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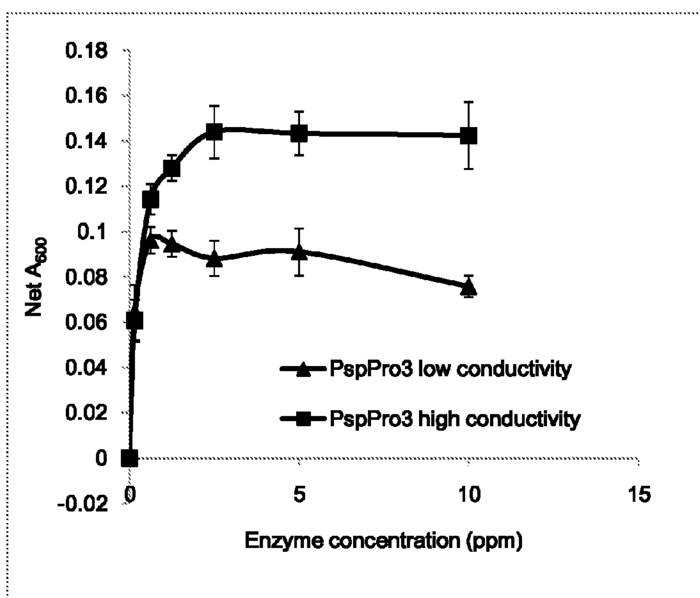

Figure 1.6. Cleaning performance of PspPro3 in liquid laundry detergent at pH 8

(57) **Abstract**: Aspects of the present compositions and methods relate to novel metalloproteases, polynucleotides encoding the novel metalloproteases, and compositions and methods for use thereof.

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NOVEL METALLOPROTEASES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority from International patent applications Serial No. PCT/CN2013/076419; Serial No. PCT/CN2013/076387; Serial No. PCT/CN2013/076401; Serial No. PCT/CN2013/076406; Serial No. PCT/CN2013/076414; Serial No. PCT/CN2013/076384; Serial No. PCT/CN2013/076398; and Serial No. PCT/CN2013/076415; all filed on 29 May 2013, the contents of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present disclosure relates to proteases and variants thereof. Compositions containing the proteases are suitable for use in cleaning, food and feed as well as in a variety of other industrial applications.

BACKGROUND

Metalloproteases (MPs) are among the hydrolases that mediate nucleophilic attack on peptide bonds using a water molecule coordinated in the active site. In their case, a divalent ion, such as zinc, activates the water molecule. This metal ion is held in place by amino acid ligands, usually 3 in number. The clan MA consists of zinc-dependent MPs in which two of the zinc ligands are the histidines in the motif: HisGluXXHis. This Glu is the catalytic residue. These are two domain proteases with the active site between the domains. In subclan MA(E), also known as Glu-zincins, the 3rd ligand is a Glu located C-terminal to the HDXXH motif. Members of the families: M1, 3, 4, 13, 27 and 34 are all secreted proteases, almost exclusively from bacteria (Rawlings and Salvessen (2013) Handbook of Proteolytic Enzymes, Elsevier Press). They are generally active at elevated temperatures and this stability is attributed to calcium binding. Thermolysin-like proteases are found in the M4 family as defined by MEROPS (Rawlings et al., (2012) Nucleic Acids Res 40:D343-D350). Although proteases have long been known in the art of industrial enzymes, there remains a need for novel proteases that are suitable for particular conditions and uses.

SUMMARY

The present disclosure provides novel metalloprotease enzymes, nucleic acids encoding the same, and compositions and methods related to the production and use thereof.

In some embodiments, the invention is a polypeptide comprising an amino acid sequence having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 3. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae, Alicyclobacillaceae, Lactobacillaceae*, or a *Bacillus, Alicyclobacillus, Geobacillus, Exiguobacterium, Lactobacillus, or Paenibacillus* spp., such as *Paenibacillus polymyxa.* In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the *Pseudococcidae*, or a *Planococcus* spp., such as *Planococcus donghaensis.* In various embodiments of the invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH 5 and 9.5. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 30ºC and 70ºC. In various embodiments of the invention, any of the above polypeptides has cleaning activity in a detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

In some embodiments, the invention is a polypeptide comprising an amino acid sequence having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 8. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae, or Brevibacillaceae,* or a *Bacillus, Brevibacillus*, or *Paenibacillus* spp., such as *Paenibacillus sp.* In some embodiments, the invention is any of the above, wherein said polypeptide is derived from Brevib*acillus sp.* In various embodiments of the invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH 5 and 10. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 35ºC and 70ºC. In various embodiments of the invention, any of the above polypeptides has cleaning activity in a detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

In some embodiments, the invention is a polypeptide comprising an amino acid sequence having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 13. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae, or Brevibacillaceae,* or a *Bacillus, Geobacillus, Brevibacillus*, or

Paenibacillus spp., such as *Paenibacillus humicus.* In some embodiments, the invention is any of the above, wherein said polypeptide is derived from *Bacillus polymyxa.* In various embodiments of the invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH 5 and 9.5. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 35°C and 70°C. In various embodiments of the invention, any of the above polypeptides has cleaning activity in a detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

In some embodiments, the invention is a polypeptide comprising an amino acid sequence having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 18. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae, or Brevibacillaceae,* or a *Bacillus, Geobacillus, Brevibacillus*, or *Paenibacillus* spp., such as *Paenibacillus ehimensis.* In some embodiments, the invention is any of the above, wherein said polypeptide is derived from Brevib*acillus sp.* In various embodiments of the invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH 5 and 10.5. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 45°C and 75°C. In various embodiments of the invention, any of the above polypeptides has cleaning activity in a detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

In some embodiments, the invention is a polypeptide comprising an amino acid sequence having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 23. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae, Alicyclobacillaceae, Lactobacillaceae*, or a *Bacillus, Geobacillus, Alicyclobacillus, Brevibacillus, Paenibacillus, or Lactobacillus* spp., such as *Paenibacillus barcinonensis.* In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the family *Pseudococcidae*, or a *Planococcus* spp., such as *Planococcus donghaensis.* In various embodiments of the invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH

5 and 10. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 35°C and 65°C. In various embodiments of the invention, any of the above polypeptides has cleaning activity in a detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

5 In some embodiments, the invention is a polypeptide comprising an amino acid sequence having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 28. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae*, or a *Bacillus, Brevibacillus, Paenibacillus, or Lactobacillus* spp., such as
10 *Paenibacillus polymyxa.* In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the family *Pseudococcidae*, or a *Planococcus* spp., such as *Planococcus donghaensis.* In various embodiments of the invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH
15 5 and 9.5. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 30°C and 65°C. In various embodiments of the invention, any of the above polypeptides has cleaning activity in a detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

In some embodiments, the invention is a polypeptide comprising an amino acid sequence
20 having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence of SEQ ID NO: 33. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae,* or a *Bacillus, Geobacillus, Brevibacillus*, or *Paenibacillus* spp., such as *Paenibacillus hunanensis.* In some embodiments, the invention is any of the above, wherein said
25 polypeptide is derived from *Bacillus polymyxa.* In various embodiments of the invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH 4.5 and 9.0. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 35°C and 70°C. In various
30 embodiments of the invention, any of the above polypeptides has cleaning activity in a detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

In some embodiments, the invention is a polypeptide comprising an amino acid sequence having at least 60%, at least 80%, or at least 95% sequence identity to the amino acid sequence

of SEQ ID NO: 38. In some embodiments, the invention is any of the above, wherein said polypeptide is derived from a member of the order Bacillales; family *Bacillaceae, Paenibacillaceae, Lactobacillaceae*, or a *Bacillus, Brevibacillus, Lactobacillus, Paenibacillus, or Geobacillus* spp., such as *Paenibacillus amylolyticus.* In various embodiments of the
5 invention, any of the above polypeptides has protease activity, such as azo-casein hydrolysis. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between pH 5.5 and 10. In various embodiments of the invention, any of the above polypeptides retains at least 50% of its maximal activity between 35ºC and 65ºC. In various embodiments of the invention, any of the above polypeptides has cleaning activity in a
10 detergent composition, such as an ADW, laundry, liquid laundry, or powder laundry detergent composition.

In some embodiments, the invention is a composition comprising any of the above, such as a cleaning or detergent composition. In some embodiments, the composition further comprises a surfactant, at least one calcium ion and/or zinc ion, at least one stabilizer, at least
15 one bleaching agent, and can contain phosphate, or be phosphate-free. In some embodiments, the composition further comprises one or more additional enzymes or enzyme derivatives selected from the group consisting of acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-
20 mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases,
25 xylan acetyl-esterases, xylanases, xyloglucanases, and xylosidases, and combinations thereof. In some embodiments, the composition is formulated at a pH of from about 5.5 to about 8.5. In some embodiments, the invention is a method of cleaning using any of the above polypeptides or compositions. In some embodiments, the invention is a textile processing composition, animal feed composition, leather processing composition, or feather processing composition.

30

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1.1 provides a plasmid map of pGX085 (aprE-PspPro3), described in Example 1.2.

Figure 1.2 provides a dose response curve of PspPro3 in the azo-casein assay.

Figure 1.3 provides the pH profile of PspPro3.

Figure 1.4 provides the temperature profile of PspPro3.

Figure 1.5A shows dose response for cleaning of PA-S-38 microswatches by PspPro3 protein in ADW detergent at pH 6 and 8.

Figure 1.5B shows dose response for cleaning of PA-S-38 microswatches shows by PspPro3 protein in ADW detergent at pH 6 and 8 in the presence of bleach.

Figure 1.6 shows cleaning performance of PspPro3 protein in liquid laundry detergent.

Figure 1.7 shows alignment of PspPro3with other protein homologs.

Figure 1.8 provides the phylogenetic tree for PspPro3 and its homologs.

Figure 2.1 provides a plasmid map of pGX084 (aprE-PspPro2), described in Example 2.2.

Figure 2.2 provides a dose response curve of PspPro2 in the azo-casein assay.

Figure 2.3 provides the pH profile of purified PspPro2.

Figure 2.4 provides the temperature profile of purified PspPro2.

Figure 2.5A shows dose response for cleaning performance of PspPro2 at pH 6 in AT dish detergent with bleach.

Figure 2.5B shows dose response for cleaning performance of purified PspPro2 at pH 8 in AT detergent with bleach.

Figure 2.6A shows cleaning performance of PspPro2 protein in liquid laundry detergent.

Figure 2.6B shows cleaning performance of PspPro2 protein in powder laundry detergent.

Figure 2.7 shows alignment of PspPro2with other protein homologs.

Figure 2.8 provides the phylogenetic tree for PspPro2 and its homologs.

Figure 3.1 provides a plasmid map of pGX150 (aprE-PhuPro2), described in Example 3.2.

Figure 3.2 provides a dose response curve of PhuPro2 in the azo-casein assay.

Figure 3.3 provides the pH profile of purified PhuPro2.

Figure 3.4 provides the temperature profile of purified PhuPro2.

Figure 3.5A shows dose response for c leaning performance of PhuPro2 in AT dish detergent at pH 6.

Figure 3.5B shows dose response for cleaning performance of PhuPro2 in AT dish detergent at pH 8.

Figure 3.6 shows alignment of PhuPro2with other protein homologs.

Figure 3.7 provides the phylogenetic tree for PhuPro2 and its homologs.

Figure 4.1 provides a plasmid map of pGX148 (aprE-PehPro1), described in Example 4.2.

Figure 4.2 provides a dose response curve of PehPro1 in the azo-casein assay.

Figure 4.3 provides the pH profile of purified PehPro1.

Figure 4.4 provides the temperature profile of purified PehPro1.

Figure 4.5A shows dose response for cleaning performance of PehPro1 at pH 6 in AT dish detergent with bleach.

Figure 4.5B shows dose response for cleaning performance of purified PehPro1 at pH 8 in AT detergent with bleach.

5 Figure 4.6 shows alignment of PehPro1with other protein homologs.

Figure 4.7 provides the phylogenetic tree for PehPro1 and its homologs.

Figure 5.1 provides a plasmid map of pGX147 (aprE-PbaPro1), described in Example 5.2.

Figure 5.2 provides a dose response curve of PbaPro1 in the azo-casein assay.

Figure 5.3 provides the pH profile of purified PbaPro1.

10 Figure 5.4 provides the temperature profile of purified PbaPro1.

Figure 5.5A shows dose response for cleaning of PA-S-38 microswatches by PbaPro1protein in ADW detergent at pH 6.

Figure 5.5B shows dose response for cleaning of PA-S-38 microswatches shows by PbaPro1protein in ADW detergent at pH 8.

15 Figure 5.6 shows the alignment of PbaPro1 with protease homologs.

Figure 5.7 provides the phylogenetic tree for PbaPro1 and its homologs.

Figure 6.1 provides a plasmid map of pGX138 (aprE-PpoPro1), described in Example 6.2.

Figure 6.2 provides a dose response curve of PpoPro1 in the azo-casein assay.

Figure 6.3 provides the pH profile of purified PpoPro1.

20 Figure 6.4 provides the temperature profile of purified PpoPro1.

Figure 6.5A shows dose response for cleaning of PA-S-38 microswatches by PpoPro1protein in ADW detergent at pH 6 in the presence of bleach.

Figure 6.5B shows dose response for cleaning of PA-S-38 microswatches shows by PpoPro1protein in ADW detergent at pH 8 in the presence of bleach.

25 Figure 6.6 shows the alignment of PpoPro1 with protease homologs.

Figure 6.7 provides the phylogenetic tree for PpoPro1 and its homologs.

Figure 7.1 provides a plasmid map of pGX149 (aprE-PhuPro1), described in Example 7.2.

Figure 7.2 provides a dose response curve of PhuPro1 in the azo-casein assay.

Figure 7.3 provides the pH profile of purified PhuPro1.

30 Figure 7.4 provides the temperature profile of purified PhuPro1.

Figure 7.5A shows dose response for cleaning of PA-S-38 microswatches by PhuPro1 protein in ADW detergent at pH 6.

Figure 7.5B shows dose response for cleaning of PA-S-38 microswatches shows by Phu Pro1protein in ADW detergent at pH 8.

Figure 7.6 shows alignment of PhuPro1with other protein homologs.

Figure 7.7 provides the phylogenetic tree for PhuPro1 and its homologs.

Figures 7.8A and 7.8B show cleaning performances of PhuPro1 and Purafect® Prime HA proteases.

5 Figure 8.1 provides a plasmid map of pGX146 (aprE-PamPro1), described in Example 8.2.

Figure 8.2 provides a dose response curve of PamPro1 in the azo-casein assay.

Figure 8.3 provides the pH profile of purified PamPro1.

Figure 8.4 provides the temperature profile of purified PamPro1.

Figure 8.5A shows dose response for cleaning of PA-S-38 microswatches by PamPro1 protein in
10 ADW detergent at pH 6.

Figure 8.5B shows dose response for cleaning of PA-S-38 microswatches shows by PamPro1 protein in ADW detergent at pH 8.

Figure 8.6 shows the alignment of PamPro1 with protease homologs.

Figure 8.7 provides the phylogenetic tree for PamPro1 and its homologs.

15 Figures 9.1A thru 9.1D show the alignment of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.

Figure 9.2 provides the phylogenetic tree of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.

20 **DETAILED DESCRIPTION**

The present invention provides novel metalloprotease enzymes, especially enzymes useful for detergent compositions cloned from various *Paenibacillus sp.* The compositions and methods are based, in part, on the observation that the novel metalloproteases of the present invention have proteolytic activity in the presence of detergent compositions. This feature
25 makes metalloproteases of the present invention particularly well suited to and useful in a variety of cleaning applications where the enzyme can hydrolyze polypeptides in the presence of surfactants and other components found in detergent compositions. The invention includes compositions comprising at least one of the novel metalloprotease enzymes set forth herein. Some such compositions comprise detergent compositions. The metalloprotease enzymes of the
30 present invention can be combined with other enzymes useful in detergent compositions. The invention also provides methods of cleaning using metalloprotease enzymes of the present invention.

Definitions and Abbreviations

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

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

Furthermore, the headings provided herein are not limitations of the various aspects or embodiments of the invention.

It is intended that every maximum numerical limitation given throughout this specification includes every lower numerical limitation, as if such lower numerical limitations
25 were expressly written herein. Every minimum numerical limitation given throughout this specification will include every higher numerical limitation, as if such higher numerical limitations were expressly written herein. Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

30 As used herein, the terms “protease” and “proteinase” refer to an enzyme that has the ability to break down proteins and peptides. A protease has the ability to conduct “proteolysis,” by hydrolysis of peptide bonds that link amino acids together in a peptide or polypeptide chain forming the protein. This activity of a protease as a protein-digesting enzyme is referred to as “proteolytic activity.” Many well known procedures exist for measuring proteolytic activity

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

As used herein, the term "variant polypeptide" refers to a polypeptide comprising an amino acid sequence that differs in at least one amino acid residue from the amino acid sequence of a parent or reference polypeptide (including but not limited to wild-type polypeptides).

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

The terms "polynucleotide" and "nucleic acid," which are used interchangeably herein, refer to a polymer of any length of nucleotide monomers covalently bonded in a chain. DNA

(deoxyribonucleic acid), a polynucleotide comprising deoxyribonucleotides, and RNA (ribonucleic acid), a polymer of ribonucleotides, are examples of polynucleotides or nucleic acids having distinct biological function. Polynucleotides or nucleic acids include, but are not limited to, a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The following are non-limiting examples of polynucleotides: genes, gene fragments, chromosomal fragments, expressed sequence tag(s) (EST(s)), exons, introns, messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), ribozymes, complementary DNA (cDNA), recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers.

As used herein, the term "mutation" refers to changes made to a reference amino acid or nucleic acid sequence. It is intended that the term encompass substitutions, insertions and deletions.

As used herein, the term "vector" refers to a nucleic acid construct used to introduce or transfer nucleic acid(s) into a target cell or tissue. A vector is typically used to introduce foreign DNA into a cell or tissue. Vectors include plasmids, cloning vectors, bacteriophages, viruses (*e.g.,* viral vector), cosmids, expression vectors, shuttle vectors, and the like. A vector typically includes an origin of replication, a multicloning site, and a selectable marker. The process of inserting a vector into a target cell is typically referred to as transformation. The present invention includes, in some embodiments, a vector that comprises a DNA sequence encoding a metalloprotease polypeptide (*e.g.,* precursor or mature metalloprotease polypeptide) that is operably linked to a suitable prosequence (*e.g.,* secretory, signal peptide sequence, etc.) capable of effecting the expression of the DNA sequence in a suitable host, and the folding and translocation of the recombinant polypeptide chain.

As used herein, the term "expression cassette," "expression plasmid" or "expression vector" refers to a nucleic acid construct or vector generated recombinantly or synthetically for the expression of a nucleic acid of interest in a target cell. An expression vector or expression cassette typically comprises a promoter nucleotide sequence that drives expression of the foreign nucleic acid. The expression vector or cassette also typically includes any other specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. A recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Many prokaryotic and eukaryotic expression vectors are commercially available.

In some embodiments, the ends of the sequence are closed such that the DNA construct forms a closed circle. The nucleic acid sequence of interest, which is incorporated into the DNA construct, using techniques well known in the art, may be a wild-type, mutant, or modified nucleic acid. In some embodiments, the DNA construct comprises one or more nucleic acid sequences homologous to the host cell chromosome. In other embodiments, the DNA construct comprises one or more non-homologous nucleotide sequences. Once the DNA construct is assembled *in vitro,* it may be used, for example, to: 1) insert heterologous sequences into a desired target sequence of a host cell; and/or 2) mutagenize a region of the host cell chromosome (*i.e*., replace an endogenous sequence with a heterologous sequence); 3) delete target genes; and/or 4) introduce a replicating plasmid into the host. “DNA construct” is used interchangeably herein with “expression cassette.”

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

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

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

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

As used herein the term “gene” refers to a polynucleotide (*e.g*., a DNA segment), that encodes a polypeptide and includes regions preceding and following the coding regions as well as intervening sequences (introns) between individual coding segments (exons).

As used herein, "recombinant" when used with reference to a cell typically indicates that the cell has been modified by the introduction of a foreign nucleic acid sequence or that the cell is derived from a cell so modified. For example, a recombinant cell may comprise a gene not found in identical form within the native (non-recombinant) form of the cell, or a recombinant cell may comprise a native gene (found in the native form of the cell) but which has been modified and re-introduced into the cell. A recombinant cell may comprise a nucleic acid endogenous to the cell that has been modified without removing the nucleic acid from the cell; such modifications include those obtained by gene replacement, site-specific mutation, and related techniques known to those of ordinary skill in the art. Recombinant DNA technology includes techniques for the production of recombinant DNA *in vitro* and transfer of the recombinant DNA into cells where it may be expressed or propagated, thereby producing a recombinant polypeptide. "Recombination," "recombining," and "recombined" of polynucleotides or nucleic acids refer generally to the assembly or combining of two or more nucleic acid or polynucleotide strands or fragments to generate a new polynucleotide or nucleic acid. The recombinant polynucleotide or nucleic acid is sometimes referred to as a chimera. A nucleic acid or polypeptide is "recombinant" when it is artificial or engineered.

A nucleic acid or polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods known to those of skill in the art, it can be transcribed and/or translated to produce the polypeptide or a fragment thereof. The anti-sense strand of such a nucleic acid is also said to encode the sequence.

"Host strain" or "host cell" refers to a suitable host for an expression vector comprising a DNA sequence of interest.

A "protein" or "polypeptide" comprises a polymeric sequence of amino acid residues. The terms "protein" and "polypeptide" are used interchangeably herein. The single and 3-letter code for amino acids as defined in conformity with the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN) is used through out this disclosure. The single letter X refers to any of the twenty amino acids. It is also understood that a polypeptide may be coded for by more than one nucleotide sequence due to the degeneracy of the genetic code. Mutations can be named by the one letter code for the parent amino acid, followed by a position number and then the one letter code for the variant amino acid. For example, mutating glycine (G) at position 87 to serine (S) is represented as "G087S" or "G87S". Mutations can also be named by using the three letter code for an amino acid followed by its position in the polypeptide chain as counted from the N-terminus; for example, Ala10 for alanine at position 10. Multiple mutations are indicated by inserting a "-" between the mutations. Mutations at positions 87 and 90 are

represented as either "G087S-A090Y" or "G87S-A90Y" or "G87S + A90Y" or "G087S + A090Y". For deletions, the one letter code "Z" is used. For an insertion relative to the parent sequence, the one letter code "Z" is on the left side of the position number. For a deletion, the one letter code "Z" is on the right side of the position number. For insertions, the position number is the position number before the inserted amino acid(s), plus 0.01 for each amino acid. For example, an insertion of three amino acids alanine (A), serine (S) and tyrosine (Y) between position 87 and 88 is shown as "Z087.01A-Z087.02S-Z087.03Y." Thus, combining all the mutations above plus a deletion at position 100 is: "G087S- Z087.01A-Z087.02S-Z087.03Y-A090Y-A100Z." When describing modifications, a position followed by amino acids listed in parentheses indicates a list of substitutions at that position by any of the listed amino acids. For example, 6(L,I) means position 6 can be substituted with a leucine or isoleucine.

A "prosequence" or "propetide sequence" refers to an amino acid sequence between the signal peptide sequence and mature protease sequence that is necessary for the proper folding and secretion of the protease; they are sometimes referred to as intramolecular chaperones. Cleavage of the prosequence or propeptide sequence results in a mature active protease. Bacterial metalloproteases are often expressed as pro-enzymes.

The term "signal sequence" or "signal peptide" refers to a sequence of amino acid residues that may participate in the secretion or direct transport of the mature or precursor form of a protein. The signal sequence is typically located N-terminal to the precursor or mature protein sequence. The signal sequence may be endogenous or exogenous. A signal sequence is normally absent from the mature protein. A signal sequence is typically cleaved from the protein by a signal peptidase after the protein is transported.

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

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

The term "wild-type" in reference to an amino acid sequence or nucleic acid sequence indicates that the amino acid sequence or nucleic acid sequence is native or naturally occurring

sequence. As used herein, the term "naturally-occurring" refers to anything (*e.g.*, proteins, amino acids, or nucleic acid sequences) that are found in nature.

As used herein, the term "non-naturally occurring" refers to anything that is not found in nature (*e.g.,* recombinant nucleic acids and protein sequences produced in the laboratory), as modification of the wild-type sequence.

As used herein with regard to amino acid residue positions, "corresponding to" or "corresponds to" or "corresponds" refers to an amino acid residue at the enumerated position in a protein or peptide, or an amino acid residue that is analogous, homologous, or equivalent to an enumerated residue in a protein or peptide. As used herein, "corresponding region" generally refers to an analogous position in a related proteins or a reference protein.

The terms "derived from" and "obtained from" refer to not only a protein produced or producible by a strain of the organism in question, but also a protein encoded by a DNA sequence isolated from such strain and produced in a host organism containing such DNA sequence. Additionally, the term refers to a protein which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the protein in question. To exemplify, "proteases derived from *Bacillus*" refers to those enzymes having proteolytic activity which are naturally produced by *Bacillus*, as well as to serine proteases like those produced by *Bacillus* sources but which through the use of genetic engineering techniques are produced by non-*Bacillus* organisms transformed with a nucleic acid encoding the serine proteases.

The term "identical" in the context of two nucleic acids or polypeptidesequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence, as measured using one of the following sequence comparison or analysis algorithms.

As used herein, "homologous genes" refers to a pair of genes from different, but usually related species, which correspond to each other and which are identical or very similar to each other. The term encompasses genes that are separated by speciation (i.e., the development of new species) (*e.g.,* orthologous genes), as well as genes that have been separated by genetic duplication (*e.g.*, paralogous genes).

As used herein, "% identity or percent identity" refers to sequence similarity. Percent identity may be determined using standard techniques known in the art (*See e.g.*, Smith and Waterman, Adv. Appl. Math. 2:482 [1981]; Needleman and Wunsch, J. Mol. Biol. 48:443 [1970]; Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 [1988]; software programs such as GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package

(Genetics Computer Group, Madison, WI); and Devereux *et al.*, Nucl. Acid Res. 12:387-395 [1984]). One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pair-wise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (*See*, Feng and Doolittle, J. Mol. Evol. 35:351-360 [1987]). The method is similar to that described by Higgins and Sharp (*See*, Higgins and Sharp, CABIOS 5:151-153 [1989]). Useful PILEUP parameters include a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps. Other useful algorithm is the BLAST algorithms described by Altschul *et al.,* (*See*, Altschul *et al.*, J. Mol. Biol. 215:403-410 [1990]; and Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787 [1993]). The BLAST program uses several search parameters, most of which are set to the default values.

The NCBI BLAST algorithm finds the most relevant sequences in terms of biological similarity but is not recommended for query sequences of less than 20 residues (Altschul, SF et al. (1997) Nucleic Acids Res. 25:3389-3402 and Schaffer, AA et al. (2001) Nucleic Acids Res. 29:2994-3005). Example default BLAST parameters for a nucleic acid sequence searches are:

- Neighboring words threshold : 11
- E-value cutoff : 10
- Scoring Matrix : NUC.3.1 (match = 1, mismatch = -3)
- Gap Opening : 5
- Gap Extension : 2

and the following parameters for amino acid sequence searches:

- Word size : 3
- E-value cutoff : 10
- Scoring Matrix : BLOSUM62
- Gap Opening : 11
- Gap extension : 1

A percent (%) amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "reference" sequence including any gaps created by the program for optimal/maximum alignment. If a sequence is 90% identical to SEQ ID NO: A, SEQ ID NO: A is is the “reference” sequence. BLAST algorithms refer the “reference” sequence as “query” sequence.

The CLUSTAL W algorithm is another example of a sequence alignment algorithm. *See* Thompson *et al.* (1994) *Nucleic Acids Res.* 22:4673-4680. Default parameters for the CLUSTAL W algorithm are:

Gap opening penalty:	10.0
Gap extension penalty:	0.05
Protein weight matrix:	BLOSUM series
DNA weight matrix:	IUB
Delay divergent sequences %:	40
Gap separation distance:	8
DNA transitions weight:	0.50
List hydrophilic residues:	GPSNDQEKR
Use negative matrix:	OFF
Toggle Residue specific penalties:	ON
Toggle hydrophilic penalties:	ON
Toggle end gap separation penalty	OFF.

In CLUSTAL algorithms, deletions occurring at either terminus are included. For example, a variant with five amino acid deletion at either terminus (or within the polypeptide) of a polypeptide of 500 amino acids would have a percent sequence identity of 99% (495/500 identical residues × 100) relative to the "reference" polypeptide. Such a variant would be encompassed by a variant having "at least 99% sequence identity" to the polypeptide.

A polypeptide of interest may be said to be "substantially identical" to a reference polypeptide if the polypeptide of interest comprises an amino acid sequence having at least about 60%, least about 65%, least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5% sequence identity to the amino acid sequence of the reference polypeptide. The percent identity between two such polypeptides can be determined manually by inspection of the two optimally aligned polypeptide sequences or by using software programs or algorithms (*e.g.,* BLAST, ALIGN, CLUSTAL) using standard parameters. One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two

peptides differ only by a conservative amino acid substitution or one or more conservative amino acid substitutions.

A nucleic acid of interest may be said to be "substantially identical" to a reference nucleic acid if the nucleic acid of interest comprises a nucleotide sequence having least about 60%, least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, at least about 99%, or at least about 99.5% sequence identity to the nucleotide sequence of the reference nucleic acid. The percent identity between two such nucleic acids can be determined manually by inspection of the two optimally aligned nucleic acid sequences or by using software programs or algorithms (*e.g.*, BLAST, ALIGN, CLUSTAL) using standard parameters. One indication that two nucleic acid sequences are substantially identical is that the two nucleic acid molecules hybridize to each other under stringent conditions (*e.g.*, within a range of medium to high stringency).

A nucleic acid or polynucleotide is "isolated" when it is at least partially or completely separated from other components, including but not limited to for example, other proteins, nucleic acids, cells, etc. Similarly, a polypeptide, protein or peptide is "isolated" when it is at least partially or completely separated from other components, including but not limited to for example, other proteins, nucleic acids, cells, etc. On a molar basis, an isolated species is more abundant than are other species in a composition. For example, an isolated species may comprise at least about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or about 100% (on a molar basis) of all macromolecular species present. Preferably, the species of interest is purified to essential homogeneity (*i.e.*, contaminant species cannot be detected in the composition by conventional detection methods). Purity and homogeneity can be determined using a number of techniques well known in the art, such as agarose or polyacrylamide gel electrophoresis of a nucleic acid or a protein sample, respectively, followed by visualization upon staining. If desired, a high-resolution technique, such as high performance liquid chromatography (HPLC) or a similar means can be utilized for purification of the material.

"Hybridization" refers to the process by which one strand of nucleic acid forms a duplex with, *i.e.*, base pairs with, a complementary strand. A nucleic acid sequence is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another under moderate to high stringency hybridization and wash conditions.

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex or probe. For example, "maximum stringency" typically occurs at about Tm-5°C (5° below the Tm of the probe); "high stringency" at about 5-10°C below the Tm; "intermediate stringency" at about 10-20°C below the Tm of the probe; and "low stringency" at about 20-25°C below the Tm. Functionally, maximum stringency conditions can be used to identify sequences having strict identity or near-strict identity with the hybridization probe; while intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

Moderate and high stringency hybridization conditions are well known in the art. Stringent hybridization conditions are exemplified by hybridization under the following conditions: 65°C and 0.1X SSC (where 1X SSC = 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.0). Hybridized, duplex nucleic acids are characterized by a melting temperature (T_m), where one half of the hybridized nucleic acids are unpaired with the complementary strand. Mismatched nucleic acids within the duplex lower the T_m. Very stringent hybridization conditions involve 68°C and 0.1X SSC. A nucleic acid encoding a variant metalloprotease can have a T_m reduced by 1°C – 3°C or more compared to a duplex formed between the nucleic acid and its identical complement.

Another example of high stringency conditions includes hybridization at about 42°C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured carrier DNA followed by washing two times in 2X SSC and 0.5% SDS at room temperature and two additional times in 0.1X SSC and 0.5% SDS at 42°C. An example of moderate stringent conditions include an overnight incubation at 37°C in a solution comprising 20% formamide, 5 x SSC (150mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1x SSC at about 37 - 50°C. Those of skill in the art know how to adjust the temperature, ionic strength, etc. to accommodate factors such as probe length and the like.

The term "purified" as applied to nucleic acids or polypeptides generally denotes a nucleic acid or polypeptide that is essentially free from other components as determined by analytical techniques well known in the art (*e.g.*, a purified polypeptide or polynucleotide forms a discrete band in an electrophoretic gel, chromatographic eluate, and/or a media subjected to density gradient centrifugation). For example, a nucleic acid or polypeptide that gives rise to essentially one band in an electrophoretic gel is "purified." A purified nucleic acid or polypeptide is at least about 50% pure, usually at least about 60%, about 65%, about 70%, about

75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, about 99.5%, about 99.6%, about 99.7%, about 99.8% or more pure (*e.g.*, percent by weight on a molar basis). In a related sense, the invention provides methods of enriching compositions for one or more molecules of the invention, such as one or more polypeptides or polynucleotides of the invention. A composition is enriched for a molecule when there is a substantial increase in the concentration of the molecule after application of a purification or enrichment technique. A substantially pure polypeptide or polynucleotide of the invention (*e.g.*, substantially pure metalloprotease polypeptide or polynucleotide encoding a metalloprotease polypeptide of the invention, respectively) will typically comprise at least about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98, about 99%, about 99.5% or more by weight (on a molar basis) of all macromolecular species in a particular composition.

The term "enriched" refers to a compound, polypeptide, cell, nucleic acid, amino acid, or other specified material or component that is present in a composition at a relative or absolute concentration that is higher than a starting composition.

In a related sense, the invention provides methods of enriching compositions for one or more molecules of the invention, such as one or more polypeptides of the invention (*e.g.*, one or more metalloprotease polypeptides of the invention) or one or more nucleic acids of the invention (*e.g.*, one or more nucleic acids encoding one or more metalloprotease polypeptides of the invention). A composition is enriched for a molecule when there is a substantial increase in the concentration of the molecule after application of a purification or enrichment technique. A substantially pure polypeptide or polynucleotide will typically comprise at least about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98, about 99%, about 99.5% or more by weight (on a molar basis) of all macromolecular species in a particular composition.

As used herein, the term "functional assay" refers to an assay that provides an indication of a protein's activity. In some embodiments, the term refers to assay systems in which a protein is analyzed for its ability to function in its usual capacity. For example, in the case of a protease, a functional assay involves determining the effectiveness of the protease to hydrolyze a proteinaceous substrate.

The terms "modified nucleic acid sequence" and "modified gene" are used interchangeably herein to refer to a nucleic acid sequence that includes a deletion, insertion or

interruption of naturally occurring (*i.e.*, wild-type) nucleic acid sequence. In some embodiments, the expression product of the modified nucleic acid sequence is a truncated protein (*e.g.*, if the modification is a deletion or interruption of the sequence). In some embodiments, the truncated protein retains biological activity. In alternative embodiments, the
5 expression product of the modified nucleic acid sequence is an elongated protein (*e.g.*, modifications comprising an insertion into the nucleic acid sequence). In some embodiments, a nucleotide insertion in the nucleic acid sequence leads to a truncated protein (*e.g.*, when the insertion results in the formation of a stop codon). Thus, an insertion may result in either a truncated protein or an elongated protein as an expression product.

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

15 As used herein, the phrase “alteration in substrate specificity” refers to changes in the substrate specificity of an enzyme. In some embodiments, a change in substrate specificity is defined as a change in k_{cat} and/or K_m for a particular substrate, resulting from mutations of the enzyme or alteration of reaction conditions. The substrate specificity of an enzyme is determined by comparing the catalytic efficiencies it exhibits with different substrates. These
20 determinations find particular use in assessing the efficiency of mutant enzymes, as it is generally desired to produce variant enzymes that exhibit greater ratios of k_{cat}/K_m for substrates of interest. However, it is not intended that the present invention be limited to any particular substrate composition or substrate specificity.

As used herein, “surface property” is used in reference to electrostatic charge, as well as
25 properties such as the hydrophobicity and hydrophilicity exhibited by the surface of a protein. As used herein, the term “net charge” is defined as the sum of all charges present in a molecule. “Net charge changes” are made to a parent protein molecule to provide a variant that has a net charge that differs from that of the parent molecule (*i.e.*, the variant has a net charge that is not the same as that of the parent molecule). For example, substitution of a neutral amino acid with a
30 negatively charged amino acid or a positively charged amino acid with a neutral amino acid results in net charge of -1 with respect to the parent molecule. Substitution of a positively charged amino acid with a negatively charged amino acid results in a net charge of -2 with respect to the parent. Substitution of a neutral amino acid with a positively charged amino acid or a negatively charged amino acid with a neutral amino acid results in net charge of +1 with

respect to the parent. Substitution of a negatively charged amino acid with a positively charged amino acid results in a net charge of +2 with respect to the parent. The net charge of a parent protein can also be altered by deletion and/or insertion of charged amino acids. A net change change applies to changes in charge of a variant versus a parent when measured at the same pH conditions.

The terms "thermally stable" and "thermostable" and "thermostability" refer to proteases that retain a specified amount of enzymatic activity after exposure to identified temperatures over a given period of time under conditions prevailing during the proteolytic, hydrolyzing, cleaning or other process of the invention, while being exposed to altered temperatures. "Altered temperatures" encompass increased or decreased temperatures. In some embodiments, the proteases retain at least about 50%, about 60%, about 70%, about 75%, about 80%, about 85%, about 90%, about 92%, about 95%, about 96%, about 97%, about 98%, or about 99% proteolytic activity after exposure to altered temperatures over a given time period, for example, at least about 60 minutes, about 120 minutes, about 180 minutes, about 240 minutes, about 300 minutes, etc.

The term "enhanced stability" in the context of an oxidation, chelator, thermal, chemical, autolytic and/or pH stable protease refers to a higher retained proteolytic activity over time as compared to other proteases (*e.g.*, thermolysin proteases) and/or wild-type enzymes.

The term "diminished stability" in the context of an oxidation, chelator, thermal and/or pH stable protease refers to a lower retained proteolytic activity over time as compared to other proteases (*e.g.*, thermolysin proteases) and/or wild-type enzymes.

The term "cleaning activity" refers to a cleaning performance achieved by a metalloprotease polypeptide or reference protease under conditions prevailing during the proteolytic, hydrolyzing, cleaning, or other process of the invention. In some embodiments, cleaning performance of a metalloprotease polypeptide or reference protease may be determined by using various assays for cleaning one or more various enzyme sensitive stains on an item or surface (*e.g.*, a stain resulting from food, grass, blood, ink, milk, oil, and/or egg protein). Cleaning performance of a variant or reference protease can be determined by subjecting the stain on the item or surface to standard wash condition(s) and assessing the degree to which the stain is removed by using various chromatographic, spectrophotometric, or other quantitative methodologies. Exemplary cleaning assays and methods are known in the art and include, but are not limited to those described in WO 99/34011 and U.S. Pat. 6,605,458, both of which are herein incorporated by reference, as well as those cleaning assays and methods included in the Examples provided below.

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

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

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

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

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

Cleaning composition or cleaning formulations, as used herein, include, unless otherwise indicated, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, granular, gel, solid, tablet, or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) detergent or heavy-duty powder detergent (HDD) types; liquid fine-fabric detergents; hand or manual dishwashing agents, including those of the high-foaming type; hand or manual dishwashing, automatic dishwashing, or dishware or tableware washing agents, including the various tablet, powder, solid, granular, liquid, gel, and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, including antibacterial hand-wash types, cleaning bars, mouthwashes, denture cleaners, car shampoos, carpet shampoos, bathroom cleaners; hair shampoos and/or hair-rinses for humans and other animals; shower gels and foam baths and metal cleaners; as well as cleaning auxiliaries, such as bleach additives and "stain-stick" or pre-treat types. In some embodiments, granular compositions are in "compact" form; in some embodiments, liquid compositions are in a "concentrated" form.

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

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

As used herein, the term "fabric and/or hard surface cleaning and/or treatment composition" is a subset of cleaning and treatment compositions that 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 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, car or carpet shampoos, bathroom cleaners including toilet bowl cleaners; fabric conditioning products including softening and/or freshening that may be in liquid, solid and/or dryer sheet form ; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types, substrate-laden products such as dryer added sheets. All of such products which are applicable may be in standard, concentrated

or even highly concentrated form even to the extent that such products may in certain aspect be non-aqueous.

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

As used herein, the term “bleaching” refers to the treatment of a material (*e.g.,* fabric, laundry, pulp, etc.) or surface for a sufficient length of time and/or under appropriate pH and/or temperature conditions to effect a brightening (*i.e.*, whitening) and/or cleaning of the material. Examples of chemicals suitable for bleaching include, but are not limited to, for example, ClO_2, H_2O_2, peracids, NO_2, etc.

