variants of the hybrid alpha-amylase comprising residues 1-33 of the alpha-amylase derived from *B. amyloliquefaciens* shown in SEQ ID NO: 6 of WO 2006/066594 and residues 36-483 of SEQ ID  
30 NO: 4 are those having the substitutions:

M197T;

H156Y+A181T+N190F+A209V+Q264S; or

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.

5 Particularly 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 SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 7 are

10 those having a substitution, a deletion or an insertion in one or more of the following positions: 140, 181, 182, 183, 184, 195, 206, 212, 243, 260, 269, 304 and 476. More preferred variants are those having a deletion in positions 181 and 182 or positions 183 and 184. Most preferred amylase variants of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 7 are those having a deletion in positions 183 and 184 and a substitution in one or more of positions 140, 195, 206, 243, 260,

15 304 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 or more of the following positions: 176, 177, 178,

20 179, 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 or more of the following positions: Q87, Q98, S125, N128, T131, T165, K178, R180, S181, T182, G183, M201, F202, N225, S243, N272, N282, Y305,

25 R309, D319, Q320, Q359, K444 and G475. More preferred variants of SEQ ID NO: 2 are those having the substitution in one or 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

30 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

35 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 or 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, 5 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 10 substitutions in all these positions. Other examples are amylase variants such as those described in WO2011/098531, 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 15 Inc./DuPont).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered variants 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.

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

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, *i.e.*, a separate additive or a combined additive, can be formulated, for example, as a granulate, liquid, slurry, etc. Preferred detergent 25 additive formulations are granulates, in particular non-dusting granulates as described above, liquids, in particular stabilized liquids, or slurries.

### Surfactants

Typically, the detergent composition comprises (by weight of the composition) one or more surfactants in the range of 0% to 50%, preferably from 2% to 40%, more preferably from 30 5% to 35%, more preferably from 7% to 30%, most preferably from 10% to 25%, even most preferably from 15% to 20%. In a preferred embodiment the detergent is a liquid or powder detergent comprising less than 40%, preferably less than 30%, more preferably less than 25%, even more preferably less than 20% by weight of surfactant. The composition may comprise from 1% to 15%, preferably from 2% to 12%, 3% to 10%, most preferably from 4% to 8%, even 35 most preferably from 4% to 6% of one or more surfactants. Preferred surfactants are anionic

surfactants, non-ionic surfactants, cationic surfactants, zwitterionic surfactants, amphoteric surfactants, and mixtures thereof. Preferably, the major part of the surfactant is anionic. Suitable anionic surfactants are well known in the art and may comprise fatty acid carboxylates (soap), branched-chain, linear-chain and random chain alkyl sulfates or fatty alcohol sulfates or primary alcohol sulfates or alkyl benzenesulfonates such as LAS and LAB or phenylalknesulfonates or alkenyl sulfonates or alkenyl benzenesulfonates or alkyl ethoxysulfates or fatty alcohol ether sulfates or alpha-olefin sulfonate or dodecenyyl/tetradecnylsuccinic acid. The anionic surfactants may be alkoxyated. The detergent composition may also comprise from 1 wt% to 10 wt% of non-ionic surfactant, preferably from 2 wt% to 8 wt%, more preferably from 3 wt% to 7 wt%, even more preferably less than 5 wt% of non-ionic surfactant. Suitable non-ionic surfactants are well known in the art and may comprise alcohol ethoxylates, and/or alkyl ethoxylates, and/or alkylphenol ethoxylates, and/or glucamides such as fatty acid N-glucosyl N-methyl amides, and/or alkyl polyglucosides and/or mono- or diethanolamides or fatty acid amides. The detergent composition may also comprise from 0 wt% to 10 wt% of cationic surfactant, preferably from 0.1 wt% to 8 wt%, more preferably from 0.5 wt% to 7 wt%, even more preferably less than 5 wt% of cationic surfactant. Suitable cationic surfactants are well known in the art and may comprise alkyl quaternary ammonium compounds, and/or alkyl pyridinium compounds and/or alkyl quaternary phosphonium compounds and/or alkyl ternary sulphonium compounds. The composition preferably comprises surfactant in an amount to provide from 100 ppm to 5,000 ppm surfactant in the wash liquor during the laundering process. The composition upon contact with water typically forms a wash liquor comprising from 0.5 g/l to 10 g/l detergent composition. Many suitable surface active compounds are available and fully described in the literature, for example, in "Surface- Active Agents and Detergents", Volumes I and 11, by Schwartz, Perry and Berch.

## 25 Builders

The main role of builder is to sequester divalent metal ions (such as calcium and magnesium ions) from the wash solution that would otherwise interact negatively with the surfactant system. Builders are also effective at removing metal ions and inorganic soils from the fabric surface, leading to improved removal of particulate and beverage stains. Builders are also a source of alkalinity and buffer the pH of the wash water to a level of 9.5 to 11. The buffering capacity is also termed reserve alkalinity, and should preferably be greater than 4.

The detergent compositions of the present invention may comprise one or more detergent builders or builder systems. Many suitable builder systems are described in the literature, for example in Powdered Detergents, Surfactant science series volume 71, Marcel Dekker, Inc. Builder may comprise from 0% to 60%, preferably from 5% to 45%, more preferably from 10% to 40%, most preferably from 15% to 35%, even more preferably from 20% to 30% builder by

weight of the subject composition. The composition may comprise from 0% to 15%, preferably from 1% to 12%, 2% to 10%, most preferably from 3% to 8%, even most preferably from 4% to 6% of builder by weight of the subject composition.

Builders include, but are not limited to, the alkali metal, ammonium and alkanolammonium salts of polyphosphates (e.g., tripolyphosphate STPP), alkali metal silicates, alkaline earth and alkali metal carbonates, aluminosilicate builders (e.g., zeolite) and polycarboxylate compounds, ether hydroxypolycarboxylates, copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1, 3, 5-trihydroxy benzene-2, 4, 6-trisulphonic acid, and carboxymethylloxysuccinic acid, the various alkali metal, ammonium and substituted ammonium salts of polyacetic acids such as ethylenediamine tetraacetic acid and nitrilotriacetic acid, as well as polycarboxylates such as mellitic acid, succinic acid, citric acid, oxydisuccinic acid, polymaleic acid, benzene 1,3,5-tricarboxylic acid, carboxymethylloxysuccinic acid, and soluble salts thereof. Ethanolamines (MEA, DEA, and TEA) may also contribute to the buffering capacity in liquid detergents.

### Bleaches

The detergent compositions of the present invention may comprise one or more bleaching agents. In particular powdered detergents may comprise one or more bleaching agents. Suitable bleaching agents include other photobleaches, pre-formed peracids, sources of hydrogen peroxide, bleach activators, hydrogen peroxide, bleach catalysts and mixtures thereof. In general, when a bleaching agent is used, the compositions of the present invention may comprise from about 0.1% to about 50% or even from about 0.1% to about 25% bleaching agent by weight of the subject cleaning composition. Examples of suitable bleaching agents include:

(1) other photobleaches for example Vitamin K3;

(2) preformed peracids: Suitable preformed peracids include, but are not limited to, compounds selected from the group consisting of percarboxylic acids and salts, percarbonic acids and salts, perimidic acids and salts, peroxymonosulfuric acids and salts, for example, Oxone, and mixtures thereof. Suitable percarboxylic acids include hydrophobic and hydrophilic peracids having the formula  $R-(C=O)O-O-M$  wherein R is an alkyl group, optionally branched, having, when the peracid is hydrophobic, from 6 to 14 carbon atoms, or from 8 to 12 carbon atoms and, when the peracid is hydrophilic, less than 6 carbon atoms or even less than 4 carbon atoms; and M is a counterion, for example, sodium, potassium or hydrogen;

(3) sources of hydrogen peroxide, for example, inorganic perhydrate salts, including alkali metal salts such as sodium salts of perborate (usually mono- or tetra-hydrate), percarbonate, persulphate, perphosphate, persilicate salts and mixtures thereof. In one aspect of the invention the inorganic perhydrate salts are selected from the group consisting of sodium salts of perborate, percarbonate and mixtures thereof. When employed, inorganic perhydrate salts



are typically present in amounts of from 0.05 to 40 wt%, or 1 to 30 wt% of the overall composition and are typically incorporated into such compositions as a crystalline solid that may be coated. Suitable coatings include inorganic salts such as alkali metal silicate, carbonate or borate salts or mixtures thereof, or organic materials such as water-soluble or dispersible polymers, waxes, oils or fatty soaps. Useful bleaching compositions are described in U.S. Patent Nos. 5,576,282, and 6,306,812;

(4) bleach activators having R-(C=O)-L wherein R is an alkyl group, optionally branched, having, when the bleach activator is hydrophobic, from 6 to 14 carbon atoms, or from 8 to 12 carbon atoms and, when the bleach activator is hydrophilic, less than 6 carbon atoms or even less than 4 carbon atoms; and L is leaving group. Examples of suitable leaving groups are benzoic acid and derivatives thereof - especially benzene sulphonate. Suitable bleach activators include dodecanoyl oxybenzene sulphonate, decanoyl oxybenzene sulphonate, decanoyl oxybenzoic acid or salts thereof, 3,5,5-trimethyl hexanoyloxybenzene sulphonate, tetraacetyl ethylene diamine (TAED) and nonanoyloxybenzene sulphonate (NOBS). Suitable bleach activators are also disclosed in WO 98/17767. While any suitable bleach activator may be employed, in one aspect of the invention the subject cleaning composition may comprise NOBS, TAED or mixtures thereof; and

(5) bleach catalysts that are capable of accepting an oxygen atom from peroxyacid and transferring the oxygen atom to an oxidizable substrate are described in WO 2008/007319. Suitable bleach catalysts include, but are not limited to: iminium cations and polyions; iminium zwitterions; modified amines; modified amine oxides; N-sulphonyl imines; N-phosphonyl imines; N-acyl imines; thiadiazole dioxides; perfluoroimines; cyclic sugar ketones and mixtures thereof. The bleach catalyst will typically be comprised in the detergent composition at a level of from 0.0005% to 0.2%, from 0.001% to 0.1%, or even from 0.005% to 0.05% by weight.

When present, the peracid and/or bleach activator is generally present in the composition in an amount of from about 0.1 to about 60 wt%, from about 0.5 to about 40 wt% or even from about 0.6 to about 10 wt% based on the composition. One or more hydrophobic peracids or precursors thereof may be used in combination with one or more hydrophilic peracid or precursor thereof.

The amounts of hydrogen peroxide source and peracid or bleach activator may be selected such that the molar ratio of available oxygen (from the peroxide source) to peracid is from 1:1 to 35:1, or even 2:1 to 10:1.

#### Adjunct materials

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.

5 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  
10 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. Any fluorescent whitening agent suitable for use  
15 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 diamino stilbene-sulphonic acid derivatives, diarylpyrazoline derivatives and bisphenyl-distyryl derivatives. Examples of the diamino stilbene-sulphonic acid derivative type of fluorescent whitening agents include the sodium salts of:

20 4,4'-bis-(2-diethanolamino-4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate,  
4,4'-bis-(2,4-dianilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate,  
4,4'-bis-(2-anilino-4(N-methyl-N-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-  
disulphonate,  
4,4'-bis-(4-phenyl-2,1,3-triazol-2-yl)stilbene-2,2'-disulphonate,  
25 4,4'-bis-(2-anilino-4(1-methyl-2-hydroxy-ethylamino)-s-triazin-6-ylamino) stilbene-2,2'-  
disulphonate and,

2-(stilbyl-4"-naphtho-1.,2':4,5)-1,2,3-triazole-2"-sulphonate. Preferred fluorescent whitening agents are Tinopal DMS and Tinopal CBS available from Ciba-Geigy AG, Basel, Switzerland. Tinopal DMS is the disodium salt of 4,4'-bis-(2-morpholino-4 anilino-s-triazin-6-ylamino) stilbene disulphonate. Tinopal CBS is the disodium salt of 2,2'-bis-(phenyl-styryl) disulphonate.  
30

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.

35 Suitable fluorescent brightener levels include lower levels of from about 0.01, from 0.05, from about 0.1 or even from about 0.2 wt% to upper levels of 0.5 or even 0.75 wt%.

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 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 1 876 226. 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.

Soil release polymers - The detergent compositions of the present invention may also include one or more soil release polymers which aid the removal of soils from fabrics such as cotton and polyester based fabrics, in particular the removal of hydrophobic soils from polyester based fabrics. The soil release polymers may for example be nonionic or anionic 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 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. 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. Other soil release polymers are substituted polysaccharide structures especially substituted cellulosic structures such as modified cellulose derivatives such as those described in EP 1 867 808 or WO 2003/040279. Suitable cellulosic polymers include cellulose, cellulose ethers, cellulose esters, cellulose amides and mixtures thereof. Suitable cellulosic polymers include anionically modified cellulose, nonionically modified cellulose, cationically modified cellulose, zwitterionically modified cellulose, and mixtures thereof. Suitable cellulosic polymers include methyl cellulose, carboxy methyl cellulose, ethyl cellulose, hydroxyl ethyl cellulose, hydroxyl propyl methyl cellulose, ester carboxy methyl cellulose, and mixtures thereof.

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, structurants for liquid detergents and/or structure elasticizing agents.

In one aspect, the detergent is a compact fluid laundry detergent composition comprising: a) at least about 10%, preferably from 20 to 80% by weight of the composition, of surfactant selected from anionic surfactants, non-ionic surfactants, soap and mixtures thereof; b) from about 1% to about 30%, preferably from 5 to 30%, by weight of the composition, of water; c) from about 1% to about 15%, preferably from 3 to 10% by weight of the composition, of non-aminofunctional solvent; and d) from about 5% to about 20%, by weight of the composition, of a performance additive selected from chelants, soil release polymers, enzymes and mixtures thereof; wherein the compact fluid laundry detergent composition comprises at least one of: (i) the surfactant has a weight ratio of the anionic surfactant to the nonionic surfactant from about 1.5:1 to about 5:1, the surfactant comprises from about 15% to about 40%, by weight of the composition, of anionic surfactant and comprises from about 5% to about 40%, by weight of the composition, of the soap; (ii) from about 0.1% to about 10%, by weight of the composition, of a suds boosting agent selected from suds boosting polymers, cationic surfactants, zwitterionic surfactants, amine oxide surfactants, amphoteric surfactants, and mixtures thereof; and (iii) both (i) and (ii). All the ingredients are described in WO 2007/130562. Further polymers useful in detergent formulations are described in WO 2007/149806.

In another aspect, the detergent is a compact granular (powdered) detergent comprising a) at least about 10%, preferably from 15 to 60% by weight of the composition, of surfactant selected from anionic surfactants, non-ionic surfactants, soap and mixtures thereof; b) from about 10 to 80% by weight of the composition, of a builder, preferably from 20% to 60% where the builder may be a mixture of builders selected from i) phosphate builder, preferably less than 20%, more preferably less than 10% even more preferably less than 5% of the total builder is a phosphate builder; ii) a zeolite builder, preferably less than 20%, more preferably less than 10% even more preferably less than 5% of the total builder is a zeolite builder; iii) citrate, preferably 0 to 5% of the total builder is a citrate builder; iv) polycarboxylate, preferably 0 to 5% of the total builder is a polycarboxylate builder v) carbonate, preferably 0 to 30% of the total builder is a

carbonate builder and vi) sodium silicates, preferably 0 to 20% of the total builder is a sodium silicate builder; c) from about 0% to 25% by weight of the composition, of fillers such as sulphate salts, preferably from 1% to 15%, more preferably from 2% to 10%, more preferably from 3% to 5% by weight of the composition, of fillers; and d) from about 0.1% to 20% by weight of the composition, of enzymes, preferably from 1% to 15%, more preferably from 2% to 10% by weight of the composition, of enzymes.