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

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

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

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

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

As used herein in connection with a numerical value, the term “about” refers to a range 20 of +/- 0.5 of the numerical value, unless the term is otherwise specifically defined in context. For instance, the phrase a “pH value of about 6” refers to pH values of from 5.5 to 6.5, unless the pH value is specifically defined otherwise.

The position of an amino acid residue in a given amino acid sequence is typically numbered herein using the numbering of the position of the corresponding amino acid residue of 25 the wild type *Paenibacillus* metalloprotease amino acid sequences shown in SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 or 38. The *Paenibacillus sp.* metalloprotease amino acid sequences, thus serves as a reference parent sequence. A given amino acid sequence, such as a metalloprotease enzyme amino acid sequence and variants thereof described herein, can be aligned with the wild type metalloprotease sequence (*e.g.*, SEQ ID NO: 3) using an alignment algorithm as described 30 herein, and an amino acid residue in the given amino acid sequence that aligns (preferably optimally aligns) with an amino acid residue in the wild type sequence can be conveniently numbered by reference to the corresponding amino acid residue in the metalloprotease sequence.

Oligonucleotide synthesis and purification steps are typically performed according to specifications. Techniques and procedures are generally performed according to conventional

methods well known in the art and various general references that are provided throughout this document. Procedures therein are believed to be well known to those of ordinary skill in the art and are provided for the convenience of the reader.

5 Metalloprotease Polypeptides of the present invention

The present invention provides novel metalloprotease enzyme polypeptides, which may be collectively referred to as “enzymes of the invention” or “polypeptides of the invention.” Polypeptides of the invention include isolated, recombinant, substantially pure, or non-naturally occurring polypeptides. In some embodiments, polypeptides of the invention are useful in
10 cleaning applications and can be incorporated into cleaning compositions that are useful in methods of cleaning an item or a surface in need of cleaning.

In some embodiments, the enzyme of the present invention has 50, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 or 38. In various embodiments, the enzyme of the present invention has 50, 60, 65, 70, 75, 80, 85,
15 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to a metalloprotease enzyme from any genus in Tables 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2 or 8.2.

In some embodiments, the enzyme of the present invention, including all embodiments supra, can be derived from a member of the order Bacillales or family *Bacillaceae, Paenibacillaceae, Alicyclobacillaceae,* or *Lactobacillaceae*. In some embodiments, the enzyme
20 of the present invention, including all embodiments supra, can be derived from a *Bacillus, Alicyclobacillus, Geobacillus, Exiguobacterium, Lactobacillus, or Paenibacillus* species. In some embodiments, the enzyme of the present invention, including all embodiments supra, can be derived from a member of the Pseudococcidae family. In some embodiments, the enzyme of the present invention, including all embodiments supra, can be derived from a *Planococcus*
25 species. Various enzyme metalloproteases have been found that have a high identity to each other and to the *Paenibacillus* enzymes as shown in SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 or 38.

In a particular embodiment, the invention is an enzyme derived from the genus *Paenibacillus*. In a particular embodiment, the invention is an enzyme derived from the genus *Paenibacillus* and from the species *Paenibacillus sp., Paenibacillus ehimensis, Paenibacillus*
30 *hunanensis, Paenibacillus barcinonensis, Paenibacillus amylolyticus, Paenibacillus humicus* and *Paenibacillus polymyxa*.

Described are compositions and methods relating to enzymes cloned from *Paenibacillus.* The compositions and methods are based, in part, on the observation that cloned and expressed enzymes of the present invention have proteolytic activity in the presence of a detergent

composition. Enzymes of the present invention also demonstrate excellent stability in detergent compositions. These features makes enzymes of the present invention well suited for use in a variety of cleaning applications, where the enzyme can hydrolyze proteins in the presence of surfactants and other components found in detergent compositions.

5 In some embodiments, the invention includes an isolated, recombinant, substantially pure, or non-naturally occurring enzyme having protease activity, which polypeptide comprises a polypeptide sequence having at least about 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a parent enzyme as provided herein.

10 In some embodiments, the polypeptide of the present invention, is a polypeptide having a specified degree of amino acid sequence homology to the exemplified polypeptides, *e.g.*, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or even at least 99% sequence homology to the amino acid sequences of SEQ ID NOs: 3, 15 8, 13, 18, 23, 28, 33 or 38. Homology can be determined by amino acid sequence alignment, *e.g.*, using a program such as BLAST, ALIGN, or CLUSTAL, as described herein.

Also provided are polypeptide enzymes of the present invention, having protease activity, said enzymes comprising an amino acid sequence which differs from the amino acid sequence of SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 or 38 by no more than 50, no more than 40, no 20 more than 30, no more than 35, no more than 25, no more than 20, no more than 19, no more than 18, no more than 17, no more than 16, no more than 15, no more than 14, no more than 13, no more than 12, no more than 11, no more than 10, no more than 9, no more than 8, no more than 7, no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 amino acid residue(s), when aligned using any of the previously described 25 alignment methods.

As noted above, the variant enzyme polypeptides of the invention have enzymatic activities (*e.g.*, protease activities) and thus are useful in cleaning applications, including but not limited to, methods for cleaning dishware items, tableware items, fabrics, and items having hard surfaces (*e.g.*, the hard surface of a table, table top, wall, furniture item, floor, ceiling, etc.). 30 Exemplary cleaning compositions comprising one or more variant metalloprotease enzyme polypeptides of the invention are described *infra*. The enzymatic activity (*e.g.*, protease enzyme activity) of an enzyme polypeptide of the invention can be determined readily using procedures well known to those of ordinary skill in the art. The Examples presented *infra* describe methods for evaluating the enzymatic activity and cleaning performance. The performance of

polypeptide enzymes of the invention in removing stains (*e.g.*, a protein stain such as blood/milk/ink or egg yolk), cleaning hard surfaces, or cleaning laundry, dishware or tableware item(s) can be readily determined using procedures well known in the art and/or by using procedures set forth in the Examples.

5 The metalloprotease polypeptides of the invention have protease activity such that they are useful in casein hydrolysis, collagen hydrolysis, elastin hydrolysis, keratin hydrolysis, soy protein hydrolysis or corn meal protein hydrolysis. Thus, the polypeptides of the invention find use in other applications such as pretreatments for food, feed, or protein degradation.

The polypeptides of the invention are also useful in pretreatment of animal feed 10 products, such as soy protein, corn meal, and other protein rich components. Pretreatment of these animal feed products with a polypeptide of the invention may help in the breakdown of complex proteins into their hydrolysates which are easily digestible by animals.

In yet other embodiments, the disclosed metalloprotease polypeptides find use in hydrolysis of corn soy protein. The disclosed metalloprotease polypeptides may be used alone or 15 in combination with other proteases, amylases or lipases to produce peptides and free amino acids from the corn or soy protein. In some embodiments, the recovered proteins, peptides or amino acids can be subsequently used in animal feed or human food products.

The polypeptides of the invention are also useful in treatment of wounds, particularly in wound debridement. Wound debridement is the removal of dead, damaged or infected tissue to 20 improve the healing potential of the remaining healthy tissue. Debridement is an important part of the healing process for burns and other serious wounds. The wounds or burns may be treated with a composition comprising a polypeptide of the invention which would result in removal of unwanted damaged tissue and improving the healthy tissue.

The metalloprotease polypeptides of the present invention can have protease activity over a 25 broad range of pH conditions. In some embodiments, the metalloprotease polypeptides have protease activity on azo-casein as a substrate, as demonstrated in Examples 3.1 to 3.8. In some embodiments, the metalloprotease polypeptides have protease activity at a pH of from about 3.0 to about 12.0. In some embodiments, the metalloprotease polypeptides have protease activity at a pH of from about 4.0 to about 10.5. In some embodiments, the metalloprotease polypeptides 30 have at least 70% of maximal protease activity at a pH of from about 5.5 to about 9.0. In some embodiments, the metalloprotease polypeptides have at least 80% of maximal protease activity at a pH of from about 6.0 to about 8.5. In some embodiments, the metalloprotease polypeptides have maximal protease activity at a pH of about 7.5.

In some embodiments, the metalloprotease polypeptides of the present invention have protease activity at a temperature range of from about 10°C to about 100°C. In some embodiments, the metalloprotease polypeptides of the present invention have protease activity at a temperature range of from about 20°C to about 90°C. In some embodiments, the metalloprotease polypeptides have at least 70% of maximal protease activity at a temperature of from about 45°C to about 60°C. In some embodiments, the metalloprotease polypeptides have maximal protease activity at a temperature of 50°C.

In some embodiments, the metalloprotease polypeptides of the present invention demonstrate cleaning performance in a cleaning composition. Cleaning compositions often include ingredients harmful to the stability and performance of enzymes, making cleaning compositions a harsh environment for enzymes, e.g. metalloproteases, to retain function. Thus, it is not trivial for an enzyme to be put in a cleaning composition and expect enzymatic function (e.g. metalloprotease activity, such as demonstrated by cleaning performance). In some embodiments, the metalloprotease polypeptides of the present invention demonstrate cleaning performance in automatic dishwashing (ADW) detergent compositions. In some embodiments, the cleaning performance in automatic dishwashing (ADW) detergent compositions includes cleaning of egg yolk stains. In some embodiments, the metalloprotease polypeptides of the present invention demonstrate cleaning performance in laundry detergent compositions. In some embodiments, the cleaning performance in laundry detergent compositions includes cleaning of blood/milk/ink stains. In each of the cleaning compositions, the metalloprotease polypeptides of the present invention demonstrate cleaning performance with or without a bleach component.

The metalloprotease polypeptides of the invention have protease activity such that they are useful in casein hydrolysis, collagen hydrolysis, elastin hydrolysis, keratin hydrolysis, soy protein hydrolysis or corn meal protein hydrolysis. Thus, the polypeptides of the invention find use in other applications such as pretreatments for food, feed, or protein degradation.

A polypeptide of the invention can be subject to various changes, such as one or more amino acid insertions, deletions, and/or substitutions, either conservative or non-conservative, including where such changes do not substantially alter the enzymatic activity of the polypeptide. Similarly, a nucleic acid of the invention can also be subject to various changes, such as one or more substitutions of one or more nucleotides in one or more codons such that a particular codon encodes the same or a different amino acid, resulting in either a silent variation (*e.g.,* when the encoded amino acid is not altered by the nucleotide mutation) or non-silent

variation, one or more deletions of one or more nucleic acids (or codons) in the sequence, one or more additions or insertions of one or more nucleic acids (or codons) in the sequence, and/or cleavage of or one or more truncations of one or more nucleic acids (or codons) in the sequence. Many such changes in the nucleic acid sequence may not substantially alter the enzymatic activity of the resulting encoded polypeptide enzyme compared to the polypeptide enzyme encoded by the original nucleic acid sequence. A nucleic acid sequence of the invention can also be modified to include one or more codons that provide for optimum expression in an expression system (*e.g.,* bacterial expression system), while, if desired, said one or more codons still encode the same amino acid(s).

In some embodiments, the present invention provides a genus of enzyme polypeptides having the desired enzymatic activity (*e.g.,* protease enzyme activity or cleaning performance activity) which comprise sequences having the amino acid substitutions described herein and also which comprise one or more additional amino acid substitutions, such as conservative and non-conservative substitutions, wherein the polypeptide exhibits, maintains, or approximately maintains the desired enzymatic activity (*e.g.*, proteolytic activity, as reflected in the cleaning activity or performance of the polypeptide enzymes of SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 and 38). Amino acid substitutions in accordance with the invention may include, but are not limited to, one or more non-conservative substitutions and/or one or more conservative amino acid substitutions. A conservative amino acid residue substitution typically involves exchanging a member within one functional class of amino acid residues for a residue that belongs to the same functional class (conservative amino acid residues are considered functionally homologous or conserved in calculating percent functional homology). A conservative amino acid substitution typically involves the substitution of an amino acid in an amino acid sequence with a functionally similar amino acid. For example, alanine, glycine, serine, and threonine are functionally similar and thus may serve as conservative amino acid substitutions for one another. Aspartic acid and glutamic acid may serve as conservative substitutions for one another. Asparagine and glutamine may serve as conservative substitutions for one another. Arginine, lysine, and histidine may serve as conservative substitutions for one another. Isoleucine, leucine, methionine, and valine may serve as conservative substitutions for one another. Phenylalanine, tyrosine, and tryptophan may serve as conservative substitutions for one another.

Other conservative amino acid substitution groups can be envisioned. For example, amino acids can be grouped by similar function or chemical structure or composition (*e.g.*, acidic, basic, aliphatic, aromatic, sulfur-containing). For instance, an aliphatic grouping may comprise: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I). Other groups

containing amino acids that are considered conservative substitutions for one another include: aromatic: Phenylalanine (F), Tyrosine (Y), Tryptophan (W); sulfur-containing: Methionine (M), Cysteine (C); Basic: Arginine (R), Lysine (K), Histidine (H); Acidic: Aspartic acid (D), Glutamic acid (E); non-polar uncharged residues, Cysteine (C), Methionine (M), and Proline (P); hydrophilic uncharged residues: Serine (S), Threonine (T), Asparagine (N), and Glutamine (Q). Additional groupings of amino acids are well-known to those of skill in the art and described in various standard textbooks. Listing of a polypeptide sequence herein, in conjunction with the above substitution groups, provides an express listing of all conservatively substituted polypeptide sequences.

More conservative substitutions exist within the amino acid residue classes described above, which also or alternatively can be suitable. Conservation groups for substitutions that are more conservative include: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

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

As described elsewhere herein in greater detail and in the Examples provided herein, polypeptides of the invention may have cleaning abilities that may be compared to known proteases, including known metalloproteases.

Nucleic Acids of the Invention

The invention provides isolated, non-naturally occurring, or recombinant nucleic acids which may be collectively referred to as “nucleic acids of the invention” or “polynucleotides of the invention”, which encode polypeptides of the invention. Nucleic acids of the invention, including all described below, are useful in recombinant production (e.g., expression) of polypeptides of the invention, typically through expression of a plasmid expression vector comprising a sequence encoding the polypeptide of interest or fragment thereof. As discussed above, polypeptides include metalloprotease polypeptides having enzymatic activity (e.g., proteolytic activity) which are useful in cleaning applications and cleaning compositions for cleaning an item or a surface (e.g., surface of an item) in need of cleaning.

In some embodiments, the invention provides an isolated, recombinant, substantially pure, or non-naturally occurring nucleic acid comprising a nucleotide sequence encoding any polypeptide (including any fusion protein, etc.) of the invention described above in the section entitled "Polypeptides of the Invention" and elsewhere herein. The invention also provides an isolated, recombinant, substantially pure, or non-naturally-occurring nucleic acid comprising a nucleotide sequence encoding a combination of two or more of any polypeptides of the invention described above and elsewhere herein. In some embodiments, the nucleic acids of the present invention has 50, 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100% identity to SEQ ID NO: 4, 9, 14, 19, 24, 29, 34 and 39.

The present invention provides nucleic acids encoding a metalloprotease polypeptide of the present invention, wherein the metalloprotease polypeptide is a mature form having proteolytic activity, wherein the amino acid positions of the variant are numbered by correspondence with the amino acid sequence of *Paenibacillus* metalloprotease polypeptides set forth as SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 or 38.

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

As indicated above, recombinant DNA techniques useful in modification of nucleic acids are well known in the art. For example, techniques such as restriction endonuclease digestion, ligation, reverse transcription and cDNA production, and polymerase chain reaction (e.g., PCR)

are known and readily employed by those of skill in the art. Nucleotides of the invention may also be obtained by screening cDNA libraries using one or more oligonucleotide probes that can hybridize to or PCR-amplify polynucleotides which encode a metalloprotease polypeptide polypeptide(s) of the invention. Procedures for screening and isolating cDNA clones and PCR amplification procedures are well known to those of skill in the art and described in standard references known to those skilled in the art. Some nucleic acids of the invention can be obtained by altering a naturally occurring polynucleotide backbone (e.g., that encodes an enzyme or parent protease) by, for example, a known mutagenesis procedure (e.g., site-directed mutagenesis, site saturation mutagenesis, and in vitro recombination).

Methods for Making Modified Metalloprotease polypeptides of the Invention

A variety of methods are known in the art that are suitable for generating modified polynucleotides of the invention that encode metalloprotease polypeptides of the invention, including, but not limited to, for example, site-saturation mutagenesis, scanning mutagenesis, insertional mutagenesis, deletion mutagenesis, random mutagenesis, site-directed mutagenesis, and directed-evolution, as well as various other recombinatorial approaches. Methods for making modified polynucleotides and proteins (e.g., metalloprotease polypeptides) include DNA shuffling methodologies, methods based on non-homologous recombination of genes, such as ITCHY (See, Ostermeier et al., 7:2139-44 [1999]), SCRACHY (See, Lutz et al. 98:11248-53 [2001]), SHIPREC (See, Sieber et al., 19:456-60 [2001]), and NRR (See, Bittker et al., 20:1024-9 [2001]; Bittker et al., 101:7011-6 [2004]), and methods that rely on the use of oligonucleotides to insert random and targeted mutations, deletions and/or insertions (See, Ness et al., 20:1251-5 [2002]; Coco et al., 20:1246-50 [2002]; Zha et al., 4:34-9 [2003]; Glaser et al., 149:3903-13 [1992]).

Vectors, Cells, and Methods for Producing Metalloprotease polypeptides of the Invention

The present invention provides vectors comprising at least one metalloprotease polynucleotide of the invention described herein (e.g., a polynucleotide encoding a metalloprotease polypeptide of the invention described herein), expression vectors or expression cassettes comprising at least one nucleic acid or polynucleotide of the invention, isolated, substantially pure, or recombinant DNA constructs comprising at least one nucleic acid or polynucleotide of the invention, isolated or recombinant cells comprising at least one polynucleotide of the invention, and compositions comprising one or more such vectors, nucleic

acids, expression vectors, expression cassettes, DNA constructs, cells, cell cultures, or any combination or mixtures thereof.

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

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

An expression vector may be derived from plasmid or viral DNA, or in alternative embodiments, contains elements of both. Exemplary vectors include, but are not limited to pC194, pJH101, pE194, pHP13 (See, Harwood and Cutting [eds.], Chapter 3, Molecular Biological Methods for Bacillus, John Wiley & Sons [1990]; suitable replicating plasmids for B. subtilis include those listed on p. 92) See also, Perego, Integrational Vectors for Genetic Manipulations in Bacillus subtilis, in Sonenshein et al., [eds.] Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology and Molecular Genetics, American Society for Microbiology, Washington, D.C. [1993], pp. 615-624), and p2JM103BBI.

For expression and production of a protein of interest (e.g., metalloprotease polypeptide) in a cell, at least one expression vector comprising at least one copy of a polynucleotide encoding the metalloprotease polypeptide, and in some instances comprising multiple copies, is transformed into the cell under conditions suitable for expression of the metalloprotease. In some embodiments of the present invention, a polynucleotide sequence encoding the metalloprotease polypeptide (as well as other sequences included in the vector) is integrated into the genome of the host cell, while in other embodiments, a plasmid vector comprising a polynucleotide sequence encoding the metalloprotease polypeptide remains as autonomous

extra-chromosomal element within the cell. The invention provides both extrachromosomal nucleic acid elements as well as incoming nucleotide sequences that are integrated into the host cell genome. The vectors described herein are useful for production of the metalloprotease polypeptides of the invention. In some embodiments, a polynucleotide construct encoding the metalloprotease polypeptide is present on an integrating vector that enables the integration and optionally the amplification of the polynucleotide encoding the metalloprotease polypeptide into the host chromosome. Examples of sites for integration are well known to those skilled in the art. In some embodiments, transcription of a polynucleotide encoding a metalloprotease polypeptide of the invention is effectuated by a promoter that is the wild-type promoter for the selected precursor protease. In some other embodiments, the promoter is heterologous to the precursor protease, but is functional in the host cell. Specifically, examples of suitable promoters for use in bacterial host cells include, but are not limited to, for example, the amyE, amyQ, amyL, pstS, sacB, pSPAC, pAprE, pVeg, pHpaII promoters, the promoter of the B. stearothermophilus maltogenic amylase gene, the B. amyloliquefaciens (BAN) amylase gene, the B. subtilis alkaline protease gene, the B. clausii alkaline protease gene the B. pumilis xylosidase gene, the B. thuringiensis cryIIIA, and the B. licheniformis alpha-amylase gene. Additional promoters include, but are not limited to the A4 promoter, as well as phage Lambda PR or PL promoters, and the E. coli lac, trp or tac promoters.

Metalloprotease polypeptides of the present invention can be produced in host cells of any suitable microorganism, including bacteria and fungi. In some embodiments, metalloprotease polypeptides of the present invention can be produced in Gram-positive bacteria. In some embodiments, the host cells are *Bacillus spp.*, *Streptomyces spp.*, *Escherichia spp.*, *Aspergillus spp.*, *Trichoderma spp.*, *Pseudomonas spp.*, *Corynebacterium spp.*, *Saccharomyces spp.*, or *Pichia spp.* In some embodiments, the metalloprotease polypeptides are produced by Bacillus sp. host cells. Examples of *Bacillus sp.* host cells that find use in the production of the metalloprotease polypeptides of the invention include, but are not limited to *B. licheniformis, B. lentus, B. subtilis, B. amyloliquefaciens, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. coagulans, B. circulans, B. pumilis, B. thuringiensis, B. clausii, and B. megaterium*, as well as other organisms within the genus Bacillus. In some embodiments, B. subtilis host cells are used for production of metalloprotease polypeptides. U.S. Patents 5,264,366 and 4,760,025 (RE 34,606) describe various Bacillus host strains that can be used for producing metalloprotease polypeptide of the invention, although other suitable strains can be used.

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

15 In some embodiments, the Bacillus host cell is a *Bacillus sp.* that includes a mutation or deletion in at least one of the following genes, degU, degS, degR and degQ. In some embodiments, the mutation is in a degU gene, and in some embodiments the mutation is degU(Hy)32 (See e.g., Msadek et al., J. Bacteriol. 172:824-834 [1990]; and Olmos et al., Mol. Gen. Genet. 253:562–567 [1997]). In some embodiments, the Bacillus host comprises a
20 mutation or deletion in scoC4 (See e.g., Caldwell et al., J. Bacteriol. 183:7329-7340 [2001]); spoIIE (See e.g., Arigoni et al., Mol. Microbiol. 31:1407-1415 [1999]); and/or oppA or other genes of the opp operon (See e.g., Perego et al., Mol. Microbiol. 5:173-185 [1991]). Indeed, it is contemplated that any mutation in the opp operon that causes the same phenotype as a mutation in the oppA gene will find use in some embodiments of the altered Bacillus strain of the
25 invention. In some embodiments, these mutations occur alone, while in other embodiments, combinations of mutations are present. In some embodiments, an altered Bacillus host cell strain that can be used to produce a metalloprotease polypeptide of the invention is a Bacillus host strain that already includes a mutation in one or more of the above-mentioned genes. In addition, Bacillus sp. host cells that comprise mutation(s) and/or deletions of endogenous
30 protease genes find use. In some embodiments, the Bacillus host cell comprises a deletion of the aprE and the nprE genes. In other embodiments, the Bacillus sp. host cell comprises a deletion of 5 protease genes, while in other embodiments, the Bacillus sp. host cell comprises a deletion of 9 protease genes (See e.g., U.S. Pat. Appln. Pub. No. 2005/0202535, incorporated herein by reference).

Host cells are transformed with at least one nucleic acid encoding at least one metalloprotease polypeptide of the invention using any suitable method known in the art. Methods for introducing a nucleic acid (e.g., DNA) into Bacillus cells or *E. coli* cells utilizing plasmid DNA constructs or vectors and transforming such plasmid DNA constructs or vectors into such cells are well known. In some embodiments, the plasmids are subsequently isolated from *E. coli* cells and transformed into Bacillus cells. However, it is not essential to use intervening microorganisms such as *E. coli*, and in some embodiments, a DNA construct or vector is directly introduced into a Bacillus host.

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

In addition to commonly used methods, in some embodiments, host cells are directly transformed with a DNA construct or vector comprising a nucleic acid encoding a metalloprotease polypeptide of the invention (i.e., an intermediate cell is not used to amplify, or otherwise process, the DNA construct or vector prior to introduction into the host cell). Introduction of the DNA construct or vector of the invention into the host cell includes those physical and chemical methods known in the art to introduce a nucleic acid sequence (e.g., DNA sequence) into a host cell without insertion into the host genome. Such methods include, but are not limited to calcium chloride precipitation, electroporation, naked DNA, liposomes and the like. In additional embodiments, DNA constructs or vector are co-transformed with a plasmid,

without being inserted into the plasmid. In further embodiments, a selective marker is deleted from the altered Bacillus strain by methods known in the art (See, Stahl et al., J. Bacteriol. 158:411-418 [1984]; and Palmeros et al., Gene 247:255 -264 [2000]).

In some embodiments, the transformed cells of the present invention are cultured in conventional nutrient media. The suitable specific culture conditions, such as temperature, pH and the like are known to those skilled in the art and are well described in the scientific literature. In some embodiments, the invention provides a culture (e.g., cell culture) comprising at least one metalloprotease polypeptide or at least one nucleic acid of the invention.

In some embodiments, host cells transformed with at least one polynucleotide sequence encoding at least one metalloprotease polypeptide of the invention are cultured in a suitable nutrient medium under conditions permitting the expression of the present protease, after which the resulting protease is recovered from the culture. In some embodiments, the protease produced by the cells is recovered from the culture medium by conventional procedures, including, but not limited to for example, separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt (e.g., ammonium sulfate), chromatographic purification (e.g., ion exchange, gel filtration, affinity, etc.).

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

Assays for detecting and measuring the enzymatic activity of an enzyme, such as a metalloprotease polypeptide of the invention, are well known. Various assays for detecting and measuring activity of proteases (e.g., metalloprotease polypeptides of the invention), are also known to those of ordinary skill in the art. In particular, assays are available for measuring

protease activity that are based on the release of acid-soluble peptides from casein or hemoglobin, measured as absorbance at 280 nm or colorimetrically using the Folin method. Other exemplary assays involve the solubilization of chromogenic substrates (See e.g., Ward, "Proteinases," in Fogarty (ed.)., Microbial Enzymes and Biotechnology, Applied Science, London, [1983], pp. 251-317). Other exemplary assays include, but are not limited to succinyl-Ala-Ala-Pro-Phe-para nitroanilide assay (suc-AAPF-pNA) and the 2,4,6-trinitrobenzene sulfonate sodium salt assay (TNBS assay). Numerous additional references known to those in the art provide suitable methods (See e.g., Wells et al., Nucleic Acids Res. 11:7911-7925 [1983]; Christianson et al., Anal. Biochem. 223:119 -129 [1994]; and Hsia et al., Anal Biochem. 242:221-227 [1999]).

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

In some other embodiments, the invention provides methods for making or producing a mature metalloprotease polypeptide of the invention. A mature metalloprotease polypeptide does not include a signal peptide or a propeptide sequence. Some methods comprise making or producing a metalloprotease polypeptide of the invention in a recombinant bacterial host cell, such as for example, a Bacillus sp. cell (e.g., a B. subtilis cell). In some embodiments, the invention provides a method of producing a metalloprotease polypeptide of the invention, the method comprising cultivating a recombinant host cell comprising a recombinant expression vector comprising a nucleic acid encoding a metalloprotease polypeptide of the invention under conditions conducive to the production of the metalloprotease polypeptide. Some such methods further comprise recovering the metalloprotease polypeptide from the culture.

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

Fabric and Home Care Products

In some embodiments, the metalloprotease polypeptides of the present invention can be used in compositions comprising an adjunct material and a metalloprotease polypeptide, wherein the composition is a fabric and home care product.

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

In some embodiments, the fabric and home care product composition is a liquid laundry detergent or a dishwashing detergent, such as an automatic dishwashing (ADW) detergent or hand dishwashing detergent.

It is intended that the fabric and home care product is provided in any suitable form, including a fluid or solid, or granular, powder, solid, bar, liquid, tablet, gel, or paste form. The fabric and home care product may be in the form of a unit dose pouch, especially when in the form of a liquid, and typically the fabric and home care product is at least partially, or even completely, enclosed by a water-soluble pouch. In addition, in some embodiments of the fabric and home care products comprising at least one metalloprotease polypeptide, the fabric and home care product may have any combination of parameters and/or characteristics detailed above.

Compositions having the metalloprotease polypeptide of the present invention

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

the invention include cleaning compositions, such as detergent compositions. In the exemplified detergent compositions, the enzymes levels are expressed by pure enzyme by weight of the total composition and unless otherwise specified, the detergent ingredients are expressed by weight of the total compositions.

5 As indicated herein, in some embodiments, the cleaning compositions of the present invention further comprise adjunct materials including, but not limited to, surfactants, builders, bleaches, bleach activators, bleach catalysts, other enzymes, enzyme stabilizing systems, chelants, optical brighteners, soil release polymers, dye transfer agents, dispersants, suds suppressors, dyes, perfumes, colorants, filler salts, hydrotropes, photoactivators, fluorescers, 10 fabric conditioners, hydrolyzable surfactants, preservatives, anti-oxidants, anti-shrinkage agents, anti-wrinkle agents, germicides, fungicides, color speckles, silvercare, anti-tarnish and/or anti-corrosion agents, alkalinity sources, solubilizing agents, carriers, processing aids, pigments, and pH control agents (See e.g., U.S. Pat. Nos. 6,610,642, 6,605,458, 5,705,464, 5,710,115, 5,698,504, 5,695,679, 5,686,014 and 5,646,101, all of which are incorporated herein by 15 reference). Embodiments of specific cleaning composition materials are exemplified in detail below. In embodiments in which the cleaning adjunct materials are not compatible with the metalloprotease polypeptides of the present invention in the cleaning compositions, then suitable methods of keeping the cleaning adjunct materials and the protease(s) separated (i.e., not in contact with each other) until combination of the two components is appropriate are used. Such 20 separation methods include any suitable method known in the art (e.g., gelcaps, encapsulation, tablets, physical separation, etc.).

The cleaning compositions of the present invention are advantageously employed for example, in laundry applications, hard surface cleaning, dishwashing applications, including automatic dishwashing and hand dishwashing, as well as cosmetic applications such as dentures, 25 teeth, hair and skin. In addition, due to the unique advantages of increased effectiveness in lower temperature solutions, the enzymes of the present invention are ideally suited for laundry applications. Furthermore, the enzymes of the present invention find use in granular and liquid compositions.

The metalloprotease polypeptides of the present invention also find use in cleaning 30 additive products. In some embodiments, low temperature solution cleaning applications find use. In some embodiments, the present invention provides cleaning additive products including at least one enzyme of the present invention is ideally suited for inclusion in a wash process when additional bleaching effectiveness is desired. Such instances include, but are not limited to low temperature solution cleaning applications. In some embodiments, the additive product is in

its simplest form, one or more proteases. In some embodiments, the additive is packaged in dosage form for addition to a cleaning process. In some embodiments, the additive is packaged in dosage form for addition to a cleaning process where a source of peroxygen is employed and increased bleaching effectiveness is desired. Any suitable single dosage unit form finds use with the present invention, including but not limited to pills, tablets, gelcaps, or other single dosage units such as pre-measured powders or liquids. In some embodiments, filler(s) or carrier material(s) are included to increase the volume of such compositions. 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. Suitable filler or carrier materials for liquid compositions include, but are not limited to water 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. In some embodiments, the compositions contain from about 5% to about 90% of such materials. Acidic fillers find use to reduce pH. Alternatively, in some embodiments, the cleaning additive includes adjunct ingredients, as more fully described below.

The present cleaning compositions and cleaning additives require an effective amount of at least one of the metalloprotease polypeptides provided herein, alone or in combination with other proteases and/or additional enzymes. The required level of enzyme is achieved by the addition of one or more metalloprotease polypeptides of the present invention. Typically the present cleaning compositions comprise at least about 0.0001 weight percent, from about 0.0001 to about 10, from about 0.001 to about 1, or from about 0.01 to about 0.1 weight percent of at least one of the metalloprotease polypeptides of the present invention.

The cleaning compositions herein are typically formulated such that, during use in aqueous cleaning operations, the wash water will have a pH of from about 4.0 to about 11.5, or even from about 5.0 to about 11.5, or even from about 5.0 to about 8.0, or even from about 7.5 to about 10.5. Liquid product formulations are typically formulated to have a pH from about 3.0 to about 9.0 or even from about 3 to about 5. Granular laundry products are typically formulated to have a pH from about 9 to about 11. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

Suitable "low pH cleaning compositions" typically have a pH of from about 3 to about 5, and are typically free of surfactants that hydrolyze in such a pH environment. Such surfactants include sodium alkyl sulfate surfactants that comprise at least one ethylene oxide moiety or even from about 1 to about 16 moles of ethylene oxide. Such cleaning compositions typically comprise a sufficient amount of a pH modifier, such as sodium hydroxide, monoethanolamine or

hydrochloric acid, to provide such cleaning composition with a pH of from about 3 to about 5. Such compositions typically comprise at least one acid stable enzyme. In some embodiments, the compositions are liquids, while in other embodiments, they are solids. The pH of such liquid compositions is typically measured as a neat pH. The pH of such solid compositions is
5 measured as a 10% solids solution of said composition wherein the solvent is distilled water. In these embodiments, all pH measurements are taken at 20°C, unless otherwise indicated.

In some embodiments, when the metalloprotease polypeptide (s) is/are employed in a granular composition or liquid, it is desirable for the metalloprotease polypeptide to be in the form of an encapsulated particle to protect the metalloprotease polypeptide from other
10 components of the granular composition during storage. In addition, encapsulation is also a means of controlling the availability of the metalloprotease polypeptide during the cleaning process. In some embodiments, encapsulation enhances the performance of the metalloprotease polypeptide (s) and/or additional enzymes. In this regard, the metalloprotease polypeptides of the present invention are encapsulated with any suitable encapsulating material known in the art.
15 In some embodiments, the encapsulating material typically encapsulates at least part of the metalloprotease polypeptide (s) of the present invention. Typically, the encapsulating material is water-soluble and/or water-dispersible. In some embodiments, the encapsulating material has a glass transition temperature (Tg) of 0°C or higher. Glass transition temperature is described in more detail in WO 97/11151. The encapsulating material is typically selected from consisting of
20 carbohydrates, natural or synthetic gums, chitin, chitosan, cellulose and cellulose derivatives, silicates, phosphates, borates, polyvinyl alcohol, polyethylene glycol, paraffin waxes, and combinations thereof. When the encapsulating material is a carbohydrate, it is typically selected from monosaccharides, oligosaccharides, polysaccharides, and combinations thereof. In some typical embodiments, the encapsulating material is a starch (See e.g., EP 0 922 499; US
25 4,977,252; US 5,354,559, and US 5,935,826). In some embodiments, the encapsulating material is a microsphere made from plastic such as thermoplastics, acrylonitrile, methacrylonitrile, polyacrylonitrile, polymethacrylonitrile and mixtures thereof; commercially available microspheres that find use include, but are not limited to those supplied by EXPANCEL® (Stockviksverken, Sweden), and PM 6545, PM 6550, PM 7220, PM 7228,
30 EXTENDOSPHERES®, LUXSIL®, Q-CEL®, and SPHERICEL® (PQ Corp., Valley Forge, PA).

As described herein, the metalloprotease polypeptides of the present invention find particular use in the cleaning industry, including, but not limited to laundry and dish detergents. These applications place enzymes under various environmental stresses. The metalloprotease

polypeptides of the present invention provide advantages over many currently used enzymes, due to their stability under various conditions.

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

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

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

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

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

In light of the foregoing, it is evident that concentrations of detergent compositions in typical wash solutions throughout the world varies from less than about 800 ppm of detergent to about 6000 ppm in high suds phosphate builder geographies.

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

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

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

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

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

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

5 Accordingly, in some embodiments, the present invention provides metalloprotease polypeptides that show surprising wash performance in at least one set of wash conditions (e.g., water temperature, water hardness, and/or detergent concentration). In some embodiments, the metalloprotease polypeptides of the present invention are comparable in wash performance to other metalloprotease polypeptide proteases. In some embodiments of the present invention, the 10 metalloprotease polypeptides provided herein exhibit enhanced oxidative stability, enhanced thermal stability, enhanced cleaning capabilities under various conditions, and/or enhanced chelator stability. In addition, the metalloprotease polypeptides of the present invention find use in cleaning compositions that do not include detergents, again either alone or in combination with builders and stabilizers.

15 In some embodiments of the present invention, the cleaning compositions comprise at least one metalloprotease polypeptide of the present invention at a level from about 0.00001 % to about 10% by weight of the composition and the balance (e.g., about 99.999% to about 90.0%) comprising cleaning adjunct materials by weight of composition. In some other embodiments of the present invention, the cleaning compositions of the present invention 20 comprises at least one metalloprotease polypeptide at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% by weight of the composition and the balance of the cleaning composition (e.g., about 99.9999% to about 90.0%, about 99.999 % to about 98%, about 99.995% to about 99.5% by weight) comprising cleaning adjunct materials.

25 In some embodiments, the cleaning compositions of the present invention comprise one or more additional detergent enzymes, which provide cleaning performance and/or fabric care and/or dishwashing benefits. Examples of suitable enzymes include, but are not limited to, acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, 30 cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases,

tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, and xylosidases, or any combinations or mixtures thereof. In some embodiments, a combination of enzymes is used (i.e., a "cocktail") comprising conventional applicable enzymes like protease, lipase, cutinase and/or cellulase in conjunction with amylase is used.

5 In addition to the metalloprotease polypeptides provided herein, any other suitable protease finds use in the compositions of the present invention. Suitable proteases include those of animal, vegetable or microbial origin. In some embodiments, microbial proteases are used. In some embodiments, chemically or genetically modified mutants are included. In some embodiments, the protease is a serine protease, preferably an alkaline microbial protease or a 10 trypsin-like protease. Examples of alkaline proteases include subtilisins, especially those derived from Bacillus (e.g., subtilisin, lentus, amyloliquefaciens, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168). Additional examples include those mutant proteases described in U.S. Pat. Nos. RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628, all of which are incorporated herein by reference. Additional protease examples include, but are not 15 limited to trypsin (e.g., of porcine or bovine origin), and the Fusarium protease described in WO 89/06270. In some embodiments, commercially available protease enzymes that find use in the present invention include, but are not limited to MAXATASE®, MAXACAL™, MAXAPEM™, OPTICLEAN®, OPTIMASE®, PROPERASE®, PURAFECT®, PURAFECT® OXP, PURAMAX™, EXCELLASE™, and PURAFAST™ (Genencor); 20 ALCALASE®, SAVINASE®, PRIMASE®, DURAZYM™, POLARZYME®, OVOZYME®, KANNASE®, LIQUANASE®, NEUTRASE®, RELASE® and ESPERASE® (Novozymes); BLAP™ and BLAP™ variants (Henkel Kommanditgesellschaft auf Aktien, Duesseldorf, Germany), and KAP (B. alkalophilus subtilisin; Kao Corp., Tokyo, Japan). Various proteases are described in WO95/23221, WO 92/21760, WO 09/149200, WO 09/149144, WO 09/149145, 25 WO 11/072099, WO 10/056640, WO 10/056653, WO 11/140364, WO 12/151534, U.S. Pat. Publ. No. 2008/0090747, and U.S. Pat. Nos. 5,801,039, 5,340,735, 5,500,364, 5,855,625, US RE 34,606, 5,955,340, 5,700,676, 6,312,936, and 6,482,628, and various other patents. In some further embodiments, metalloproteases find use in the present invention, including but not limited to the neutral metalloprotease described in WO 07/044993.

30 In addition, any suitable lipase finds use in the present invention. Suitable lipases include, but are not limited to those of bacterial or fungal origin. Chemically or genetically modified mutants are encompassed by the present invention. Examples of useful lipases include Humicola lanuginosa lipase (See e.g., EP 258 068, and EP 305 216), Rhizomucor miehei lipase (See e.g., EP 238 023), Candida lipase, such as C. antarctica lipase (e.g., the C. antarctica lipase

A or B; See e.g., EP 214 761), Pseudomonas lipases such as P. alcaligenes lipase and P. pseudoalcaligenes lipase (See e.g., EP 218 272), P. cepacia lipase (See e.g., EP 331 376), P. stutzeri lipase (See e.g., GB 1,372,034), P. fluorescens lipase, Bacillus lipase (e.g., B. subtilis lipase [Dartois et al., Biochem. Biophys. Acta 1131:253-260 [1993]); B. stearothermophilus
5 lipase [See e.g., JP 64/744992]; and B. pumilus lipase [See e.g., WO 91/16422]).

Furthermore, a number of cloned lipases find use in some embodiments of the present invention, including but not limited to Penicillium camembertii lipase (See, Yamaguchi et al., Gene 103:61-67 [1991]), Geotricum candidum lipase (See, Schimada et al., J. Biochem., 106:383-388 [1989]), and various Rhizopus lipases such as R. delemar lipase (See, Hass et al.,
10 Gene 109:117-113 [1991]), a R. niveus lipase (Kugimiya et al., Biosci. Biotech. Biochem. 56:716-719 [1992]) and R. oryzae lipase.