#### **Use of the M4 metalloprotease in detergents**

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.

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

In a preferred aspect of the present invention, the metalloprotease of the invention useful according to the invention may be combined with at least two enzymes. These additional enzymes are described in details in the section "other enzymes", 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. The enzyme combination may for example be a metalloprotease of the invention with another stain removing enzyme, e.g., a metalloprotease of the invention and a protease, a metalloprotease of the invention and a serine protease, a metalloprotease of the invention and another metalloprotease, a metalloprotease of the invention and an amylase, a metalloprotease of the invention and a cellulase, a metalloprotease of the invention and a hemicellulase, a metalloprotease of the invention and a lipase, a metalloprotease of the invention and a cutinase, a metalloprotease of the invention and a pectinase or a metalloprotease of the invention and an anti-redeposition enzyme. More preferably, the metalloprotease of the invention is combined

with at least two other stain removing enzymes, e.g., a metalloprotease of the invention, a lipase and an amylase; or a metalloprotease of the invention, a protease and an amylase; or a metalloprotease of the invention, a protease and a lipase; or a metalloprotease of the invention, a protease and a pectinase; or a metalloprotease of the invention, a protease and a cellulase; or  
5 a metalloprotease of the invention, a protease and a hemicellulase; or a metalloprotease of the invention, a protease and a cutinase; or a metalloprotease of the invention, an amylase and a pectinase; or a metalloprotease of the invention, an amylase and a cutinase; or a metalloprotease of the invention, an amylase and a cellulase; or a metalloprotease of the invention, an amylase and a hemicellulase; or a metalloprotease of the invention, a lipase and a pectinase; or a  
10 metalloprotease of the invention, a lipase and a cutinase; or a metalloprotease of the invention, a lipase and a cellulase; or a metalloprotease of the invention, a lipase and a hemicellulase. Even more preferably, a metalloprotease of the invention may be combined with at least three other stain removing enzymes, e.g., a metalloprotease of the invention, a protease, a lipase and an amylase; or a metalloprotease of the invention, a protease, an amylase and a pectinase; or a  
15 metalloprotease of the invention, a protease, an amylase and a cutinase; or a metalloprotease of the invention, a protease, an amylase and a cellulase; or a metalloprotease of the invention, a protease, an amylase and a hemicellulase; or a metalloprotease of the invention, an amylase, a lipase and a pectinase; or a metalloprotease of the invention, an amylase, a lipase and a cutinase; or a metalloprotease of the invention, an amylase, a lipase and a cellulase; or a metalloprotease of the invention, an amylase, a lipase and a hemicellulase; or a metalloprotease of the invention, a protease, a lipase and a pectinase; or a metalloprotease of the invention, a protease, a lipase and a cutinase; or a metalloprotease of the invention, a protease, a lipase and a cellulase; or a metalloprotease of the invention, a protease, a lipase and a hemicellulase. A metalloprotease according to the present invention may be combined with any of the enzymes  
25 selected from the non-exhaustive list comprising: carbohydrases, such as an amylase, a hemicellulase, a pectinase, a cellulase, a xanthanase or a pullulanase, a peptidase, a protease or a lipase.

In a preferred embodiment, a metalloprotease of the invention is combined with a serine protease, e.g., an S8 family protease such as Savinase®.

30 In another embodiment of the present invention, a metalloprotease of the invention useful according to the present invention may be combined with one or more other metalloproteases, such as another M4 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,  
35 a dishwashing process or cleaning of hard surfaces such as bathroom tiles, floors, table tops, drains, sinks and washbasins. Laundry processes can for example be household laundering,

but it may also be industrial laundering. Furthermore, the invention relates to a process for laundering of fabrics and/or garments where the process comprises treating fabrics with a washing solution containing a detergent composition, and at least one metalloprotease of the invention. The cleaning process or a textile care process can for example be carried out in a machine washing process or in a manual washing process. The washing solution can for example be an aqueous washing solution containing a detergent composition.

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 cellulose, including cotton, flax, linen, jute, ramie, sisal or coir or manmade cellulose (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 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).

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

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

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 discolor often by oxidation and many bleaches also have strong bactericidal properties, and are used for disinfecting

and sterilizing. Yet other components like builder and chelator softens, e.g., the wash water by removing the metal ions from the liquid.

In a particular embodiment, the invention concerns the use of a composition comprising a metalloprotease 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 component in laundry or dish wash.

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 metalloprotease 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 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 metalloprotease of the invention, such as a conventional amount of such component. In one aspect, the metalloprotease 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

The detergent compositions 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. 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 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.

The metalloprotease from *Kribbella aluminosa* is active at alkaline pH as shown in Example 4. In a preferred embodiment the metalloprotease is used in a detergent composition for application in a washing method conducted at a pH of 7 to 11, a pH of 8 to 10, e.g., a pH of around 9.5.

10 In particular embodiments, the washing method is conducted at a degree of hardness of 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,  
15 about 25°dH, about 26°dH, about 27°dH, about 28°dH, about 29°dH, about 30°dH. Under typical European wash conditions, 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 metalloprotease of the invention.

20 A preferred embodiment concerns a method of cleaning, said method comprising the steps of: contacting an object with a cleaning composition comprising a metalloprotease 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.

25 Still another embodiment relates to a method for removing stains from fabric which comprises contacting said a fabric with a composition comprising a metalloprotease 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  
30 an enzyme selected from the group consisting of carbohydrases, 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.

Low temperature uses

One embodiment of the invention concerns a method of doing laundry, dish wash or industrial cleaning comprising contacting a surface to be cleaned with a metalloprotease of the invention, and wherein said laundry, dish wash, industrial or institutional cleaning is performed at a temperature of about 40°C or below. One embodiment of the invention relates to the use of a metalloprotease in laundry, dish wash or a cleaning process wherein the temperature in laundry, dish wash, industrial cleaning is about 40°C or below

In another embodiment, the invention concerns the use of a metalloprotease according to the invention in a protein removing process, wherein the temperature in the protein removing process is about 40°C or below.

The present invention also relates to the use in laundry, dish wash or industrial cleaning process of a metalloprotease having at least one improved property compared to a commercial metalloprotease such as Neutrase® and wherein the temperature in laundry, dish wash or cleaning process is performed at a temperature of about 40°C or below.

In each of the above-identified methods and uses, the wash temperature is about 40°C or below, such as about 39°C or below, such as about 38°C or below, such as about 37°C or below, such as about 36°C or below, such as about 35°C or below, such as about 34°C or below, such as about 33°C or below, such as about 32°C or below, such as about 31°C or below, such as about 30°C or below, such as about 29°C or below, such as about 28°C or below, such as about 27°C or below, such as about 26°C or below, such as about 25°C or below, such as about 24°C or below, such as about 23°C or below, such as about 22°C or below, such as about 21°C or below, such as about 20°C or below, such as about 19°C or below, such as about 18°C or below, such as about 17°C or below, such as about 16°C or below, such as about 15°C or below, such as about 14°C or below, such as about 13°C or below, such as about 12°C or below, such as about 11°C or below, such as about 10°C or below, such as about 9°C or below, such as about 8°C or below, such as about 7°C or below, such as about 6°C or below, such as about 5°C or below, such as about 4°C or below, such as about 3°C or below, such as about 2°C or below, such as about 1°C or below.

In another preferred embodiment, the wash temperature is in the range of about 5-40°C, such as about 5-30°C, about 5-20°C, about 5-10°C, about 10-40°C, about 10-30°C, about 10-20°C, about 15-40°C, about 15-30°C, about 15-20°C, about 20-40°C, about 20-30°C, about 25-40°C, about 25-30°C, or about 30-40°C. In particular preferred embodiments the wash temperature is about 20°C, about 30°C, or about 40°C.

In particular embodiments, the low temperature 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, about 5.5 to about 9, preferably about 6 to about 8.5, and more preferably about 6 to about 8.

In particular embodiments, the low temperature washing method is conducted at a degree of hardness of 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, 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.

#### Use in removing egg stains

Another particular embodiment of the invention concerns removal of egg stains. These types of stain are often very difficult to remove completely. Egg stains are particularly problematic in hard surface cleaning such as dish wash where the stains often remain on the plates and cutlery after washing. The metalloproteases of the invention are particularly suitable for removing egg stains.

Thus, the invention further concerns methods for removing egg stains from textiles, fabrics and/or hard surfaces like dishes and cutlery in particular from fabrics and textiles. A preferred aspect of the invention concerns a method of removing egg stains from textiles and/or fabrics comprising contacting a surface in need of removal of an egg stain with a metalloprotease of the invention. In one embodiment, the invention comprises a method of removing egg stains from textiles and/or fabrics comprising contacting a surface in need of removal of an egg stain with a detergent composition comprising a metalloprotease of the invention. The invention also concerns a method of removing egg stains comprising adding a metalloprotease of the invention to a laundry and/or washing process wherein said textiles and/or fabric comprises various egg stains.

One embodiment of the present invention relates to a method for removal of egg stains from a hard surface or from laundry, the method comprising contacting the egg stain-containing hard surface or the egg stain-containing laundry with a cleaning or detergent composition, preferably a laundry or dish wash composition, containing a metalloprotease of the invention.

Another embodiment relates a method for removing egg stains from fabric or textile which comprises contacting the fabric or textile with a cleaning or detergent composition, preferably a laundry or dish wash composition, comprising a metalloprotease of the invention.

5 A still further embodiment relates to a method for removing egg stains from fabric or textile which comprises contacting said a fabric or textile with a composition comprising a metalloprotease of the invention, wherein said composition 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 a carbohydrase, a peptidase, a protease, a lipase, a cellulase, a xylanase, a cutinase or a combination hereof.

10 In particular embodiments, the egg removing 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  
15 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, about 5.5 to about 9, preferably about 6 to about 8.5, and more preferably about 6 to about 8.

20 In particular embodiments, the egg removing method is conducted at a degree of hardness of 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  
25 typical European wash conditions, 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.

All documents cited herein are incorporated by reference in the entirety.

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

30

## EXAMPLES

### Materials and methods

#### **Test material for wash assays**

The grass stain swatch 062KC was obtained from Warwick Equest Ltd, Unit 55, Consett Business Park, Consett County Durham, DH8 6BN England,

The remaining swatches were obtained from Center for Testmaterials BV, P.O. Box 120, 3133 KT Vlaardingen, the Netherlands.

5

<b>Table 1. Swatch types used</b>
062KC, Whole scrubbed grass on cotton
C-05, Blood/milk/ink on cotton
C-H010, Cocoa, cooked up with milk on cotton
CS-01, Aged blood on cotton
CS-37, Full egg/pigment on cotton
CS-38, Egg yolk/pigment with heating on cotton
EMPA117EH, Blood/milk/ink on cotton/polyester, extra heated
PC-03, Chocolate/milk/soot on cotton/polyester
PC-05, Blood/milk/ink on cotton/polyester

### Model detergents

The model detergents B, J, M, and Y were used. Model detergent B is similar to a European type liquid detergent composition. Model detergent J is similar to a US type liquid detergent composition. Model detergent M is similar to a Chinese type powder detergent composition. Model detergent Y is similar to a Developing and Emerging (D&E) Asia Pacific market type powder detergent composition.

10

Table 2a. Laundry liquid model detergent B		
<b>Compound</b>	<b>Content of compound (% w/w)</b>	<b>% active component (% w/w)</b>
LAS	7.20	7.0
AEOS, SLES	10.58	3.00
Soy fatty acid	2.75	2.48
Coco fatty acid	2.75	2.80
AEO	6.60	6.60
Sodium hydroxide	1.05	1.1
Ethanol / Propan-2-ol	3.00	2.70/0.30
MPG	6.00	6.00

Glycerol	1.71	1.70
TEA	3.33	3.30
Sodium formate	1.00	1.00
Sodium citrate	2.00	2.00
DTMPA	0.48	0.20
PCA	0.46	0.18
Phenoxy ethanol	0.50	0.50
H <sub>2</sub> O, ion exchanged	50.59	50.6

Table 2b. Laundry liquid model detergent J

Compound	Content of compound (% w/w)	% active component (% w/w)
LAS	5.15	5.00
AS	5.00	4.50
AEOS	14.18	10.00
Coco fatty acid	1.00	1.00
AEO	5.00	5.00
MEA	0.30	0.30
MPG	3.00	3.00
Ethanol	1.50	1.35
DTPA (as Na <sub>5</sub> salt)	0.25	0.10
Sodium citrate	4.00	4.00
Sodium formate	1.00	1.00
Sodium hydroxide	0.66	0.66
H <sub>2</sub> O, ion exchanged	58.95	58.95

Table 2c. Laundry powder model detergent M

Compound	Content of compound (% w/w)
Sodium sulfate	41.23
Sodium carbonate	15
Zeolite	20
Sodium silicate	6
LAS	13.6
AEO (7)	1.92
Polycarboxylate (Dow 445N)	1
FWA (CBS-X)	0.05
Perfume	0.15

Table 2d Laundry powder model detergent Y

Compound	Content of compound (% w/w)
Sodium sulfate	42.1
Sodium carbonate	20
Zeolite 4A (Silkem)	15
Sodium disilicate (Britesil H 265 LC)	12
Na-LAS granules (Marlon ARL)	9.9
Sokalan CP 5 Gran.	1

**Wash assays**

Automatic Mechanical Stress Assay (AMSA) for laundry

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

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

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

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

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

The AMSA wash performance experiments were conducted under the experimental conditions specified below:

Table 3 AMSA Experimental conditions	
Detergents	EU-type liquid model detergent B US-type liquid model detergent J Chinese-type powder model detergent M Developing and Emerging market-type powder detergent model Y
Detergent dose	3.33 g/L for liquid model detergent B 0.79 g/L for liquid model detergent J

	3.33 g/L for powder model detergent M 1.75 g/L for powder model detergent Y (with 0.035 g/L AEO added)
Test solution volume	160 micro L
pH	pH is used "as is" in the current detergent solution and is not adjusted.
Wash time	20 minutes
Temperature	20°C, 30°C, or 40°C
Water hardness	For wash with EU-type liquid model detergent B: 15°dH, adjusted by adding CaCl <sub>2</sub> *2H <sub>2</sub> O, MgCl <sub>2</sub> *6H <sub>2</sub> O and NaHCO <sub>3</sub> (4:1:7.5) to milli-Q water.  For wash with US-type liquid model detergent J: 6°dH, adjusted by adding CaCl <sub>2</sub> *2H <sub>2</sub> O, MgCl <sub>2</sub> *6H <sub>2</sub> O and NaHCO <sub>3</sub> (2:1:4.5) to milli-Q water.  For wash with Chinese-type powder model detergent M: 14°dH, adjusted by adding CaCl <sub>2</sub> *2H <sub>2</sub> O, MgCl <sub>2</sub> *6H <sub>2</sub> O and NaHCO <sub>3</sub> (2:1:4.5) to milli-Q water.  For wash with D&E market-type powder detergent model Y: 12°dH, adjusted by adding CaCl <sub>2</sub> *2H <sub>2</sub> O, MgCl <sub>2</sub> *6H <sub>2</sub> O and NaHCO <sub>3</sub> (2:1:4.5) to milli-Q water.
Enzymes	M4 metalloprotease from <i>Kribbella aluminosa</i> M4 metalloprotease from <i>Bacillus amyloliquefaciens</i> , also known as Neutrase® S8 serine protease known as Savinase®
Enzyme concentration	60 nM for model detergent B and for model detergent J 60 nM, 100 nM, and 140 nM for model detergent Y 100 nM, 500 nM, and 1000 nM for model detergent M

#### Mini wash assay

Wash performance is assessed in laundry wash experiment using a Mini wash assay, which is a test method where soiled textile is continuously is lifted up and down into the test solution and subsequently rinsed.