Other types of lipase polypeptide enzymes such as cutinases also find use in some embodiments of the present invention, including but not limited to the cutinase derived from Pseudomonas mendocina (See, WO 88/09367), and the cutinase derived from Fusarium solani
15 pisi (See, WO 90/09446).

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

20 In some embodiments of the present invention, the cleaning compositions of the present invention further comprise lipases at a level from about 0.00001 % to about 10% of additional lipase by weight of the composition and the balance of cleaning adjunct materials by weight of composition. In some other embodiments of the present invention, the cleaning compositions of the present invention also comprise lipases at a level of about 0.0001 % to about 10%, about
25 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% lipase by weight of the composition.

In some embodiments of the present invention, any suitable amylase finds use in the present invention. In some embodiments, any amylase (e.g., alpha and/or beta) suitable for use in alkaline solutions also find use. Suitable amylases include, but are not limited to those of
30 bacterial or fungal origin. Chemically or genetically modified mutants are included in some embodiments. Amylases that find use in the present invention, include, but are not limited to α-amylases obtained from B. licheniformis (See e.g., GB 1,296,839). Additional suitable amylases include those found in W09510603, WO9526397, WO9623874, WO9623873, WO9741213, WO9919467, WO0060060, WO0029560, WO9923211, WO9946399, WO0060058,

WO0060059, WO9942567, WO0114532, WO02092797, WO0166712, WO0188107, WO0196537, WO0210355, WO9402597, WO0231124, WO9943793, WO9943794, WO2004113551, WO2005001064, WO2005003311, WO0164852, WO2006063594, WO2006066594, WO2006066596, WO2006012899, WO2008092919, WO2008000825, WO2005018336, WO2005066338, WO2009140504, WO2005019443, WO2010091221, WO2010088447, WO0134784, WO2006012902, WO2006031554, WO2006136161, WO2008101894, WO2010059413, WO2011098531, WO2011080352, WO2011080353, WO2011080354, WO2011082425, WO2011082429, WO2011076123, WO2011087836, WO2011076897, WO94183314, WO9535382, WO9909183, WO9826078, WO9902702, WO9743424, WO9929876, WO9100353, WO9605295, WO9630481, WO9710342, WO2008088493, WO2009149419, WO2009061381, WO2009100102, WO2010104675, WO2010117511, and WO2010115021. Commercially available amylases that find use in the present invention include, but are not limited to DURAMYL®, TERMAMYL®, FUNGAMYL®, STAINZYME®, STAINZYME PLUS®, STAINZYME ULTRA®, and BAN™ (Novozymes), as well as POWERASE™, RAPIDASE® and MAXAMYL® P (Genencor).

In some embodiments of the present invention, the cleaning compositions of the present invention further comprise amylases at a level from about 0.00001 % to about 10% of additional amylase by weight of the composition and the balance of cleaning adjunct materials by weight of composition. In some other embodiments of the present invention, the cleaning compositions of the present invention also comprise amylases at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% amylase by weight of the composition.

In some further embodiments, any suitable cellulase finds used in the cleaning compositions of the present invention. Suitable cellulases include, but are not limited to those of bacterial or fungal origin. Chemically or genetically modified mutants are included in some embodiments. Suitable cellulases include, but are not limited to Humicola insolens cellulases (See e.g., U.S. Pat. No. 4,435,307). Especially suitable cellulases are the cellulases having color care benefits (See e.g., EP 0 495 257). Commercially available cellulases that find use in the present include, but are not limited to CELLUZYME, CELLUCLEAN, CAREZYME (Novozymes), PURADEX AND REVITALENZ (Danisco US Inc.), and KAC-500(B) (Kao Corporation). In some embodiments, cellulases are incorporated as portions or fragments of mature wild-type or variant cellulases, wherein a portion of the N-terminus is deleted (See e.g., U.S. Pat. No. 5,874,276). Additional suitable cellulases include those found in WO2005054475,

WO2005056787, U.S. Pat. Nos. 7,449,318, and 7,833,773. In some embodiments, the cleaning compositions of the present invention further comprise cellulases at a level from about 0.00001 % to about 10% of additional cellulase by weight of the composition and the balance of cleaning adjunct materials by weight of composition. In some other embodiments of the present invention, the cleaning compositions of the present invention also comprise cellulases at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% cellulase by weight of the composition.

Any mannanase suitable for use in detergent compositions also finds use in the present invention. Suitable mannanases include, but are not limited to those of bacterial or fungal origin. Chemically or genetically modified mutants are included in some embodiments. Various mannanases are known which find use in the present invention (See e.g., U.S. Pat. Nos. 6,566,114; 6,602,842; 5,476,775 and 6,440,991, and US Prov. App. Serial No. 61/739267; all of which are incorporated herein by reference). Commercially available mannanases that find use in the present invention include, but are not limited to MANNASTAR, PURABRITE, and MANNAWAY. In some embodiments, the cleaning compositions of the present invention further comprise mannanases at a level from about 0.00001 % to about 10% of additional mannanase by weight of the composition and the balance of cleaning adjunct materials by weight of composition. In some embodiments of the present invention, the cleaning compositions of the present invention also comprise mannanases at a level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% mannanase by weight of the composition.

In some embodiments, peroxidases are used in combination with hydrogen peroxide or a source thereof (e.g., a percarbonate, perborate or persulfate) in the compositions of the present invention. In some alternative embodiments, oxidases are used in combination with oxygen. Both types of enzymes are used for "solution bleaching" (i.e., to prevent transfer of a textile dye from a dyed fabric to another fabric when the fabrics are washed together in a wash liquor), preferably together with an enhancing agent (See e.g., WO 94/12621 and WO 95/01426). Suitable peroxidases/oxidases include, but are not limited to those of plant, bacterial or fungal origin. Chemically or genetically modified mutants are included in some embodiments. In some embodiments, the cleaning compositions of the present invention further comprise peroxidase and/or oxidase enzymes at a level from about 0.00001 % to about 10% of additional peroxidase and/or oxidase by weight of the composition and the balance of cleaning adjunct materials by weight of composition. In some other embodiments of the present invention, the cleaning compositions of the present invention also comprise, peroxidase and/or oxidase enzymes at a

level of about 0.0001 % to about 10%, about 0.001% to about 5%, about 0.001% to about 2%, about 0.005% to about 0.5% peroxidase and/or oxidase enzymes by weight of the composition.

In some embodiments, additional enzymes find use, including but not limited to perhydrolases (See e.g., WO 05/056782). In addition, in some embodiments, mixtures of the 5 above mentioned enzymes are encompassed herein, in particular one or more additional protease, amylase, lipase, mannanase, and/or at least one cellulase. Indeed, it is contemplated that various mixtures of these enzymes will find use in the present invention. It is also contemplated that the varying levels of the metalloprotease polypeptide (s) and one or more additional enzymes may both independently range to about 10%, the balance of the cleaning 10 composition being cleaning adjunct materials. The specific selection of cleaning adjunct materials are 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 (e.g., through the wash detergent use).

Examples of suitable cleaning adjunct materials include, but are not limited to, 15 surfactants, builders, bleaches, bleach activators, bleach catalysts, other enzymes, enzyme stabilizing systems, chelants, optical brighteners, soil release polymers, dye transfer agents, dye transfer inhibiting agents, catalytic materials, hydrogen peroxide, sources of hydrogen peroxide, preformed peracids, polymeric dispersing agents, clay soil removal agents, structure elasticizing agents, dispersants, suds suppressors, dyes, perfumes, colorants, filler salts, hydrotropes, 20 photoactivators, fluorescers, fabric conditioners, fabric softeners, carriers, hydrotropes, processing aids, solvents, pigments, hydrolyzable surfactants, preservatives, anti-oxidants, anti-shrinkage agents, anti-wrinkle agents, germicides, fungicides, color speckles, silvercare, anti-tarnish and/or anti-corrosion agents, alkalinity sources, solubilizing agents, carriers, processing aids, pigments, and pH control agents (See e.g., U.S. Pat. Nos. 6,610,642; 6,605,458; 5,705,464; 25 5,710,115; 5,698,504; 5,695,679; 5,686,014 and 5,646,101, all of which are incorporated herein by reference). Embodiments of specific cleaning composition materials are exemplified in detail below. In embodiments in which the cleaning adjunct materials are not compatible with the metalloprotease polypeptides of the present invention in the cleaning compositions, then suitable methods of keeping the cleaning adjunct materials and the protease(s) separated (i.e., not in 30 contact with each other) until combination of the two components is appropriate are used. Such separation methods include any suitable method known in the art (e.g., gelcaps, encapsulation, tablets, physical separation, etc.).

In some embodiments, an effective amount of one or more metalloprotease polypeptide (s) provided herein is included in compositions useful for cleaning a variety of surfaces in need

of proteinaceous stain removal. Such cleaning compositions include cleaning compositions for such applications as cleaning hard surfaces, fabrics, and dishes. Indeed, in some embodiments, the present invention provides fabric cleaning compositions, while in other embodiments, the present invention provides non-fabric cleaning compositions. Notably, the present invention also provides cleaning compositions suitable for personal care, including oral care (including dentrifices, toothpastes, mouthwashes, etc., as well as denture cleaning compositions), skin, and hair cleaning compositions. It is intended that the present invention encompass detergent compositions in any form (i.e., liquid, granular, bar, semi-solid, gels, emulsions, tablets, capsules, etc.).

By way of example, several cleaning compositions wherein the metalloprotease polypeptides of the present invention find use are described in greater detail below. In some embodiments in which the cleaning compositions of the present invention are formulated as compositions suitable for use in laundry machine washing method(s), the compositions of the present invention preferably contain at least one surfactant and at least one builder compound, as well as one or more cleaning adjunct materials preferably selected from organic polymeric compounds, bleaching agents, additional enzymes, suds suppressors, dispersants, lime-soap dispersants, soil suspension and anti-redeposition agents and corrosion inhibitors. In some embodiments, laundry compositions also contain softening agents (i.e., as additional cleaning adjunct materials). The compositions of the present invention also find use in detergent additive products in solid or liquid form. Such additive products are intended to supplement and/or boost the performance of conventional detergent compositions and can be added at any stage of the cleaning process. In some embodiments, the density of the laundry detergent compositions herein ranges from about 400 to about 1200 g/liter, while in other embodiments, it ranges from about 500 to about 950 g/liter of composition measured at 20°C.

In embodiments formulated as compositions for use in manual dishwashing methods, the compositions of the invention preferably contain at least one surfactant and preferably at least one additional cleaning adjunct material selected from organic polymeric compounds, suds enhancing agents, group II metal ions, solvents, hydrotropes and additional enzymes.

In some embodiments, various cleaning compositions such as those provided in U.S, Pat. No. 6,605,458, find use with the metalloprotease polypeptides of the present invention. Thus, in some embodiments, the compositions comprising at least one metalloprotease polypeptide of the present invention is a compact granular fabric cleaning composition, while in other embodiments, the composition is a granular fabric cleaning composition useful in the laundering of colored fabrics, in further embodiments, the composition is a granular fabric cleaning

composition which provides softening through the wash capacity, in additional embodiments, the composition is a heavy duty liquid fabric cleaning composition. In some embodiments, the compositions comprising at least one metalloprotease polypeptide of the present invention are fabric cleaning compositions such as those described in U.S. Pat. Nos. 6,610,642 and 6,376,450.
5 In addition, the metalloprotease polypeptides of the present invention find use in granular laundry detergent compositions of particular utility under European or Japanese washing conditions (See e.g., U.S. Pat. No. 6,610,642).

In some alternative embodiments, the present invention provides hard surface cleaning compositions comprising at least one metalloprotease polypeptide provided herein. Thus, in
10 some embodiments, the compositions comprising at least one metalloprotease polypeptide of the present invention is a hard surface cleaning composition such as those described in U.S. Pat. Nos. 6,610,642; 6,376,450, and 6,376,450.

In yet further embodiments, the present invention provides dishwashing compositions comprising at least one metalloprotease polypeptide provided herein. Thus, in some
15 embodiments, the compositions comprising at least one metalloprotease polypeptide of the present invention is a hard surface cleaning composition such as those in U.S. Pat. Nos. 6,610,642 and 6,376,450. In some still further embodiments, the present invention provides dishwashing compositions comprising at least one metalloprotease polypeptide provided herein. In some further embodiments, the compositions comprising at least one metalloprotease
20 polypeptide of the present invention comprise oral care compositions such as those in U.S. Pat. No. 6,376,450, and 6,376,450. The formulations and descriptions of the compounds and cleaning adjunct materials contained in the aforementioned US Pat. Nos. 6,376,450; 6,605,458; 6,605,458, and 6,610,642, find use with the metalloprotease polypeptides provided herein.

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

30 In some embodiments, the cleaning compositions of the present invention can be formulated to have an alkaline pH under wash conditions, such as a pH of from about 8.0 to about 12.0, or from about 8.5 to about 11.0, or from about 9.0 to about 11.0. In some embodiments, the cleaning compositions of the present invention can be formulated to have a neutral pH under wash conditions, such as a pH of from about 5.0 to about 8.0, or from about

5.5 to about 8.0, or from about 6.0 to about 8.0, or from about 6.0 to about 7.5. In some embodiments, the neutral pH conditions can be measured when the cleaning composition is dissolved 1:100 (wt:wt) in de-ionised water at 20°C., measured using a conventional pH meter.

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

In some embodiments, the cleaning compositions according to the present invention comprise an acidifying particle or an amino carboxylic builder. Examples of an amino carboxylic builder include aminocarboxylic acids, salts and derivatives thereof. In some embodiment, the amino carboxylic builder is an aminopolycarboxylic builder, such as glycine-N,N-diacetic acid or derivative of general formula MOOC-CHR-N $(CH_2COOM)_2$ where R is C_{1-12} alkyl and M is alkali metal. In some embodiments, the amino carboxylic builder can be methylglycine diacetic acid (MGDA), GLDA (glutamic-N,N-diacetic acid), iminodisuccinic acid (IDS), carboxymethyl inulin and salts and derivatives thereof, aspartic acid-N-monoacetic acid (ASMA), aspartic acid-N,N-diacetic acid (ASDA), aspartic acid-N-monopropionic acid (ASMP), iminodisuccinic acid (IDA), N-(2-sulfomethyl) aspartic acid (SMAS), N-(2-sulfoethyl)aspartic acid (SEAS), N-(2-sulfomethyl)glutamic acid (SMGL), N-(2-sulfoethyl) glutamic acid (SEGL), IDS (iminodiacetic acid) and salts and derivatives thereof such as N-methyliminodiacetic acid (MIDA) , alpha-alanine-N,N-diacetic acid (alpha-ALDA) , serine-

N,N-diacetic acid (SEDA), isoserine-N,Ndiacetic acid (ISDA) , phenylalanine-N,N-diacetic acid (PHDA), anthranilic acid-N,N-diacetic acid (ANDA), sulfanilic acid-N,N-diacetic acid (SLDA), taurine-N,N-diacetic acid (TUDA) and sulfomethyl-N,N-diacetic acid (SMDA) and alkali metal salts and derivative thereof. In some embodiments, the acidifying particle has a weight
5 geometric mean particle size of from about 400 μ to about 1200 μ and a bulk density of at least 550 g/L. In some embodiments, the acidifying particle comprises at least about 5% of the builder.

In some embodiments, the acidifying particle can comprise any acid, including organic acids and mineral acids. Organic acids can have one or two carboxyls and in some instances up
10 to 15 carbons, especially up to 10 carbons, such as formic, acetic, propionic, capric, oxalic, succinic, adipic, maleic, fumaric, sebacic, malic, lactic, glycolic, tartaric and glyoxylic acids. In some embodiments, the acid is citric acid. Mineral acids include hydrochloric and sulphuric acid. In some instances, the acidifying particle of the invention is a highly active particle comprising a high level of amino carboxylic builder. Sulphuric acid has been found to further
15 contribute to the stability of the final particle.

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

In some embodiments, the cleaning compositions of the present invention comprise one or more detergent builders or builder systems. In some embodiments incorporating at least one builder, the cleaning compositions comprise at least about 1%, from about 3% to about 60% or even from about 5% to about 40% builder by weight of the cleaning composition. Builders
30 include, but are not limited to, the alkali metal, ammonium and alkanolammonium salts of polyphosphates, alkali metal silicates, alkaline earth and alkali metal carbonates, aluminosilicates, polycarboxylate compounds, ether hydroxypolycarboxylates, copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1, 3, 5-trihydroxy benzene-2, 4, 6-trisulphonic acid, and carboxymethyloxysuccinic acid, the various alkali metal, ammonium and

substituted ammonium salts of polyacetic acids such as ethylenediamine tetraacetic acid and nitrilotriacetic acid, as well as polycarboxylates such as mellitic acid, succinic acid, citric acid, oxydisuccinic acid, polymaleic acid, benzene 1,3,5-tricarboxylic acid, carboxymethyloxysuccinic acid, and soluble salts thereof. Indeed, it is contemplated that any
5 suitable builder will find use in various embodiments of the present invention.

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

In some embodiments, builders for use herein include phosphate builders and non-phosphate builders. In some embodiments, the builder is a phosphate builder. In some embodiments, the builder is a non-phosphate builder. If present, builders are used in a level of from 0.1% to 80%, or from 5 to 60%, or from 10 to 50% by weight of the composition. In some
15 embodiments the product comprises a mixture of phosphate and non-phosphate builders. Suitable phosphate builders include mono-phosphates, di-phosphates, tri-polyphosphates or oligomeric-poylphosphates, including the alkali metal salts of these compounds, including the sodium salts. In some embodiments, a builder can be sodium tripolyphosphate (STPP). Additionally, the composition can comprise carbonate and/or citrate, preferably citrate that helps
20 to achieve a neutral pH composition of the invention. Other suitable non-phosphate builders include homopolymers and copolymers of polycarboxylic acids and their partially or completely neutralized salts, monomeric polycarboxylic acids and hydroxycarboxylic acids and their salts. In some embodiments, salts of the above mentioned compounds include the ammonium and/or alkali metal salts, i.e. the lithium, sodium, and potassium salts, including sodium salts. Suitable
25 polycarboxylic acids include acyclic, alicyclic, hetero-cyclic and aromatic carboxylic acids, wherein in some embodiments, they can contain at least two carboxyl groups which are in each case separated from one another by, in some instances, no more than two carbon atoms.

In some embodiments, the cleaning compositions of the present invention contain at least one chelating agent. Suitable chelating agents include, but are not limited to copper, iron and/or
30 manganese chelating agents and mixtures thereof. In embodiments in which at least one chelating agent is used, the cleaning compositions of the present invention comprise from about 0.1% to about 15% or even from about 3.0% to about 10% chelating agent by weight of the subject cleaning composition.

In some still further embodiments, the cleaning compositions provided herein contain at least one deposition aid. Suitable deposition aids include, but are not limited to, polyethylene glycol, polypropylene glycol, polycarboxylate, soil release polymers such as polytelephthalic acid, clays such as kaolinite, montmorillonite, atapulgite, illite, bentonite, halloysite, and mixtures thereof.

As indicated herein, in some embodiments, anti-redeposition agents find use in some embodiments of the present invention. In some embodiments, non-ionic surfactants find use. For example, in automatic dishwashing embodiments, non-ionic surfactants find use for surface modification purposes, in particular for sheeting, to avoid filming and spotting and to improve shine. These non-ionic surfactants also find use in preventing the re-deposition of soils. In some embodiments, the anti-redeposition agent is a non-ionic surfactant as known in the art (See e.g., EP 2 100 949). In some embodiments, the non-ionic surfactant can be ethoxylated nonionic surfactants, epoxy-capped poly(oxyalkylated) alcohols and amine oxides surfactants.

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

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

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

In some further embodiments, the enzymes used in the cleaning compositions are stabilized by any suitable technique. In some embodiments, the enzymes employed herein are stabilized by the presence of water-soluble sources of calcium and/or magnesium ions in the finished compositions that provide such ions to the enzymes. In some embodiments, the enzyme

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

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

In some embodiments, bleach activators are used in the compositions of the present invention. Bleach activators are typically organic peracid precursors that enhance the bleaching
25 action in the course of cleaning at temperatures of 60°C and below. Bleach activators suitable for use herein include compounds which, under perhydrolysis conditions, give aliphatic peroxoycarboxylic acids having preferably from about 1 to about 10 carbon atoms, in particular from about 2 to about 4 carbon atoms, and/or optionally substituted perbenzoic acid. Additional bleach activators are known in the art and find use in the present invention (See e.g., EP 2 100
30 949).

In addition, in some embodiments and as further described herein, the cleaning compositions of the present invention further comprise at least one bleach catalyst. In some embodiments, the manganese triazacyclononane and related complexes find use, as well as cobalt, copper, manganese, and iron complexes. Additional bleach catalysts find use in the

present invention (See e.g., US 4,246,612, 5,227,084, 4,810410, WO 99/06521, and EP 2 100 949).

In some embodiments, the cleaning compositions of the present invention contain one or more catalytic metal complexes. In some embodiments, a metal-containing bleach catalyst finds use. In some embodiments, the metal bleach catalyst comprises a catalyst system comprising a transition metal cation of defined bleach catalytic activity, (e.g., copper, iron, titanium, ruthenium, tungsten, molybdenum, or manganese cations), an auxiliary metal cation having little or no bleach catalytic activity (e.g., zinc or aluminum cations), and a sequestrate having defined stability constants for the catalytic and auxiliary metal cations, particularly ethylenediaminetetraacetic acid, ethylenediaminetetra (methylenephosphonic acid) and water-soluble salts thereof are used (See e.g., US Patent No. 4,430,243). In some embodiments, the cleaning compositions of the present invention are catalyzed by means of a manganese compound. Such compounds and levels of use are well known in the art (See e.g., US Patent No. 5,576,282). In additional embodiments, cobalt bleach catalysts find use in the cleaning compositions of the present invention. Various cobalt bleach catalysts are known in the art (See e.g., US Patent Nos. 5,597,936 and 5,595,967) and are readily prepared by known procedures.

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

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

In some embodiments, the cleaning compositions of the present invention comprise metal care agents. Metal care agents find use in preventing and/or reducing the tarnishing, corrosion, and/or oxidation of metals, including aluminum, stainless steel, and non-ferrous metals (e.g., silver and copper). Suitable metal care agents include those described in EP 2 100 949, WO 9426860 and WO 94/26859). In some embodiments, the metal care agent is a zinc

salt. In some further embodiments, the cleaning compositions of the present invention comprise from about 0.1% to about 5% by weight of one or more metal care agent.

In some embodiments, the cleaning composition is a high density liquid (HDL) composition having a variant metalloprotease polypeptide protease. The HDL liquid laundry detergent can comprise a detersive surfactant (10%-40%) comprising anionic detersive surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxylated sulphate, alkyl phosphates, alkyl phosphonates, alkyl carboxylates, and/or mixtures thereof); and optionally non-ionic surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl alkoxylated alcohol, for example a C_8-C_{18} alkyl ethoxylated alcohol and/or C_6-C_{12} alkyl phenol alkoxylates), optionally wherein the weight ratio of anionic detersive surfactant (with a hydrophilic index (HIc) of from 6.0 to 9) to non-ionic detersive surfactant is greater than 1: 1.

The composition can comprise optionally, a surfactancy boosting polymer consisting of amphiphilic alkoxylated grease cleaning polymers (selected from a group of alkoxylated polymers having branched hydrophilic and hydrophobic properties, such as alkoxylated polyalkylenimines in the range of 0.05wt%-10wt%) and/or random graft polymers (typically comprising of hydrophilic backbone comprising monomers selected from the group consisting of: unsaturated C_1-C_6 carboxylic acids, ethers, alcohols, aldehydes, ketones, esters, sugar units, alkoxy units, maleic anhydride, saturated polyalcohols such as glycerol, and mixtures thereof; and hydrophobic side chain(s) selected from the group consisting of: C_4-C_{25} alkyl group, polypropylene, polybutylene, vinyl ester of a saturated C-C_6 mono-carboxylic acid, C_1-C_6 alkyl ester of acrylic or methacrylic acid, and mixtures thereof.

The composition can comprise additional polymers such as soil release polymers (include anionically end-capped polyesters, for example SRP1, polymers comprising at least one monomer unit selected from saccharide, dicarboxylic acid, polyol and combinations thereof, in random or block configuration, ethylene terephthalate-based polymers and co-polymers thereof in random or block configuration, for example Repel-o-tex SF, SF-2 and SRP6, Texcare SRA100, SRA300, SRN100, SRN170, SRN240, SRN300 and SRN325, Marloquest SL), anti-redeposition polymers (0.1 wt% to 10wt%, include carboxylate polymers, such as polymers comprising at least one monomer selected from acrylic acid, maleic acid (or maleic anhydride), fumaric acid, itaconic acid, aconitic acid, mesaconic acid, citraconic acid, methylenemalonic acid, and any mixture thereof, vinylpyrrolidone homopolymer, and/or polyethylene glycol, molecular weight in the range of from 500 to 100,000 Da); cellulosic polymer (including those

selected from alkyl cellulose, alkyl alkoxyalkyl cellulose, carboxyalkyl cellulose, alkyl carboxyalkyl cellulose examples of which include carboxymethyl cellulose, methyl cellulose, methyl hydroxyethyl cellulose, methyl carboxymethyl cellulose, and mixures thereof) and polymeric carboxylate (such as maleate/acrylate random copolymer or polyacrylate homopolymer).

The composition can further comprise saturated or unsaturated fatty acid, preferably saturated or unsaturated C_{12}-C_{24} fatty acid (0 wt% to 10 wt%); deposition aids (examples for which include polysaccharides, preferably cellulosic polymers, poly diallyl dimethyl ammonium halides (DADMAC), and co-polymers of DAD MAC with vinyl pyrrolidone, acrylamides, imidazoles, imidazolinium halides, and mixtures thereof, in random or block configuration, cationic guar gum, cationic cellulose such as cationic hydoxyethyl cellulose, cationic starch, cationic polyacylamides, and mixtures thereof.

The composition can further comprise dye transfer inhibiting agents examples of which include manganese phthalocyanine, peroxidases, polyvinylpyrrolidone polymers, polyamine N-oxide polymers, copolymers of N-vinylpyrrolidone and N-vinylimidazole, polyvinyloxazolidones and polyvinylimidazoles and/or mixtures thereof; chelating agents examples of which include ethylene-diamine-tetraacetic acid (EDTA); diethylene triamine penta methylene phosphonic acid (DTPMP); hydroxy-ethane diphosphonic acid (HEDP); ethylenediamine N,N'-disuccinic acid (EDDS); methyl glycine diacetic acid (MGDA); diethylene triamine penta acetic acid (DTPA); propylene diamine tetracetic acid (PDT A); 2-hydroxypyridine-N-oxide (HPNO); or methyl glycine diacetic acid (MGDA); glutamic acid N,N-diacetic acid (N,N-dicarboxymethyl glutamic acid tetrasodium salt (GLDA); nitrilotriacetic acid (NTA); 4,5-dihydroxy-m-benzenedisulfonic acid; citric acid and any salts thereof; N-hydroxyethylethylenediaminetri-acetic acid (HEDTA), triethylenetetraaminehexaacetic acid (TTHA), N-hydroxyethyliminodiacetic acid (HEIDA), dihydroxyethylglycine (DHEG), ethylenediaminetetrapropionic acid (EDTP) and derivatives thereof.

The composition can further comprise enzymes (0.01 wt% active enzyme to 0.03wt% active enzyme) selected from a group of acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases,

pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, and xylosidases, and any mixture thereof. The composition may comprise an enzyme stabilizer (examples of which include polyols such as propylene glycol or glycerol, sugar or sugar alcohol, lactic acid, reversible protease inhibitor, boric acid, a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, peptides or formate).

The composition can further comprise silicone or fatty-acid based suds suppressors; heuing dyes, calcium and magnesium cations, visual signaling ingredients, anti-foam (0.001 wt% to about 4.0wt%), and/or structurant/thickener (0.01 wt% to 5wt%, selected from the group consisting of diglycerides and triglycerides, ethylene glycol distearate, microcrystalline cellulose, cellulose based materials, microfiber cellulose, biopolymers, xanthan gum, gellan gum, and mixtures thereof).

Suitable detersive surfactants also include cationic detersive surfactants (selected from a group of alkyl pyridinium compounds, alkyl quarternary ammonium compounds, alkyl quarternary phosphonium compounds, alkyl ternary sulphonium compounds, and/or mixtures thereof); zwitterionic and/or amphoteric detersive surfactants (selected from a group of alkanolamine sulpho-betaines); ampholytic surfactants; semi-polar non-ionic surfactants and mixtures thereof.

The composition can be any liquid form, for example a liquid or gel form, or any combination thereof. The composition may be in any unit dose form, for example a pouch.

In some embodiments, the cleaning composition is a high density powder (HDD) composition having a variant metalloprotease polypeptide protease. The HDD powder laundry detergent can comprise a detersive surfactant including anionic detersive surfactants (selected from a group of linear or branched or random chain, substituted or unsubstituted alkyl sulphates, alkyl sulphonates, alkyl alkoxylated sulphate, alkyl phosphates, alkyl phosphonates, alkyl carboxylates and/or mixtures thereof), non-ionic detersive surfactant (selected from a group of linear or branched or random chain, substituted or unsubstituted C_8-C_{18} alkyl ethoxylates, and/or C_6-C_{12} alkyl phenol alkoxylates), cationic detersive surfactants (selected from a group of alkyl pyridinium compounds, alkyl quaternary ammonium compounds, alkyl quaternary phosphonium compounds, alkyl ternary sulphonium compounds, and mixtures thereof), zwitterionic and/or amphoteric detersive surfactants (selected from a group of alkanolamine sulpho-betaines); ampholytic surfactants; semi-polar non-ionic surfactants and mixtures thereof; builders (phosphate free builders [for example zeolite builders examples of which include zeolite A, zeolite X, zeolite P and zeolite MAP in the range of 0wt% to less than 10wt%]; phosphate

builders [examples of which include sodium tri-polyphosphate in the range of 0wt% to less than 10wt%]; citric acid, citrate salts and nitrilotriacetic acid or salt thereof in the range of less than 15 wt%); silicate salt (sodium or potassium silicate or sodium meta-silicate in the range of 0wt% to less than 10wt%, or layered silicate (SKS-6)); carbonate salt (sodium carbonate and/or
5 sodium bicarbonate in the range of 0 wt% to less than 10 wt%); and bleaching agents (photobleaches, examples of which include sulfonated zinc phthalocyanines, sulfonated aluminum phthalocyanines, xanthenes dyes, and mixtures thereof; hydrophobic or hydrophilic bleach activators (examples of which include dodecanoyl oxybenzene sulfonate, decanoyl oxybenzene sulfonate, decanoyl oxybenzoic acid or salts thereof, 3,5,5-trimethy hexanoyl
10 oxybenzene sulfonate, tetraacetyl ethylene diamine-TAED, and nonanoyloxybenzene sulfonate-NOBS, nitrile quats, and mixtures thereof; hydrogen peroxide; sources of hydrogen peroxide (inorganic perhydrate salts examples of which include mono or tetra hydrate sodium salt of perborate, percarbonate, persulfate, perphosphate, or persilicate); preformed hydrophilic and/or hydrophobic peracids (selected from a group consisting of percarboxylic acids and salts,
15 percarbonic acids and salts, perimidic acids and salts, peroxymonosulfuric acids and salts) & mixtures thereof and/or bleach catalyst (such as imine bleach boosters examples of which include 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; metal-containing bleach catalyst for
20 example copper, iron, titanium, ruthenium, tungsten, molybdenum, or manganese cations along with an auxiliary metal cations such as zinc or aluminum and a sequestrate such as ethylenediaminetetraacetic acid, ethylenediaminetetra(methylenephos-phonic acid) and water-soluble salts thereof).

The composition can further comprise enzymes selected from a group of acyl
25 transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, pectate lyases, pectin acetyl esterases,
30 pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, and xylosidases and any mixture thereof.

The composition can further comprise additional detergent ingredients including perfume microcapsules, starch encapsulated perfume accord, hueing agents, additional polymers including fabric integrity and cationic polymers, dye lock ingredients, fabric-softening agents, brighteners (for example C.I. Fluorescent brighteners), flocculating agents, chelating agents,
5 alkoxylated polyamines, fabric deposition aids, and/or cyclodextrin.

In some embodiments, the cleaning composition is an automatic dishwashing (ADW) detergent composition having a metalloprotease of the present invention. The ADW detergent composition can comprise two or more non-ionic surfactants selected from a group of ethoxylated non-ionic surfactants, alcohol alkoxylated surfactants, epoxy-capped
10 poly(oxyalkylated) alcohols, or amine oxide surfactants present in amounts from 0 to 10% by weight; builders in the range of 5-60% comprising either phosphate (mono-phosphates, di-phosphates, tri-polyphosphates or oligomeric-poylphosphates, preferred sodium tripolyphosphate-STPP or phosphate-free builders [amino acid based compounds, examples of which include MGDA (methyl-glycine-diacetic acid), and salts and derivatives thereof, GLDA
15 (glutamic-N,Ndiacetic acid) and salts and derivatives thereof, IDS (iminodisuccinic acid) and salts and derivatives thereof, carboxy methyl inulin and salts and derivatives thereof and mixtures thereof, nitrilotriacetic acid (NTA), diethylene triamine penta acetic acid (DTPA), B-alaninediacetic acid (B-ADA) and their salts], homopolymers and copolymers of poly-carboxylic acids and their partially or completely neutralized salts, monomeric polycarboxylic
20 acids and hydroxycarboxylic acids and their salts in the range of 0.5% to 50% by weight; sulfonated/carboxylated polymers (provide dimensional stability to the product) in the range of about 0.1 % to about 50% by weight; drying aids in the range of about 0.1 % to about 10% by weight (selected from polyesters, especially anionic polyesters optionally together with further monomers with 3 to 6 functionalities which are conducive to polycondensation, specifically
25 acid, alcohol or ester functionalities, polycarbonate-, polyurethane- and/or polyurea-polyorganosiloxane compounds or precursor compounds thereof of the reactive cyclic carbonate and urea type); silicates in the range from about 1 % to about 20% by weight (sodium or potassium silicates for example sodium disilicate, sodium meta-silicate and crystalline phyllosilicates); bleach-inorganic (for example perhydrate salts such as perborate, percarbonate,
30 perphosphate, persulfate and persilicate salts) and organic (for example organic peroxyacids including diacyl and tetraacylperoxides, especially diperoxydodecanedioc acid, diperoxytetradecanedioc acid, and diperoxyhexadecanedioc acid); bleach activators- organic peracid precursors in the range from about 0.1% to about 10% by weight; bleach catalysts (selected from manganese triazacyclononane and related complexes, Co, Cu, Mn and Fe

bispyridylamine and related complexes, and pentamine acetate cobalt(III) and related complexes); metal care agents in the range from about 0.1% to 5% by weight (selected from benzatriazoles, metal salts and complexes, and/or silicates); enzymes in the range from about 0.01 to 5.0mg of active enzyme per gram of automatic dishwashing detergent composition (acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, and xylosidases, and any mixture thereof); and enzyme stabilizer components (selected from oligosaccharides, polysaccharides and inorganic divalent metal salts).

The metalloproteases are normally incorporated into the detergent composition at a level of from 0.000001 % to 5% of enzyme protein by weight of the composition, or from 0.00001 % to 2 %, or from 0.0001% to 1%, or from 0.001 % to 0.75% of enzyme protein by weight of the composition.

Metalloprotease polypeptides of the present invention for use in Animal Feed

In a further aspect of the invention, the metalloprotease polypeptides of the present invention can be used as a compontent of an animal feed composition, animal feed additive and/or pet food comprising a metalloprotease and variants thereof. The present invention further relates to a method for preparing such an animal feed composition, animal feed additive composition and/or pet food comprising mixing the metalloprotease polypeptide with one or more animal feed ingredients and/or animal feed additive ingredients and/or pet food ingredients. Furthermore, the present invention relates to the use of the metalloprotease polypeptide in the preparation of an animal feed composition and/or animal feed additive composition and/or pet food.

The term “animal” includes all non-ruminant and ruminant animals. In a particular embodiment, the animal is a non-ruminant animal, such as a horse and a mono-gastric animal. Examples of mono-gastric animals include, but are not limited to, pigs and swine, such as piglets, growing pigs, sows; poultry such as turkeys, ducks, chicken, broiler chicks, layers; fish such as salmon, trout, tilapia, catfish and carps; and crustaceans such as shrimps and prawns. In

a further embodiment the animal is a ruminant animal including, but not limited to, cattle, young calves, goats, sheep, giraffes, bison, moose, elk, yaks, water buffalo, deer, camels, alpacas, llamas, antelope, pronghorn and nilgai.

In the present context, it is intended that the term "pet food" is understood to mean a food for a household animal such as, but not limited to, dogs, cats, gerbils, hamsters, chinchillas, fancy rats, guinea pigs; avian pets, such as canaries, parakeets, and parrots; reptile pets, such as turtles, lizards and snakes; and aquatic pets, such as tropical fish and frogs.

The terms "animal feed composition," "feedstuff" and "fodder" are used interchangeably and can comprise one or more feed materials selected from the group comprising a) cereals, such as small grains (*e.g.*, wheat, barley, rye, oats and combinations thereof) and/or large grains such as maize or sorghum; b) by products from cereals, such as corn gluten meal, Distillers Dried Grain Solubles (DDGS) (particularly corn based Distillers Dried Grain Solubles (cDDGS), wheat bran, wheat middlings, wheat shorts, rice bran, rice hulls, oat hulls, palm kernel, and citrus pulp; c) protein obtained from sources such as soya, sunflower, peanut, lupin, peas, fava beans, cotton, canola, fish meal, dried plasma protein, meat and bone meal, potato protein, whey, copra, sesame; d) oils and fats obtained from vegetable and animal sources; e) minerals and vitamins.

Metalloprotease polypeptides of the present invention for use in Textile Desizing

Also contemplated are compositions and methods of treating fabrics (*e.g.*, to desize a textile) using a metalloprotease polypeptide of the present invention. Fabric-treating methods are well known in the art (*see*, *e.g.*, U.S. Patent No. 6,077,316). For example, the feel and appearance of a fabric can be improved by a method comprising contacting the fabric with a metalloprotease in a solution. The fabric can be treated with the solution under pressure.

A metalloprotease of the present invention can be applied during or after the weaving of a textile, or during the desizing stage, or one or more additional fabric processing steps. During the weaving of textiles, the threads are exposed to considerable mechanical strain. Prior to weaving on mechanical looms, warp yarns are often coated with sizing starch or starch derivatives to increase their tensile strength and to prevent breaking. A metalloprotease of the present invention can be applied during or after the weaving to remove these sizing starch or starch derivatives. After weaving, the metalloprotease can be used to remove the size coating before further processing the fabric to ensure a homogeneous and wash-proof result.

A metalloprotease of the present invention can be used alone or with other desizing chemical reagents and/or desizing enzymes to desize fabrics, including cotton-containing

fabrics, as detergent additives, *e.g.*, in aqueous compositions. An amylase also can be used in compositions and methods for producing a stonewashed look on indigo-dyed denim fabric and garments. For the manufacture of clothes, the fabric can be cut and sewn into clothes or garments, which are afterwards finished. In particular, for the manufacture of denim jeans, different enzymatic finishing methods have been developed. The finishing of denim garment normally is initiated with an enzymatic desizing step, during which garments are subjected to the action of proteolytic enzymes to provide softness to the fabric and make the cotton more accessible to the subsequent enzymatic finishing steps. The metalloprotease can be used in methods of finishing denim garments (*e.g.*, a "bio-stoning process"), enzymatic desizing and providing softness to fabrics, and/or finishing process.

Metalloprotease polypeptides of the present invention for use in Paper Pulp Bleaching

The metalloprotease polypeptides described herein find further use in the enzyme aided bleaching of paper pulps such as chemical pulps, semi-chemical pulps, kraft pulps, mechanical pulps or pulps prepared by the sulfite method. In general terms, paper pulps are incubated with a metalloprotease polypeptide of the present invention under conditions suitable for bleaching the paper pulp.

In some embodiments, the pulps are chlorine free pulps bleached with oxygen, ozone, peroxide or peroxyacids. In some embodiments, the metalloprotease polypeptides are used in enzyme aided bleaching of pulps produced by modified or continuous pulping methods that exhibit low lignin contents. In some other embodiments, the metalloprotease polypeptides are applied alone or preferably in combination with xylanase and/or endoglucanase and/or alpha-galactosidase and/or cellobiohydrolase enzymes.

Metalloprotease polypeptides of the present invention for use in Protein degradation

The metalloprotease polypeptides described herein find further use in the enzyme aided removal of proteins from animals and their subsequent degradation or disposal, such as feathers, skin, hair, hide, and the like. In some instances, immersion of the animal carcass in a solution comprising a metalloprotease polypeptide of the present invention can act to protect the skin from damage in comparison to the traditional immersion in scalding water or the defeathering process. In one embodiment, feathers can be sprayed with an isolated metalloprotase polypeptide of the present invention under conditions suitable for digesting or initiating degradation of the plumage. In some embodiments, a metalloprotease of the present invention can be used, as above, in combination with an oxidizing agent.

In some embodiments, removal of the oil or fat associated with raw feathers is assisted by using a metalloprotease polypeptide of the present invention. In some embodiments, the metalloprotease polypeptides are used in compositions for cleaning the feathers as well as to sanitize and partially dehydrate the fibers. In some other embodiments, the metalloprotease polypeptides are applied in a wash solution in combination with 95% ethanol or other polar organic solvent with or without a surfactant at about 0.5% (v/v).

In yet other embodiments, the disclosed metalloprotease polypeptides find use in recovering protein from plumage. The disclosed metalloprotease polypeptides may be used alone or in combination in suitable feather processing and proteolytic methods, such as those disclosed in PCT/EP2013/065362, PCT/EP2013/065363, and PCT/EP2013/065364, which are hereby incorporated by reference. In some embodiments, the recovered protein can be subsequently used in animal or fish feed.

EXPERIMENTAL

The claimed invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXAMPLE 1.1

Cloning of *Paenibacillus sp.* metalloprotease PspPro3

A strain of *Paenibacillus sp.* was selected as a potential source for enzymes which may be useful for various industrial applications. Genomic DNA for sequencing was obtained by first growing the strain on Heart Infusion agar plates (Difco) at 37°C for 24 hours. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The genomic DNA was used for genome sequencing. The entire genome of the *Paenibacillus sp.* strain was sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified after annotation in *Paenibacillus sp.* encodes a metalloprotease and the sequence of this gene, called *PspPro3,* is provided in SEQ ID NO: 1. The corresponding protein encoded by the *PspPro3* gene is shown in SEQ ID NO: 2. At the N-terminus, the protein has a signal peptide with a length of 26 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786). The presence of a signal sequence suggests that PspPro3 is a secreted enzyme. The propeptide region was predicted based on protein sequence alignment with the *Paenibacillus polymyxa* Npr protein (Takekawa *et al.* (1991) Journal of Bacteriology,

173 (21): 6820-6825). The predicted mature region of PspPro3 protein is shown on SEQ ID NO: 3.

The nucleotide sequence of the *PspPro3* gene isolated from *Paenibacillus sp.* is set forth as SEQ ID NO: 1. The sequence encoding the predicted native signal peptide is shown in italics:

ATGTTAATGAAAAAAGTATGGGTTTCGCTTCTTGGAGGAGCGATGTTATTAGGGTCTGTAG
*CGTCTGGTGCATCAGCA*GCGGAGAGTTCCGTTTCGGGGCCGGCTCAGCTTACGCCAA
CCTTCCATGCCGAACAATGGAAAGCACCTTCATCGGTATCGGGTGATGACATCGTAT
GGAGCTATTTAAATCGGCAAAAGAAAACGTTGCTGGGTACGGACAGCACCAGTGTC
CGTGATCAATTCCGTATCGTAGATCGCACAAGCGACAAATCCGGCGTGAGCCATTA
TCGGCTGAAGCAATATGTAAACGGAATTCCCGTATATGGAGCTGAACAGACCATTC
ATGTGGGCAAATCCGGTGAAGTGACCTCTTATCTGGGAGCCGTGATTACTGAGGAT
CAGCAAGAAGAAGCTACGCAAGGTACAACTCCGAAAATCAGCGCTTCTGAAGCGGT
CCATACCGCATATCAGGAGGCAGCTACACGGGTTCAAGCCCTCCCTACCTCCGATG
ATACGATTTCTAAAGATGCGGAGGAGCCAAGCAGTGTAAGCAAAGACACTTACTCC
GAAGCAGCTAACAACGGAAAAACGAGTTCTGTTGAAAAGGACAAGCTCAGCCTTG
AGAAAGCGGCTGACCTGAAAGATAGCAAAATTGAAGCGGTGGAGGCAGAGCCAAA
CTCCATTGCCAAAATCGCCAACCTGCAGCCTGAGGTAGATCCTAAAGCCGAACTAT
ATTTCTATGCGAAGGGCGATGCATTGCAGCTGGTTTATGTGACTGAGGTTAATATTT
TGCAGCCTGCGCCGCTGCGTACACGCTACATCATTGACGCCAATGATGGCAAAATC
GTATCCCAGTATGACATCATTAATGAAGCGACAGGCACAGGCAAAGGTGTACTCGG
TGATACCAAAACATTCAACACTACTGCTTCCGGCAGCAGCTACCAGTTAAGAGATA
CGACTCGCGGGAATGGAATCGTGACTTACACGGCCTCCAACCGTCAAAGCATCCCA
GGTACGATCCTGACCGATGCCGATAACGTATGGAATGATCCAGCCGGCGTGGATGC
CCACGCTTATGCAGCCAAAACCTATGATTATTATAAGGAAAAGTTCAATCGCAACA
GCATTGACGGACGAGGCCTGCAGCTCCGTTCGACAGTTCATTACGGCAATCGTTAC
AACAACGCCTTCTGGAACGGCTCCCAAATGACTTATGGAGACGGAGACGGCACCAC
ATTTATCGCTTTTAGCGGTGATCCGGATGTAGTTGGTCATGAACTCACACACGGTGT
TACGGAGTATACTTCCAATTTGGAATATTACGGAGAATCCGGTGCGTTGAACGAGG
CCTTCTCGGACATCATCGGCAATGACATCCAGCGTAAAAACTGGCTTGTAGGCGAT
GATATTTACACGCCACGCATTGCGGGTGATGCACTTCGTTCTATGTCCAATCCTACG
CTGTACGATCAACCGGATCACTATTCGAACTTGTACAGAGGCAGCTCCGATAACGG
CGGCGTTCATACGAACAGCGGTATTATAAATAAAGCCTATTATCTGTTGGCACAAG
GCGGCACCTTCCATGGTGTAACTGTCAATGGGATTGGCCGCGATGCAGCGGTTCAA
ATTTACTACAGCGCCTTTACGAACTACCTGACTTCTTCTTCTGACTTCTCCAATGCAC

GTGATGCCGTTGTACAAGCGGCAAAAGATCTCTACGGCGCGAGCTCGGCACAAGCT
ACCGCAGCAGCCAAATCTTTTGATGCTGTAGGCGTTAAC

The amino acid sequence of the PspPro3 precursor protein is set forth as SEQ ID NO: 2. The predicted signal peptide is shown in italics, and the predicted pro-peptide is shown in underlined text:

*MLMKKVWVSLLGGAMLLGSVASGASA*AESSVSGPAQLTPTFHAEQWKAPSSVSGDDIVW
SYLNRQKKTLLGTDSTSVRDQFRIVDRTSDKSGVSHYRLKQYVNGIPVYGAEQTIHVGK
SGEVTSYLGAVITEDQQEEATQGTTPKISASEAVHTAYQEAATRVQALPTSDDTISKDAE
EPSSVSKDTYSEAANNGKTSSVEKDKLSLEKAADLKDSKIEAVEAEPNSIAKIANLQPEV
DPKAELYFYAKGDALQLVYVTEVNILQPAPLRTRYIIDANDGKIVSQYDIINEATGTGKG
VLGDTKTFNTTASGSSYQLRDTTRGNGIVTYTASNRQSIPGTILTDADNVWNDPAGVDA
HAYAAKTYDYYKEKFNRNSIDGRGLQLRSTVHYGNRYNNAFWNGSQMTYGDGDGTT
FIAFSGDPDVVGHELTHGVTEYTSNLEYYGESGALNEAFSDIIGNDIQRKNWLVGDDIYT
PRIAGDALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGTFHG
VTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAVVQAAKDLYGASSAQATAAAKSF
DAVGVN

The amino acid sequence of the predicted mature form of PspPro3 is set forth as SEQ ID NO: 3:

ATGTGKGVLGDTKTFNTTASGSSYQLRDTTRGNGIVTYTASNRQSIPGTILTDADNVWN
DPAGVDAHAYAAKTYDYYKEKFNRNSIDGRGLQLRSTVHYGNRYNNAFWNGSQMTY
GDGDGTTFIAFSGDPDVVGHELTHGVTEYTSNLEYYGESGALNEAFSDIIGNDIQRKNW
LVGDDIYTPRIAGDALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLA
QGGTFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAVVQAAKDLYGASSAQA
TAAAKSFDAVGVN

EXAMPLE 1.2

Expression of *Paenibacillus sp.* metalloprotease PspPro3

The DNA sequence of the propeptide-mature form of PspPro3 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX085 (AprE-PspPro3) (Figure 1.1). Ligation of the gene encoding the PspPro3 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3’ end of the *Bacillus*

subtilis AprE signal sequence and the 5' end of the predicted PspPro3 native propeptide. The gene has an alternative start codon (GTG). As shown in Figure 1.1, pGX085(AprE-PspPro3) contains an AprE promoter, an AprE signal sequence used to direct target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature
5 region of PspPro3 (SEQ ID NO: 4). The translation product of the synthetic *AprE- PspPro3* gene is shown in SEQ ID NO: 5.

B. subtilis cells (*degU*^{Hy}*32*, Δ*scoC*) were transformed with the pGX085(AprE- PspPro3) plasmid and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm Chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear
10 halos on the plates were selected and subjected to fermentation in a 250 ml shake flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$). The broth from the shake flasks was concentrated and buffer-exchanged into the loading buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM $CaCl_2$ and 10% propylene glycol using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to a 150
15 mL Q Sepharose High Performance column pre-equilibrated with the loading buffer above and PspPro3 was then eluted from the column via the loading buffer supplemented with a linear NaCl gradient from 0 to 0.7 M. The corresponding active purified protein fractions were further pooled and concentrated via 10K Amicon Ultra for further analyses.

The nucleotide sequence of the synthesized *PspPro3* gene in plasmid pGX085(AprE-
20 PspPro3) is depicted in SEQ ID NO: 4. The sequence encoding the predicted native signal peptide is shown in italics and the region encoding the three residue addition (AGK) is shown in bold:

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACGATGG
*CGTTCAGCAACATGAGCGCGCAGGCT***GCTGGAAAA**GCAGAATCATCAGTGTCAGGAC
25 CGGCTCAGCTTACGCCGACGTTTCATGCAGAGCAGTGGAAAGCACCGAGCAGCGTT
AGCGGAGATGACATCGTGTGGAGCTACCTGAACAGACAGAAGAAAACGCTTCTTGG
CACGGACAGCACGAGCGTCAGAGACCAGTTCAGAATCGTGGATAGAACAAGCGAC
AAAAGCGGCGTCAGCCATTATAGACTGAAGCAGTATGTGAACGGAATCCCGGTTTA
TGGCGCAGAACAAACAATCCATGTCGGAAAGAGCGGCGAAGTTACGAGCTATCTGG
30 GCGCGGTTATTACAGAGGACCAGCAAGAGGAGGCTACACAAGGCACGACACCGAA
AATTTCAGCATCAGAGGCAGTTCATACGGCCTACCAAGAAGCTGCAACGAGAGTTC
AAGCCCTGCCTACGTCAGATGATACAATCAGCAAAGACGCTGAGGAACCTAGCTCA
GTTAGCAAGGACACGTATAGCGAAGCCGCGAACAATGGCAAGACGTCAAGCGTGG
AAAAAGACAAGCTTTCACTGGAGAAGGCCGCTGATCTGAAAGACTCAAAGATCGA

GGCTGTGGAAGCGGAACCGAATAGCATTGCAAAGATTGCCAACCTGCAACCGGAG
GTGGACCCGAAGGCGGAGCTGTATTTCTACGCTAAAGGCGATGCACTGCAACTGGT
TTACGTCACGGAGGTTAACATCCTGCAGCCGGCACCGCTTAGAACGAGATACATCA
TTGACGCGAACGACGGCAAGATCGTGAGCCAGTACGACATTATCAACGAGGCCACG
GGAACGGGCAAGGGAGTCCTTGGCGACACGAAGACATTCAATACAACGGCCTCAG
GCTCATCATACCAGCTGAGAGACACGACGAGAGGCAACGGAATCGTCACGTACACG
GCTAGCAATAGACAGAGCATTCCGGGCACAATCCTTACGGACGCAGACAATGTGTG
GAATGACCCGGCAGGCGTGGACGCACATGCCTACGCAGCGAAGACGTACGACTACT
ACAAGGAGAAGTTCAACAGAAACAGCATCGACGGAAGAGGACTGCAACTTAGAAG
CACGGTGCATTACGGCAACAGATACAACAACGCTTTCTGGAACGGCAGCCAAATGA
CGTATGGAGACGGCGATGGAACAACGTTTATCGCATTCTCAGGCGACCCTGACGTT
GTGGGACATGAACTGACGCATGGAGTCACAGAATACACGAGCAATCTGGAGTATTA
CGGAGAATCAGGCGCACTTAATGAGGCCTTCAGCGACATCATCGGAAACGACATCC
AGAGAAAGAACTGGCTGGTTGGCGATGATATCTACACGCCGAGAATTGCGGGCGAC
GCGCTGAGATCAATGAGCAACCCTACGCTGTACGATCAGCCGGATCATTACAGCAA
CCTGTATAGAGGCTCAAGCGATAATGGCGGCGTGCATACAAACAGCGGCATCATCA
ACAAAGCCTATTATCTGCTGGCGCAAGGCGGCACATTCCATGGCGTTACAGTTAAT
GGCATTGGCAGAGACGCAGCCGTGCAGATCTACTACAGCGCATTCACGAATTACCT
GACATCAAGCAGCGACTTTTCAAATGCAAGAGATGCAGTGGTGCAGGCGGCTAAAG
ACCTTTATGGAGCTTCAAGCGCTCAGGCCACAGCTGCGGCAAAAAGCTTCGACGCG
GTTGGAGTGAAT

The amino acid sequence of the PspPro3 precursor protein expressed from plasmid pGX085(AprE- PspPro3) is depicted in SEQ ID NO: 5. The predicted signal sequence is shown in italics, the three residue addition (AGK) shown in bold and the predicted pro-peptide is shown in underlined text. :

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**AESSVSGPAQLTPTFHAEQWKAPSSVSGD
DIVWSYLNRQKKTLLGTDSTSVRDQFRIVDRTSDKSGVSHYRLKQYVNGIPVYGAEQTI
HVGKSGEVTSYLGAVITEDQQEEATQGTTPKISASEAVHTAYQEAATRVQALPTSDDTI
SKDAEEPSSVSKDTYSEAANNGKTSSVEKDKLSLEKAADLKDSKIEAVEAEPNSIAKIAN
LQPEVDPKAELYFYAKGDALQLVYVTEVNILQPAPLRTRYIIDANDGKIVSQYDIINEAT
GTGKGVLGDTKTFNTTASGSSYQLRDTTRGNGIVTYTASNRQSIPGTILTDADNVWNDP
AGVDAHAYAAKTYDYYKEKFNRNSIDGRGLQLRSTVHYGNRYNNAFWNGSQMTYGD
GDGTTFIAFSGDPDVVGHELTHGVTEYTSNLEYYGESGALNEAFSDIIGNDIQRKNWLV
GDDIYTPRIAGDALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQG

GTFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAVVQAAKDLYGASSAQATA
AAKSFDAVGVN

The amino acid sequence of the PspPro3 recombinant protein isolated from *Bacillus subtilis* culture was determined by tandem mass spectrometry, and shown below. It is the same
5 as predicted and depicted in SEQ ID NO: 3.

ATGTGKGVLGDTKTFNTTASGSSYQLRDTTRGNGIVTYTASNRQSIPGTILTDADNVWN
DPAGVDAHAYAAKTYDYYKEKFNRNSIDGRGLQLRSTVHYGNRYNNAFWNGSQMTY
GDGDGTTFIAFSGDPDVVGHELTHGVTEYTSNLEYYGESGALNEAFSDIIGNDIQRKNW
LVGDDIYTPRIAGDALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLA
10 QGGTFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAVVQAAKDLYGASSAQA
TAAAKSFDAVGVN

EXAMPLE 1.3

Proteolytic Activity of metalloprotease PspPro3

15 The proteolytic activity of purified PspPro3 was measured in 50 mM Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To initiate the reaction, 50 μL of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-
20 binding 96-well microtiter Plate (96-MTP) (Corning Life Sciences, #3641) placed on ice, followed by the addition of 50 μL of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40°C and 650 rpm for 10 min. The reaction was terminated by adding 100 μL of 5% Trichloroacetic Acid (TCA). Following equilibration (5 min at the room temperature) and subsequent centrifugation (2000 g for 10 min at 4 °C) , 120 μL
25 supernatant was transferred to a new 96-MTP, and absorbance of the supernatant was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of duplicate assays, and the value varies no more than 5%. The proteolytic activity is shown as Net A_{440}. The proteolytic assay with azo-
30 casein as the substrate (Figure 1.2) indicates that PspPro3 is an active protease.

EXAMPLE 1.4

pH profile of metalloprotease PspPro3

With azo-casein as the substrate, the pH profile of PspPro3 was studied in 12.5 mM
5 acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 4 to 11). To initiate the assay, 50 µL of 25 mM acetate/Bis-Tris/HEPES/CHES buffer with a specific pH was first mixed with 2 µL diluted enzyme (250 ppm in Milli-Q H_2O) in a 96-MTP placed on ice, followed by the addition of 48 µL of 1.5% (w/v) azo-casein prepared in H_2O. The reaction was performed and analyzed as described in Example 1.3. Enzyme activity at each pH was reported
10 as relative activity where the activity at the optimal pH was set to be 100%. The pH values tested were 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10 and 11. Each value was the mean of triplicate assays. As shown in Figure 1.3, the optimal pH of PspPro3 is 7.5, with greater than 70% of maximal activity retained between pH 5.5 and 9.

15

EXAMPLE 1.5

Temperature profile of metalloprotease PspPro3

The temperature profiles of PspPro3 were analyzed in 50 mM Tris buffer (pH 7) using the azo-casein assay. The enzyme sample and azo-casein substrate were prepared as in Example 3. Prior to the reaction, 50 µL of 1.5% azo-casein and 45 µl Milli-Q H_2O were mixed in a 200µL
20 PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler (BioRad) at desired temperatures (i.e. 20~90°C) for 5 min. After the incubation, 5 µL of diluted PspPro3 (100 ppm) or H_2O (the blank control) was added to the substrate mixture, and the reaction was carried out in the Peltier Thermal Cycle for 10 min at different temperatures. To terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 µL of 5% TCA per
25 well. Subsequent centrifugation and absorbance measurement were performed as described in Example 1.3. The activity was reported as relative activity where the activity at the optimal temperature was set to be 100%. The tested temperatures were 20, 30, 40, 50, 60, 70, 80, and 90°C. Each value was the mean of triplicate assays. The data in Figure 1.4 suggest that PspPro3 showed an optimal temperature at 50°C, and retained greater than 70% of its maximal activity
30 between 45°C and 60°C.

EXAMPLE 1.6

Cleaning Performance of metalloprotease PspPro3 in Automatic Dishwashing (ADW) conditions

5 The cleaning performance of PspPro3 in automatic dishwashing (ADW) conditions was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 or 8 using a model automatic dishwashing (ADW) detergent. Prior to the reaction, purified PspPro3 were diluted with a dilution solution containing 10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol to the desired 10 concentrations. The reactions were performed in AT detergent (composition shown in Table 1.1) with 100 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1), in the absence or presence of a bleach component (Peracid *N*,*N*-phthaloylaminoperoxycaproic acid-PAP). To initiate the reaction, 180 µL of AT detergent buffered at pH 6 or 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the addition of 20 µL of diluted enzymes (or the dilution solution as 15 the blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 30 min at 50°C and 1150 rpm. After incubation, 100µL of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 405 nm (A_{405}) (referred here as the "Initial performance") using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the microswatches were rinsed once with 200 µL water. Following the addition of 20 180 µL of 0.1 M CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50 °C and 1150 rpm for 10 min. One hundred microliter of the resulting wash liquid was transferred to a new 96-MTP, and its absorbance measured at 405 nm (referred here as "Wash-off"). The sum of two absorbance measurements ("Initial performance" plus "Wash-off") gives the "Total performance", which measures the protease activity on the model 25 stain. Dose response in cleaning the PA-S-38 microswatches at pH 6 and pH 8 for PspPro3 in AT detergent, in the absence or presence of bleach, is shown in Figures 5A and 5B, respectively.

Table 1.1. Composition of AT dish detergent formula with bleach

Ingredient	Concentration (mg/ml)
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
PAP (peracid *N*,*N*-phthaloylaminoperoxycaproic acid)	0.057
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029
PEG 6000	0.043
PEG 1500	0.1

*The pH of the AT detergent is adjusted to the desired value (pH 6 or 8) by the addition of 0.9 M citric acid.

5

EXAMPLE 1.7

Cleaning performance of metalloprotease PspPro3 in laundry conditions

The cleaning performance of PspPro3 protein in liquid laundry detergent was tested using EMPA-116 (cotton soiled with blood/milk/ink) microswatches (obtained from CFT
10 Vlaardingen, The Netherlands) at pH 8.2 using a commercial detergent. Prior to the reaction, purified PspPro3 protein samples were diluted with a dilution solution (10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol) to the desired concentrations; and the commercial detergent (Tide®, Clean Breeze®, Proctor & Gamble, USA, purchased September 2011) was incubated at 95°C for 1 hour to inactivate the enzymes present in the detergent.
15 Proteolytic assays were subsequently performed to confirm the inactivation of proteases in the commercial detergent. The heat treated detergent was further diluted with 5 mM HEPES (pH 8.2) to a final concentration of 0.788 g/L. Meanwhile, the water hardness of the buffered liquid detergent was adjusted to 103 ppm (Ca^{2+} : Mg^{2+} = 3 : 1). The specific conductivity of the buffered detergent was adjusted to either 0.62 mS/cm (low conductivity) or 3.5 mS/cm (high
20 conductivity) by adjusting the NaCl concentration in the buffered detergent. To initiate the reaction, 190 µl of either the high or low conductivity buffered detergent was added to a 96-MTP containing the EMPA-116 microswatches, followed by the addition of 10 µl of diluted enzyme (or the dilution solution as blank control). The 96-MTP was sealed and incubated in an

incubator/shaker for 20 min at 32°C and 1150 rpm. After incubation, 150 µl of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 600 nm using a spectrophotometer, which indicates the protease activity on the model stain; and Net A_{600} was subsequently calculated by subtracting the A_{600} of the blank control from that of the enzyme..
5 Dose response for the cleaning of EMPA-116 microswatches in liquid laundry detergent at high or low conductivity is shown in Figure 1.6.

EXAMPLE 1.8

Comparison of PspPro3 to other metalloproteases

10 **A. Identification of Homologous Proteases**

Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res, 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest Patent database with search parameters set to default values. The mature protein amino acid sequence for PspPro3 (SEQ ID NO: 3) was used as the query sequence. Percent identity (PID) 15 for both search sets is defined as the number of identical residues divided by the number of aligned residues in the pairwise alignment. Tables 1.2A and 1.2B provide a list of sequences with the percent identity to PspPro3. The length in Table 1.2 refers to the entire sequence length of the homologous proteases.

Table 1.2A: List of sequences with percent identity to PspPro3 protein identified from the NCBI non-redundant protein database

Accession #	PID to PspPro3	Organism	Length
ZP_10321515.1	55	Bacillus macauensis ZFHKF-1	552
AAC43402.1	57	Alicyclobacillus acidocaldarius	546
P00800	57	Bacillus thermoproteolyticus	548
AAA22621.1	58	Geobacillus stearothermophilus	548
ZP_01862236.1	59	Bacillus sp. SG-1	560
YP_002884504.1	59	Exiguobacterium sp. AT1b	509
AEI46285.1	60	Paenibacillus mucilaginosus KNP414	525
ZP_08093424	60	Planococcus donghaensis MPA1U2	553
ZP_10324092.1	61	Bacillus macauensis ZFHKF-1	533
YP_006792441.1	61	Exiguobacterium antarcticum B7	498
AAK69076.1	63	Bacillus thuringiensis serovar finitimus	566
NP_976992.1	64	Bacillus cereus ATCC 10987	566
ZP_04321694	64	Bacillus cereus	566
BAA06144	64	Lactobacillus sp.	566
ZP_10241029.1	78	Paenibacillus peoriae KCTC 3763	599
YP_005073223	93	Paenibacillus terrae HPL-003	591
YP_003872179	94	Paenibacillus polymyxa E681	592
ZP_09775364	100	Paenibacillus sp. Aloe-11	593

Table 1.2B: List of sequences with percent identity to PspPro3 protein identified from the Genome Quest Patent database

Patent #	PID to PspPro3	Organism	Length
US20120107907-0184	57.88	Bacillus caldoyticus	319
US20120107907-0177	57.88	Bacillus caldolyticus	544
WO2012110563-0002	58.2	Bacillus caldolyticus	319
EP2390321-0176	58.52	Bacillus stearothermophilis	548
US6518054-0002	59.22	Bacillus sp.	316
WO2004011619-0044	60.6	*Empty*	507
WO2004011619-0047	62.14	*Empty*	532
WO2004011619-0046	62.26	*Empty*	536
WO2012110563-0004	63.02	Bacillus megaterium	320
JP2002272453-0003	63.67	*Empty*	562
US8114656-0186	64.24	Bacillus brevis	304
WO2012110562-0005	64.52	Bacillus cereus	320
WO2007044993-0178	64.74	Bacillus thuringiensis	566
EP2178896-0184	65.38	Bacillus anthracis	566
WO2012110563-0005	65.48	Bacillus cereus	320
JP1995184649-0001	65.71	Lactobacillus sp.	566
US5962264-0004	65.81	*Empty*	566
US20120107907-0185	66.13	Bacillus cereus	317
US8114656-0187	93.36	Bacillus polymyxa	302

Table 1.2B: List of sequences with percent identity to PspPro3 protein identified from the Genome Quest Patent database			
JP2005229807-0019	93.38	Paenibacillus polymyxa	566

B. Alignment of Homologous Protease Sequences

The amino acid sequence for mature PspPro3 (SEQ ID NO: 3) was aligned with thermolysin (P00800, *Bacillus thermoproteolyticus*) and protease from *Paenibacillus* sp. Aloe-11 (ZP_09775364) using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 1.7 shows the alignment of PspPro3 with these protease sequences.

C. Phylogenetic Tree

A phylogenetic tree for full length sequence of PspPro3 (SEQ ID NO: 2) was built using sequences of representative homologs from Tables 2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 1.8.

EXAMPLE 1.9

Terg-o-Tometer performance evaluation of PspPro3

The wash performance of PspPro3 was tested in a laundry detergent application using a Terg-o-Tometer (Instrument Marketing Services, Inc, Fairfield, NJ). The performance evaluation was conducted at 32°C and 16°C. The soil load consisted of two of each of the following stain swatches: EMPA116 Blood, Milk, Ink on cotton (Test materials AG, St. Gallen, Switzerland), EMPA117 Blood, Milk, Ink on polycotton (Test materials AG, St. Gallen, Switzerland), EMPA112 Cocoa on cotton (Test materials AG, St. Gallen, Switzerland), and CFT C-10 Pigment, Oil, and Milk content on cotton (Center for Testmaterials BV, Vlaardingen, Netherlands), plus extra white interlock knit fabric to bring the total fabric load to 40 g per beaker of the Terg-o-Tometer, which was filled with 1 L of deionized water. The water hardness was adjusted to 6 grains per gallon, and the pH in the beaker was buffered with 5 mM HEPES, pH 8.2. Heat inactivated Tide Regular HDL (Procter & Gamble), a commercial liquid detergent purchased in a local US supermarket, was used at 0.8 g/L. The detergent was inactivated before use by treatment at 92°C in a water bath for 2-3 hours followed by cooling to

room temperature. Heat inactivation of commercial detergents serves to destroy the activity of enzymatic components while retaining the properties of the non-enzymatic components. Enzyme activity in the heat inactivated detergent was measured using the Suc-AAPF-pNA assay for measuring protease activity. The Purafect® Prime HA, (Genencor Int'l) and PspPro3
5 proteases were each added to final concentrations of 0, 0.2, 0.5, 1, and 1.5 ppm. The wash time was 12 minutes. After the wash treatment, all swatches were rinsed for 3 minutes and machine-dried at low heat.

Four of each types of swatch were measured before and after treatment by optical reflectance using a Tristimulus Minolta Meter CR- 400. The difference in the L, a, b values was
10 converted to total color difference (dE), as defined by the CIE-LAB color space. Cleaning of the stains is expressed as percent stain removal index (%SRI) by taking a ratio between the color difference before and after washing, and comparing it to the difference of unwashed soils (before wash) to unsoiled fabric, and averaging the eight values obtained by reading two different regions of each washed swatch and is reported in Tables 1.9A and 1.9B as Average %
15 SRI (dE) ±95CI. Table 1.9A summarizes the cleaning performance of PspPro3 at 32°C and Table 1.9B at 16°C.

Table 1.9A: Cleaning performance of PspPro3 at 32°C

	EMPA-116				**EMPA-117**			
ppm enzyme	**Purafect Prime HA**		**PspPro3**		**Purafect Prime HA**		**SprPro3**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.19	0.01	0.19	0.01	0.17	0.01	0.17	0.01
0.2	0.27	0.02	0.27	0.02	0.25	0.03	0.30	0.02
0.5	0.28	0.03	0.31	0.01	0.30	0.03	0.31	0.02
1	0.30	0.01	0.32	0.02	0.35	0.02	0.34	0.03
1.5	0.31	0.02	0.31	0.01	0.37	0.01	0.37	0.03
	EMPA-112				**CFT C-10**			
ppm enzyme	**Purafect Prime HA**		**PspPro3**		**Purafect Prime HA**		**PspPro3**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.11	0.03	0.11	0.03	0.07	0.01	0.07	0.01
0.2	0.11	0.05	0.18	0.04	0.12	0.01	0.11	0.01
0.5	0.13	0.04	0.17	0.03	0.15	0.01	0.16	0.01
1	0.18	0.03	0.19	0.04	0.17	0.01	0.21	0.01
1.5	0.19	0.03	0.18	0.04	0.18	0.01	0.23	0.01

Table 1.9B: Cleaning performance of PspPro3 at 16°C								
	EMPA-116				**EMPA-117**			
ppm enzyme	**Purafect Prime HA**		**PspPro3**		**Purafect Prime HA**		**PspPro3**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.15	0.02	0.15	0.02	0.13	0.01	0.13	0.01
0.2	0.19	0.02	0.20	0.03	0.15	0.02	0.15	0.02
0.5	0.20	0.02	0.19	0.02	0.21	0.02	0.20	0.02
1	0.24	0.04	0.21	0.02	0.22	0.02	0.20	0.01
1.5	0.19	0.02	0.25	0.04	0.23	0.03	0.20	0.01
	EMPA-112				**CFT C-10**			
ppm enzyme	**Purafect Prime HA**		**PspPro3**		**Purafect Prime HA**		**PspPro3**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.08	0.03	0.08	0.03	0.04	0.08	0.04	0.08
0.2	0.12	0.02	0.09	0.01	0.06	0.12	0.06	0.09
0.5	0.08	0.02	0.11	0.02	0.08	0.08	0.08	0.11
1	0.11	0.02	0.10	0.03	0.08	0.11	0.09	0.10
1.5	0.13	0.02	0.11	0.03	0.11	0.13	0.10	0.11

EXAMPLE 2.1

Cloning of Metalloprotease *PspPro2* from *Paenibacillus sp.*

5 A strain of *Paenibacillus sp.* was selected as a potential source for enzymes which may be useful for various industrial applications. Genomic DNA for sequencing was obtained by first growing the strain on Heart Infusion agar plates (Difco) at 37°C for 24 hr. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The genomic DNA was used for genome 10 sequencing. The entire genome of the *Paenibacillus sp.* strain was sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified after annotation in *Paenibacillus sp.* encodes a metalloprotease and the sequence of this gene, called *PspPro2,* is provided in SEQ ID NO: 6. The corresponding protein encoded by 15 the *PspPro2* gene is shown in SEQ ID NO: 7. At the N-terminus, the protein has a signal peptide with a length of 24 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786). The presence of a signal sequence suggests that PspPro2 is a secreted enzyme. The propeptide region of PspPro2 was predicted based on protein sequence alignment with the *Paenibacillus polymyxa* Npr protein (Takekawa *et al.* (1991) Journal of

Bacteriology, **173** (21): 6820-6825). The predicted mature region of PspPro2 is shown in SEQ ID NO: 8.

The nucleotide sequence of the *PspPro2* gene isolated from *Paenibacillus sp.* is set forth as SEQ ID NO: 6. The sequence encoding the predicted native signal peptide is shown in italics:

ATGAAAAAAGTATGGGTTTCACTTCTTGGAGGAGCGATGTTATTAGGGGCTGTAGCACCA
*GGTGCATCAGCA*GCAGAGCATTCTGTTCCTGATCCTACTCAGCTAACACCGACCTTTC
ACGCCGAGCAATGGAAGGCTCCTTCCACGGTAACCGGCGACAATATTGTATGGAGC
TATTTGAATCGACAAAAGAAAACCTTATTGAATACAGACAGCACCAGTGTGCGTGA
TCAGTTCCGCATCATTGATCGTACAAGCGACAAATCCGGTGCAAGCCATTATCGGCT
CAAGCAATATGTAAACGGGATCCCCGTATATGGGGCTGAACAGACCATTCATGTGA
ACAACGCCGGTAAAGTAACCTCTTATTTGGGTGCTGTCATTTCAGAGGATCAGCAGC
AAGACGCGACCGAAGATACCACTCCAAAAATCAGCGCGACTGAAGCCGTTTATACC
GCATATGCAGAAGCCGCTGCCCGGATTCAATCCTTCCCTTCCATCAATGATAGTCTT
TCTGAGGCTAGTGAGGAACAAGGGAGTGAGAATCAAGGCAATGAGATTCAAAACA
TTGGGATTAAAAGCAGTGTAAGTAATGACACTTACGCAGAGGCGCATAACAACGTA
CTTTTAACCCCCGTTGACCAAGCAGAGCAAAGTTACATTGCCAAAATTGCTAATCTG
GAGCCAAGTGTAGAGCCCAAAGCAGAATTATACATCTATCCAGATGGTGAGACTAC
ACGACTGGTTTATGTAACAGAGGTTAATATTCTTGAACCTGCGCCTCTGCGCACACG
CTACTTCATTGATGCGAAAACCGGCAAAATCGTATTCCAGTATGACATCCTCAACCA
CGCAACAGGCACCGGCCGCGGCGTGGATGGCAAAACAAAATCATTTACGACTACAG
CTTCAGGCAACCGGTATCAGTTGAAAGACACGACTCGCAGCAATGGAATCGTGACT
TACACCGCTGGCAATCGCCAGACGACGCCAGGTACGATTTTGACCGATACAGATAA
TGTATGGGAGGACCCTGCGGCTGTTGATGCCCATGCCTACGCCATTAAAACCTATGA
CTATTATAAGAATAAATTCGGTCGCGACAGTATTGATGGACGTGGCATGCAAATTC
GTTCGACAGTCCATTACGGCAAAAAATATAACAATGCCTTCTGGAACGGCTCGCAA
ATGACCTACGGAGACGGAGACGGGTCCACATTTACCTTCTTCAGCGGCGATCCCGA
TGTCGTGGGGCATGAGCTCACCCACGGCGTCACCGAGTTCACCTCCAATTTGGAGTA
TTATGGTGAGTCCGGTGCATTGAACGAAGCCTTCTCGGATATTATCGGTAATGATAT
AGATGGCACCAGTTGGCTTCTTGGCGACGGCATTTATACGCCTAATATTCCAGGCGA
CGCTCTGCGTTCCCTGTCCGATCCTACACGATTCGGCCAGCCGGATCACTACTCCAA
TTTCTATCCGGACCCCAACAATGATGATGAAGGCGGAGTCCATACGAACAGCGGTA
TTATCAACAAAGCCTATTATTTGCTGGCACAAGGCGGTACGTCCCATGGTGTAACGG
TAACTGGTATCGGACGCGAAGCGGCTGTATTCATTTACTACAATGCCTTTACCAACT

ATTTGACCTCTACCTCCAACTTCTCTAACGCACGCGCTGCTGTTATACAGGCAGCCA
AGGATTTTTATGGTGCTGATTCGCTGGCAGTAACCAGTGCTATTCAATCCTTTGATG
CGGTAGGAATCAAA

The amino acid sequence of the PspPro2 precursor protein is set forth as SEQ ID NO: 7. The predicted signal peptide is shown in italics, and the predicted pro-peptide is shown in underlined text:

*MKKVWVSLLGGAMLLGAVAPGASA*AEHSVPDPTQLTPTFHAEQWKAPSTVTGDNIVWSY
LNRQKKTLLNTDSTSVRDQFRIIDRTSDKSGASHYRLKQYVNGIPVYGAEQTIHVNNAG
KVTSYLGAVISEDQQQDATEDTTPKISATEAVYTAYAEAAARIQSFPSINDSLSEASEEQ
GSENQGNEIQNIGIKSSVSNDTYAEAHNNVLLTPVDQAEQSYIAKIANLEPSVEPKAELYI
YPDGETTRLVYVTEVNILEPAPLRTRYFIDAKTGKIVFQYDILNHATGTGRGVDGKTKSF
TTTASGNRYQLKDTTRSNGIVTYTAGNRQTTPGTILTDTDNVWEDPAAVDAHAYAIKT
YDYYKNKFGRDSIDGRGMQIRSTVHYGKKYNNAFWNGSQMTYGDGDGSTFTFFSGDP
DVVGHELTHGVTEFTSNLEYYGESGALNEAFSDIIGNDIDGTSWLLGDGIYTPNIPGDAL
RSLSDPTRFGQPDHYSNFYPDPNNDDEGGVHTNSGIINKAYYLLAQGGTSHGVTVTGIG
REAAVFIYYNAFTNYLTSTSNFSNARAAVIQAAKDFYGADSLAVTSAIQSFDAVGIK

The amino acid sequence of the predicted mature form of PspPro2 is set forth as SEQ ID NO: 8.

ATGTGRGVDGKTKSFTTTASGNRYQLKDTTRSNGIVTYTAGNRQTTPGTILTDTDNVW
EDPAAVDAHAYAIKTYDYYKNKFGRDSIDGRGMQIRSTVHYGKKYNNAFWNGSQMT
YGDGDGSTFTFFSGDPDVVGHELTHGVTEFTSNLEYYGESGALNEAFSDIIGNDIDGTS
WLLGDGIYTPNIPGDALRSLSDPTRFGQPDHYSNFYPDPNNDDEGGVHTNSGIINKAYY
LLAQGGTSHGVTVTGIGREAAVFIYYNAFTNYLTSTSNFSNARAAVIQAAKDFYGADSL
AVTSAIQSFDAVGIK

EXAMPLE 2.2

Expression of *Paenibacillus sp.* Metalloprotease PspPro2

The DNA sequence of the propeptide-mature form of PspPro2 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX084 (AprE-PspPro2) (Figure 2.1). Ligation of this gene encoding the PspPro2 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3’ end of the *Bacillus*

subtilis AprE signal sequence and the 5' end of the predicted PspPro2 native propeptide. The gene has an alternative start codon (GTG). As shown in Figure 2.1, pGX084(AprE-PspPro2) contains an AprE promoter, an AprE signal sequence used to direct target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature
5 regions of PspPro2, (SEQ ID NO: 9). The translation product of the synthetic *AprE-PspPro2* gene is shown in SEQ ID NO: 10.

The pGX084(AprE-PspPro2) plasmid was transformed into *B. subtilis* cells (*degU*^{Hy}*32*, Δ*scoC*) and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear halos
10 on the plates were selected and subjected to fermentation in a 250 ml shake flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$).