5

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

Detergent	Liquid laundry detergent B
Detergent dose	3.33 g/L
pH	pH was measured to be 7.6, but is used "as is" in the current detergent solution and is not adjusted
Water hardness	15°dH, adjusted by adding CaCl <sub>2</sub> *2H <sub>2</sub> O, MgCl <sub>2</sub> *6H <sub>2</sub> O and NaHCO <sub>3</sub> (4:1:7.5) to milli-Q water.
Enzymes	M4 metalloprotease from <i>Kribbella aluminosa</i> M4 metalloprotease from <i>Bacillus amyloliquefaciens</i> , also known as Neutrase® S8 serine protease from <i>Bacillus clausii</i> known as Savinase®
Enzyme conc.	2.5 nM, 5 nM, 10 nM
Test solution volume	50 mL
Test material	C-05 (Blood/milk/ink on cotton) (3 cm x 150 cm)
Temperature	20°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 0.4 sec, down-time 0.4 sec, lift time 0.4 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.

The washed textiles are subsequently air-dried and the wash performance is measured as the brightness of the colour 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 is measured at 460 nm using a Zeiss MCS 521 VIS spectrophotometer. The measurements are done according to the manufacturer's protocol.

The performance of the new enzyme is compared to the performance of reference enzymes at 2.5 nM, 5 nM, 10 nM, 30 nM, or 60 nM protease concentration by calculating the relative performance:

$$RP = (R_{\text{ENZYME}} - R_{\text{BLANK}}) / (R_{\text{REFERENCE}} - R_{\text{BLANK}})$$

An enzyme is considered to exhibit improved wash performance, if it performs better than the reference (RP > 1) in at least one detergent composition.

15 Terg-O-tometer (TOM) wash assay

The Tergo-O-Tometer (TOM) is a medium scale model wash system that can be applied to test 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 will contain a solution of a specific detergent/enzyme system and the soiled and unsoiled fabrics its performance is tested on. Mechanical stress is achieved by a rotating stirring arm, which stirs the liquid within each beaker - 500 or 1200mL of detergent solution per beaker. Because the TOM beakers have no lid, it is possible to withdraw samples during a TOM experiment and assay for information on-line during wash.

25 In a TOM experiment, factors such as the ballast to soil ratio and the fabric to wash liquor ratio can be varied. Therefore, the TOM provides the link between small scale experiments, such as AMSA and mini-wash, and the more time consuming full scale experiments in full scale washing machines.

30 The TOM wash experiment is conducted under the experimental conditions specified below:

Table 5. TOM wash conditions	
Detergent	Liquid laundry detergent B

Detergent dose	3.33 g/L
pH	pH was measured to be 7.6, but is used "as is" in the current detergent solution and is not adjusted.
Water hardness	15°dH, adjusted by adding CaCl <sub>2</sub> *2H <sub>2</sub> O, MgCl <sub>2</sub> *6H <sub>2</sub> O and NaHCO <sub>3</sub> (4:1:7.5) to Milli-Q water.
Enzymes	M4 metalloprotease from <i>Kribbella aluminosa</i> M4 metalloprotease from <i>Bacillus amyloliquefaciens</i> , also known as Neutrase® S8 serine protease from <i>Bacillus clausii</i> known as Savinase®
Enzyme conc.	30 nM
Test solution volume	1000 mL
Test material	2 swatches, each 5x5 cm, of each of the 6 soiled textile types per beaker (i.e. 12 soiled swatches per beaker): PC-03 (Chocolate-milk/ink on cotton/polyester) C-H010 (Cocoa, cooked up with milk on cotton) C-05 (Blood/milk/soot on cotton) CS-01 (Aged blood on cotton) CS-37 (Full egg/pigment on cotton) 062KC (Scrubbed grass on cotton)  Cotton ballast swatches (50%:50% WFK10A:WFK80A, 5x5 cm) added to give a total weight of 30 g of soiled textile swatches + ballast per TOM beaker.
Temperature	20°C
Wash time	30 min
Rinse time	5 min
Rotation	120 rpm

## Protease assays

### Protazyme AK purification activity assay:

Substrate: Protazyme AK tablet (AZCL-casein, Megazyme T-PRAK 1000).

5 Temperature: 37°C

Assay buffer: 50mM HEPES/NaOH, pH 7.0.

A Protazyme AK tablet is suspended in 2.0ml 0.01% Triton X-100 by gentle stirring. 500µl of this suspension and 500µl assay buffer are dispensed in an Eppendorf tube and placed on ice. 20µl protease sample (diluted in 0.01% Triton X-100) is added to the ice cold tube. The assay is initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which is set to the assay temperature. The tube is incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate (1400 rpm). The incubation is stopped by transferring the tube back to the ice bath. Then the tube is centrifuged in an ice cold centrifuge for a few minutes and 200µl supernatant is transferred to a microtiter plate. OD<sub>650</sub> is read as a measure of protease activity. A buffer blind is included in the assay (instead of enzyme).

Characterization activity assays:Protazyme OL characterization assay:

Substrate: Protazyme OL tablet (AZCL-collagen, Megazyme T-PROL 1000).

Temperature: Controlled (assay temperature).

- 5 Assay buffers: 100mM succinic acid, 100mM HEPES, 100mM CHES, 100mM CABS, 1mM CaCl<sub>2</sub>, 150mM KCl, 0.01% Triton X-100 adjusted to pH-values 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 with HCl or NaOH.

A Protazyme OL tablet is suspended in 2.0ml 0.01% Triton X-100 by gentle stirring. 500µl of this suspension and 500µl assay buffer are dispensed in an Eppendorf tube and placed  
10 on ice. 20µl protease sample (diluted in 0.01% Triton X-100) is added to the ice cold tube. The assay is initiated by transferring the Eppendorf tube to an Eppendorf thermomixer, which is set to the assay temperature. The tube is incubated for 15 minutes on the Eppendorf thermomixer at its highest shaking rate (1400 rpm). The incubation is stopped by transferring the tube back to the ice bath. Then the tube is centrifuged in an ice cold centrifuge for a few minutes and  
15 200µl supernatant is transferred to a microtiter plate. OD<sub>650</sub> is read as a measure of protease activity. A buffer blind is included in the assay (instead of enzyme).

**Example 1. Cloning and expression of a M4 metalloprotease from *Kribbella aluminosa***

The metalloprotease was derived from a *Kribbella aluminosa* strain isolated from a soil sample collected in China 2009.

20 Chromosomal DNA from a pure culture was purified and subjected to full genome sequencing using Illumina technology. The assembled genome sequence and subsequent analysis of the 16S ribosomal subunit gene sequences indicated that the correct taxonomical identification was as a species of *Kribbella aluminosa*.

The genome sequence was analyzed for metalloproteases from the MEROPS family M4  
25 by comparison to the M4 protease NprE from *B. subtilis* 168 (Uniprot P68736) by search using the BLAST program. This analysis identified a gene encoding a putative M4 metalloprotease with the nucleotide sequence given in SEQ ID: 1.

The gene encoding the M4 metalloprotease was amplified by PCR and fused with regulatory elements and homology regions for recombination into the *B. subtilis* genome.