The broth from the shake flasks was concentrated and buffer-exchanged into the loading buffer containing 20 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to a 150 ml Q
15 Sepharose High Performance column pre-equilibrated with the loading buffer above, PspPro2 was eluted from the column with a linear salt gradient from 0 to 0.5 M NaCl in the loading buffer. The corresponding active fractions were collected, concentrated and buffer-exchanged again into the loading buffer described above. The sample was loaded onto a 20 ml DEAE Fast Flow column pre-equilibrated with the same loading buffer. PspPro2 was eluted from the
20 column with a linear salt gradient from 0 to 0.3 M NaCl in the loading buffer. The corresponding active purified protein fractions were further pooled and concentrated via 10K Amicon Ultra for further analyses. The nucleotide sequence of the synthesized *PspPro2* gene in plasmid pGX084 (AprE-PspPro2) is depicted in SEQ ID NO: 9. The sequence encoding the predicted native signal peptide is shown in italics and the oligo-nucleotide encoding the three
25 residue addition (AGK) is shown in bold:

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACGATGG
*CGTTCAGCAACATGAGCGCGCAGGCT***GCTGGAAAA**GCAGAGCATTCAGTTCCTGACC
CGACGCAACTTACACCGACATTTCATGCTGAGCAGTGGAAGGCACCGAGCACGGTC
ACGGGCGACAACATCGTGTGGAGCTACCTGAACAGACAGAAAAAGACGCTGCTGA
30 ACACGGACTCAACGAGCGTGAGAGACCAGTTCAGAATCATCGACAGAACGAGCGA
CAAGTCAGGCGCGTCACATTATAGACTGAAGCAGTACGTGAACGGCATCCCGGTCT
ACGGAGCCGAGCAAACGATCCATGTGAATAATGCGGGCAAAGTTACATCATACCTG
GGCGCCGTCATCTCAGAAGACCAGCAGCAAGATGCAACGGAGGATACAACACCGA

AGATCAGCGCCACAGAAGCGGTCTATACGGCTTACGCCGAAGCGGCTGCAAGAATC
CAGAGCTTCCCGTCAATTAATGACAGCCTGAGCGAAGCATCAGAGGAACAAGGCAG
CGAGAACCAGGGCAATGAAATCCAAAACATCGGCATCAAGAGCAGCGTGTCAAAC
GACACGTATGCGGAGGCTCATAACAACGTTCTGCTGACACCGGTCGATCAGGCCGA
ACAGAGCTATATTGCAAAGATCGCGAATCTGGAGCCGTCAGTCGAGCCGAAGGCCG
AGCTGTATATCTATCCGGACGGCGAGACGACGAGACTGGTGTACGTTACGGAGGTC
AACATCCTTGAGCCTGCGCCGCTGAGAACAAGATACTTTATCGACGCCAAGACGGG
CAAGATCGTGTTTCAGTACGATATCCTGAACCATGCGACGGGAACAGGCAGAGGCG
TGGACGGCAAAACAAAATCATTCACGACAACGGCAAGCGGCAACAGATACCAGCT
GAAGGACACAACAAGATCAAATGGCATCGTCACATACACGGCCGGAAATAGACAG
ACGACGCCGGGAACGATTCTGACGGATACAGATAACGTGTGGAAGATCCGGCAG
CAGTTGATGCACATGCATACGCGATCAAGACGTACGACTACTACAAGAACAAATTC
GGAAGAGATTCAATCGATGGAAGAGGCATGCAAATCAGATCAACGGTTCATTATGG
CAAAAAGTACAACAATGCCTTCTGGAACGGCAGCCAAATGACATACGGCGATGGA
GACGGCTCAACGTTTACATTCTTTTCAGGCGACCCGGACGTCGTCGGCCATGAACTG
ACGCATGGCGTTACAGAGTTCACGAGCAACCTGGAGTATTACGGCGAATCAGGCGC
ACTGAATGAGGCTTTCAGCGACATCATTGGCAACGACATTGATGGCACATCATGGC
TGCTTGGCGACGGCATTTACACACCTAACATTCCGGGCGATGCACTGAGAAGCCTG
TCAGACCCTACGAGATTCGGCCAACCTGACCATTACAGCAACTTCTACCCGGATCCT
AATAACGATGATGAGGGCGGAGTGCATACGAACAGCGGCATTATCAACAAAGCGT
ACTATCTGCTGGCACAAGGCGGAACGTCACATGGAGTGACGGTGACAGGAATCGGC
AGAGAGGCGGCAGTGTTTATCTACTACAACGCCTTCACAAACTACCTGACGAGCAC
GTCAAATTTCAGCAACGCTAGAGCGGCGGTCATCCAGGCAGCAAAGGACTTTTATG
GAGCAGACTCACTGGCAGTTACGTCAGCAATTCAGTCATTCGACGCAGTTGGAATT
AAG

The amino acid sequence of the PspPro2 precursor protein expressed from plasmid pGX084(AprE-PspPro2) is depicted in SEQ ID NO: 10. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold, and the predicted pro-peptide is shown in underlined text:

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**AEHSVPDPTQLTPTFHAEQWKAPSTVTG
DNIVWSYLNRQKKTLLNTDSTSVRDQFRIIDRTSDKSGASHYRLKQYVNGIPVYGAEQT
IHVNNAGKVTSYLGAVISEDQQQDATEDTTPKISATEAVYTAYAEAAARIQSFPSINDSL
SEASEEQGSENQGNEIQNIGIKSSVSNDTYAEAHNNVLLTPVDQAEQSYIAKIANLEPSV

EPKAELYIYPDGETTRLVYVTEVNILEPAPLRTRYFIDAKTGKIVFQYDILNHATGTGRG
VDGKTKSFTTTASGNRYQLKDTTRSNGIVTYTAGNRQTTPGTILTDTDNVWEDPAAVD
AHAYAIKTYDYYKNKFGRDSIDGRGMQIRSTVHYGKKYNNAFWNGSQMTYGDGDGS
TFTFFSGDPDVVGHELTHGVTEFTSNLEYYGESGALNEAFSDIIGNDIDGTSWLLGDGIY
TPNIPGDALRSLSDPTRFGQPDHYSNFYPDPNNDDEGGVHTNSGIINKAYYLLAQGGTS
HGVTVTGIGREAAVFIYYNAFTNYLTSTSNFSNARAAVIQAAKDFYGADSLAVTSAIQS
FDAVGIK

The amino acid sequence of the recombinant PspPro2 protein isolated from *Bacillus subtilis* culture was determined by tandem mass spectrometry, and shown below. It is the same as predicted and depicted in SEQ ID NO: 8.

ATGTGRGVDGKTKSFTTTASGNRYQLKDTTRSNGIVTYTAGNRQTTPGTILTDTDNVW
EDPAAVDAHAYAIKTYDYYKNKFGRDSIDGRGMQIRSTVHYGKKYNNAFWNGSQMT
YGDGDGSTFTFFSGDPDVVGHELTHGVTEFTSNLEYYGESGALNEAFSDIIGNDIDGTS
WLLGDGIYTPNIPGDALRSLSDPTRFGQPDHYSNFYPDPNNDDEGGVHTNSGIINKAYY
LLAQGGTSHGVTVTGIGREAAVFIYYNAFTNYLTSTSNFSNARAAVIQAAKDFYGADSL
AVTSAIQSFDAVGIK

EXAMPLE 2.3

Proteolytic Activity of Metalloprotease PspPro2

The proteolytic activity of purified PspPro2 was measured in 50 mM Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To initiate the reaction, 50 μl of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-binding 96-well Microtiter Plate (96-MTP) (Corning Life Sciences, #3641) placed on ice, followed by the addition of 50 μl of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40°C and 650 rpm for 10 min. The reaction was terminated by adding 100 μl of 5% Trichloroacetic Acid (TCA). Following equilibration (5 min at the room temperature) and subsequent centrifugation (2000 g for 10 min at 4°C) , 120 μl supernatant was transferred to a new 96-MTP, and absorbance of the supernatant was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of duplicate assays, and the value varies

no more than 5%. The proteolytic activity is shown as Net A_{440}. The proteolytic assays with azo-casein as the substrate (Figure 2.2) indicate that PspPro2 is an active protease.

EXAMPLE 2.4

5 **pH profile of Metalloprotease PspPro2**

With azo-casein as the substrate, the pH profile of PspPro2 was studied in 12.5 mM acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 4 to 11). To initiate the assay, 50 μl of 25 mM acetate/Bis-Tris/HEPES/CHES buffer with a specific pH was first mixed with 2 μl diluted enzyme (500 ppm in Milli-Q H_2O) in a 96-MTP placed on ice,
10 followed by the addition of 48 μl of 1.5% (w/v) azo-casein prepared in H_2O. The reaction was performed and analyzed as described in Example 2.3. Enzyme activity at each pH was reported as the relative activity, where the activity at the optimal pH was set to be 100%. The pH values tested were 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10 and 11. Each result was the mean of triplicate assays. As shown in Figure 2.3, the optimal pH of PspPro2 is 7.5 with greater than 70% of its
15 maximal activity retained between pH 5.5 and 9.5.

EXAMPLE 2.5

Temperature profile of Metalloprotease PspPro2

The temperature profile of PspPro2 was analyzed in 50 mM Tris buffer (pH 7) using the
20 azo-casein assay. The enzyme sample and azo-casein substrate were prepared as in Example 2.3. Prior to the reaction, 50 μl of 1.5% azo-casein and 45 μl Milli-Q H_2O were mixed in a 200 μl PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler (BioRad) at desired temperatures (i.e. 20~90°C) for 5 min. After the incubation, 5 μl of diluted PspPro2 (200 ppm) or H_2O (the blank control) was added to the substrate mixture, and the reaction was carried
25 out in the Peltier Thermal Cycle for 10 min at different temperatures. To terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 μl of 5% TCA per well. Subsequent centrifugation and absorbance measurement were performed as described in Example 3. The activity was reported as the relative activity, where the activity at the optimal temperature was set to be 100%. The tested temperatures are 20, 30, 40, 50, 60, 70, 80 and 90°C.
30 Each result was the mean of triplicate assays. The data in Figure 2.4 suggest that PspPro2 showed an optimal temperature at 50°C, and retained greater than 70% of its maximal activity between 40 and 65°C.

EXAMPLE 2.6

Cleaning performance of Metalloprotease PspPro2 in Automatic Dishwashing (ADW) conditions

The cleaning performance of PspPro2 protein in automatic dishwashing (ADW) conditions was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 and 8 using a model automatic dishwashing (ADW) detergent. Prior to the reaction, purified PspPro2 protein samples were diluted with the dilution solution containing 10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol to the desired concentrations. The reactions were performed in AT detergent with 100 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1), in the presence of a bleach component (Peracid *N*,*N*-phthaloylaminoperoxycaproic acid-PAP) (AT detergent composition shown in Table 1). To initiate the reaction, 180 µl of the AT detergent solution at pH 6 or pH 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the addition of 20 µl of diluted enzymes (or the dilution solution as the blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 30 min at 50°C and 1150 rpm. After incubation, 100 µl of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 405 nm (referred here as the "Initial performance") using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the microswatches were rinsed once with 200 µl water. Following the addition of 180 µl of 0.1 M CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50 °C and 1150 rpm for 10 min. One hundred microliters of the resulting wash liquid was transferred to a new 96-MTP, and its absorbance was measured at 405 nm (referred here as "Wash-off"). The sum of two absorbance measurements ("Initial performance" plus "Wash-off") gives the "Total performance", which measures the protease activity on the model stain. Dose response for cleaning of PA-S-38 microswatches at pH 6 and pH 8 for PspPro2 in AT detergent in the presence of bleach, is shown in Figure 2.5A and 2.5B, respectively.

Table 2.1. Composition of AT dish detergent with bleach	
Ingredient	**Concentration (mg/ml)**
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
PAP (peracid *N*,*N*-phthaloylaminoperoxycaproic acid)	0.057
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029
PEG 6000	0.043
PEG 1500	0.1

* The pH of the AT formula detergent is adjusted to the desired pH value (pH 6 or 8) by the addition of 0.9 M citric acid.

5

EXAMPLE 2.7

Cleaning performance of Metalloprotease PspPro2 in laundry conditions

A. Cleaning performance in liquid laundry detergent

The cleaning performance of PspPro2 protein in liquid laundry detergent was tested
10 using EMPA-116 (cotton soiled with blood/milk/ink) microswatches (obtained from CFT Vlaardingen, The Netherlands) at pH 8.2 using a commercial detergent. Prior to the reaction, purified PspPro2 protein samples were diluted with a dilution solution (10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol) to the desired concentrations; and the commercial detergent (Tide®, Clean Breeze®, Proctor & Gamble, USA, purchased September
15 2011) was incubated at 95°C for 1 hour to inactivate the enzymes present in the detergent. Proteolytic assays were subsequently performed to confirm the inactivation of proteases in the commercial detergent. The heat treated detergent was further diluted with 5 mM HEPES (pH 8.2) to a final concentration of 0.788 g/L. Meanwhile, the water hardness of the buffered liquid detergent was adjusted to 103 ppm (Ca^{2+} : Mg^{2+} = 3 : 1). The specific conductivity of the
20 buffered detergent was adjusted to either 0.62 mS/cm (low conductivity) or 3.5 mS/cm (high conductivity) by adjusting the NaCl concentration in the buffered detergent. To initiate the reaction, 190 µl of either the high or low conductivity buffered detergent was added to a 96-

MTP containing the EMPA-116 microswatches, followed by the addition of 10 μl of diluted enzyme (or the dilution solution as blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 20 min at 32°C and 1150 rpm. After incubation, 150 μl of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 600 nm using a spectrophotometer, which indicates the protease activity on the model stain; and Net A_{600} was subsequently calculated by subtracting the A_{600} of the blank control from that of the enzyme. Dose response for the cleaning of EMPA-116 microswatches in liquid laundry detergent at high or low conductivity is shown in Figure 2.6A.

B. Cleaning performance in powder laundry detergent

The cleaning performance of PspPro2 protein in powder laundry detergent was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) using a commercial detergent. Prior to the reaction, purified PspPro2 protein samples were diluted with a dilution solution (10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol) to the desired concentrations. The powder laundry detergent (Tide®, Bleach Free, Proctor & Gamble, China, purchased in December 2011) was dissolved in water with 103 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1) to a final concentration of 2 g/L (with conductivity of 2.3 mS/cm-low conductivity) or 5 g/L (with conductivity of 5.5 mS/cm-high conductivity). The detergents of different conductivities were subsequently heated in a microwave to near boiling in order to inactivate the enzymes present in the detergent. Proteolytic activity was measured following treatment to ensure that proteases in the commercial detergent had been inactivated. To initiate the reaction, 190 μl of either the high or low conductivity heat-treated detergent was added to a 96-MTP containing the PA-S-38 microswatches, followed by the addition of 10 μl of diluted enzyme (or the dilution solution as blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 15 minutes at 32°C and 1150 rpm. After incubation, 150 μl of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 405 nm using a spectrophotometer, which indicates the protease activity on the model stain; and Net A_{405} was subsequently calculated by subtracting the A_{405} of the blank control from that of the enzyme. Dose response for the cleaning of PA-S-38 microswatches in powder laundry detergent at high or low conductivity is shown in Figure 2.6B.

EXAMPLE 2.8
Comparison of PspPro2 to Other Metalloproteases

Identification of Homologous Proteases

5 Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res, 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest Patent database with search parameters set to default values. The mature protein amino acid sequence for PspPro2 (SEQ ID NO: 8) is used as query sequence. Percent identity (PID) for both search sets is defined as the number of identical residues divided by the number of aligned 10 residues in the pairwise alignment. Tables 2.2A and 2.2B provide a list of sequences with the percent identity to PspPro2. The length in Table 2.2 refers to the entire sequence length of the homologous proteases.

Table 2.2A: List of sequences with percent identity to PspPro2 protein identified from the NCBI non-redundant protein database

Accession #	PID to PspPro2	Organism	Length
AAB02774.1	55	Geobacillus stearothermophilus	552
AAA22623.1	56	Bacillus caldolyticus	544
P00800	56	Bacillus thermoproteolyticus	548
YP_003670279.1	57	Geobacillus sp. C56-T3	546
BAD60997.1	57	Bacillus megaterium	562
ZP_02326503.1	58	Paenibacillus larvae subsp. larvae BRL-230010	520
ZP_08640523.1	58	Brevibacillus laterosporus LMG 15441	564
YP_003597483.1	58	Bacillus megaterium DSM 319	562
ZP_09069025.1	59	Paenibacillus larvae subsp. larvae B-3650	520
YP_001373863.1	59	Bacillus cytotoxicus NVH 391-98	565
ZP_04149724.1	59	Bacillus pseudomycoides DSM 12442	566
CAA43589.1	60	Brevibacillus brevis	527
ZP_10738945.1	60	Brevibacillus sp. CF112	528
ZP_04216147.1	60	Bacillus cereus Rock3-44	566
ZP_10575942.1	61	Brevibacillus sp. BC25	528

Table 2.2A: List of sequences with percent identity to PspPro2 protein identified from the NCBI non-redundant protein database			
Accession #	**PID to PspPro2**	**Organism**	**Length**
YP_002770810.1	62	Brevibacillus brevis NBRC 100599	528
ZP_08511445.1	63	Paenibacillus sp. HGF7	525
ZP_09077634.1	64	Paenibacillus elgii B69	524
ZP_09071078.1	67	Paenibacillus larvae subsp. larvae B-3650	529
YP_003872180.1	73	Paenibacillus polymyxa E681	587
YP_005073223.1	78	Paenibacillus terrae HPL-003	591
ZP_09775364.1	78	Paenibacillus sp. Aloe-11	593
YP_003948511.1	80	Paenibacillus polymyxa SC2	592
YP_005073224.1	94	Paenibacillus terrae HPL-003	595
ZP_10241029.1	96	Paenibacillus peoriae KCTC 3763	599
ZP_09775365.1	100	Paenibacillus sp. Aloe-11	580

Table 2.2B: List of sequences with percent identity to PspPro2 protein identified from the Genome Quest database			
Patent #	**PID to PspPro2**	**Organism**	**Length**
JP2002272453-0002	57.01	Bacillus megaterium	562
US6518054-0001	57.19	Bacillus sp.	319
EP2390321-0177	57.19	Bacillus caldolyticus	544
US20120107907-0176	57.19	Bacillus stearothermophilis	548
WO9520663-0003	57.51	*empty*	319
WO2012110562-0003	57.51	Geobacillus stearothermophilus	319
WO2012110563-0002	57.51	Bacillus caldolyticus	319
WO2004011619-0056	57.51	*empty*	546
WO2004011619-0003	57.51	*empty*	546
JP2002272453-0003	57.64	*empty*	562
US6518054-0002	57.88	Bacillus sp.	316
EP2178896-0184	58.15	Bacillus anthracis	566
WO2012110563-0004	58.28	Bacillus megaterium	320
JP1995184649-0001	58.79	Lactobacillus sp.	566
JP1994014788-0003	58.84	*empty*	317

Table 2.2B: List of sequences with percent identity to PspPro2 protein identified from the Genome Quest database			
Patent #	**PID to PspPro2**	**Organism**	**Length**
US8114656-0178	59.42	Bacillus thuringiensis	566
WO2012110562-0005	59.49	Bacillus cereus	320
US5962264-0004	59.81	*empty*	566
US20120107907-0185	59.81	Bacillus cereus	317
US20120107907-0179	59.81	Bacillus cereus	566
WO2012110563-0005	60.13	Bacillus cereus	320
EP2390321-0186	60.33	Bacillus brevis	304
JP2005229807-0018	78.62	Paenibacillus polymyxa	566
EP2390321-0187	79.21	Bacillus polymyxa	302

B. Alignment of Homologous Protease Sequences

The amino acid sequence of mature PspPro2 (SEQ ID NO: 8) was aligned with thermolysin (P00800, *Bacillus thermoproteolyticus*) and protease from *Paenibacillus sp. Aloe-*
5 *11* (ZP_09775365.1) sequences using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 2.7 shows the alignment of PspPro2 with these protease sequences.

C. Phylogenetic Tree

A phylogenetic tree for precursor PspPro2 protein sequence (SEQ ID NO: 7) was built using
10 sequences of representative homologs from Table 2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software
15 (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 2.8.

EXAMPLE 3.1

Cloning of *Paenibacillus humicus* metalloprotease *PhuPro2*

20 A strain (DSM18784) of *Paenibacillus humicus* was selected as a potential source of enzymes which may be useful in various industrial applications. Genomic DNA for sequencing

was obtained by first growing the strain on Heart Infusion agar plates (Difco) at 37°C for 24 hr. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The genomic DNA was used for genome sequencing. The entire genome of the *Paenibacillus humicus* strain was sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified after annotation in *Paenibacillus humicus* encodes a metalloprotease and the sequence of this gene, called *PhuPro2*, is provided in SEQ ID NO: 11. The corresponding protein encoded by the *PhuPro2* gene is shown in SEQ ID NO: 12. At the N-terminus, the protein has a signal peptide with a length of 23 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786). The presence of a signal sequence suggests that PhuPro2 is a secreted enzyme. The propeptide region was predicted based on its protein sequence alignment with the *Paenibacillus polymyxa* Npr protein (Takekawa *et al.* (1991) Journal of Bacteriology, **173** (21): 6820-6825). The predicted mature region of PhuPro2 protein in shown in SEQ ID NO: 13. The nucleotide sequence of the *PhuPro2* gene isolated from *Paenibacillus humicus* is set forth as SEQ ID NO: 11. The sequence encoding the predicted native signal peptide is shown in italics:

ATGAAAAAAATGATTCCTACTCTGCTCGGTACCGTATTGCTGCTTTCTTCCGCTTCCGCTG
*TCGCTGCT*GAATCGCCAAGCCTCGGAGCGGCCGGAACTCCCGGGGTCAGCGTCGTG
AACAATCAGCTCGTGACTCAATTCATCGAGGCTTCCAAGGATGCCAAGATTGTCCC
GGGCTCTTCCGAGGATAAAATCTGGGCTTTCCTTGAAGGCCAGCAAGCAAAGCTGG
GTGTATCCGCAGCGGATGTAAAAACCTCGTTCCTGATCCAGAAGAAGGAAGTCGAT
CCGACTTCGGGCGTCGAGCATTTCCGCCTGCAGCAATATGTGAATGGCATCCCGGTA
TATGGCGGTGACCAAACCATTCACATCGACAAGGCCGGCCAGGTTACGTCGTTCGT
AGGAGCTGTTCTGCCGGCTCAAAATCAAATCACGGCAAAATCCAGCGTACCAGCCA
TAAGCGCATCCGACGCTCTGGCTATCGCGGCGAAGGAAGCCAGTTCCCGCATCGGC
GAGCTGGGAGCACAGGAGAAGACTCCGTCGGCTCAGCTGTACGTATATCCGGAAGG
CAACGGGTCGCGTCTCGTCTACCAGACGGAAGTGAATGTGCTTGAGCCGCAGCCTC
TGCGCACCCGCTATCTTATCGATGCGGCCGACGGCCATATCGTGCAGCAGTACGATC
TGATCGAGACGGCGACCGGTTCGGGCACGGGCGTGCTGGGCGACAATAAGACGTTC
CAGACGACTCTTTCCGGCAGCACGTACCAGCTGAAAGACACCACTCGCGGCAACGG
CATCTACACCTACACAGCCAGCAATCGGACCACGATTCCGGGCACGCTGCTGACGG
ACGCCGACAACGTATGGACGGATGGAGCCGCCGTCGATGCCCATACTTATGCCGGA
AAAGTATATGATTTCTACAAAACGAAGTTCGGACGCAACAGCCTCGACGGCAACGG

CCTGCTGATCCGTTCCTCGGTCCACTACAGCAGCAGGTACAACAATGCCTTCTGGAA
CGGCACCCAGATTGTATTCGGCGACGGCGACGGCTCGACGTTCATTCCGCTGTCGG
GCGATCTCGACGTGGTCGGCCATGAGCTGTCCCACGGAGTCATCGAGTACACGTCC
AACCTTCAATACCTCAATGAATCCGGCGCGCTGAACGAGTCCTATGCCGACGTCCTC
GGCAACTCGATCCAGGCGAAAAACTGGCTTATCGGCGACGATGTCTATACGCCTGG
CATCTCCGGAGATGCTCTCCGTTCCATGTCCAACCCGACGCTTTACGGGCAGCCGGA
CAACTATGCCAACCGCTATACGGGATCTTCCGACAACGGCGGCGTTCATACGAACA
GCGGCATCACGAACAAAGCGTTCTACCTGCTCGCCCAAGGCGGCACCCAGAACGGC
GTTACCGTCGCCGGCATCGGGCGCGACGCAGCCGTGAACATTTTCTACAACACAGT
GGCCTATTACCTTACTTCCACTTCCAACTTCGCCGCGGCGAAGAACGCCTCGATCCA
GGCAGCCAAAGACCTGTACGGAACGGGCTCCTCTTATGTCACCTCGGTGACCAATG
CATTCAGAGCCGTAGGCCTG

The amino acid sequence of the PhuPro2 precursor protein is set forth as SEQ ID NO: 12. The predicted signal sequence is shown in italics, and the predicted propeptide is shown in underlined text:

*MKKMIPTLLGTVLLLSSASAVAA*ESPSLGAAGTPGVSVVNNQLVTQFIEASKDAKIVPGSSE
DKIWAFLEGQQAKLGVSAADVKTSFLIQKKEVDPTSGVEHFRLQQYVNGIPVYGGDQTI
HIDKAGQVTSFVGAVLPAQNQITAKSSVPAISASDALAIAAKEASSRIGELGAQEKTPSA
QLYVYPEGNGSRLVYQTEVNVLEPQPLRTRYLIDAADGHIVQQYDLIETATGSGTGVLG
DNKTFQTTLSGSTYQLKDTTRGNGIYTYTASNRTTIPGTLLTDADNVWTDGAAVDAHT
YAGKVYDFYKTKFGRNSLDGNGLLIRSSVHYSSRYNNAFWNGTQIVFGDGDGSTFIPLS
GDLDVVGHELSHGVIEYTSNLQYLNESGALNESYADVLGNSIQAKNWLIGDDVYTPGIS
GDALRSMSNPTLYGQPDNYANRYTGSSDNGGVHTNSGITNKAFYLLAQGGTQNGVTV
AGIGRDAAVNIFYNTVAYYLTSTSNFAAAKNASIQAAKDLYGTGSSYVTSVTNAFRAV
GL

The amino acid sequence of the predicted mature form of PhuPro2 is set forth as SEQ ID NO: 13:

ATGSGTGVLGDNKTFQTTLSGSTYQLKDTTRGNGIYTYTASNRTTIPGTLLTDADNVWT
DGAAVDAHTYAGKVYDFYKTKFGRNSLDGNGLLIRSSVHYSSRYNNAFWNGTQIVFG
DGDGSTFIPLSGDLDVVGHELSHGVIEYTSNLQYLNESGALNESYADVLGNSIQAKNWL
IGDDVYTPGISGDALRSMSNPTLYGQPDNYANRYTGSSDNGGVHTNSGITNKAFYLLA
QGGTQNGVTVAGIGRDAAVNIFYNTVAYYLTSTSNFAAAKNASIQAAKDLYGTGSSYV
TSVTNAFRAVGL

EXAMPLE 3.2

Expression of *Paenibacillus humicus s* metalloprotease PhuPro2

The DNA sequence of the propeptide-mature form of PhuPro2 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX150(AprE-PhuPro2) (Figure 1). Ligation of this gene encoding the PhuPro2 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3' end of the *B. subtilis* AprE signal sequence and the 5' end of the predicted PhuPro2 native propeptide. The gene has an alternative start codon (GTG). The resulting plasmid shown in Figure 1 was labeled pGX150(AprE-PhuPro2). As shown in Figure 3.1, pGX150(AprE-PhuPro2) contains an AprE promoter, an AprE signal sequence used to direct target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature regions of PhuPro2 (SEQ ID NO: 14). The translation product of the synthetic AprE- PhuPro2 gene is shown in SEQ ID NO: 15.

The pGX150 (AprE-PhuPro2) plasmid was then transformed into *B. subtilis* cells ($degU^{Hy}32$, *ΔscoC*) and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm Chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250 ml shake flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$).

The broth from the shake flasks was concentrated and buffer-exchanged into the loading buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM CaCl2 and 10% propylene glycol using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to an 80 ml Q Sepharose High Performance column pre-equilibrated with the loading buffer above; and the active flow-through fractions were collected and concentrated. The sample was loaded onto a 320 ml Superdex 75 gel filtration column pre-equilibrated with the loading buffer described above containing 0.15 M NaCl. The corresponding active purified protein fractions were further pooled and concentrated via 10K Amicon Ultra for further analyses.

The nucleotide sequence of the synthesized PhuPro2 gene in plasmid pGX150(AprE-PhuPro2) is depicted in SEQ ID NO: 14. The sequence encoding the three residue addition (AGK) is shown in bold:

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACG
ATGGCGTTCAGCAACATGAGCGCGCAGGCT**GCTGGAAAA**GAATCACCGAGCCTTG
GCGCTGCAGGAACACCGGGCGTTAGCGTTGTGAATAACCAACTGGTCACGCAGTTC

ATCGAAGCATCAAAAGACGCGAAAATTGTCCCTGGATCAAGCGAAGATAAGATTTG
GGCATTTCTGGAAGGCCAGCAAGCAAAGCTTGGCGTCTCAGCTGCCGACGTGAAGA
CGAGCTTCCTGATCCAGAAGAAGGAGGTTGACCCGACATCAGGCGTTGAGCACTTT
AGACTGCAACAGTACGTCAACGGCATCCCGGTTTATGGAGGCGATCAAACAATCCA
TATTGATAAGGCAGGCCAGGTCACATCATTCGTCGGAGCTGTCCTGCCGGCTCAGA
ACCAAATTACAGCAAAATCATCAGTTCCGGCAATTTCAGCCTCAGACGCTCTGGCA
ATCGCTGCCAAGGAGGCAAGCTCAAGAATTGGCGAACTGGGCGCACAAGAAAAGA
CACCGAGCGCCCAACTTTATGTCTATCCGGAGGGCAACGGAAGCAGACTGGTGTAC
CAGACAGAGGTCAATGTTCTGGAGCCGCAACCGCTGAGAACGAGATACCTTATCGA
TGCTGCGGATGGCCACATTGTTCAGCAATACGACCTGATTGAGACAGCAACAGGAA
GCGGAACGGGCGTGCTGGGCGACAACAAGACGTTTCAGACAACACTTAGCGGCAG
CACGTACCAACTTAAGGACACGACGAGAGGCAATGGCATTTACACGTACACGGCCT
CAAACAGAACGACAATCCCAGGCACACTGCTGACGGATGCAGACAATGTTTGGACG
GACGGCGCAGCAGTTGACGCACACACGTACGCCGGCAAGGTGTACGACTTTTACAA
GACGAAGTTCGGCAGAAACAGCCTTGATGGAAATGGACTGCTGATCAGAAGCAGC
GTCCACTACAGCAGCAGATACAATAACGCCTTCTGGAACGGCACACAAATCGTCTT
TGGCGATGGAGACGGATCAACATTCATCCCGCTGTCAGGCGACCTGGACGTTGTGG
GCCACGAGCTGAGCCACGGCGTCATCGAGTACACGAGCAACCTGCAGTACCTGAAT
GAAAGCGGCGCACTGAACGAGTCATATGCTGATGTGCTTGGCAATAGCATCCAGGC
CAAGAACTGGCTTATCGGAGACGACGTCTACACACCTGGCATCAGCGGCGATGCTC
TGAGAAGCATGAGCAATCCTACACTTTACGGCCAACCGGACAACTACGCGAATAGA
TATACGGGCAGCAGCGACAATGGCGGCGTTCATACAAACTCAGGCATCACGAACAA
GGCGTTCTACCTGCTGGCACAGGGAGGCACGCAAAACGGCGTTACAGTTGCGGGCA
TTGGCAGAGATGCGGCCGTCAACATCTTCTACAACACAGTCGCCTACTACCTGACG
AGCACGTCAAACTTCGCAGCGGCAAAGAACGCATCAATTCAAGCAGCAAAGGATCT
GTACGGAACAGGCAGCTCATATGTCACGTCAGTTACGAATGCGTTTAGAGCCGTCG
GCCTTTAA

The amino acid sequence of the PhuPro2 precursor protein expressed from plasmid pGX150(AprE- PhuPro2) is depicted in SEQ ID NO: 15. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold, and the predicted propeptide is shown in underlined text.

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**ESPSLGAAGTPGVSVVNNQLVTQFIEASK
DAKIVPGSSEDKIWAFLEGQQAKLGVSAADVKTSFLIQKKEVDPTSGVEHFRLQQYVN
GIPVYGGDQTIHIDKAGQVTSFVGAVLPAQNQITAKSSVPAISASDALAIAAKEASSRIGE

LGAQEKTPSAQLYVYPEGNGSRLVYQTEVNVLEPQPLRTRYLIDAADGHIVQQYDLIET
ATGSGTGVLGDNKTFQTTLSGSTYQLKDTTRGNGIYTYTASNRTTIPGTLLTDADNVWT
DGAAVDAHTYAGKVYDFYKTKFGRNSLDGNGLLIRSSVHYSSRYNNAFWNGTQIVFG
DGDGSTFIPLSGDLDVVGHELSHGVIEYTSNLQYLNESGALNESYADVLGNSIQAKNWL
5 IGDDVYTPGISGDALRSMSNPTLYGQPDNYANRYTGSSDNGGVHTNSGITNKAFYLLA
QGGTQNGVTVAGIGRDAAVNIFYNTVAYYLTSTSNFAAAKNASIQAAKDLYGTGSSYV
TSVTNAFRAVGL (SEQ ID NO: 15).

EXAMPLE 3.3

10 **Proteolytic Activity of Metalloprotease PhuPro2**

The proteolytic activity of purified metalloprotease PhuPro2 was measured in 50 mM Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To
15 initiate the reaction, 50 μl of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-binding 96-well Microtiter Plate (96-MTP) (Corning Life Sciences, #3641) placed on ice, followed by the addition of 50 μl of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40°C and 650 rpm for 10 min. The reaction was terminated by adding 100 μl of 5% Trichloroacetic Acid (TCA). Following
20 equilibration (5 min at the room temperature) and subsequent centrifugation (2000 g for 10 min at 4°C) , 120 μl supernatant was transferred to a new 96-MTP, and absorbance of the supernatant was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of triplicate assays.
25 The proteolytic activity is shown as Net A_{440}. The proteolytic assay with azo-casein as the substrate (shown in Figure 3.2) indicates that PhuPro2 is an active protease.

EXAMPLE 3.4

30 **pH Profile of Metalloprotease PhuPro2**

With azo-casein as the substrate, the pH profile of metalloprotease PhuPro2 was studied in 12.5 mM acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 4 to 11). To initiate the assay, 50 μl of 25 mM acetate/Bis-Tris/HEPES/CHES buffer with a specific pH was first mixed with 2 μl Milli-Q H_2O diluted enzyme (125 ppm) in a 96-MTP
35 placed on ice, followed by the addition of 48 μl of 1.5% (w/v) azo-casein prepared in H_2O. The

reaction was performed and analyzed as described in Example 3.3. Enzyme activity at each pH was reported as the relative activity, where the activity at the optimal pH was set to be 100%. The pH values tested were 4, 5, 6, 7, 8, 9, 10 and 11. Each value was the mean of triplicate assays. As shown in Figure 3.3, the optimal pH of PhuPro2 is 6, with greater than 70% of
5 maximal activity retained between 5.5 and 8.5.

EXAMPLE 3.5

Temperature Profile of Metalloprotease PhuPro2

The temperature profile of metalloprotease PhuPro2 was analyzed in 50 mM Tris buffer (pH 7) using the azo-casein assays. The enzyme sample and azo-casein substrate were prepared
10 as in Example 3.3. Prior to the reaction, 50 μl of 1.5% azo-casein and 45 μl Milli-Q H_2O were mixed in a 200 μl PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler (BioRad) at desired temperatures (i.e. 20~90°C) for 5 min. After the incubation, 5 μl of diluted enzyme (50 ppm) or H_2O (the blank control) was added to the substrate mixture, and the reaction was carried out in the Peltier Thermal Cycle for 10 min at different temperatures. To
15 terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 μl of 5% TCA per well. Subsequent centrifugation and absorbance measurement were performed as described in Example 3.3. The activity was reported as the relative activity, where the activity at the optimal temperature was set to be 100%. The tested temperatures are 20, 30, 40, 50, 60, 70, 80, and 90°C. Each value was the mean of duplicate assays (the value varies no more than 5%).
20 The data in Figure 3.4 suggests that PhuPro2 showed an optimal temperature at 50°C, and retained greater than 70% of its maximum activity between 45 and 65°C.

EXAMPLE 3.6

Cleaning performance of metalloprotease PhuPro2

25 The cleaning performance of PhuPro2 was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 and 8 using a model automatic dishwashing (ADW) detergent. Prior to the reaction, purified protease samples were diluted with a dilution solution containing 10 mM NaCl, 0.1mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol to the desired concentrations. The reactions were
30 performed in AT detergent with 100 ppm water hardness ($Ca^{2+} : Mg^{2+} = 3 : 1$) (detergent composition shown in Table 3.1). To initiate the reaction, 180 μl of the AT detergent buffered at pH 6 or pH 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the addition of 20 μl of diluted enzymes (or the dilution solution as the blank control). The 96-MTP

was sealed and incubated in an incubator/shaker for 30 min at 50 °C and 1150 rpm. After incubation, 100 µl of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 405 nm (referred here as the "Initial performance") using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the
5 microswatches were rinsed once with 200 µl water. Following the addition of 180 µl of 0.1 M CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50 °C and 1150 rpm for 10 min. One hundred microliters of the resulting wash liquid was transferred to a new 96-MTP, and its absorbance measured at 405 nm (referred here as the "Wash-off"). The sum of two absorbance measurements ("Initial performance" plus "Wash-off") gives the "Total
10 performance", which measures the protease activity on the model stain; and Net A_{405} was subsequently calculated by subtracting the A_{405} of the "Total performance" of the blank control from that of the enzyme. Dose response in cleaning the PA-S-38 microswatches at pH 6 and pH 8 in AT dish detergent for PhuPro2 is shown in Figures 3.5A and 3.5B.

Table 3.1. Composition of AT dish detergent

Ingredient	Concentration (mg/ml)
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029
PEG 6000	0.043
PEG 1500	0.1

15 *The pH of the AT formula detergent is adjusted to the desired value (pH 6 or 8) by the addition of 0.9 M citric acid.

EXAMPLE 3.7

20 **Comparison of PhuPro2 to Other Proteases**

A. Identification of Homologous Proteases

Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res, 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest

Patent database with search parameters set to default values. The predicted mature protein amino acid sequence for PhuPro2 (SEQ ID NO: 13) is used as the query sequences. Percent identity (PID) for both search sets is defined as the number of identical residues divided by the number of aligned residues in the pairwise alignment. Tables 3.2A and 3.2B provide a list of sequences
5 with the percent identity to PhuPro2. The length in Table 3.2 refers to the entire sequence length of the homologous proteases.

Table 3.2A: List of sequences with percent identity to PhuPro2 protein identified from the NCBI non-redundant protein database

Accession #	PID to PhuPro2	Organism	Length
P00800	59	Bacillus thermoproteolyticus	548
YP_003872180.1	59	Paenibacillus polymyxa E681	587
ZP_10575942.1	59	Brevibacillus sp. BC25	528
ZP_02326602.1	60	Paenibacillus larvae subsp. larvae BRL-230010	520
ADM87306.1	61	Bacillus megaterium	562
ZP_09069025.1	61	Paenibacillus larvae subsp. larvae B-3650	520
ZP_09069194.1	62	Paenibacillus larvae subsp. Larvae B-3650	502
ZP_10738945.1	63	Brevibacillus sp. CF112	528
ZP_08511445.1	64	Paenibacillus sp. HGF7	525
ZP_09077634.1	65	Paenibacillus elgii B69	524
ZP_09775365.1	65	Paenibacillus sp. Aloe-11	580
ZP_09775364.1	70	Paenibacillus sp. Aloe-11	593
P29148	71	Paenibacillus polymyxa	590
ZP_10241030.1	71	Paenibacillus peoriae KCTC 3763	593
ZP_09071078.1	71	Paenibacillus larvae subsp. larvae B-3650	529
YP_003872179.1	72	Paenibacillus polymyxa E681	592
YP_005073223.1	72	Paenibacillus terrae HPL-003	591

Table 3.2B: List of sequences with percent identity to PhuPro2 protein identified from the Genome Quest Patent database

Patent ID #	PID to PhuPro2	Organism	Length
US20090208474-0030	59.22	Bacillus thermoproteolyticus	316
JP2002272453-0002	59.42	Bacillus megaterium	562
JP2006124323-0003	59.55	Bacillus thermoproteolyticus	316
US8114656-0183	59.87	Bacillus stearothermophilis	316
JP1989027475-0001	59.87	Bacillus subtilis	316
US20120009651-0002	59.87	Geobacillus caldoproteolyticus	548
JP2002272453-0003	60.45	*empty*	562
WO2012110563-0004	60.77	Bacillus megaterium	320
EP2390321-0186	62.25	Bacillus brevis	304
JP2005229807-0018	71.85	Paenibacillus polymyxa	566
US8114656-0187	72.09	Bacillus polymyxa	302

B. Alignment of Homologous Protease Sequences

The amino acid sequence of predicted mature PhuPro2 (SEQ ID NO: 13) protein was aligned with thermolysin (P00800, *Bacillus thermoproteolyticus*), and protease from *Paenibacillus terrae* HPL-003 (YP_005073223.1) sequences using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 3.6 shows the alignment of PhuPro2 with these protease sequences.

C. Phylogenetic Tree

A phylogenetic tree for full length sequence of PhuPro2 (SEQ ID NO: 12) was built using sequences of representative homologs from Table 2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 3.7.