30 The linear integration construct was a SOE-PCR fusion product (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*) made by fusion of the gene between two *Bacillus subtilis* chromosomal regions along with strong promoters and a

chloramphenicol resistance marker. The SOE PCR method is also described in patent application WO 2003095658.

5 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 was expressed with a *Bacillus clausii* secretion signal (encoding the following amino acid sequence: MKKPLGKIVASTALLISVAFSSSIASA) replacing the native secretion signal.

10 The SOE-PCR product was transformed into *Bacillus subtilis* and integrated in the chromosome by homologous recombination into the pectate lyase locus. Subsequently a recombinant *Bacillus subtilis* clone containing the integrated expression construct was grown in liquid culture. The culture broth was centrifuged (20000 x g, 20 min) and the supernatant was carefully decanted from the precipitate and filtered through a Nalgene 0.2µm filtration unit in  
15 order to remove the rest of the *Bacillus* host cells. The 0.2µm filtrate was concentrated by ultrafiltration on a Sartorius 10kDa cut-off membrane to a minimal volume and the concentrate was transferred to 20mM MES/NaOH, 1mM CaCl<sub>2</sub>, pH 6.0 on a G25 Sephadex column (from GE Healthcare). The G25 transferred protease solution was applied to a Bacitracin agarose column (from Upfront chromatography) equilibrated in 20mM MES, 1mM CaCl<sub>2</sub>, pH 6.0. After  
20 washing the column extensively with the equilibration buffer, the protease was eluted with 100mM H<sub>3</sub>BO<sub>3</sub>, 10mM MES, 2mM CaCl<sub>2</sub>, 1M NaCl, pH 6.0 with 25%(v/v) 2-propanol. The elution peak (containing the protease activity) was transferred to 20mM MES, 2mM CaCl<sub>2</sub>, 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.

25

### Example 2. SDS-PAGE and N-terminal sequencing

When the purified protease preparation was analysed by SDS-PAGE and the gel was stained with coomassie three bands at approx. 55 kDa, 47 kDa and 8 kDa were seen. EDMAN degradation, Intact MS and in-gel peptide map analysis showed that all three bands originated  
30 from the M4 protease, suggesting that the 47 kDa and 8 kDa bands represented nicked molecules where the full length M4 protease was cleaved once in a loop and the two peptide chains remained bound to each other and giving fully active molecules. The relative intensity of the 47 kDa band and the 8 kDa band suggested a 1:1 molar ratio between the two bands supporting this hypothesis.

35 N-terminal sequencing of the purified protein was performed and the mature enzyme was found to start at GTGNSMY confirming the identity of the enzyme and the presence of an

N-terminal propeptide. Intact molecular weight analysis of the purified protein was done by mass spectroscopy. The analysis indicated two predominant molecular weight forms of 50.8 kDa and 42.7 kDa for the purified proteins ending at different C-terminal residues corresponding to the polypeptides GTGNSMY-----LTAS (amino acids 1 to 489 of SEQ ID NO:2 ) and GTGNSMY-----  
5 TAET (amino acids 1 to 414 of SEQ ID NO:2).

### Example 3. Stability of the *Kribbella aluminosa* protease in detergents

#### *Materials:*

*SpectraMax M2 fluorescence/UV-VIS plate-reader*

10 *96 well nunc-plates (florescence Maxi Sorp)*

*PST 100HL shaker (Biosan)*

#### *Enzyme and chemicals*

Buffer: 10mM MES, 0.01% TritonX, 1mM CaCl<sub>2</sub>, pH 6

Detergent: Model B detergent

15 Detergent solutions: 50 % and 90 % (w/w) model B detergent in milli Q water. 90 % model B detergent with 2 mM CaCl<sub>2</sub> and 2 mM ZnCl<sub>2</sub>. 10 % model B in 15 °dH water.

Substrate solution: One ampule EnzCheck-red substrate mixed with 20 ml 0.3 % model B detergent in 15 °dH water.

Protease: *Kribbella aluminosa* 0.30 mg / ml in buffer

20 Neutrase® 0.8 L product, buffer exchanged to 10mM MES, 0.01% TritonX, 1mM CaCl<sub>2</sub>.

#### *Storage stability of proteases in model B detergent*

The storage stability of the *Kribbella aluminosa* metallo-protease and Neutrase was measured in different detergent solutions based on model B detergent as outlined above. Storage stability was measured as residual activity (see below).  
25

#### *Incubation protocol:*

20 µl enzyme solution (as defined above) was mixed with 180 µl detergent solution and incubated for 30 minutes at 25 °C in 96 well plates. As a reference, 20 µl enzyme solution was mixed with 180 µl buffer (in case of experiments with 2mM CaCl<sub>2</sub> and 2 mM ZnCl<sub>2</sub>, these salts were also added to the buffer in the reference experiment), and activity was measured immediately after (see below for assay protocol). After incubation in detergent solutions, 20 µl sample were mixed with 180 µl buffer after which 7 times 2-fold dilutions were made of each sample.  
30

This resulted in 8 concentrations for each enzyme sample ranging from 10X-1280X dilution. The reason for this high span of concentrations is to ensure that data is obtained within the approximate linear range of the assay. After dilution, 20 µl sample was mixed with 80 µl buffer and 100 µl substrate and assayed as described under “activity assay” below.

#### 5 Activity assay:

The commercial available EnzCheck assay kit (red fluorescence) from Invitrogen™ was used for quantification of protease residual activity. Briefly, enzyme sample was mixed with substrate solution as described above in 96 well microtiter plates and fluorescence was measured for 10 minutes at 25 °C. Excitation was set to 589 nm and emission to 617nm. The fluorescent value after 10 min was used as activity measurement.

Residual activity was calculated from the ratio of activity after incubation ( $A_{Inc}$ ) with the reference activity (no incubation) ( $A_{Ref}$ )

$$\text{Residual activity} = (A_{Inc}/A_{Ref}) * 100$$

Results are shown in the table below.

<i>Table 6: Residual activity of Neutrase and the Kribbella aluminosa metalloprotease after 30 min incubation in different detergent solutions (model B).</i>				
	<b>10 % model B in 15 °dH water</b>	<b>50 % model B in milli Q</b>	<b>90 % model B in milli Q</b>	<b>90 % model B + 2mM CaCl<sub>2</sub> + 2 mM ZnCl<sub>2</sub></b>
<b>Neutrase</b>	0	2	5	26
<b><i>K. aluminosa</i> protease</b>	62	51	39	75

The *Kribbella aluminosa* protease was much more stable in the tested detergent compositions than the reference metalloprotease Neutrase.

#### 20 Example 4. AMSA wash performance of M4 protease from *Kribbella aluminosa*.

The wash performance of the M4 protease from *Kribbella aluminosa* was tested using different liquid detergents and different wash temperatures on different technical stains using the Automatic Mechanical Stress Assay.

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

The M4 protease from *Kribbella aluminosa* surpassed the performance by Neutrase® on the PC-03 (Chocolate/milk/soot on cotton/polyester) swatch, and matched the performance by Savinase®. When the water hardness was adjusted to 30 °dH, the *Kribbella aluminosa* metalloprotease outperformed Neutrase® on the PC-03 swatch to an even larger extent, displaying more than double the performance by Neutrase® under these conditions (table 7).

On blood stains PC-05 (Blood/milk/ink on cotton/polyester) and C-05 (Blood/milk/ink on cotton), the *Kribbella aluminosa* metalloprotease surpassed the wash performance by Savinase® on both blood stains at 20 °C, and showed a similar wash performance to both Neutrase® and Savinase® at 40 °C. The M4 protease from *Kribbella aluminosa* surpassed the performance by Neutrase® on the PC-05 swatch at both temperatures tested (table 7).