EXAMPLE 4.1

Cloning of *Paenibacillus ehimensis* Metalloprotease *PehPro1*

A strain (DSM11029) of *Paenibacillus ehimensis* was selected as a potential source of enzymes which may be useful in various industrial applications. Genomic DNA for sequencing was obtained by first growing the strain on Heart Infusion agar plates (Difco) at 37°C for 24 hr. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The genomic DNA was used for genome sequencing. The entire genome of the *Paenibacillus ehimensis* strain was sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified after annotation in *Paenibacillus ehimensis* encodes a metalloprotease and the sequence of this gene, called *PehPro1*, is provided in SEQ ID NO: 16. The corresponding protein encoded by the *PehPro1* gene is shown in SEQ ID NO: 17. At the N-terminus, the protein has a signal peptide with a length of 23 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786). The presence of a signal sequence suggests that PehPro1 is a secreted enzyme. The propeptide region was predicted based on protein sequence alignment with the *Paenibacillus polymyxa* Npr protein

(Takekawa *et al.* (1991) Journal of Bacteriology, **173** (21): 6820-6825). The predicted mature region of PehPro1 protein is shown in SEQ ID NO: 18.

The nucleotide sequence of the *PehPro1* gene isolated from *Paenibacillus ehimensis* is set forth as SEQ ID NO: 16. The sequence encoding the predicted native signal peptide is shown in italics:

ATGTTAAAAGTATGGGCATCGATTATTACAGGAGCATTTTTGCTCGGGAGCGTGCAAGGG
*GTGCAAGCT*GCTCCACAAGATCAAGCTGCTCCCTTCGGAGGATTCACCCCTCAATTG
ATTACCGGGGAAAGCTGGAGTGCGCCGCAAGGAGTATCGGGAGAGGAAAAAATCT
GGAAGTATCTCGAATCCAAGCAGGAAAGCTTCCAAATCGGCCAAACCGTTGATCTG
AAAAAGCAATTGAAAATTATCGGCCAAACGACCGACGAGAAAACGGGAACCACGC
ATTACCGTCTACAGCAGTATGTGGGAGGCGTCCCCGTATACGGCGGCGTACAAACG
ATCCATGTCAACAAAGAAGGACAAGTTACCTCGCTGATCGGCAGCCTGCTTCCCGA
CCAGCAGCAGCAAGTTTCGAAAAGCTTGAATTCGCAAATCAGCGAAGCGCAAGCCA
TCGCCGTGGCCCAGAAAGATACCGAGGCCGCCGTCGGCAAGCTGGGTGAACCGCAA
AAGACACCGGAAGCGGATCTGTACGTTTATTTACACAACGGACAACCGGTCCTCGC
TTATGTGACCGAGGTTAACGTTCTCGAACCGGAGGCAATCCGGACGCGCTACTTCAT
CAGCGCCGAAGACGGCAGCATTTTATTCAAGTACGACATCCTCGCTCACGCTACAG
GTACCGGAAAAGGCGTGCTCGGAGATACGAAATCGTTCACGACCACGCAATCCGGC
TCCACTTATCAATTGAAGGATACGACGCGCGGGCAAGGTATCGTCACTTACAGCGC
TGGCAACCGGTCCTCTCTGCCGGGAACGCTGCTCACCAGCTCCAGCAATATTTGGAA
CGACGGCGCGGCGGTCGATGCGCATGCCTATACCGCCAAAGTGTACGATTACTATA
AAAACAAATTTGGCCGCAACAGCATTGACGGCAACGGCTTCCAGCTTAAATCGACC
GTGCACTATTCCTCCAGATACAACAACGCCTTCTGGAACGGTGTGCAAATGGTGTAC
GGCGACGGCGACGGCGTAACCTTCATTCCGTTCTCCGCCGATCCGGACGTCATCGGC
CACGAATTGACCCACGGCGTTACGGAACATACGGCCGGCCTGGAATACTACGGCGA
ATCCGGAGCGCTGAACGAATCGATCTCCGATATTATCGGCAACGCGATCGACGGCA
AAAACTGGCTGATCGGCGACTTGATTTATACGCCGAATACTCCCGGGGACGCCCTC
CGCTCTATGGAGAACCCCAAGCTGTATAACCAACCCGACCGCTATCAAGACCGCTA
TACGGGACCTTCCGATAACGGCGGCGTGCATATTAACAGCGGTATCAACAACAAAG
CCTTCTACCTGATCGCCCAAGGCGGCACGCACTATGGCGTCACCGTGAACGGGATC
GGACGCGATGCGGCTGTGCAAATTTTCTATGACGCCCTCATCAATTACCTGACTCCA
ACTTCGAACTTCTCGGCGATGCGCGCAGCAGCCATTCAAGCGGCAACCGACCTGTA
CGGAGCGAATTCTTCTCAAGTAAACGCTGTCAAAAAAGCGTATACTGCCGTCGGCG
TGAAC

The amino acid sequence of the PehPro1 precursor protein is set forth as SEQ ID NO: 17. The predicted signal sequence is shown in italics, and the predicted propeptide is shown in underlined text:

MLKVWASIITGAFLLGSVQGVQAAPQDQAAPFGGFTPQLITGESWSAPQGVSGEEKIWK
YLESKQESFQIGQTVDLKKQLKIIGQTTDEKTGTTHYRLQQYVGGVPVYGGVQTIHVNK
EGQVTSLIGSLLPDQQQQVSKSLNSQISEAQAIAVAQKDTEAAVGKLGEPQKTPEADLY
VYLHNGQPVLAYVTEVNVLEPEAIRTRYFISAEDGSILFKYDILAHATGTGKGVLGDTK
SFTTTQSGSTYQLKDTTRGQGIVTYSAGNRSSLPGTLLTSSSNIWNDGAAVDAHAYTAK
VYDYYKNKFGRNSIDGNGFQLKSTVHYSSRYNNAFWNGVQMVYGDGDGVTFIPFSAD
PDVIGHELTHGVTEHTAGLEYYGESGALNESISDIIGNAIDGKNWLIGDLIYTPNTPGDAL
RSMENPKLYNQPDRYQDRYTGPSDNGGVHINSGINNKAFYLIAQGGTHYGVTVNGIGR
DAAVQIFYDALINYLTPTSNFSAMRAAAIQAATDLYGANSSQVNAVKKAYTAVGVN

The amino acid sequence of the predicted mature form of PehPro1 is set forth as SEQ ID NO: 18:

ATGTGKGVLGDTKSFTTTQSGSTYQLKDTTRGQGIVTYSAGNRSSLPGTLLTSSSNIWN
DGAAVDAHAYTAKVYDYYKNKFGRNSIDGNGFQLKSTVHYSSRYNNAFWNGVQMV
YGDGDGVTFIPFSADPDVIGHELTHGVTEHTAGLEYYGESGALNESISDIIGNAIDGKNW
LIGDLIYTPNTPGDALRSMENPKLYNQPDRYQDRYTGPSDNGGVHINSGINNKAFYLIA
QGGTHYGVTVNGIGRDAAVQIFYDALINYLTPTSNFSAMRAAAIQAATDLYGANSSQV
NAVKKAYTAVGVN

EXAMPLE 4.2

Expression of *Paenibacillus ehimensis* Metalloprotease PehPro1

The DNA sequence of the propeptide-mature form of PehPro1 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX148(AprE-PehPro1) (Figure 4.1). Ligation of this gene encoding the PehPro1 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3' end of the *B. subtilis* AprE signal sequence and the 5' end of the predicted PehPro1 native propeptide. The gene has an alternative start codon (GTG). The resulting plasmid shown in Figure 1 was labeled pGX148(AprE-PehPro1). As shown in Figure 1, pGX148(AprE-PehPro1) contains an AprE promoter, an AprE signal sequence used to direct target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature regions of PehPro1

(SEQ ID NO: 19). The translation product of the synthetic AprE-PehPro1 gene is shown in SEQ ID NO: 20.

The pGX148(AprE-PehPro1) plasmid was then transformed into *B. subtilis* cells (*degU^{Hy}32, ΔscoC*) and the transformed cells were spread on Luria Agar plates supplemented
5 with 5 ppm Chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250 ml shake flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$).

The broth from the shake flasks was concentrated and buffer-exchanged into the loading
10 buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM $CaCl_2$ and 10% propylene glycol using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to an 80 ml Q Sepharose High Performance column pre-equilibrated with the loading buffer above, PehPro1 was eluted from the column with a linear salt gradient from 0 to 0.3 M NaCl in the loading buffer. The corresponding active fractions were collected, concentrated and buffer-
15 exchanged again into the loading buffer described above. The sample was loaded onto a 40 ml DEAE Fast Flow column pre-equilibrated with the same loading buffer. PehPro1 was eluted from the column with a linear salt gradient from 0 to 0.15 M NaCl in the loading buffer. The corresponding active purified protein fractions were further pooled and concentrated via 10K Amicon Ultra for further analyses.

20 The nucleotide sequence of the synthesized *PehPro1* gene in plasmid pGX148(AprE-PehPro1) is depicted in SEQ ID NO: 19. The sequence encoding the three residue addition (AGK) is shown in bold:

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACG
ATGGCGTTCAGCAACATGAGCGCGCAGGCT**GCTGGAAAA**GCACCTCAAGATCAGG
25 CAGCACCTTTTGGAGGCTTTACACCGCAACTTATCACAGGCGAATCATGGTCAGCAC
CGCAGGGCGTTTCAGGCGAGGAAAAGATCTGGAAGTACCTTGAGAGCAAGCAGGA
GTCATTTCAAATCGGCCAGACAGTCGACCTGAAAAAGCAACTGAAGATCATCGGCC
AAACAACGGACGAAAAGACGGGCACGACGCATTATAGACTGCAACAATATGTTGG
CGGCGTGCCGGTTTATGGAGGCGTGCAAACAATCCACGTGAACAAGGAAGGACAG
30 GTCACGTCACTGATCGGCAGCCTGCTGCCGGATCAGCAGCAACAAGTCTCAAAGAG
CCTGAACTCACAAATTAGCGAGGCACAAGCGATTGCAGTTGCACAAAAGGACACGG
AAGCAGCTGTCGGCAAGCTGGGCGAACCGCAAAAAACACCTGAGGCTGACCTTTAC
GTCTACCTGCATAACGGCCAGCCGGTCCTTGCGTACGTTACGGAAGTTAACGTGCTG
GAGCCGGAGGCCATCAGAACGAGATACTTCATTAGCGCGGAGGATGGAAGCATTCT

GTTTAAGTACGATATTCTTGCTCACGCGACAGGCACAGGCAAGGGCGTCCTTGGCG
ACACAAAAAGCTTCACGACAACGCAGAGCGGATCAACGTACCAGCTGAAAGATAC
AACAAGAGGACAAGGCATCGTTACGTATTCAGCGGGCAATAGATCAAGCCTGCCGG
GCACACTGCTGACATCAAGCTCAAACATTTGGAATGACGGCGCAGCAGTTGATGCC
CATGCGTACACAGCCAAGGTGTACGACTACTATAAGAACAAGTTTGGCAGAAATAG
CATCGACGGAAATGGATTTCAACTTAAATCAACGGTGCACTACTCATCAAGATATA
ACAATGCGTTTTGGAACGGAGTGCAGATGGTCTACGGAGACGGCGACGGCGTGACA
TTTATTCCGTTTAGCGCCGACCCGGACGTGATTGGACATGAACTGACACATGGAGTG
ACAGAGCATACGGCGGGACTGGAATATTACGGCGAAAGCGGCGCACTGAACGAAA
GCATCTCAGACATTATTGGAAACGCAATCGATGGCAAAAACTGGCTGATTGGCGAT
CTGATTTATACGCCGAATACACCGGGCGATGCACTGAGATCAATGGAGAATCCGAA
GCTGTACAACCAACCGGACAGATACCAAGATAGATACACAGGACCGTCAGACAAC
GGCGGAGTCCATATCAACAGCGGAATCAATAACAAAGCCTTTTACCTGATCGCCCA
AGGCGGAACGCACTATGGCGTTACAGTCAATGGCATCGGAAGAGATGCCGCAGTTC
AGATTTTCTATGACGCGCTGATCAACTATCTGACGCCTACAAGCAATTTCTCAGCAA
TGAGAGCCGCAGCAATCCAAGCAGCCACGGATCTGTATGGAGCCAATTCATCACAA
GTTAATGCTGTTAAGAAGGCTTATACGGCAGTGGGAGTTAACTAA

The amino acid sequence of the PehPro1 precursor protein expressed from plasmid pGX148(AprE- PehPro1) is depicted in SEQ ID NO: 20. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold, and the predicted pro-peptide is shown in underlined text.

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**APQDQAAPFGGFTPQLITGESWSAPQGVS
GEEKIWKYLESKQESFQIGQTVDLKKQLKIIGQTTDEKTGTTHYRLQQYVGGVPVYGG
VQTIHVNKEGQVTSLIGSLLPDQQQQVSKSLNSQISEAQAIAVAQKDTEAAVGKLGEPQ
KTPEADLYVYLHNGQPVLAYVTEVNVLEPEAIRTRYFISAEDGSILFKYDILAHATGTGK
GVLGDTKSFTTTQSGSTYQLKDTTRGQGIVTYSAGNRSSLPGTLLTSSSNIWNDGAAVD
AHAYTAKVYDYYKNKFGRNSIDGNGFQLKSTVHYSSRYNNAFWNGVQMVYGDGDG
VTFIPFSADPDVIGHELTHGVTEHTAGLEYYGESGALNESISDIIGNAIDGKNWLIGDLIY
TPNTPGDALRSMENPKLYNQPDRYQDRYTGPSDNGGVHINSGINNKAFYLIAQGGTHY
GVTVNGIGRDAAVQIFYDALINYLTPTSNFSAMRAAAIQAATDLYGANSSQVNAVKKA
YTAVGVN.

EXAMPLE 4.3

Proteolytic Activity of Metalloprotease PehPro1

The proteolytic activity of purified metalloprotease PehPro1 was measured in 50 mM
5 Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To initiate the reaction, 50 µl of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-binding 96-well Microtiter Plate (96-MTP) (Corning Life Sciences, #3641)
10 placed on ice, followed by the addition of 50 µl of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40°C and 650 rpm for 10 min. The reaction was terminated by adding 100 µl of 5% Trichloroacetic Acid (TCA). Following equilibration (5 min at the room temperature) and subsequent centrifugation (2000 g for 10 min at 4°C) , 120 µl supernatant was transferred to a new 96-MTP, and absorbance of the supernatant
15 was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of triplicate assays. The proteolytic activity is shown as Net A_{440}. The proteolytic assay with azo-casein as the substrate (shown in Figure 4.2) indicates that PehPro1 is an active protease.

20

EXAMPLE 4.4

pH Profile of Metalloprotease PehPro1

With azo-casein as the substrate, the pH profile of metalloprotease PehPro1 was studied in 12.5 mM acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 4 to 11).
25 To initiate the assay, 50 µl of 25 mM acetate/Bis-Tris/HEPES/CHES buffer with a specific pH was first mixed with 2 µl Milli-Q H_2O diluted enzyme (250 ppm) in a 96-MTP placed on ice, followed by the addition of 48 µl of 1.5% (w/v) azo-casein prepared in H_2O. The reaction was performed and analyzed as described in Example 4.3. Enzyme activity at each pH was reported as the relative activity, where the activity at the optimal pH was set to be 100%. The pH values
30 tested were 4, 5, 6, 7, 8, 9, 10 and 11. Each value was the mean of triplicate assays. As shown in Figure 4.3, the optimal pH of PehPro1 is 7, with greater than 70% of maximal activity retained between 5.5 and 9.5.

EXAMPLE 4.5

Temperature Profile of Metalloprotease PehPro1

The temperature profile of metalloprotease PehPro1 was analyzed in 50 mM Tris buffer (pH 7)
5 using the azo-casein assays. The enzyme sample and azo-casein substrate were prepared as in Example 4.3. Prior to the reaction, 50 μl of 1.5% azo-casein and 45 μl Milli-Q H_2O were mixed in a 200 μl PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler (BioRad) at desired temperatures (i.e. 20~90 °C) for 5 min. After the incubation, 5 μl of diluted enzyme (100 ppm) or H_2O (the blank control) was added to the substrate mixture, and the
10 reaction was carried out in the Peltier Thermal Cycle for 10 min at different temperatures. To terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 μl of 5% TCA per well. Subsequent centrifugation and absorbance measurement were performed as described in Example 4.3. The activity was reported as the relative activity, where the activity at the optimal temperature was set to be 100%. The tested temperatures are 20, 30, 40, 50, 60, 70,
15 80, and 90 °C. Each value was the mean of duplicate assays (the value varies no more than 5%). The data in Figure 4.4 suggest that PehPro1 showed an optimal temperature at 70°C, and retained greater than 70% of its maximum activity between 60 and 75°C.

EXAMPLE 4.6

20 **Cleaning performance of Metalloprotease PehPro1**

The cleaning performance of PehPro1 was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 and 8 using a model automatic dishwashing (ADW) detergent. Prior to the reaction, purified protease samples were diluted with a dilution solution containing 10 mM NaCl, 0.1mM $CaCl_2$, 0.005%
25 TWEEN® 80 and 10% propylene glycol to the desired concentrations. The reactions were performed in AT detergent with 100 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1) (detergent composition shown in Table 4.1). To initiate the reaction, 180 μl of the AT detergent buffered at pH 6 or pH 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the addition of 20 μl of diluted enzymes (or the dilution solution as the blank control). The 96-MTP
30 was sealed and incubated in an incubator/shaker for 30 min at 50 °C and 1150 rpm. After incubation, 100 μl of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 405 nm (referred here as the "Initial performance") using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the microswatches were rinsed once with 200 μl water. Following the addition of 180 μl of 0.1 M

CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50 °C and 1150 rpm for 10 min. One hundred microliters of the resulting wash liquid was transferred to a new 96-MTP, and its absorbance measured at 405 nm (referred here as the "Wash-off"). The sum of two absorbance measurements ("Initial performance" plus "Wash-off") gives the "Total performance", which measures the protease activity on the model stain; and Net A_{405} was subsequently calculated by subtracting the A_{405} of the "Total performance" of the blank control from that of the enzyme. Dose response in cleaning the PA-S-38 microswatches at pH 6 and pH 8 for PehPro1 in AT detergent is shown in Figure 4.5A and 4.5B.

Table 4.1. Composition of AT dish detergent

Ingredient	Concentration (mg/ml)
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029
PEG 6000	0.043
PEG 1500	0.1

*The pH of the AT formula detergent is adjusted to the desired value (pH 6 or 8) by the addition of 0.9 M citric acid.

EXAMPLE 4.7

Comparison of PehPro1 to Other Proteases

A. Identification of Homologous Proteases

Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res, 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest Patent database with search parameters set to default values. The mature protein amino acid sequence for PehPro1 (SEQ ID NO: 18) was used as the query sequence. Percent identity (PID) for both search sets is defined as the number of identical residues divided by the number of aligned residues in the pairwise alignment. Tables 4.2A and 4.2B provide a list of sequences with the percent identity to PehPro1. The length in Table 4.2 refers to the entire sequence length of the homologous proteases.

Table 4.2A: List of sequences with percent identity to PehPro1 protein identified from the NCBI non-redundant protein database			
Accession #	**PID to PehPro1**	**Organism**	**Length**
ZP_09077634.1	88	Paenibacillus elgii B69	524
ZP_09071078.1	74	Paenibacillus larvae subsp. larvae B-3650	529
YP_003872179.1	74	Paenibacillus polymyxa E681	592
P29148	73	Paenibacillus polymyxa	590
P43263	68	Brevibacillus brevis	527
ZP_09775365.1	68	Paenibacillus sp. Aloe-11	580
ZP_10241029.1	67	Paenibacillus peoriae KCTC 3763	599
ZP_10575942.1	66	Brevibacillus sp. BC25	528
YP_002770810.1	67	Brevibacillus brevis NBRC 100599	528
ZP_08640523.1	64	Brevibacillus laterosporus LMG 15441	564
YP_004646155.1	63	Paenibacillus mucilaginosus KNP414	525
ZP_08093424.1	60	Planococcus donghaensis MPA1U2	553
YP_003670279.1	59	Geobacillus sp. C56-T3	546
P00800	59	Bacillus thermoproteolyticus	548

Table 4.2B: List of sequences with percent identity to PehPro1 protein identified from the Genome Quest Patent database			
Patent ID #	**PID to PehPro1**	**Organism**	**Length**
JP2005229807-0019	74.5	Paenibacillus polymyxa	566
US20120107907-0187	74.09	Bacillus polymyxa	302
US8114656-0186	68.21	Bacillus brevis	304
WO2004011619-0044	63.25	*empty*	507
EP2390321-0185	62.9	Bacillus cereus	317
WO2012110563-0004	62.7	Bacillus megaterium	320
WO2012110563-0005	62.58	Bacillus cereus	320
JP1995184649-0001	62.5	Lactobacillus sp.	566
JP2005333991-0002	62.38	*empty*	562
EP2178896-0184	62.18	Bacillus anthracis	566
JP1994014788-0003	61.94	*empty*	317
EP2390321-0178	61.86	Bacillus thuringiensis	566
US6518054-0002	60.84	Bacillus sp.	316
US8114656-0176	60.13	Bacillus stearothermophilus	548
US6103512-0003	59.81	*empty*	319
US20120107907-0184	59.49	Bacillus caldoyticus	319

B. Alignment of Homologous Protease Sequences

The amino acid sequence of predicted mature PehPro1 (SEQ ID NO: 18) was aligned with thermolysin (P00800, *Bacillus thermoproteolyticus*) and protease from *Paenibacillus elgii* B69 (ZP_09077634.1) using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 4.6 shows the alignment of PehPro1 with these protease sequences.

C. Phylogenetic Tree

A phylogenetic tree for precursor protein PehPro1 (SEQ ID NO: 17) was built using sequences of representative homologs from Table 2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 4.7.

EXAMPLE 5.1

Cloning of *Paenibacillus barcinonensis* metalloprotease *PbaPro1*

A strain (DSM15478) of *Paenibacillus barcinonensis* was selected as a potential source of enzymes which may be useful in various industrial applications. Genomic DNA for sequencing was obtained by first growing the strain on Heart Infusion agar plates (Difco) at 37°C for 24 hr. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The genomic DNA was used for genome sequencing. The entire genome of the *Paenibacillus barcinonensis* strain was sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified after annotation in *Paenibacillus barcinonensis* encodes a metalloprotease and the sequence of this gene, called *PbaPro1*, is provided in SEQ ID NO: 21. The corresponding protein encoded by the *PbaPro1* gene is shown in SEQ ID NO: 22. At the N-terminus, the protein has a signal peptide with a length of 25 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786). The presence of a signal sequence suggests that PbaPro1 is a secreted enzyme. The propeptide region was predicted based on protein sequence alignment with the *Paenibacillus polymyxa* Npr protein (Takekawa *et al.* (1991) Journal of Bacteriol*ogy, **173** (21): 6820-6825).* The predicted mature region of PbaPro1 protein is shown in SEQ ID NO: 23.

The nucleotide sequence of the *PbaPro1* gene isolated from *Paenibacillus barcinonensis* is set forth as SEQ ID NO: 21. The sequence encoding the predicted native signal peptide is shown in italics:

*ATGAAATTGACCAAAATTATGCCAACAATTCTTGCAGGAGCTCTTTTGCTCACATCCCTGTC CTCTGCAGCAGCA*ATGCCGTTATCTGACTCATCCATTCCATTTGAGGGCCCCTACACC TCCGAGGAGAGTATTCTGTTGAACAACAACCCGGACGAAATGATTTATAATTTTCTT GCACAACAAGAGCAATTTCTGAATGCCGACGTCAAAGGACAGCTCAAAATCATTAA ACGCAACACAGACACTTCCGGCATCAGACACTTTCGTCTGAAGCAATACATCAAAG GTGTTCCGGTTTACGGCGCAGAACAAACGATCCATCTGGACAAGAACGGAGCTGTA ACTTCCGCACTCGGCGATCTTCCGCCAATTGAAGAACAGGCTGTTCCGAATGATGGC GTTCCCGCAATCAGTGCAGACGATGCCATCCGTGCCGCCGAGAATGAAGCCACCTC CCGTCTTGGAGAGCTTGGCGCACCAGAGCTTGAGCCAAAGGCCGAATTAAACATTT ATCATCATGAAGATGACGGACAAACCTACCTCGTTTACATTACGGAAGTTAACGTG CTTGAGCCTTCCCCGCTACGGACCAAATATTTTATTAACGCCCTTGATGGAAGCATC GTATCTCAATACGATATTATCAACTTTGCCACAGGCACCGGTACAGGCGTGCATGGT GATACCAAAACACTGACGACAACTCAATCCGGCAGCACCTATCAGCTGAAAGATAC AACTCGTGGAAAAGGCATTCAAACCTATACTGCGAACAATCGCTCCTCGCTTCCAG GCAGCTTGTCTACCAGTTCCAATAACGTATGGACAGACCGTGCAGCTGTAGATGCG CACGCCTATGCTGCCGCCACATATGACTTCTACAAAAACAAATTCAATCGCAACGG CATTGACGGAAACGGGCTGTTGATTCGCTCTACAGTGCATTATGGCTCCAACTATAA AAACGCCTTCTGGAACGGAGCACAGATTGTCTATGGAGATGGCGATGGCATCGAGT TCGGTCCCTTCTCCGGTGATCTCGATGTTGTCGGACATGAATTGACACACGGGGTGA TTGAATATACAGCCAATCTCGAATATCGCAATGAGCCGGGTGCTTTAAACGAAGCT TTTGCCGACATTATGGGGAACACCATCGAAAGCAAAAACTGGCTGCTTGGCGACGG AATCTATACTCCAAACATTCCAGGTGATGCCCTGCGCTCGTTATCCGACCCTACGCT GTATAACCAGCCTGACAAATACAGTGATCGCTACACTGGCTCTCAGGATAATGGCG GTGTGCATATCAACAGCGGGATCATTAACAAAGCATATTATCTTGCAGCCCAAGGC GGTACTCATAACGGGGTAACCGTTAGCGGCATCGGCCGGGATAAAGCAGTACGTAT TTTCTATAGCACGCTGGTGAACTACCTGACGCCAACCTCCAAATTTGCAGCAGCCAA AACAGCGACAATTCAGGCAGCCAAGGACCTGTACGGTGCCAATTCCGCTGAAGCTA CGGCAATCACCAAAGCTTATCAAGCGGTAGGTTTG

The amino acid sequence of the PbaPro1 precursor protein is set forth as SEQ ID NO: 22. The predicted signal sequence is shown in italics, and the predicted pro-peptide is shown in underlined text:

MKLTKIMPTILAGALLLTSLSSAAA<u>MPLSDSSIPFEGPYTSEESILLNNNPDEMIYNFLAQQE QFLNADVKGQLKIIKRNTDTSGIRHFRLKQYIKGVPVYGAEQTIHLDKNGAVTSALGDL PPIEEQAVPNDGVPAISADDAIRAAENEATSRLGELGAPELEPKAELNIYHHEDDGQTYL</u>

VYITEVNVLEPSPLRTKYFINALDGSIVSQYDIINFATGTGTGVHGDTKTLTTTQSGSTYQ
LKDTTRGKGIQTYTANNRSSLPGSLSTSSNNVWTDRAAVDAHAYAAATYDFYKNKFN
RNGIDGNGLLIRSTVHYGSNYKNAFWNGAQIVYGDGDGIEFGPFSGDLDVVGHELTHG
VIEYTANLEYRNEPGALNEAFADIMGNTIESKNWLLGDGIYTPNIPGDALRSLSDPTLYN
QPDKYSDRYTGSQDNGGVHINSGIINKAYYLAAQGGTHNGVTVSGIGRDKAVRIFYSTL
VNYLTPTSKFAAAKTATIQAAKDLYGANSAEATAITKAYQAVGL

The amino acid sequence of the predicted mature form of PbaPro1 is set forth as SEQ ID NO: 23:

ATGTGTGVHGDTKTLTTTQSGSTYQLKDTTRGKGIQTYTANNRSSLPGSLSTSSNNVWT
DRAAVDAHAYAAATYDFYKNKFNRNGIDGNGLLIRSTVHYGSNYKNAFWNGAQIVYG
DGDGIEFGPFSGDLDVVGHELTHGVIEYTANLEYRNEPGALNEAFADIMGNTIESKNWL
LGDGIYTPNIPGDALRSLSDPTLYNQPDKYSDRYTGSQDNGGVHINSGIINKAYYLAAQ
GGTHNGVTVSGIGRDKAVRIFYSTLVNYLTPTSKFAAAKTATIQAAKDLYGANSAEAT
AITKAYQAVGL

EXAMPLE 5.2

Expression of *Paenibacillus barcinonensis* metalloprotease PbaPro1

The DNA sequence of the propeptide-mature form of PbaPro1 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX147(AprE-PbaPro1) (Figure 5.1). Ligation of this gene encoding the PbaPro1 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3' end of the *B. subtilis* AprE signal sequence and the 5' end of the predicted PbaPro1 native propeptide. The gene has an alternative start codon (GTG). The resulting plasmid shown in Figure 1 was labeled pGX147(AprE-PbaPro1). As shown in Figure 5.1, pGX147(AprE-PbaPro1) contains an AprE promoter, an AprE signal sequence used to direct target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature regions of PbaPro1 (SEQ ID NO: 24). The translation product of the synthetic AprE- PbaPro1 gene is shown in SEQ ID NO: 25.

The pGX147(AprE-PbaPro1) plasmid was then transformed into *B. subtilis* cells ($degU^{Hy}32$, $\Delta scoC$) and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm Chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250 ml shake

flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$).

The broth from the shake flasks was concentrated and buffer-exchanged into the loading buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM $CaCl_2$ and 10% propylene glycol using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to an 80 ml Q Sepharose High Performance column pre-equilibrated with the loading buffer above; and the active flow-through fractions were collected and concentrated. The sample was loaded onto a 320 ml Superdex 75 gel filtration column pre-equilibrated with the loading buffer described above containing 0.15 M NaCl. The corresponding active purified protein fractions were further pooled and concentrated via 10K Amicon Ultra for further analyses.

The nucleotide sequence of the synthesized *PbaPro1* gene in plasmid pGX147(AprE-PbaPro1) is depicted in SEQ ID NO: 24. The sequence encoding the three residue addition (AGK) is shown in bold:

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACG
ATGGCGTTCAGCAACATGAGCGCGCAGGCT**GCTGGAAAA**ATGCCTCTGTCAGACA
GCAGCATTCCGTTTGAGGGCCCGTACACATCAGAAGAAAGCATCCTGCTGAACAAC
AACCCGGACGAGATGATCTACAATTTCCTGGCACAGCAGGAGCAGTTCCTGAACGC
AGACGTGAAGGGCCAGCTGAAAATCATCAAAAGAAACACAGACACGAGCGGCATC
AGACACTTCAGACTGAAGCAGTACATCAAGGGCGTCCCGGTTTACGGCGCTGAGCA
GACAATCCACCTGGACAAAAATGGCGCAGTGACGAGCGCACTTGGAGATCTGCCGC
CGATTGAAGAGCAAGCAGTCCCGAACGATGGCGTTCCGGCGATTAGCGCTGATGAC
GCTATCAGAGCCGCGGAAAACGAAGCGACGTCAAGACTGGGAGAACTTGGCGCAC
CGGAACTTGAACCGAAGGCGGAACTGAACATCTATCACCACGAAGACGATGGACA
GACGTACCTGGTGTACATCACGGAGGTGAATGTGCTGGAGCCGTCACCGCTGAGAA
CAAAATACTTCATCAATGCGCTGGATGGCAGCATCGTTAGCCAATACGACATCATT
AACTTCGCCACAGGCACGGGCACAGGCGTTCATGGCGACACAAAAACGCTTACGAC
AACACAGTCAGGCTCAACGTACCAGCTGAAAGACACAACAAGAGGCAAGGGCATC
CAGACGTATACAGCCAATAACAGAAGCTCACTTCCGGGCTCACTGTCAACAAGCAG
CAATAATGTCTGGACGGACAGAGCTGCAGTGGACGCGCACGCGTATGCTGCGGCCA
CGTACGACTTCTACAAGAACAAGTTCAACAGAAACGGCATTGATGGCAACGGCCTG
CTTATTAGAAGCACGGTCCACTACGGCTCAAACTACAAGAATGCGTTTTGGAACGG
CGCCCAAATTGTTTATGGCGATGGAGACGGCATCGAGTTCGGACCTTTTAGCGGCG
ACCTGGATGTGGTCGGACATGAACTGACGCACGGCGTTATCGAGTATACGGCGAAT
CTGGAATACAGAAATGAACCGGGCGCTCTGAATGAGGCCTTCGCGGATATCATGGG

CAACACAATTGAGAGCAAAAACTGGCTTCTGGGCGACGGAATCTACACGCCGAACA
TTCCGGGAGATGCACTGAGATCACTGAGCGACCCTACGCTGTACAACCAGCCGGAC
AAATACAGCGACAGATACACGGGATCACAGGACAATGGCGGCGTCCATATTAACTC
AGGCATCATCAACAAAGCGTATTATCTGGCAGCTCAAGGCGGCACGCATAATGGCG
TCACAGTTAGCGGAATCGGCAGAGACAAGGCCGTCAGAATTTTCTACTCAACGCTG
GTGAACTACCTGACACCGACAAGCAAGTTTGCAGCCGCCAAAACAGCCACGATTCA
GGCAGCAAAGGACCTGTACGGAGCGAACTCAGCAGAGGCCACAGCGATTACGAAG
GCTTATCAAGCCGTGGGACTGTAA

The amino acid sequence of the PbaPro1 precursor protein expressed from plasmid pGX147(AprE-PbaPro1) is depicted in SEQ ID NO: 25. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold, and the predicted pro-peptide is shown in underlined text.

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**MPLSDSSIPFEGPYTSEESILLNNNPDEMIY
NFLAQQEQFLNADVKGQLKIIKRNTDTSGIRHFRLKQYIKGVPVYGAEQTIHLDKNGAV
TSALGDLPPIEEQAVPNDGVPAISADDAIRAAENEATSRLGELGAPELEPKAELNIYHHE
DDGQTYLVYITEVNVLEPSPLRTKYFINALDGSIVSQYDIINFATGTGTGVHGDTKTLTT
TQSGSTYQLKDTTRGKGIQTYTANNRSSLPGSLSTSSNNVWTDRAAVDAHAYAAATYD
FYKNKFNRNGIDGNGLLIRSTVHYGSNYKNAFWNGAQIVYGDGDGIEFGPFSGDLDVV
GHELTHGVIEYTANLEYRNEPGALNEAFADIMGNTIESKNWLLGDGIYTPNIPGDALRSL
SDPTLYNQPDKYSDRYTGSQDNGGVHINSGIINKAYYLAAQGGTHNGVTVSGIGRDKA
VRIFYSTLVNYLTPTSKFAAAKTATIQAAKDLYGANSAEATAITKAYQAVGL

EXAMPLE 5.3

Proteolytic activity of metalloprotease PbaPro1

The proteolytic activity of purified metalloprotease PbaPro1 was measured in 50 mM Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To initiate the reaction, 50 µl of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-binding 96-well Microtiter Plate (96-MTP) (Corning Life Sciences, #3641) placed on ice, followed by the addition of 50 µl of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40°C and 650 rpm for 10 min. The reaction was terminated by adding 100 µl of 5% Trichloroacetic Acid (TCA). Following equilibration (5 min at the room temperature) and subsequent centrifugation (2000 g for 10 min

at 4 °C) , 120 μl supernatant was transferred to a new 96-MTP, and absorbance of the supernatant was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of
5 triplicate assays.

The proteolytic activities are shown as Net A_{440}. The proteolytic assay with azo-casein as the substrate (shown in Figure 5.2) indicates that PbaPro1 is an active protease.

EXAMPLE 5.4

10

pH profile of metalloprotease PbaPro1

With azo-casein as the substrate, the pH profile of metalloprotease PbaPro1 was studied in 12.5 mM acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 5 to 11). To initiate the assay, 50 μl of 25 mM acetate/Bis-Tris/HEPES/CHES buffer with a specific pH was first mixed with 2 μl Milli-Q H_2O diluted enzyme (125 ppm) in a 96-MTP
15 placed on ice, followed by the addition of 48 μl of 1.5% (w/v) azo-casein prepared in H_2O. The reaction was performed and analyzed as described in Example 5.3. Enzyme activity at each pH was reported as the relative activity, where the activity at the optimal pH was set to be 100%. The pH values tested were 5, 6, 7, 8, 9, 10 and 11. Each value was the mean of triplicate assays. As shown in Figure 5.3, the optimal pH of PbaPro1 is 8, with greater than 70% of maximal
20 activity retained between 7 and 9.

EXAMPLE 5.5

Temperature profile of metalloprotease PbaPro1

The temperature profiles of metalloprotease PbaPro1 was analyzed in 50 mM Tris buffer
25 (pH 7) using the azo-casein assays. The enzyme sample and azo-casein substrate were prepared as in Example 5.3. Prior to the reaction, 50 μl of 1.5% azo-casein and 45 μl Milli-Q H_2O were mixed in a 200 μl PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler (BioRad) at desired temperatures (i.e. 20~90°C) for 5 min. After the incubation, 5 μl of diluted enzyme (50 ppm) or H_2O (the blank control) was added to the substrate mixture, and the
30 reaction was carried out in the Peltier Thermal Cycle for 10 min at different temperatures. To terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 μl of 5% TCA per well. Subsequent centrifugation and absorbance measurement were performed as described in Example 5.3. The activity was reported as the relative activity, where the activity at the optimal temperature was set to be 100%. The tested temperatures are 20, 30, 40, 50, 60, 70,

80, and 90°C. Each value was the mean of duplicate assays (the value varies no more than 5%). The data in Figure 5.4 suggest that PbaPro1 showed an optimal temperature at 50°C, and retained greater than 70% of its maximum activity between 45 and 55°C.

5

EXAMPLE 5.6

Cleaning performance of metalloprotease PbaPro1

The cleaning performance of PbaPro1 was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 and 8 using a model automatic dishwashing (ADW) detergent. Prior to the reaction, purified protease
10 samples were diluted with a dilution solution containing 10 mM NaCl, 0.1mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol to the desired concentrations. The reactions were performed in AT detergent with 100 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1) (detergent composition shown in Table 5.1). To initiate the reaction, 180 µl of the AT detergent buffered at pH 6 or pH 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the
15 addition of 20 µl of diluted enzymes (or the dilution solution as the blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 30 min at 50 °C and 1150 rpm. After incubation, 100 µl of wash liquid from each well was transferred to a new 96-MTP, and its absorbance was measured at 405 nm (referred here as the "Initial performance") using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the
20 microswatches were rinsed once with 200 µl water. Following the addition of 180 µl of 0.1 M CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50°C and 1150 rpm for 10 min. One hundred microliters of the resulting wash liquid was transferred to a new 96-MTP, and its absorbance measured at 405 nm (referred here as the "Wash-off"). The sum of two absorbance measurements ("Initial performance" plus "Wash-off") gives the "Total
25 performance", which measures the protease activity on the model stain; and Net A_{405} was subsequently calculated by subtracting the A_{405} of the "Total performance" of the blank control from that of the enzyme. Dose response in cleaning the PA-S-38 microswatches at pH 6 and pH 8 in AT detergent for PbaPro1 is shown in Figures 5.5A and 5.5B.

Table 5.1. Composition of AT dish detergent formula with bleach

Ingredient	Concentration (mg/ml)
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
PAP (peracid *N*,*N*-phthaloylaminoperoxycaproic acid)	0.057
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029
PEG 6000	0.043
PEG 1500	0.1

*The pH of the AT formula detergent is adjusted to the desired value (pH 6 or 8) by the addition of 0.9 M citric acid.

5

EXAMPLE 5.7

Comparison of PbaPro1 to Other Proteases

A. Identification of Homologous Proteases

Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res,
10 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest Patent database with search parameters set to default values. The predicted mature protein amino acid sequence for PbaPro1 (SEQ ID NO: 23) was used as the query sequence. Percent identity (PID) for both search sets is defined as the number of identical residues divided by the number of aligned residues in the pairwise alignment. Tables 5.2A and 5.2B provide a list of sequences
15 with the percent identity to PbaPro1. The length in Table 5.2 refers to the entire sequence length of the homologous proteases.