Table 7. AMSA data for *Kribbella aluminosa* protease in Delta Intensity Units and relative to prior art proteases Neutrase® and Savinase®.

Conditions are 15 °dH (4:1:7.5 Ca<sup>2+</sup>:Mg<sup>2+</sup>:HCO<sub>3</sub><sup>-</sup>), 60 nM protease, EU type liquid laundry detergent (model B - 60% surfactant level) 3.33 g/L, 20 min. (In one specified case on PC-03, there was used 30 °dH (4:1:7.5 Ca<sup>2+</sup>:Mg<sup>2+</sup>:HCO<sub>3</sub><sup>-</sup>), with all other parameters unchanged and as specified above.)

	<b>PC-03 40 °C</b>	<b>PC-03 40 °C 30 °dH</b>	<b>PC-05 20 °C</b>	<b>PC-05 40 °C</b>	<b>C-05 20 °C</b>
<b>Delta intensity</b>					
<i>Kribbella aluminosa</i> protease	25	9	27	47	23
<b>Relative performance</b>					
Relative to Neutrase®	1.1	2.3	1.5	1.2	0.9
Relative to Savinase®	1.0	0.7	1.5	0.9	1.2

On the egg stains CS-37 (full egg/pigment on cotton) and CS-38 (egg yolk/pigment with heating on cotton), the *Kribbella aluminosa* metalloprotease displayed a higher wash performance than Savinase® at both temperatures tested, and displayed wash performances not far from those by Neutrase® (table 8).

Table 8. AMSA data for *Kribbella aluminosa* protease in Delta Intensity Units and relative to prior art proteases Neutrase® and Savinase®. Conditions are 15 °dH (4:1:7.5 Ca<sup>2+</sup>:Mg<sup>2+</sup>:HCO<sub>3</sub><sup>-</sup>), 60 nM protease, EU type liquid laundry detergent (model B - 60% surfactant level) 3.33 g/L, 20 min.

	<b>CS-37</b>	<b>CS-37</b>	<b>CS-38</b>
<b>Delta intensity</b>	<b>20 °C</b>	<b>40 °C</b>	<b>20 °C</b>
<i>Kribbella aluminosa</i> protease	39	54	10
<b>Relative performance</b>			
Relative to Neutrase®	0.7	0.9	0.8
Relative to Savinase®	3.1	2.6	1.1

In US type liquid laundry detergent at 30 °C, the M4 protease from *Kribbella aluminosa* matched or surpassed the wash performance by Savinase® on all three stain types tested; chocolate stain (PC-03), blood stain (PC-05), and egg stain (CS-37) (table 9).

5

Table 9. AMSA data for *Kribbella aluminosa* protease in Delta Intensity Units and relative to prior art proteases Neutrase® and Savinase®. Conditions are 6 °dH (2:1:4.5 Ca<sup>2+</sup>:Mg<sup>2+</sup>:HCO<sub>3</sub><sup>-</sup>), 60 nM protease, US type liquid laundry detergent (model J - 100% surfactant level) 0.79 g/L, 20 min., 30 °C.

	<b>PC-03</b>	<b>PC-05</b>	<b>CS-37</b>
<b>Delta intensity</b>	<b>30 °C</b>	<b>30 °C</b>	<b>30 °C</b>
<i>Kribbella aluminosa</i> protease	21	29	42
<b>Relative performance</b>			
Relative to Neutrase®	0.7	1.1	0.7
Relative to Savinase®	1.1	1.0	4.1

Surprisingly, in powder laundry detergent, the M4 protease from *Kribbella aluminosa* surpassed the wash performance by Neutrase® by up to more than seven times. This shows potential for use in alkaline detergent systems, as well as potential for use in powder-derived detergent systems (table 10). Astonishingly, despite the alkalinity of the wash liquor from the Chinese powder detergent being at a basic pH ca. 10.0, the *Kribbella aluminosa* metalloprotease afforded more than 26 delta intensity units on PC-05 (Blood/milk/ink on cotton/polyester), which is over 22 delta intensity units more than Neutrase® afforded.

10



Table 10. AMSA data for <i>Kribbella aluminosa</i> protease in Delta Intensity Units and relative to prior art proteases Neutrase® and Savinase®. Conditions are 14 °dH (2:1:4.5 Ca <sup>2+</sup> :Mg <sup>2+</sup> :HCO <sub>3</sub> <sup>-</sup> ), Chinese type powder laundry detergent (model M - 80% surfactant level) 3.33 g/L, 20 min., 20 °C.			
	<b>PC-05, 20 °C</b>		
<b>Delta intensity</b>	<b>100 nM protease</b>	<b>500 nM protease</b>	<b>1000 nM protease</b>
<i>Kribbella aluminosa</i> protease	4	17	26
<b>Relative performance</b>			
Relative to Neutrase®	5.8	6.2	7.1
Relative to Savinase®	0.1	0.3	0.4

In a D&E market type powder laundry detergent on PC-05 (Blood/milk/ink on cotton/polyester), the *Kribbella aluminosa* metalloprotease surpassed the wash performance by Neutrase by at least 17 times (table 11) at all concentrations tested. This remarkable wash performance by the *Kribbella aluminosa* metalloprotease in a powder detergent-derived wash solution is very surprising.

Table 11. AMSA data for <i>Kribbella aluminosa</i> protease in Delta Intensity Units and relative to prior art proteases Neutrase® and Savinase®. Conditions are 12 °dH (2:1:4.5 Ca <sup>2+</sup> :Mg <sup>2+</sup> :HCO <sub>3</sub> <sup>-</sup> ), D&E market type powder laundry detergent (model Y - 60% surfactant level) 1.75 g/L with 0.035 g/L AEO, 20 min., 20 °C.			
	<b>PC-05, 20 °C</b>		
<b>Delta intensity</b>	<b>60 nM protease</b>	<b>100 nM protease</b>	<b>140 nM protease</b>
<i>Kribbella aluminosa</i> protease	12	16	17
<b>Relative performance</b>			
Relative to Neutrase®	Neutrase® lacked wash performance	17.3	Neutrase® lacked wash performance
Relative to Savinase®	0.3	0.3	0.3

On the EMPA117EH (Blood/milk/ink on cotton/polyester, extra heated) swatch the *Kribbella aluminosa* metalloprotease displayed wash performances not far from those of Neutrase® and Savinase® at standard pH of the model B detergent (around pH 7.5). When the pH was adjusted to 9.5, however, Neutrase® lost almost all wash performance while the wash performance of the *Kribbella aluminosa* metalloprotease surprisingly increased (table 12). This indicates potential for use in alkaline detergent systems.

Table 12. AMSA data for *Kribbella aluminosa* protease in Delta Intensity Units and relative to prior art proteases Neutrase® and Savinase®. 15 °dH (4:1:7.5 Ca<sup>2+</sup>:Mg<sup>2+</sup>:HCO<sub>3</sub><sup>-</sup>), 60 nM protease, EU type liquid laundry detergent (model B - 60% surfactant level) 3.33 g/L, 20 min, 20 °C.

	EMPA117EH, 20 °C	EMPA117EH, 20 °C, pH 9.5
<b>Delta intensity</b>		
<i>Kribbella aluminosa</i> protease	27	33
<b>Relative performance</b>		
Relative to Neutrase®	0.8	34.1
Relative to Savinase®	0.9	0.8

**Example 5. Mini-wash assay performance of M4 protease from *Kribbella aluminosa*.**

Wash performance was assessed in laundry wash experiment using the Mini-wash assay as described above.

The results show that the *Kribbella aluminosa* metalloprotease surpassed the performance of Neutrase® and was almost on par with Savinase® on the C-05 swatch (Blood/milk/ink on cotton) under the tested conditions (table 13).