Table 5.2A: List of sequences with percent identity to PbaPro1 protein identified from the NCBI non-redundant protein database			
Accession #	PID to PbaPro1	Organism	Length
AAB02774.1	56	*Geobacillus stearothermophilus*	552
P00800	56	*Bacillus stermoproteolyticus*	548

Table 5.2A: List of sequences with percent identity to PbaPro1 protein identified from the NCBI non-redundant protein database			
AAA22623.1	57	*Bacillus caldolyticus*	544
YP_003670279.1	57	*Geobacillus sp. C56-T3*	546
AAC43402.1	57	*Alicyclobacillus acidocaldarius*	546
YP_003597483.1	57	*Bacillus megaterium DSM 319*	562
ZP_08093424.1	57	*Planococcus donghaensis MPA1U2*	553
ZP_08640523.1	59	*Brevibacillus laterosporus LMG 15441*	564
ZP_04216147.1	59	*Bacillus cereus Rock3-44*	566
YP_001373863.1	60	*Bacillus cytotoxicus NVH 391-98*	565
YP_004646155.1	60	*Paenibacillus mucilaginosus KNP414*	525
ZP_10738945.1	61	*Brevibacillus sp. CF112*	528
CAA43589.1	63	*Brevibacillus brevis*	527
ZP_02326602.1	64	*Paenibacillus larvae subsp. larvae BRL-230010*	520
ZP_02326503.1	65	*Paenibacillus larvae subsp. larvae B-3650*	520
ZP_09077634.1	66	*Paenibacillus elgii B69*	524
ZP_08511445.1	68	*[Paenibacillus sp. HGF7*	525
ZP_09775364.1	70	*Paenibacillus sp. Aloe-11*	593
YP_005073223.1	70	*Paenibacillus terrae HPL-003*	591
ZP_10241030.1	70	*Paenibacillus peoriae KCTC 3763*	593
YP_003948511.1|	71	*Paenibacillus polymyxa SC2*	592

Table 5.2B: List of sequences with percent identity to PbaPro1 protein identified from the Genome Quest Patent database			
Patent #	**PID to PbaPro1**	**Organism**	**Length**
JP2005333991-0002	56.91		562
WO2012110562-0007	56.96	*Bacillus cereus*	320
WO2012110562-0006	57.23	*Bacillus megaterium*	320
EP2390321-0178	57.23	*Bacillus thuringiensis*	566
EP2390321-0184	57.56	*Bacillus caldoyticus*	319
WO2007044993-0184	57.56	*Bacillus sp.*	319
US20120107907-0177	57.56	*Bacillus caldolyticus*	544
CN102168095-0002	57.88		319
WO2012110562-0004	57.88	*Bacillus caldolyticus*	319
WO2012110562-0003	57.88	*Geobacillus stearothermophilus*	319
WO2004011619-0056	57.88		546
JP1995184649-0001	57.88	*Lactobacillus sp.*	566
JP2010535248-0240	57.88	*Bacillus anthracis*	566
US6518054-0001	58.2	*Bacillus sp.*	319
US6103512-0003	58.2		319
WO2011163237-0001	58.2	*Geobacillus stearothermophilus*	548
JP1994014788-0003	58.25		317
US8114656-0185	58.9	*Bacillus cereus*	317

Table 5.2B: List of sequences with percent identity to PbaPro1 protein identified from the Genome Quest Patent database			
Patent #	**PID to PbaPro1**	**Organism**	**Length**
US20120107907-0179	58.9	*Bacillus cereus*	566
WO2012110563-0005	59.22	*Bacillus cereus*	320
WO2004011619-0044	59.6		507
US20120107907-0186	63.25	*Bacillus brevis*	304
JP2005229807-0018	70.86	*Paenibacillus polymyxa*	566
EP2390321-0187	71.1	*Bacillus polymyxa*	302
JP2009511072-0203	71.1	*Paenibacillus polymyxa*	302

B. Alignment of Homologous Protease Sequences

The amino acid sequence of the predicted mature PbaPro1 (SEQ ID NO: 23) was aligned with Thermolysin (P00800, *Bacillus thermoproteolyticus*), and protease from *Paenibacillus*
5 *polymyxa* SC2 (YP_003948511.1) using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 5.6 shows the alignment of PbaPro1 with these protease sequences.

C. Phylogenetic Tree

A phylogenetic tree for full length sequence of PbaPro1 (SEQ ID NO: 22) was built
10 using sequences of representative homologs from Table 2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software
15 (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 5.7.

EXAMPLE 6.1

Cloning of *Paenibacillus polymyxa* SC2 metalloprotease *PpoPro1*

20 The nucleic acid sequence for the *PpoPro1* gene was identified in the NCBI database (NCBI Reference Sequence: NC_014622.1 from 4536397-4538175) and is provided in SEQ ID NO: 26. The corresponding protein encoded by the *PpoPro1* gene is shown in SEQ ID NO: 27. At the N-terminus, the protein has a signal peptide with a length of 24 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786).
25 The presence of a signal sequence suggests that PpoPro1 is a secreted enzyme. The propeptide

region was predicted based on protein sequence alignment with the *Paenibacillus polymyxa* Npr protein (Takekawa *et al.* (1991) Journal of Bacteriology, **173** (21): 6820-6825). The predicted mature region of PpoPro1 protein is shown in SEQ ID NO: 28.

The nucleotide sequence of the *PpoPro1* gene identified from NCBI database is set forth as SEQ ID NO: 26. The sequence encoding the predicted native signal peptide is shown in italics:

*ATGAAAAAAGTATGGGTTTCGCTTCTTGGAGGAGCTATGTTATTAGGGTCTGTCGCGTCTG GTGCATCTGCG*GAGAGTTCCGTTTCGGGGCCAGCTCAGCTTACACCGACCTTCCACG CCGAGCAATGGAAAGCACCTACCTCGGTATCGGGGGATGACATTGTATGGAGCTAT TTAAATCGACAAAAGAAATCGTTGCTGGGTGTGGATAGCTCCAGTGTACGTGAACA ATTCCGAATCGTTGATCGCACAAGCGACAAATCCGGTGTAAGCCATTATCGACTGA AGCAGTATGTAAACGGAATTCCCGTGTATGGAGCTGAACAAACTATTCATGTGGGC AAATCTGGTGAGGTCACCTCTTACTTAGGAGCGGTGGTTAATGAGGATCAGCAGGC AGAAGCTACGCAAGGTACAACTCCAAAAATCAGCGCTTCTGAAGCGGTCTACACCG CATATAAAGAAGCAGCTGCACGGATTGAAGCCCTCCCTACCTCCGACGATACTATTT CTAAAGACGCTGAGGAGCCAAGCAGTGTAAGTAAAGATACTTACGCCGAAGCAGCT AACAACGAAAAAACGCTTTCTGTTGATAAGGACGAGCTGAGTCTTGATCAGGCATC TGTCCTGAAAGATAGCAAAATTGAAGCAGTGGAACCAGAAAAAAGTTCCATTGCCA AAATCGCTAATCTGCAGCCTGAAGTAGATCCTAAAGCAGAACTCTACTACTACCCT AAGGGGGATGACCTGCTGCTGGTTTATGTAACAGAAGTTAATGTTTTAGAACCTGCC CCACTGCGTACCCGCTACATTATTGATGCCAATGACGGCAGCATCGTATTCCAGTAT GACATCATTAATGAAGCGACAGGCACAGGTAAAGGTGTGCTTGGTGATTCCAAATC GTTCACTACTACCGCTTCCGGCAGTAGCTACCAGTTAAAAGATACAACACGCGGTA ACGGAATCGTGACTTACACGGCCTCCAACCGTCAAAGCATCCCAGGTACCATTTTG ACAGATGCCGATAATGTATGGAATGATCCAGCTGGTGTGGACGCCCATGCGTATGC TGCTAAAACCTATGATTACTATAAAGCCAAATTTGGACGCAACAGCATTGACGGAC GCGGTCTGCAACTTCGTTCGACGGTCCATTACGGTAGTCGCTACAACAATGCCTTCT GGAACGGCTCCCAAATGACTTATGGAGATGGAGATGGTAGCACATTTATCGCCTTC AGCGGGGACCCCGATGTAGTAGGACATGAACTTACGCATGGTGTCACAGAGTATAC TTCGAATTTGGAATATTACGGAGAGTCCGGCGCATTGAATGAAGCTTTCTCAGACGT TATCGGGAATGACATTCAGCGCAAAAACTGGCTTGTAGGCGATGATATTTACACGC CAAACATTGCAGGCGATGCCCTTCGCTCAATGTCCAATCCAACCCTGTACGATCAAC CAGATCACTATTCCAACCTGTACAGAGGCAGCTCCGATAACGGCGGTGTTCACACC AACAGCGGTATTATCAATAAAGCTTACTACTTGTTAGCACAAGGTGGTAATTTCCAT

GGCGTAACTGTAAATGGAATTGGCCGTGATGCAGCGGTGCAAATTTACTACAGTGC
CTTTACGAACTACCTGACTTCTTCTTCCGACTTCTCCAACGCACGTGCTGCTGTGATC
CAAGCCGCAAAAGATCTGTACGGGGCGAACTCAGCAGAAGCAACTGCAGCTGCCA
AGTCTTTTGACGCTGTAGGCGTAAACTAA

The amino acid sequence of the PpoPro1 precursor protein is set forth as SEQ ID NO: 27. The predicted signal sequence is shown in italics, and the predicted propeptide is shown in underlined text:

*MKKVWVSLLGGAMLLGSVASGASA*ESSVSGPAQLTPTFHAEQWKAPTSVSGDDIVWSYL
NRQKKSLLGVDSSSVREQFRIVDRTSDKSGVSHYRLKQYVNGIPVYGAEQTIHVGKSGE
VTSYLGAVVNEDQQAEATQGTTPKISASEAVYTAYKEAAARIEALPTSDDTISKDAEEP
SSVSKDTYAEAANNEKTLSVDKDELSLDQASVLKDSKIEAVEPEKSSIAKIANLQPEVDP
KAELYYYPKGDDLLLVYVTEVNVLEPAPLRTRYIIDANDGSIVFQYDIINEATGTGKGVL
GDSKSFTTTASGSSYQLKDTTRGNGIVTYTASNRQSIPGTILTDADNVWNDPAGVDAHA
YAAKTYDYYKAKFGRNSIDGRGLQLRSTVHYGSRYNNAFWNGSQMTYGDGDGSTFIA
FSGDPDVVGHELTHGVTEYTSNLEYYGESGALNEAFSDVIGNDIQRKNWLVGDDIYTP
NIAGDALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGNFHGV
TVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAVIQAAKDLYGANSAEATAAAKSFD
AVGVN

The amino acid sequence of the predicted mature form of PpoPro1 is set forth as SEQ ID NO: 28:

ATGTGKGVLGDSKSFTTTASGSSYQLKDTTRGNGIVTYTASNRQSIPGTILTDADNVWN
DPAGVDAHAYAAKTYDYYKAKFGRNSIDGRGLQLRSTVHYGSRYNNAFWNGSQMTY
GDGDGSTFIAFSGDPDVVGHELTHGVTEYTSNLEYYGESGALNEAFSDVIGNDIQRKNW
LVGDDIYTPNIAGDALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLA
QGGNFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAVIQAAKDLYGANSAEA
TAAAKSFDAVGVN

EXAMPLE 6.2

Expression of *Paenibacillus polymyxa* SC2 metalloprotease *PpoPro1*

The DNA sequence of the propeptide-mature form of PpoPro1 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX138(AprE-

PpoPro1) (Figure 1). Ligation of this gene encoding the PpoPro1 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3' end of the *B. subtilis* AprE signal sequence and the 5' end of the predicted PpoPro1 native propeptide. The gene has an alternative start codon (GTG). The resulting plasmid shown in Figure 6.1, labeled pGX138(AprE-PpoPro1 contains an AprE promoter, an AprE signal sequence used to direct target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature regions of PpoPro1 (SEQ ID NO: 29). The translation product of the synthetic *AprE-PpoPro1* gene is shown in SEQ ID NO: 30.

The pGX138(AprE-PpoPro1) plasmid was then transformed into *B. subtilis* cells ($degU^{Hy}32$, *ΔscoC*) and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm Chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250 ml shake flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$).

The broth from the shake flasks was concentrated and buffer-exchanged into the loading buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM $CaCl_2$ and 10% propylene glycol using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to an 80 ml Q Sepharose High Performance column pre-equilibrated with the loading buffer above, PpoPro1 was eluted from the column with a linear salt gradient from 0 to 0.25 M NaCl in the loading buffer. The corresponding active fractions were collected and concentrated. The sample was loaded onto a 320 ml Superdex 75 gel filtration column pre-equilibrated with the loading buffer described above containing 0.15 M NaCl. The corresponding active purified protein fractions were further pooled and concentrated via 10K Amicon Ultra for further analyses.

The nucleotide sequence of the synthesized *PpoPro1* gene in plasmid pGX138(AprE-PpoPro1) is depicted in SEQ ID NO: 29. The sequence encoding the three residue addition (AGK) is shown in bold:

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACG
ATGGCGTTCAGCAACATGAGCGCGCAGGCT**GCTGGAAAA**GAATCATCAGTGTCAG
GACCGGCTCAGCTTACACCGACATTTCACGCAGAACAATGGAAGGCTCCGACGTCA
GTTTCAGGAGACGACATCGTGTGGAGCTACCTGAATAGACAGAAGAAAAGCCTGCT
GGGAGTGGATAGCAGCAGCGTCAGAGAGCAGTTCAGAATCGTTGACAGAACGAGC
GACAAAAGCGGAGTCAGCCATTATAGACTGAAGCAGTACGTGAATGGCATCCCGGT
TTATGGCGCAGAGCAGACAATTCATGTTGGCAAGAGCGGAGAAGTCACAAGCTATC

TGGGCGCTGTGGTCAATGAAGATCAACAAGCCGAGGCTACACAGGGAACAACGCC
GAAAATTAGCGCCTCAGAGGCAGTCTACACGGCGTACAAAGAAGCGGCTGCAAGA
ATCGAAGCCCTGCCGACATCAGACGATACAATTTCAAAAGATGCGGAGGAGCCGAG
CTCAGTTAGCAAGGATACATACGCGGAAGCCGCAAACAATGAGAAAACACTGAGC
GTGGACAAGGACGAGCTGTCACTTGATCAGGCTAGCGTCCTTAAAGACAGCAAGAT
CGAGGCCGTTGAGCCTGAAAAGTCATCAATTGCGAAAATCGCCAATCTGCAACCTG
AAGTCGACCCGAAGGCGGAACTGTACTACTACCCGAAAGGCGATGACCTGCTTCTG
GTGTACGTCACGGAAGTGAACGTCCTGGAACCGGCACCGCTGAGAACAAGATACAT
CATCGACGCGAACGACGGAAGCATCGTCTTCCAGTATGACATTATCAACGAAGCAA
CGGGAACGGGCAAAGGCGTTCTTGGAGACTCAAAGAGCTTCACGACAACGGCTTCA
GGAAGCAGCTACCAGCTGAAAGACACGACGAGAGGAAACGGAATCGTCACATATA
CGGCGTCAAACAGACAAAGCATCCCTGGCACAATCCTGACGGATGCTGACAACGTT
TGGAATGATCCGGCTGGCGTGGATGCCCATGCTTATGCGGCAAAAACGTATGACTA
TTACAAGGCGAAGTTCGGCAGAAATTCAATCGATGGCAGAGGACTGCAGCTTAGAA
GCACGGTGCACTACGGATCAAGATATAACAATGCCTTCTGGAACGGCAGCCAGATG
ACATACGGAGACGGAGATGGAAGCACATTTATTGCATTCAGCGGCGACCCTGATGT
GGTTGGCCATGAGCTGACGCATGGCGTTACAGAATATACGAGCAATCTTGAATACT
ACGGCGAGTCAGGCGCTCTGAACGAGGCATTTAGCGATGTTATCGGCAATGACATC
CAGAGAAAAAACTGGCTGGTGGGCGACGATATTTACACGCCTAATATCGCTGGCGA
TGCCCTTAGATCAATGTCAAACCCGACGCTGTATGATCAGCCTGACCACTACTCAAA
CCTGTATAGAGGCTCATCAGATAACGGAGGCGTCCATACGAATAGCGGCATCATTA
ACAAGGCATATTATCTTCTGGCCCAGGGCGGCAATTTTCATGGAGTGACGGTTAATG
GAATTGGAAGAGACGCAGCCGTCCAAATCTACTACAGCGCTTTCACGAACTACCTT
ACATCAAGCTCAGACTTTAGCAATGCCAGAGCTGCTGTTATCCAGGCAGCGAAGGA
TCTTTACGGCGCCAACTCAGCCGAAGCTACGGCCGCAGCTAAATCATTTGATGCAGT
GGGCGTTAAT

The amino acid sequence of the PpoPro1 precursor protein expressed from plasmid pGX138(AprE-PpoPro1) is depicted in SEQ ID NO: 30. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold, and the predicted pro-peptide is shown in underlined text.

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**ESSVSGPAQLTPTFHAEQWKAPTSVSGDD
IVWSYLNRQKKSLLGVDSSSVREQFRIVDRTSDKSGVSHYRLKQYVNGIPVYGAEQTIH
VGKSGEVTSYLGAVVNEDQQAEATQGTTPKISASEAVYTAYKEAAARIEALPTSDDTIS
KDAEEPSSVSKDTYAEAANNEKTLSVDKDELSLDQASVLKDSKIEAVEPEKSSIAKIANL

QPEVDPKAELYYYPKGDDLLLVYVTEVNVLEPAPLRTRYIIDANDGSIVFQYDIINEATG
TGKGVLGDSKSFTTTASGSSYQLKDTTRGNGIVTYTASNRQSIPGTILTDADNVWNDPA
GVDAHAYAAKTYDYYKAKFGRNSIDGRGLQLRSTVHYGSRYNNAFWNGSQMTYGDG
DGSTFIAFSGDPDVVGHELTHGVTEYTSNLEYYGESGALNEAFSDVIGNDIQRKNWLVG
5 DDIYTPNIAGDALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGG
NFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAVIQAAKDLYGANSAEATAA
AKSFDAVGVN

EXAMPLE 6.3

10 **Proteolytic Activity of metalloprotease PpoPro1**

The proteolytic activity of purified PpoPro1 was measured in 50 mM Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To initiate the reaction, 50
15 μL of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-binding 96-well microtiter Plate (96-MTP) (Corning Life Sciences, #3641) placed on ice, followed by the addition of 50 μL of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40 °C and 650 rpm for 10 min. The reaction was terminated by adding 100 μL of 5% Trichloroacetic Acid (TCA). Following equilibration (5
20 min at the room temperature) and subsequent centrifugation (2000 g for 10 min at 4 °C) , 120 μL supernatant was transferred to a new 96-MTP, and absorbance of the supernatant was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of duplicate assays, and the value varies
25 no more than 5%. The proteolytic activity is shown as Net A_{440}. The proteolytic assay with azo-casein as the substrate (Figure 6.2) indicates PpoPro1 is an active protease.

EXAMPLE 4

pH profile of metalloprotease PpoPro1

30 With azo-casein as the substrate, the pH profile of PpoPro1 was studied in 12.5 mM acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 4 to 11). To initiate the assay, 50 μL of 25 mM acetate/Bis-Tris/HEPES/CHES buffer with a specific pH was first mixed with 2 μL diluted enzyme (250 ppm in Milli-Q H_2O) in a 96-MTP placed on ice,

followed by the addition of 48 μL of 1.5% (w/v) azo-casein prepared in H_2O. The reaction was performed and analyzed as described in Example 6.3. Enzyme activity at each pH was reported as relative activity where the activity at the optimal pH was set to be 100%. The pH values tested were 4, 5, 6, 7, 8, 9, 10 and 11. Each value was the mean of triplicate assays. As shown in
5 Figure 6.3, the optimal pH of PpoPro1 is about 7, with greater than 70% of maximal activity retained between 5.5 and 8.5.

EXAMPLE 6.5

Temperature profile of metalloprotease PpoPro1

10 The temperature profile of PpoPro1 was analyzed in 50 mM Tris buffer (pH 7) using the azo-casein assay. The enzyme sample and azo-casein substrate were prepared as in Example 6.3. Prior to the reaction, 50 μL of 1.5% azo-casein and 45 μl Milli-Q H_2O were mixed in a 200μL PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler (BioRad) at desired temperatures (i.e. 20~90 °C) for 5 min. After the incubation, 5 μL of diluted PpoPro1
15 (100 ppm) or H_2O (the blank control) was added to the substrate mixture, and the reaction was carried out in the Peltier Thermal Cycle for 10 min at different temperatures. To terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 μL of 5% TCA per well. Subsequent centrifugation and absorbance measurement were performed as described in Example 6.3. The activity was reported as relative activity where the activity at the optimal
20 temperature was set to be 100%. The tested temperatures were 20, 30, 40, 50, 60, 70, 80, and 90 °C. Each value was the mean of duplicate assays (the value varies no more than 5%). The data in Figure 6.4 suggests that PpoPro1 showed an optimal temperature at 50°C, and retained greater than 70% of its maximum activity between 40 and 55°C.

25 **EXAMPLE 6.6**

Cleaning Performance of metalloprotease PpoPro1

The cleaning performance of PpoPro1 was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 or 8 using a model automatic dishwashing (ADW) detergent (AT detergent). Prior to the reaction,
30 purified PpoPro1 was diluted with a dilution solution containing 10 mM NaCl, 0.1mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol to the desired concentrations. The reactions were performed in AT detergent (composition shown in Table 6.1) with 100 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1), in the presence of a bleach component ((Peracid *N,N*-phthaloylaminoperoxycaproic acid-PAP). To initiate the reaction, 180 μL of AT detergent

buffered at pH 6 or 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the addition of 20 µL of diluted enzymes (or the dilution solution as the blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 30 min at 50 °C and 1150 rpm. After incubation, 100 µL of wash liquid from each well was transferred to a new 96-MTP, and its
5 absorbance was measured at 405 nm (referred here as the "Initial performance") using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the microswatches were rinsed once with 200 µL water. Following the addition of 180 µL of 0.1 M CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50 °C and 1150 rpm for 10 min. One hundred microliter of the resulting wash liquid was transferred to a
10 new 96-MTP, and its absorbance measured at 405 nm (referred here as "Wash-off"). The sum of two absorbance measurements ("Initial performance" plus "Wash-off") gives the "Total performance", which measures the protease activity on the model stain; and Net A_{405} was subsequently calculated by subtracting the A_{405} of the "Total performance" of the blank control from that of the enzyme. Dose response in cleaning the PA-S-38 microswatches at pH 6 and pH
15 8 for PpoPro1 in AT dish detergent, in the presence of bleach, is shown in Figures 6.5A and 6.5B.

Table 6.1. Composition of AT dish detergent formula with bleach

Ingredient	Concentration (mg/ml)
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
PAP (peracid *N*,*N*-phthaloylaminoperoxycaproic acid)	0.057
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029
PEG 6000	0.043
PEG 1500	0.1

*The pH of the AT formula detergent is adjusted to the desired value (pH 6 or 8) by the addition of 0.9 M citric acid.

20

EXAMPLE 6.7

Comparison of PpoPro1 to other metalloproteases

Identification of Homologous Proteases

5 Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res, 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest Patent database with search parameters set to default values. The predicted mature protein amino acid sequence for PpoPro1 (SEQ ID NO: 28) was used as the query sequence. Percent identity (PID) for both search sets is defined as the number of identical residues divided by the number 10 of aligned residues in the pairwise alignment. Tables 6.2A and 6.2B provide a list of sequences with the percent identity to PpoPro1. The length in Table 6.2 refers to the entire sequence length of the homologous proteases.

Table 6.2A: List of sequences with percent identity to PpoPro1 protein identified from the NCBI non-redundant protein database

Accession #	PID to PpoPro1	Organism	Length
P00800	56	Bacillus thermoproteolyticus	548
ZP_08640523.1	57	Brevibacillus laterosporus LMG 15441	564
AAA22623.1	57	Bacillus caldolyticus	544
ZP_08093424.1	59	Planococcus donghaensis MPA1U2	553
ZP_10738945.1	60	Brevibacillus sp. CF112	528
CAA43589.1	62	Brevibacillus brevis	527
ZP_02326503.1	62	Paenibacillus larvae subsp. larvae BRL-230010	520
YP_005495105.1	63	Bacillus megaterium WSH-002	562
YP_001373863.1	64	Bacillus cytotoxicus NVH 391-98	565
ZP_04310163.1	64	Bacillus cereus BGSC 6E1	581
BAA06144.1	64	Lactobacillus sp.]	566
ZP_08511445.1	65	Paenibacillus sp. HGF7	525
ZP_04216147.1	65	Bacillus cereus Rock3-44	566
ZP_09071078.1	68	Paenibacillus larvae subsp. larvae B-3650	
ZP_09077634.1	69	Paenibacillus elgii B69	524
YP_005073224.1	79	Paenibacillus terrae HPL-003	595
ZP_10241029.1	80	Paenibacillus peoriae KCTC 3763	599
YP_005073223.1	93	Paenibacillus terrae HPL-003	591
ZP_10241030.1	95	Paenibacillus peoriae KCTC 3763	593
ZP_09775364.1	95	Paenibacillus sp. Aloe-11	593
YP_003872179.1	97	Paenibacillus polymyxa E681	592
YP_003948511.1	100	Paenibacillus polymyxa SC2	592

Table 6.2B: List of sequences with percent identity to PpoPro1 protein identified from the Genome Quest Patent database			
Patent #	**PID to PpoPro1**	**Organism**	**Length**
US20120107907-0187	97.34	Bacillus polymyxa	302
US5962264-0004	65.48	*empty*	566
WO2012110563-0005	65.16	Bacillus cereus	320
JP1994070791-0002	64.52	*empty*	317
WO2012110562-0005	64.19	Bacillus cereus	320
WO2012110563-0004	63.34	Bacillus megaterium	320
JP2002272453-0002	61.98	Bacillus megaterium	562
WO2004011619-0047	61.49	*empty*	532
EP2390321-0186	62.58	Bacillus brevis	304
US6518054-0002	59.22	Bacillus sp.	316
US6518054-0001	58.52	Bacillus sp.	319
US20120107907-0176	58.52	Bacillus stearothermophilis	548
JP2005229807-0019	93.05	Paenibacillus polymyxa	566
WO2012110562-0003	58.2	Geobacillus stearothermophilus	319
WO2004011619-0044	59.27	*empty*	507
EP2390321-0185	66.13	Bacillus cereus	317
JP1995184649-0001	65.71	Lactobacillus sp.	566
EP2178896-0184	65.38	Bacillus anthracis	566

Alignment of Homologous Protease Sequences

The amino acid sequence of predicted mature PpoPro1 (SEQ ID NO: 28) was aligned with thermolysin (P00800, *Bacillus thermoproteolyticus*) and protease from *Paenibacillus polymyxa* SC2 (YP_003948511.1) using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 6.6 shows the alignment of PpoPro1 with these protease sequences.

Phylogenetic Tree

A phylogenetic tree for precursor PpoPro1 (SEQ ID NO: 27) was built using sequences of representative homologs from Tables 6.2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software

(http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 6.7.

EXAMPLE 7.1

Cloning of *Paenibacillus hunanensis* metalloprotease *PhuPro1*

A strain (DSM22170) of *Paenibacillus hunanensis* was selected as a potential source of enzymes which may be useful in various industrial applications. Genomic DNA for sequencing was obtained by first growing the strain on Heart Infusion agar plates (Difco) at 37°C for 24 hr. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The genomic DNA was used for genome sequencing. The entire genome of the *Paenibacillus hunanensis* strain was sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified after annotation in *Paenibacillus hunanensis* encodes a metalloprotease and the sequence of this gene, called *PhuPro1*, is provided in SEQ ID NO: 31. This gene has an alternative start codon (TTG). The corresponding protein encoded by the *PhuPro1* gene is shown in SEQ ID NO: 32. At the N-terminus, the protein has a signal peptide with a length of 23 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786). The presence of a signal sequence suggests that PhuPro1 is a secreted enzyme. The propeptide region was predicted based on protein sequence alignment with the *Paenibacillus polymyxa* Npr protein (Takekawa *et al.* (1991) Journal of Bacteriology, **173** (21): 6820-6825). The predicted mature region of PhuPro1 protein is shown in SEQ ID NO: 33.

The nucleotide sequence of the *PhuPro1* gene isolated from *Paenibacillus hunanensis* is set forth as SEQ ID NO: 31. The sequence encoding the predicted native signal peptide is shown in italics:

TTGAAAAAAACAGTTGGTCTTTTACTTGCAGGTAGCTTGCTCGTTGGTGCTACAACGTCCG
*CTTTCGCA*GCAGAAGCAAATGATCTGGCACCACTCGGTGATTACACGCCAAAATTGA
TTACGCAAGCAACAGGCATCACTGGCGCTAGTGGCGATGCTAAAGTATGGAAGTTC
CTGGAGAAGCAAAAACGTACCATCGTAACCGATGATGCAGCTTCTGCTGATGTGAA
GGAATTGTTTGAGATCACAAAACGTCAATCCGATTCTCAAACCGGTACAGAGCACT
ATCGCCTGAACCAAACCTTTAAAGGCATCCCAGTCTATGGCGCAGAGCAAACACTG
CACTTTGACAAATCCGGCAATGTATCTCTGTACATGGGTCAGGTTGTTGAGGATGTG
TCCGCTAAACTGGAAGCTTCCGATTCCAAAAAAGGCGTAACTGAGGATGTATACGC

TTCGGATACGAAAAATGATCTGGTAACACCAGAAATCAGCGCTTCTCAAGCCATCT
CGATTGCTGAAAAGGATGCAGCTTCCAAAATCGGCTCCCTCGGCGAAGCACAAAAA
ACGCCAGAAGCGAAGCTGTATATCTACGCTCCTGAGGATCAAGCAGCACGTCTGGC
TTATGTGACAGAAGTAAACGTACTGGAGCCATCTCCGCTGCGTACTCGCTATTTTGT
AGATGCAAAAACAGGTTCGATCCTGTTCCAATATGATCTGATTGAGCATGCAACAG
GTACAGGTAAAGGGGTACTGGGTGATACCAAGTCCTTCACTGTAGGTACTTCCGGTT
CTTCCTATGTGATGACTGATAGCACGCGTGGAAAAGGTATCCAAACCTACACGGCG
TCTAACCGCACATCACTGCCAGGTAGCACTGTAACGAGCAGCAGCAGCACATTTAA
CGATCCAGCATCTGTCGATGCCCATGCGTATGCACAAAAAGTATATGATTTCTACAA
ATCCAACTTTAACCGCAACAGCATCGACGGTAATGGTCTGGCTATCCGCTCCACTAC
GCACTATTCCACACGTTATAACAATGCGTTCTGGAATGGTTCCCAAATGGTATACGG
TGATGGCGATGGTTCGCAATTCATCGCATTCTCCGGCGACCTTGACGTAGTAGGTCA
CGAGCTGACACACGGTGTAACCGAGTACACAGCGAACCTGGAATACTATGGTCAAT
CCGGTGCACTGAACGAATCCATTTCGGATATCTTTGGTAACACAATCGAAGGTAAA
AACTGGATGGTAGGCGATGCGATCTACACACCAGGCGTATCCGGCGATGCTCTTCG
CTACATGGATGATCCAACAAAAGGTGGACAACCAGCGCGTATGGCAGATTACAACA
ACACAAGCGCTGATAATGGCGGTGTACACACAAACAGTGGTATCCCGAATAAAGCA
TACTACTTGCTGGCACAGGGTGGCACATTTGGCGGTGTAAATGTAACAGGTATCGG
TCGCTCGCAAGCGATCCAGATCGTTTACCGTGCACTAACATACTACCTGACATCCAC
ATCTAACTTCTCGAACTACCGTTCTGCAATGGTGCAAGCATCTACAGACCTGTACGG
TGCAAACTCTACACAAACAACAGCGGTGAAAAACTCGCTGAGCGCAGTAGGCATTA
AC

The amino acid sequence of the PhuPro1 precursor protein is set forth as SEQ ID NO: 32. The predicted signal sequence is shown in italics, and the predicted pro-peptide is shown in underlined text:

*MKKTVGLLLAGSLLVGATTSAFA*AEANDLAPLGDYTPKLITQATGITGASGDAKVWKFLE
KQKRTIVTDDAASADVKELFEITKRQSDSQTGTEHYRLNQTFKGIPVYGAEQTLHFDKS
GNVSLYMGQVVEDVSAKLEASDSKKGVTEDVYASDTKNDLVTPEISASQAISIAEKDA
ASKIGSLGEAQKTPEAKLYIYAPEDQAARLAYVTEVNVLEPSPLRTRYFVDAKTGSILFQ
YDLIEHATGTGKGVLGDTKSFTVGTSGSSYVMTDSTRGKGIQTYTASNRTSLPGSTVTS
SSSTFNDPASVDAHAYAQKVYDFYKSNFNRNSIDGNGLAIRSTTHYSTRYNNAFWNGS
QMVYGDGDGSQFIAFSGDLDVVGHELTHGVTEYTANLEYYGQSGALNESISDIFGNTIE
GKNWMVGDAIYTPGVSGDALRYMDDPTKGGQPARMADYNNTSADNGGVHTNSGIPN

KAYYLLAQGGTFGGVNVTGIGRSQAIQIVYRALTYYLTSTSNFSNYRSAMVQASTDLY
GANSTQTTAVKNSLSAVGIN

The amino acid sequence of the predicted mature form of PhuPro1 is set forth as SEQ
5 ID NO: 33:
ATGTGKGVLGDTKSFTVGTSGSSYVMTDSTRGKGIQTYTASNRTSLPGSTVTSSSSTFN
DPASVDAHAYAQKVYDFYKSNFNRNSIDGNGLAIRSTTHYSTRYNNAFWNGSQMVYG
DGDGSQFIAFSGDLDVVGHELTHGVTEYTANLEYYGQSGALNESISDIFGNTIEGKNWM
VGDAIYTPGVSGDALRYMDDPTKGGQPARMADYNNTSADNGGVHTNSGIPNKAYYLL
10 AQGGTFGGVNVTGIGRSQAIQIVYRALTYYLTSTSNFSNYRSAMVQASTDLYGANSTQT
TAVKNSLSAVGIN

EXAMPLE 7.2

Expression of *Paenibacillus hunanensis* metalloprotease PhuPro1

15 The DNA sequence of the propeptide-mature form of PhuPro1 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX149(AprE-PhuPro1) (Figure 7.1). Ligation of this gene encoding the PhuPro1 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3' end of the *B.*
20 *subtilis* AprE signal sequence and the 5' end of the predicted PhuPro1 native propeptide. The gene has an alternative start codon (GTG). The resulting plasmid shown in Figure 1, labeled pGX149(AprE-PhuPro1) contains an AprE promoter, an AprE signal sequence used to direct target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature regions of PhuPro1 (SEQ ID NO: 34). The translation product
25 of the synthetic AprE- PhuPro1 gene is shown in SEQ ID NO: 35.

The pGX149(AprE-PhuPro1) plasmid was then transformed into *B. subtilis* cells (*degU*^{Hy}*32*, Δ*scoC*) and the transformed cells were spread on Luria Agar plates supplemented with 5 ppm Chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250 ml shake
30 flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$).

The broth from the shake flasks was concentrated and buffer-exchanged into the loading buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM $CaCl_2$ and 10% propylene glycol using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to a 80 ml
35 Q Sepharose High Performance column pre-equilibrated with the loading buffer above; and the

active flow-through fractions were collected and concentrated via 10K Amicon Ultra for further analyses.

The nucleotide sequence of the synthesized *PhuPro1* gene in plasmid pGX149(AprE-PhuPro1) is depicted in SEQ ID NO: 34. The sequence encoding the three residue addition
5 (AGK) is shown in bold:
GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACG
ATGGCGTTCAGCAACATGAGCGCGCAGGCT**GCTGGAAAA**GCAGAAGCTAATGATC
TTGCCCCGCTTGGCGATTATACACCGAAGCTTATTACACAGGCAACGGGAATTACA
GGCGCATCAGGCGATGCGAAGGTGTGGAAGTTCCTGGAGAAGCAGAAGAGAACGA
10 TTGTCACGGACGACGCCGCAAGCGCGGATGTCAAGGAGCTGTTCGAGATCACGAAG
AGACAGAGCGATAGCCAGACGGGAACGGAGCATTACAGACTGAACCAGACGTTCA
AGGGCATTCCGGTCTACGGAGCTGAACAAACGCTGCATTTTGATAAAAGCGGCAAC
GTCTCACTGTACATGGGCCAAGTCGTTGAGGACGTTAGCGCCAAACTTGAGGCTAG
CGACAGCAAGAAAGGCGTCACAGAAGATGTCTACGCGTCAGACACGAAAAACGAC
15 CTGGTTACACCGGAAATCTCAGCTTCACAGGCCATCTCAATTGCAGAGAAAGACGC
AGCGTCAAAAATCGGCTCACTGGGCGAGGCTCAGAAAACGCCGGAGGCGAAACTTT
ACATCTACGCCCCTGAGGACCAGGCTGCGAGACTGGCTTACGTGACAGAAGTTAAT
GTGCTGGAGCCGTCACCGCTTAGAACGAGATATTTCGTGGACGCAAAGACGGGCAG
CATTCTGTTTCAGTACGATCTTATCGAACACGCGACAGGCACAGGAAAGGGAGTTC
20 TGGGAGACACAAAAAGCTTCACGGTTGGCACGTCAGGCAGCAGCTACGTGATGACA
GACAGCACGAGAGGCAAGGGCATTCAAACGTATACAGCGAGCAACAGAACAAGCC
TGCCGGGAAGCACAGTCACGAGCTCATCATCAACGTTTAATGACCCGGCCTCAGTG
GATGCTCACGCATACGCGCAGAAAGTGTACGACTTCTACAAAAGCAACTTCAATAG
AAACAGCATCGACGGAAACGGCCTTGCGATCAGAAGCACGACGCACTACAGCACA
25 AGATACAACAACGCCTTCTGGAACGGCAGCCAAATGGTTTACGGCGATGGCGACGG
ATCACAGTTTATCGCATTTAGCGGAGACCTGGACGTCGTTGGCCATGAGCTGACAC
ATGGCGTTACGGAGTACACAGCAAACCTGGAATACTATGGCCAGTCAGGCGCCCTT
AACGAGAGCATCAGCGACATTTTTGGCAATACGATCGAAGGAAAGAACTGGATGGT
CGGCGACGCAATCTACACACCGGGCGTTTCAGGCGATGCACTGAGATATATGGACG
30 ACCCGACAAAGGGCGGACAGCCGGCCAGAATGGCGGATTACAATAATACGTCAGC
AGATAACGGCGGCGTGCATACAAATAGCGGCATCCCTAACAAAGCATATTACCTGC
TTGCGCAAGGAGGAACATTTGGCGGCGTGAATGTTACGGGCATTGGCAGATCACAA
GCGATTCAGATCGTTTACAGAGCGCTGACGTACTACCTTACGAGCACGAGCAATTTT

AGCAACTACAGAAGCGCAATGGTGCAGGCAAGCACGGATCTGTATGGCGCAAATTC
AACACAAACGACGGCGGTCAAGAATAGCCTTTCAGCAGTGGGCATTAACTAA

The amino acid sequence of the PhuPro1 precursor protein expressed from plasmid
5 pGX149(AprE- PhuPro1) is depicted in SEQ ID NO: 35. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold, and the predicted pro-peptide is shown in underlined text.

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**AEANDLAPLGDYTPKLITQATGITGASGD
AKVWKFLEKQKRTIVTDDAASADVKELFEITKRQSDSQTGTEHYRLNQTFKGIPVYGAE
10 QTLHFDKSGNVSLYMGQVVEDVSAKLEASDSKKGVTEDVYASDTKNDLVTPEISASQA
ISIAEKDAASKIGSLGEAQKTPEAKLYIYAPEDQAARLAYVTEVNVLEPSPLRTRYFVDA
KTGSILFQYDLIEHATGTGKGVLGDTKSFTVGTSGSSYVMTDSTRGKGIQTYTASNRTSL
PGSTVTSSSSTFNDPASVDAHAYAQKVYDFYKSNFNRNSIDGNGLAIRSTTHYSTRYNN
AFWNGSQMVYGDGDGSQFIAFSGDLDVVGHELTHGVTEYTANLEYYGQSGALNESISD
15 IFGNTIEGKNWMVGDAIYTPGVSGDALRYMDDPTKGGQPARMADYNNTSADNGGVH
TNSGIPNKAYYLLAQGGTFGGVNVTGIGRSQAIQIVYRALTYYLTSTSNFSNYRSAMVQ
ASTDLYGANSTQTTAVKNSLSAVGIN

EXAMPLE 7.3

20 **Proteolytic activity of metalloprotease PhuPro1**

The proteolytic activity of purified metalloprotease PhuPro1 was measured in 50 mM Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To
25 initiate the reaction, 50 µl of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-binding 96-well Microtiter Plate (96-MTP) (Corning Life Sciences, #3641) placed on ice, followed by the addition of 50 µl of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40 °C and 650 rpm for 10 min. The reaction was terminated by adding 100 µl of 5% Trichloroacetic Acid (TCA). Following
30 equilibration (5 min at the room temperature) and subsequent centrifugation (2000 g for 10 min at 4 °C) , 120 µl supernatant was transferred to a new 96-MTP, and absorbance of the supernatant was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of

triplicate assays. The proteolytic activity is shown as Net A_{440}. The proteolytic assay with azo-casein as the substrate (shown in Figure 7.2) indicates that PhuPro1 is an active protease.