Table 13. Mini-wash data for *Kribbella aluminosa* protease in Delta Remission Units and relative to prior art proteases Neutrase® and Savinase®. Conditions are 15 °dH (4:1:7.5 Ca<sup>2+</sup>:Mg<sup>2+</sup>:HCO<sub>3</sub><sup>-</sup>), EU type liquid laundry detergent (model B - 60% surfactant level) 3.33 g/L, 20 min., 20 °C (50 lifts/min).

	C-05 (Blood/milk/ink on cotton) at 20 °C.		
<b>Delta remission</b>	2.5 nM	5 nM	10 nM

	protease	protease	protease
<i>Kribbella aluminosa</i> protease	1.8	2.0	2.5
<b>Relative performance</b>			
Relative to Neutrase®	2.7	1.4	1.3
Relative to Savinase	0.9	0.8	0.8

**Example 6. TOM wash performance of M4 protease from *Kribbella aluminosa*.**

Wash performance was assessed in laundry wash experiment using the TOM wash assay as described above.

- 5 The results show that the *Kribbella aluminosa* metalloprotease surpassed the wash performance by Savinase® on the four stain types; chocolate (PC-03), blood (C-05), egg (CS-37), and grass (062KC). On PC-03 (Chocolate/milk/soot on cotton/polyester) and on C-05 (Blood/milk/ink on cotton) stains, the M4 protease from *Kribbella aluminosa* showed similar wash performance to Neutrase®. On grass stain (062KC), the *Kribbella aluminosa* metalloprotease displayed almost double the wash performance of Neutrase® (table 14).
- 10

Table 14. TOM wash data for <i>Kribbella aluminosa</i> protease in Delta Remission Units and relative to prior art proteases Neutrase® and Savinase®. Conditions are 15 °dH (4:1:7.5 Ca <sup>2+</sup> :Mg <sup>2+</sup> :HCO <sub>3</sub> <sup>-</sup> ), 30 nM protease, EU type liquid laundry detergent (model B - 60% surfactant level) 3.33 g/L, 20 °C, 30 g ballast+stains per liter, 30 min. (120 rpm)				
Delta remission	PC-03	C-05	CS-37	062KC
<i>Kribbella aluminosa</i> protease	6.5	6.0	11.6	11.6
<b>Relative performance</b>				
Relative to Neutrase®	1.0	1.0	0.7	1.7
Relative to Savinase®	1.4	1.1	1.3	1.4

**CLAIMS**

1. An isolated polypeptide having protease activity selected from the group consisting of:
- 5 a. a polypeptide having at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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 of SEQ ID NO: 2;
- 10 b. a polypeptide encoded by a polynucleotide that hybridizes under high stringency conditions with (i) the mature polypeptide coding sequence of SEQ ID NO: 1 or (ii) the full-length complementary strand of (i);
- 15 c. a polypeptide encoded by a polynucleotide having at least 85% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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;
- 20 d. a variant comprising a substitution, deletion, and/or insertion of one or more (e.g. several) amino acids of the mature polypeptide of SEQ ID NO: 2; and
- 25 e. a fragment of a polypeptide of (a), (b), (c), or (d) that has protease activity.
2. The polypeptide of claim 1, comprising or consisting of SEQ ID NO: 2 or the mature polypeptide of SEQ ID NO: 2.
3. The polypeptide of claim 2, wherein the mature polypeptide is amino acids 1 to 489 of SEQ ID NO: 2 and/or amino acids 1 to 414 of SEQ ID NO: 2.
- 30 4. The polypeptide of any of claims 1-3, which is a variant of the mature polypeptide of SEQ ID NO: 2 comprising a substitution, deletion, and/or insertion at one or more positions.

5. A composition comprising an isolated polypeptide having protease activity selected from the group consisting of:

- 5 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% sequence identity, at least 86% sequence identity, at least 87% sequence identity, at least 88% sequence identity, at least 89% sequence identity, at least 90% sequence identity, at least 91% 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 of SEQ ID NO: 2;
- 10 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);
- 15 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 91% 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;
- 20 d. a variant comprising a substitution, deletion, and/or insertion of one or more (e.g. several) amino acids of the mature polypeptide of SEQ ID NO: 2; and
- 25 e. a fragment of a polypeptide of (a), (b), (c), or (d) that has protease activity.

6. The composition of claim 5, which is a detergent composition.

7. The detergent composition of claim 6, further comprising one or more additional enzymes selected among protease, lipase, cutinase, an amylase, carbohydrase, cellulase, pectinase, mannanase, arabinase, galactanase, xylanase, oxidase, e.g., a laccase, and/or peroxidase.

8. The detergent composition of claim 6 or 7, further comprising a detergent component selected from builders, surfactants, thickeners, enzyme stabilizing systems, bleaching compounds, bleaching agents, bleach activators, bleach catalysts, brighteners, dyes, hueing

agents, dye transfer inhibiting agents, chelating agents, suds suppressors, softeners, perfumes or mixtures thereof.

- 5 9. The detergent composition of any of claim 6-8, 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.
- 10 10. Use of a composition according to any of the claims 5-9 in a cleaning process, such as laundry, hard surface cleaning, dish wash or automated dish wash.
11. A method of cleaning comprising contacting a surface and/or a fabric with the composition of any of the claims 5-9.
- 15 12. The method of claim 11 wherein said a surface and/or a fabric comprises a grass stain and/or an egg stain.
13. An isolated polynucleotide encoding the polypeptide of any of claims 1-4.
- 20 14. The polynucleotide of claim 13, wherein the polynucleotide is modified to correspond to the codon usage of a host cell intended for recombinant production of the enzyme.
- 25 15. A nucleic acid construct or expression vector comprising the polynucleotide of any of claims 13 or 14 operably linked to one or more control sequences that direct the production of the polypeptide in a host cell.
- 30 16. A recombinant host cell comprising the polynucleotide of any of claims 13 or 14 operably linked to one or more control sequences that direct the production of the polypeptide.
17. A method of producing a polypeptide having protease activity, comprising:
- (a) cultivating the host cell of claim 16 under conditions conducive for production of the polypeptide; and
- (b) recovering the polypeptide.
- 35

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2015/063847

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N9/52 C11D3/386 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, BIOSIS, EMBASE, Sequence Search		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/026796 A1 (NOVOZYMES AS [DK]; SJOEHOLM CARSTEN [DK]; OESTERGAARD PETER RAHBEK [DK]) 28 February 2013 (2013-02-28) Seq.4, examples 1-3, page 44-49 -----	1-17
A	WO 2014/071410 A1 (DANISCO US INC [US]) 8 May 2014 (2014-05-08) para. [021], p.154, 1.8 -----	1-17
A	WO 2007/044993 A2 (GENENCOR INT [US]; PROCTER & GAMBLE [US]; SHAW ANDREW [US]; WALLACE LO) 19 April 2007 (2007-04-19) Source of "Neutrase". See whole doc., in particular examples, p.51-55 ----- -/--	1-17
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search  6 August 2015	Date of mailing of the international search report  11/09/2015	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Roscoe, Richard	

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2015/063847

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE UniProt [Online]</p> <p>18 September 2013 (2013-09-18),  "SubName: Full=Uncharacterized protein  {ECO:0000313 EMBL:EPD64728.1}";  XP002743058,  retrieved from EBI accession no.  UNIPROT:S2YJW6  Database accession no. S2YJW6  sequence</p> <p style="text-align: center;">-----</p>	1-17
A	<p>DATABASE UniProt [Online]</p> <p>2 March 2010 (2010-03-02),  "SubName: Full=Bacillolysin  {ECO:0000313 EMBL:ADB34616.1};  EC=3.4.24.28  {ECO:0000313 EMBL:ADB34616.1}; Flags:  Precursor";  XP002743059,  retrieved from EBI accession no.  UNIPROT:D2PND2  Database accession no. D2PND2  sequence</p> <p style="text-align: center;">-----</p>	1-17



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Information on patent family members

International application No PCT/EP2015/063847
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