EXAMPLE 7.4

5 **pH profile of metalloprotease PhuPro1**

With azo-casein as the substrate, the pH profile of metalloprotease PhuPro1 was studied in 12.5 mM acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 4 to 11). To initiate the assay, 50 µl of 25 mM acetate/Bis-Tris/HEPES/CHES buffer with a specific pH was first mixed with 2 µl Milli-Q H_2O diluted enzyme (125 ppm) in a 96-MTP
10 placed on ice, followed by the addition of 48 µl of 1.5% (w/v) azo-casein prepared in H_2O. The reaction was performed and analyzed as described in Example 3. Enzyme activity at each pH was reported as the relative activity, where the activity at the optimal pH was set to be 100%. The pH values tested were 4, 5, 6, 7, 8, 9, 10 and 11. Each value was the mean of triplicate assays. As shown in Figure 7.3, the optimal pH of PhuPro1 is about 6, with greater than 70% of
15 maximal activity retained between 5 and 8.

EXAMPLE 7.5

Temperature profile of metalloprotease PhuPro1

The temperature profile of metalloprotease PhuPro1 was analyzed in 50 mM Tris buffer
20 (pH 7) using the azo-casein assays. The enzyme sample and azo-casein substrate were prepared as in Example 7.3. Prior to the reaction, 50 µl of 1.5% azo-casein and 45 µl Milli-Q H_2O were mixed in a 200 µl PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler (BioRad) at desired temperatures (i.e. 20~90 ^{o}C) for 5 min. After the incubation, 5 µl of diluted enzyme (50 ppm) or H_2O (the blank control) was added to the substrate mixture, and the
25 reaction was carried out in the Peltier Thermal Cycle for 10 min at different temperatures. To terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 µl of 5% TCA per well. Subsequent centrifugation and absorbance measurement were performed as described in Example 7.3. The activity was reported as the relative activity, where the activity at the optimal temperature was set to be 100%. The tested temperatures are 20, 30, 40, 50, 60, 70,
30 80, and 90°C. Each value was the mean of duplicate assays (the value varies no more than 5%). The data in Figure 7.4 suggests that PhuPro1 showed an optimal temperature at 60°C, and retained greater than 70% of its maximum activity between 45 and 65°C.

EXAMPLE 7.6

Cleaning performance of metalloprotease PhuPro1

The cleaning performance of PhuPro1 was tested using PA-S-38 (egg yolk, with
5 pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 and 8 using a model automatic dishwashing (ADW) detergent. Prior to the reaction, purified protease samples were diluted with a dilution solution containing 10 mM NaCl, 0.1mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol to the desired concentrations. The reactions were performed in AT detergent with 100 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1) (detergent
10 composition shown in Table 7.1). To initiate the reaction, 180 μl of the AT detergent buffered at pH 6 or pH 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the addition of 20 μl of diluted enzymes (or the dilution solution as the blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 30 min at 50 °C and 1150 rpm. After incubation, 100 μl of wash liquid from each well was transferred to a new 96-MTP, and its
15 absorbance was measured at 405 nm (referred here as the “Initial performance”) using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the microswatches were rinsed once with 200 μl water. Following the addition of 180 μl of 0.1 M CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50 °C and 1150 rpm for 10 min. One hundred microliters of the resulting wash liquid was transferred to a
20 new 96-MTP, and its absorbance measured at 405 nm (referred here as the “Wash-off”). The sum of two absorbance measurements (“Initial performance” plus “Wash-off”) gives the “Total performance”, which measures the protease activity on the model stain; and Net A_{405} was subsequently calculated by subtracting the A_{405} of the “Total performance” of the blank control from that of the enzyme. Dose response in cleaning the PA-S-38 microswatches at pH 6 and pH
25 8 in AT detergent for PhuPro1 is shown in Figures 7.5A and 7.5B.

Table 7.1. Composition of AT detergent

Ingredient	Concentration (mg/ml)
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029

Ingredient	Concentration (mg/ml)
PEG 6000	0.043
PEG 1500	0.1

*The pH of the AT formula detergent is adjusted to the desired value (pH 6 or 8) by the addition of 0.9 M citric acid.

EXAMPLE 7.7

5

Comparison of PhuPro1 to Other Proteases

A. Identification of Homologous Proteases

Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res, 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest
10 Patent database with search parameters set to default values. The predicted mature protein amino acid sequence for PhuPro1 (SEQ ID NO: 33) was used as the query sequence. Percent identity (PID) for both search sets is defined as the number of identical residues divided by the number of aligned residues in the pairwise alignment. Tables 7.2A and 7.2B provide a list of sequences with the percent identity to PhuPro1. The length in Table 7.2 refers to the entire sequence length
15 of the homologous proteases.

Table 7.2A: List of sequences with percent identity to PhuPro1 protein identified from the NCBI non-redundant protein database

Accession #	PID to PhuPro1	Organism	Length
P00800	55	Bacillus thermoproteolyticus	548
AAB02774.1	55	Geobacillus stearothermophilus	552
EJS73098.1	56	Bacillus cereus BAG2X1-3	566
BAD60997.1	56	Bacillus megaterium	562
ZP_04216147.1	57	Bacillus cereus Rock3-44	566
YP_893436.1	56	Bacillus thuringiensis str. Al Hakam	566
ZP_08640523.1	58	Brevibacillus laterosporus	564
ZP_09069194.1	59	Paenibacillus larvae subsp. larvae B-3650	502
YP_002770810.1	60	Brevibacillus brevis	528
ZP_08511445.1	61	Paenibacillus sp. HGF7	525
P43263	61	Brevibacillus brevis	527
ZP_09775365.1	62	Paenibacillus sp. Aloe-11	580
ZP_09077634.1	66	Paenibacillus elgii B69	524
P29148	68	NPRE_PAEPO	590
ZP_09775364.1	69	Paenibacillus sp. Aloe-11	593
ZP_10241030.1	69	Paenibacillus peoriae KCTC 3763	593
YP_005073223.1	69	Paenibacillus terrae HPL-003	591

Table 7.2B: List of sequences with percent identity to PhuPro1 protein identified from the Genome Quest Patent database			
Patent ID #	**PID to PhuPro1**	**Organism**	**Length**
WO2012110562-0003	56.23	Geobacillus stearothermophilus	319
US6518054-0001	56.55	Bacillus sp.	319
JP2002272453-0002	56.69	Bacillus megaterium	562
US20090123467-0184	56.73	Bacillus anthracis	566
US6103512-0003	56.87		319
EP0867512-0002	56.96		316
WO2012110562-0005	57.1	Bacillus cereus	320
WO2012110563-0005	58.06	Bacillus cereus	320
US20120107907-0187	68.44	Bacillus polymyxa	302

B. Alignment of Homologous Protease Sequences

The amino acid sequence of predicted mature PhuPro1 (SEQ ID NO: 33) protein was aligned with Proteinase T (P00800, *Bacillus thermoproteolyticus*), and protease from Paenibacillus terrae HPL-003 (YP_005073223.1) using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 7.6 shows the alignment of PhuPro1 with these protease sequences.

C. Phylogenetic Tree

A phylogenetic tree for full length sequence of PhuPro1 (SEQ ID NO: 2) was built using sequences of representative homologs from Table 2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 7.7.

EXAMPLE 7.8

Terg-o-Tometer performance evaluation of PhuPro1

The wash performance of PhuPro1 was tested in a laundry detergent application using a Terg-o-Tometer (Instrument Marketing Services, Inc, Fairfield, NJ). The performance evaluation was conducted at 32°C and 16°C. The soil load consisted of two of each of the following stain swatches: EMPA116 Blood, Milk, Ink on cotton (Test materials AG, St. Gallen, Switzerland), EMPA117 Blood, Milk, Ink on polycotton (Test materials AG, St. Gallen,

Switzerland), EMPA112 Cocoa on cotton (Test materials AG, St. Gallen, Switzerland), and CFT C-10 Pigment, Oil, and Milk content on cotton (Center for Testmaterials BV, Vlaardingen, Netherlands), plus extra white interlock knit fabric to bring the total fabric load to 40 g per beaker of the Terg-o-Tometer, which was filled with 1 L of deionized water. The water hardness was adjusted to 6 grains per gallon, and the pH in the beaker was buffered with 5 mM HEPES, pH 8.2. Heat inactivated Tide Regular HDL (Proctor & Gamble), a commercial liquid detergent purchased in a local US supermarket, was used at 0.8 g/L. The detergent was inactivated before use by treatment at 92°C in a water bath for 2-3 hours followed by cooling to room temperature. Heat inactivation of commercial detergents serves to destroy the activity of enzymatic components while retaining the properties of the non-enzymatic components. Enzyme activity in the heat inactivated detergent was measured using the Suc-AAPF-pNA assay for measuring protease activity. The Purafect® Prime HA, (Genencor Int'l) and PhuPro1 proteases were each added to final concentrations of 1ppm. A control sample with no enzyme was included. The wash time was 12 minutes. After the wash treatment, all swatches were rinsed for 3 minutes and machine-dried at low heat.

Four of each type of swatch were measured before and after treatment by optical reflectance using a Tristimulus Minolta Meter CR- 400. The difference in the L, a, b values was converted to total color difference (dE), as defined by the CIE-LAB color space. Cleaning of the stains is expressed as percent stain removal index (%SRI) by taking a ratio between the color difference before and after washing, and comparing it to the difference of unwashed soils (before wash) to unsoiled fabric, and averaging the eight values obtained by reading two different regions of each washed swatch. Cleaning performances of PhuPro1 and Purafect® Prime HA proteases at 32°C are shown in Tables 7.8A and Figure 7.8A and at 16°C are shown in Table 7.8B and Figure 7.8B.

Table 7.8A: Cleaning performance of PhuPro1 at 32℃								
	EMPA-116				**EMPA-117**			
ppm enzyme	**Purafect Prime HA**		**PhuPro1**		**Purafect Prime HA**		**PhuPro1**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.25	0.02	0.25	0.02	0.19	0.02	0.19	0.02
0.2	0.31	0.02	0.31	0.01	0.31	0.03	0.32	0.04
0.5	0.34	0.02	0.33	0.03	0.34	0.02	0.37	0.02
1	0.35	0.03	0.36	0.02	0.38	0.03	0.42	0.03
1.5	0.36	0.02	0.37	0.03	0.35	0.03	0.43	0.03
	EMPA-112				**CFT C-10**			
ppm enzyme	**Purafect Prime HA**		**PhuPro1**		**Purafect Prime HA**		**PhuPro1**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.15	0.03	0.15	0.03	0.07	0.01	0.07	0.01
0.2	0.17	0.04	0.14	0.02	0.11	0.01	0.15	0.01
0.5	0.19	0.02	0.19	0.04	0.13	0.01	0.16	0.03
1	0.20	0.03	0.22	0.03	0.17	0.01	0.17	0.01
1.5	0.24	0.03	0.25	0.04	0.17	0.02	0.20	0.02

Table 7.8B: Cleaning performance of PhuPro1 at 16℃								
	EMPA-116				**EMPA-117**			
ppm enzyme	**Purafect Prime HA**		**PhuPro1**		**Purafect Prime HA**		**PhuPro1**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.14	0.02	0.14	0.02	0.12	0.01	0.12	0.01
0.2	0.19	0.02	0.17	0.03	0.17	0.02	0.14	0.03
0.5	0.22	0.03	0.28	0.04	0.20	0.03	0.22	0.01
1	0.24	0.02	0.26	0.02	0.20	0.01	0.24	0.04
1.5	0.23	0.03	0.26	0.03	0.23	0.02	0.25	0.02
	EMPA-112				**CFT C-10**			
ppm enzyme	**Purafect Prime HA**		**PhuPro1**		**Purafect Prime HA**		**PhuPro1**	
	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]	Average %SRI (dE)	95CI [%SRI (dE)]
0	0.09	0.03	0.09	0.03	0.07	0.01	0.07	0.01
0.2	0.07	0.01	0.09	0.02	0.08	0.02	0.06	0.01
0.5	0.11	0.02	0.12	0.03	0.10	0.01	0.09	0.01
1	0.11	0.02	0.12	0.02	0.13	0.01	0.15	0.01
1.5	0.13	0.03	0.19	0.03	0.13	0.01	0.11	0.01

EXAMPLE 8.1

Cloning of *Paenibacillus amylolyticus* metalloprotease *PamPro1*

A strain (DSM11747) of *Paenibacillus amylolyticus* was selected as a potential source of
5 enzymes which may be useful in various industrial applications. Genomic DNA for sequencing was obtained by first growing the strain on Heart Infusion agar plates (Difco) at 37°C for 24 hr. Cell material was scraped from the plates and used to prepare genomic DNA with the ZF Fungal/Bacterial DNA miniprep kit from Zymo (Cat No. D6005). The genomic DNA was used for genome sequencing. The entire genome of the *Paenibacillus amylolyticus* strain was
10 sequenced by BaseClear (Leiden, The Netherlands) using the Illumina's next generation sequencing technology. After assembly of the data, contigs were annotated by BioXpr (Namur, Belgium). One of the genes identified after annotation in *Paenibacillus amylolyticus* encodes a metalloprotease and the sequence of this gene, called *PamPro1*, is provided in SEQ ID NO: 36. The corresponding protein encoded by the *PamPro1* gene is shown in SEQ ID NO: 37. At the
15 N-terminus, the protein has a signal peptide with a length of 25 amino acids as predicted by SignalP version 4.0 (Nordahl Petersen *et al.* (2011) Nature Methods, **8:**785-786). The presence of a signal sequence suggests that PamPro1 is a secreted enzyme. The propeptide region was predicted based on protein sequence alignment with the *Paenibacillus polymyxa* Npr protein (Takekawa *et al.* (1991) Journal of Bacteriol*ogy, **173** (21): 6820-6825).* The predicted mature
20 region of PamPro1 protein is shown in SEQ ID NO: 3.

The nucleotide sequence of the *PamPro1* gene isolated from *Paenibacillus amylolyticus* is set forth as SEQ ID NO: 36. The sequence encoding the predicted native signal peptide is shown in italics*:*

ATGAAATTCGCCAAAGTTATGCCAACAATTCTTGGAGGAGCTCTTTTGCTCGCTTCCGTAT
25 *CCTCTGCTACTGCA*GCTCCAGTG*TCTGATC*AATCCATTCCACTTCAGGCCCCTTATGCC
TCTGAGGGGGGTATTCCATTGAACAGTGGAACAGATGACACTA*TCTTTAA*TTATCTT
GGACAGCAGGAACAATTTCTGAATTCCGATGTGAAATCCCAGCTCAAAATTGTCAA
AAGAAACACAGATACATCTGGCGTAAGACACTTCCGCCTGAAACAGTATATTAAAG
GTATCCCGGTTTATGGTGCAGAACAGACGGTCCACCTGGACAAAACCGGA*GCCGTG*
30 AGCTCCGCACTTGGCGATCTTCCACCGATTGAAGAGCAGGCCATTCCGAATGATGG
TGTAGCCGAGATCAGCGGAGAAGACGCGATCCAGATTGCAACCGAAGAAGCAACC
TCCCGGATTGGAGAGCTTGGTGCCGCGGAAATCACGCCTCAAGCTGAATTGAACAT
CTATCATCATGAAGAAGATGGTCAGACATA*TCTGGTTTACATTACGGAAGTA*AACGTA
CTGGAACCTGCCCCTCTA*CGGAC*CAAATATTTCATTAACGCAGTGGATGGCAGTATC

GTATCCCAGTTTGACCTCATTAACTTCGCTACTGGAACAGGTACAGGTGTACTCGGT
GATACCAAAACCCTGACAACCACCCAATCCGGCAGCACCTTCCAACTGAAAGACAC
CACTCGTGGCAATGGCATCCAAACGTATACGGCAAACAATGGCTCCTCACTGCCTG
GTAGCTTGCTTACAGATTCGGATAATGTATGGACCGATCGTGCAGGTGTAGATGCTC
ATGCTCATGCCGCTGCTACGTATGATTTCTACAAAAACAAATTCAACCGTAACGGTA
TTAATGGTAACGGATTGTTGATCAGATCAACCGTGCACTACGGCTCCAATTACAATA
ACGCCTTCTGGAACGGGGCACAGATTGTCTTTGGTGACGGAGATGGAACGATGTTC
CGATCCCTGTCTGGTGATCTGGATGTTGTGGGTCATGAATTGACGCATGGTGTTATT
GAATATACAGCCAATCTGGAATATCGCAATGAACCAGGTGCACTCAATGAAGCCTT
TGCCGATATTTTCGGTAATACGATCCAAAGCAAAAACTGGCTGCTCGGTGATGATAT
CTACACACCTAACACTCCAGGAGATGCGCTGCGCTCCCTCTCCAACCCTACATTGTA
TGGTCAACCTGACAAATACAGCGATCGCTACACAGGCTCACAGGACAACGGCGGTG
TCCATATCAACAGTGGTATCATCAATAAAGCCTATTTCCTTGCTGCTCAAGGCGGAA
CACATAATGGTGTGACTGTTACCGGAATCGGCCGGGATAAAGCGATCCAGATTTTC
TACAGCACACTGGTGAACTACCTGACACCAACGTCCAAATTTGCCGCTGCCAAAAC
AGCTACCATTCAAGCAGCCAAAGATCTGTACGGAGCAACTTCCGCTGAAGCTACTG
CTATTACCAAAGCATATCAAGCTGTAGGCCTG

The amino acid sequence of the PamPro1 precursor protein is set forth as SEQ ID NO: 37. The predicted signal sequence is shown in italics, and the predicted propeptide is shown in underlined text:

*MKFAKVMPTILGGALLLASVSSATA*APVSDQSIPLQAPYASEGGIPLNSGTDDTIFNYLGQQ
EQFLNSDVKSQLKIVKRNTDTSGVRHFRLKQYIKGIPVYGAEQTVHLDKTGAVSSALGD
LPPIEEQAIPNDGVAEISGEDAIQIATEEATSRIGELGAAEITPQAELNIYHHEEDGQTYLV
YITEVNVLEPAPLRTKYFINAVDGSIVSQFDLINFATGTGTGVLGDTKTLTTTQSGSTFQL
KDTTRGNGIQTYTANNGSSLPGSLLTDSDNVWTDRAGVDAHAHAAATYDFYKNKFNR
NGINGNGLLIRSTVHYGSNYNNAFWNGAQIVFGDGDGTMFRSLSGDLDVVGHELTHG
VIEYTANLEYRNEPGALNEAFADIFGNTIQSKNWLLGDDIYTPNTPGDALRSLSNPTLYG
QPDKYSDRYTGSQDNGGVHINSGIINKAYFLAAQGGTHNGVTVTGIGRDKAIQIFYSTL
VNYLTPTSKFAAAKTATIQAAKDLYGATSAEATAITKAYQAVGL

The amino acid sequence of the predicted mature form of PamPro1 is set forth as SEQ ID NO: 38:

ATGTGTGVLGDTKTLTTTQSGSTFQLKDTTRGNGIQTYTANNGSSLPGSLLTDSDNVWT
DRAGVDAHAHAAATYDFYKNKFNRNGINGNGLLIRSTVHYGSNYNNAFWNGAQIVFG
DGDGTMFRSLSGDLDVVGHELTHGVIEYTANLEYRNEPGALNEAFADIFGNTIQSKNW

LLGDDIYTPNTPGDALRSLSNPTLYGQPDKYSDRYTGSQDNGGVHINSGIINKAYFLAA
QGGTHNGVTVTGIGRDKAIQIFYSTLVNYLTPTSKFAAAKTATIQAAKDLYGATSAEAT
AITKAYQAVGL

5

EXAMPLE 8.2

Expression of *Paenibacillus amylolyticus* metalloprotease PamPro1

The DNA sequence of the propeptide-mature form of PamPro1 was synthesized and inserted into the *Bacillus subtilis* expression vector p2JM103BBI (Vogtentanz, *Protein Expr Purif*, 55:40-52, 2007) by Generay (Shanghai, China), resulting in plasmid pGX146(AprE-
10 PamPro1) (Figure 1). Ligation of this gene encoding the PamPro1 protein into the digested vector resulted in the addition of three codons (Ala-Gly-Lys) between the 3' end of the *B. subtilis* AprE signal sequence and the 5' end of the predicted PamPro1 native propeptide. The gene has an alternative start codon (GTG). The resulting plasmid shown in Figure 8.1, labeled pGX146(AprE-PamPro1) contains an AprE promoter, an AprE signal sequence used to direct
15 target protein secretion in *B. subtilis*, and the synthetic nucleotide sequence encoding the predicted propeptide and mature regions of PamPro1 (SEQ ID NO: 39). The translation product of the synthetic AprE- PamPro1 gene is shown in SEQ ID NO: 40.

The pGX146(AprE-PamPro1) plasmid was then transformed into *B. subtilis* cells (*degU*^{Hy}*32, ΔscoC*) and the transformed cells were spread on Luria Agar plates supplemented
20 with 5 ppm Chloramphenicol and 1.2% skim milk (Cat#232100, Difco). Colonies with the largest clear halos on the plates were selected and subjected to fermentation in a 250 ml shake flask with MBD medium (a MOPS based defined medium, supplemented with additional 5 mM $CaCl_2$).

The broth from the shake flasks was concentrated and buffer-exchanged into the
25 loading buffer containing 20 mM Tris-HCl (pH 8.5), 1 mM $CaCl_2$ and 10% propylene glycol using a VivaFlow 200 ultra filtration device (Sartorius Stedim). After filtering, this sample was applied to an 80 ml Q Sepharose High Performance column pre-equilibrated with the loading buffer above; and the active flow-through fractions were collected and concentrated. The sample was loaded onto a 320 ml Superdex 75 gel filtration column pre-equilibrated with the loading
30 buffer described above containing 0.15 M NaCl. The corresponding active purified protein fractions were further pooled and concentrated via 10K Amicon Ultra for further analyses.

The nucleotide sequence of the synthesized *PamPro1* gene in plasmid pGX146(AprE-PamPro1) is depicted in SEQ ID NO: 39. The sequence encoding the three residue addition (AGK) is shown in bold:

GTGAGAAGCAAAAAATTGTGGATCAGCTTGTTGTTTGCGTTAACGTTAATCTTTACG
ATGGCGTTCAGCAACATGAGCGCGCAGGCT**GCTGGAAAA**GCTCCGGTTAGCGACC
AGTCAATCCCTCTTCAAGCACCGTATGCCAGCGAAGGAGGCATTCCGCTTAACAGC
GGCACGGACGACACGATTTTCAATTACCTGGGCCAACAGGAGCAGTTCCTGAACAG
5 CGACGTCAAGAGCCAGCTGAAGATCGTCAAAAGAAACACAGACACATCAGGCGTG
AGACACTTCAGACTGAAGCAATACATCAAGGGCATCCCGGTTTATGGCGCTGAACA
AACGGTTCACCTGGACAAAACAGGCGCAGTTTCATCAGCACTGGGAGATCTGCCGC
CGATTGAAGAGCAAGCAATCCCGAATGATGGAGTTGCGGAAATTAGCGGCGAGGA
TGCAATCCAAATCGCGACGGAGGAGGCTACATCAAGAATTGGAGAACTTGGCGCAG
10 CGGAGATTACACCGCAGGCTGAACTGAACATCTATCACCATGAGGAAGACGGCCAG
ACGTACCTGGTTTACATTACGGAAGTGAACGTGCTGGAACCGGCACCTCTGAGAAC
AAAGTACTTTATCAACGCGGTTGACGGCAGCATCGTCTCACAGTTCGACCTGATTAA
CTTCGCCACGGGAACAGGAACGGGCGTTCTTGGAGACACAAAGACGCTGACGACG
ACGCAGTCAGGCAGCACATTCCAGCTGAAGGACACAACAAGAGGCAACGGCATCC
15 AAACGTACACGGCGAACAATGGATCATCACTGCCGGGCTCACTGCTGACGGATTCA
GATAACGTGTGGACGGATAGAGCTGGCGTTGACGCGCATGCTCACGCTGCTGCGAC
GTACGACTTCTACAAGAACAAGTTCAACAGAAACGGCATTAACGGAAATGGCCTGC
TGATCAGAAGCACGGTGCATTATGGCTCAAACTACAACAACGCTTTTTGGAACGGC
GCACAGATCGTGTTTGGCGACGGCGATGGCACAATGTTTAGAAGCCTGTCAGGAGA
20 CCTGGATGTGGTGGGCCACGAACTGACGCACGGCGTGATCGAGTATACGGCGAACC
TTGAATATAGAAACGAGCCGGGAGCACTGAATGAGGCGTTCGCGGACATTTTCGGC
AACACAATCCAGAGCAAAAACTGGCTGCTGGGCGACGATATCTATACACCGAACAC
ACCGGGCGATGCACTGAGATCACTGTCAAATCCGACGCTGTATGGCCAACCGGATA
AGTACTCAGACAGATATACGGGCAGCCAAGACAATGGCGGCGTTCACATCAACTCA
25 GGCATCATCAACAAGGCTTACTTCCTTGCGGCCCAAGGAGGAACACATAACGGCGT
TACAGTTACAGGCATTGGCAGAGACAAGGCGATCCAGATCTTTTACAGCACGCTGG
TGAACTACCTGACACCTACGTCAAAGTTTGCCGCAGCGAAAACAGCAACAATTCAG
GCGGCTAAAGACCTGTACGGAGCGACATCAGCCGAGGCCACAGCAATTACAAAAG
CATATCAAGCAGTTGGCCTTTAA

30 The amino acid sequence of the PamPro1 precursor protein expressed from plasmid pGX146(AprE- PamPro1) is depicted in SEQ ID NO: 40. The predicted signal sequence is shown in italics, the three residue addition (AGK) is shown in bold, and the predicted pro-peptide is shown in underlined text.

*MRSKKLWISLLFALTLIFTMAFSNMSAQA***AGK**APVSDQSIPLQAPYASEGGIPLNSGTDDTI
FNYLGQQEQFLNSDVKSQLKIVKRNTDTSGVRHFRLKQYIKGIPVYGAEQTVHLDKTG
AVSSALGDLPPIEEQAIPNDGVAEISGEDAIQIATEEATSRIGELGAAEITPQAELNIYHHE
EDGQTYLVYITEVNVLEPAPLRTKYFINAVDGSIVSQFDLINFATGTGTGVLGDTKTLTT
TQSGSTFQLKDTTRGNGIQTYTANNGSSLPGSLLTDSDNVWTDRAGVDAHAHAAATY
DFYKNKFNRNGINGNGLLIRSTVHYGSNYNNAFWNGAQIVFGDGDGTMFRSLSGDLDV
VGHELTHGVIEYTANLEYRNEPGALNEAFADIFGNTIQSKNWLLGDDIYTPNTPGDALR
SLSNPTLYGQPDKYSDRYTGSQDNGGVHINSGIINKAYFLAAQGGTHNGVTVTGIGRDK
AIQIFYSTLVNYLTPTSKFAAAKTATIQAAKDLYGATSAEATAITKAYQAVGL

EXAMPLE 8.3

Proteolytic activity of metalloprotease PamPro1

The proteolytic activity of purified metalloprotease PamPro1 was measured in 50 mM Tris (pH 7), using azo-casein (Cat# 74H7165, Megazyme) as a substrate. Prior to the reaction, the enzyme was diluted with Milli-Q water (Millipore) to specific concentrations. The azo-casein was dissolved in 100 mM Tris buffer (pH 7) to a final concentration of 1.5% (w/v). To initiate the reaction, 50 µl of the diluted enzyme (or Milli-Q H_2O alone as the blank control) was added to the non-binding 96-well Microtiter Plate (96-MTP) (Corning Life Sciences, #3641) placed on ice, followed by the addition of 50 µl of 1.5% azo-casein. After sealing the 96-MTP, the reaction was carried out in a Thermomixer (Eppendorf) at 40°C and 650 rpm for 10 min. The reaction was terminated by adding 100 µl of 5% Trichloroacetic Acid (TCA). Following equilibration (5 min at the room temperature) and subsequent centrifugation (2000 g for 10 min at 4°C) , 120 µl supernatant was transferred to a new 96-MTP, and absorbance of the supernatant was measured at 440 nm (A_{440}) using a SpectraMax 190. Net A_{440} was calculated by subtracting the A_{440} of the blank control from that of enzyme, and then plotted against different protein concentrations (from 1.25 ppm to 40 ppm). Each value was the mean of triplicate assays. The proteolytic activity is shown as Net A_{440}. The proteolytic assay with azo-casein as the substrate (shown in Figure 8.2) indicates that PamPro1 is an active protease.

EXAMPLE 8.4

pH profiles of metalloprotease PamPro1

With azo-casein as the substrate, the pH profiles of metalloprotease PamPro1 were studied in 12.5 mM acetate/Bis-Tris/HEPES/CHES buffer with different pH values (ranging from pH 4 to 11). To initiate the assay, 50 µl of 25 mM acetate/Bis-Tris/HEPES/CHES buffer

with a specific pH was first mixed with 2 µl Milli-Q H_2O diluted enzyme (125 ppm) in a 96-MTP placed on ice, followed by the addition of 48 µl of 1.5% (w/v) azo-casein prepared in H_2O. The reaction was performed and analyzed as described in Example 8.3. Enzyme activity at each pH was reported as the relative activity, where the activity at the optimal pH was set to be 100%. 5 The pH values tested were 4, 5, 6, 7, 8, 9, 10 and 11. Each value was the mean of triplicate assays. As shown in Figure 8.3, the optimal pH of PamPro1 is about 8, with greater than 70% of maximal activity retained between 7 and 9.5.

EXAMPLE 8.5

10 **Temperature profile of metalloprotease PamPro1**

The temperature profile of metalloprotease PamPro1 was analyzed in 50 mM Tris buffer (pH 7) using the azo-casein assays. The enzyme sample and azo-casein substrate were prepared as in Example 8.3. Prior to the reaction, 50 µl of 1.5% azo-casein and 45 µl Milli-Q H_2O were mixed in a 200 µl PCR tube, which was then subsequently incubated in a Peltier Thermal Cycler 15 (BioRad) at desired temperatures (i.e. 20~90°C) for 5 min. After the incubation, 5 µl of diluted enzyme (50 ppm) or H_2O (the blank control) was added to the substrate mixture, and the reaction was carried out in the Peltier Thermal Cycle for 10 min at different temperatures. To terminate the reaction, each assay mixture was transferred to a 96-MTP containing 100 µl of 5% TCA per well. Subsequent centrifugation and absorbance measurement were performed as 20 described in Example 8.3. The activity was reported as the relative activity, where the activity at the optimal temperature was set to be 100%. The tested temperatures are 20, 30, 40, 50, 60, 70, 80, and 90°C. Each value was the mean of duplicate assays (the value varies no more than 5%). The data in Figure 8.4 suggest that PamPro1 showed an optimal temperature at about 50°C, and retained greater than 70% of its maximum activity between 45 and 55°C.

25

EXAMPLE 8.6

Cleaning performance of metalloprotease PamPro1

The cleaning performance of PamPro1 was tested using PA-S-38 (egg yolk, with pigment, aged by heating) microswatches (CFT-Vlaardingen, The Netherlands) at pH 6 and 8 30 using a model automatic dishwashing (ADW) detergent. Prior to the reaction, purified protease samples were diluted with a dilution solution containing 10 mM NaCl, 0.1mM $CaCl_2$, 0.005% TWEEN® 80 and 10% propylene glycol to the desired concentrations. The reactions were performed in AT detergent with 100 ppm water hardness (Ca^{2+} : Mg^{2+} = 3 : 1) (detergent composition shown in Table 8.1). To initiate the reaction, 180 µl of the AT detergent buffered at

pH 6 or pH 8 was added to a 96-MTP placed with PA-S-38 microswatches, followed by the addition of 20 µl of diluted enzymes (or the dilution solution as the blank control). The 96-MTP was sealed and incubated in an incubator/shaker for 30 min at 50 °C and 1150 rpm. After incubation, 100 µl of wash liquid from each well was transferred to a new 96-MTP, and its
5 absorbance was measured at 405 nm (referred here as the "Initial performance") using a spectrophotometer. The remaining wash liquid in the 96-MTP was discarded and the microswatches were rinsed once with 200 µl water. Following the addition of 180 µl of 0.1 M CAPS buffer (pH 10), the second incubation was carried out in the incubator/shaker at 50°C and 1150 rpm for 10 min. One hundred microliters of the resulting wash liquid was transferred to a
10 new 96-MTP, and its absorbance measured at 405 nm (referred here as the "Wash-off"). The sum of two absorbance measurements ("Initial performance" plus "Wash-off") gives the "Total performance", which measures the protease activity on the model stain; and Net A_{405} was subsequently calculated by subtracting the A_{405} of the "Total performance" of the blank control from that of the enzyme. Dose response in cleaning the PA-S-38 microswatches at pH 6 and pH
15 8 in AT dish detergent for PamPro1 is shown in Figures 5A and 5B.

Table 8.1. Composition of AT dish detergent formula with bleach

Ingredient	**Concentration (mg/ml)**
MGDA (methylglycinediacetic acid)	0.143
Sodium citrate	1.86
Citric acid*	varies
PAP (peracid *N*,*N*-phthaloylaminoperoxycaproic acid)	0.057
Plurafac® LF 18B (a non-ionic surfactant)	0.029
Bismuthcitrate	0.006
Bayhibit® S (Phosphonobutantricarboxylic acid sodium salt)	0.006
Acusol™ 587 (a calcium polyphosphate inhibitor)	0.029
PEG 6000	0.043
PEG 1500	0.1

*The pH of the AT formula detergent is adjusted to the desired value (pH 6 or 8) by the addition of 0.9 M citric acid.

EXAMPLE 8.7

Comparison of PamPro1 to Other Proteases

A. Identification of Homologous Proteases

5 Homologs were identified by a BLAST search (Altschul et al., Nucleic Acids Res, 25:3389-402, 1997) against the NCBI non-redundant protein database and the Genome Quest Patent database with search parameters set to default values. The predicted mature protein amino acid sequence for PamPro1 (SEQ ID NO: 38) was used as the query sequence. Percent identity (PID) for both search sets is defined as the number of identical residues divided by the number 10 of aligned residues in the pairwise alignment. Tables 8.2A and 8.2B provide a list of sequences with the percent identity to PamPro1. The length in Table 8.2 refers to the entire sequence length of the homologous proteases.

Table 8.2A: List of sequences with percent identity to PamPro1 protein identified from the NCBI non-redundant protein database

Accession #	PID to PamPro1	Organism	Length
P23384	56	*Bacillus caldolyticus*	544
P00800	56	*Bacillus thermoproteolyticus*	548
ZP_08640523.1	57	*Brevibacillus laterosporus LMG 15441*	564
BAA06144.1	57	*Lactobacillus sp.*	566
YP_003872180.1	58	*Paenibacillus polymyxa E681*	587
ZP_04149724.1	59	*Bacillus pseudomycoides DSM 12442*	566
EJR46541.1	60	*Bacillus cereus VD107*	566
YP_001373863.1	60	*Bacillus cytotoxicus NVH 391-98*	565
ZP_10738945.1	61	*Brevibacillus sp. CF112*	528
YP_004646155.1	61	*Paenibacillus mucilaginosus KNP414*	525
ZP_02326602.1	62	*Paenibacillus larvae subsp. larvae BRL-230010*	520
P43263	63	*Brevibacillus brevis*	527
ZP_09775365.1	64	*Paenibacillus sp. Aloe-11*	580
ZP_09077634.1	65	*Paenibacillus elgii B69*	529
ZP_09071078.1	68	*Paenibacillus larvae subsp. larvae B-3650*	529
ZP_08511445.1	69	*Paenibacillus sp. HGF7*	525
YP_005073223.1	70	*Paenibacillus terrae HPL-003*	591
YP_003948511.1	71	*Paenibacillus polymyxa SC2*	592
ZP_10241030.1	71	*Paenibacillus peoriae KCTC 3763*	593

Table 8.2B: List of sequences with percent identity to PamPro1 protein identified from the Genome Quest Patent database			
Patent #	**PID to PamPro1**	**Organism**	**Length**
US7335504-0030	56.63	*Bacillus thermoproteolyticus*	316
US20120107907-0184	56.91	*Bacillus caldoyticus*	319
JP2006124323-0003	56.96	*Bacillus thermoproteolyticus*	316
JP1993199872-0001	56.96	*Bacillus sp.*	316
JP1997000255-0001	56.96	*empty*	548
US6518054-0001	57.23	*Bacillus sp.*	319
US20120107907-0176	57.23	*Bacillus stearothermophilis*	548
US8114656-0183	57.28	*Bacillus stearothermophilis*	316
US20120009651-0002	57.28	*Geobacillus caldoproteolyticus*	548
JP2011103791-0020	57.28	*Geobacillus stearothermophilus*	552
WO2012110562-0006	57.88	*Bacillus megaterium*	320
EP2390321-0178	57.88	*Bacillus thuringiensis*	566
US6518054-0002	57.93	*Bacillus sp.*	316
WO2012110562-0007	58.25	*Bacillus cereus*	320
JP1995184649-0001	58.52	*Lactobacillus sp.*	566
EP2178896-0184	58.52	*Bacillus anthracis*	566
EP2390321-0195	59.55	*Bacillus cereus*	317
WO2012110563-0005	59.87	*Bacillus cereus*	320
US20080293610-0186	63.25	*Bacillus brevis*	304
JP2005229807-0018	71.19	*Paenibacillus polymyxa*	566
US8114656-0187	71.43	*Bacillus polymyxa*	302

B. Alignment of Homologous Protease Sequences

The amino acid sequence of the predicted mature PamPro1 (SEQ ID NO: 38) was aligned with thermolysin (P00800, *Bacillus thermoproteolyticus*), and protease from *Paenibacillus peoriae* KCTC 3763 (YP_005073223.1) using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 8.6 shows the alignment of PamPro1 with these protease sequences.

C. Phylogenetic Tree

A phylogenetic tree for full length sequences of PamPro1 (SEQ ID NO: 37) was built using sequences of representative homologs from Table 8.2A and the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software

(http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 8.7.

EXAMPLE 9

Comparison of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs

A. Alignment of Homologous Protease Sequences

The amino acid sequence of the predicted mature sequences for the Paenibacillus proteases described in Examples 1.1 to 8.7 were aligned with related bacterial metalloproteases using CLUSTALW software (Thompson et al., Nucleic Acids Research, 22:4673-4680, 1994) with the default parameters. Figure 9.1 shows the alignment of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.

B. Phylogenetic Tree

A phylogenetic tree for full length sequences of the metalloproteases aligned in Figure 9.1 was created using the Neighbor Joining method (NJ) (Saitou, N.; and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing Guide Trees. MolBiol.Evol. 4, 406-425). The NJ method works on a matrix of distances between all pairs of sequences to be analyzed. These distances are related to the degree of divergence between the sequences. The phylodendron-phylogenetic tree printer software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html) was used to display the phylogenetic tree shown in Figure 9.2, where one can observe the clustering of the sequences from Paenibacillus genus.

While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein can be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS:

1. A polypeptide comprising an amino acid sequence having at least 60% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 and 38.

2. The polypeptide of claim 1, wherein said polypeptide has at least 80% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 and 38.

3. The polypeptide of of any of claims 1 or 2, wherein said polypeptide has at least 95% sequence identity to the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 and 38.

4. The polypeptide of any of the above claims, wherein said amino acid sequence is the amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33 and 38.

5. The polypeptide of any of the above claims, wherein said polypeptide is derived from a member of the order Bacillales.

6. The polypeptide of any of the above claims, wherein said Bacillales member is a *Paenibacillaceae* family member.

7. The polypeptide of claim 6, wherein said Bacillales member is a *Paenibacillus* spp.

8. The polypeptide of any of claims 1-4, wherein said polypeptide is derived from a *Planococcus* species.

9. The polypeptide of any of the above claims, wherein said polypeptide has protease activity.

10. The polypeptide of claim 9, wherein said protease activity comprises casein hydrolysis, collagen hydrolysis, elastin hydrolysis, keratin hydrolysis, soy protein hydrolysis or corn meal protein hydrolysis.

11. The polypeptide of any of the above claims, wherein said polypeptide retains at least 50% of its maximal activity between pH 4.5 and 10.

12. The polypeptide of any of the above claims, wherein said polypeptide retains at least 50% of its maximal activity between 30ºC and 70ºC.

13. The polypeptide of any of the above claims, wherein said polypeptide has cleaning activity in a detergent composition.

14. The polypeptide of claim 13, wherein said detergent composition is an ADW detergent composition.

15. The polypeptide of claim 13, wherein said detergent composition is a laundry detergent composition.

16. The polypeptide of claim 15, wherein said detergent composition is a liquid laundry detergent composition.

17. The polypeptide of claim 15, wherein said detergent composition is a powder laundry detergent composition.

18. The polypeptide of claim 13, wherein said detergent composition comprises a bleach component.

19. The polypeptide of any of the above claims, wherein said polypeptide is a recombinant polypeptide.

20. A composition comprising the polypeptide of any of the above claims.

21. The composition of claim 20, wherein said composition is a cleaning composition.

22. The composition of claim 21, wherein said composition is a detergent composition.

23. The composition of claim 22, wherein said detergent composition is selected from the group consisting of a laundry detergent, a fabric softening detergent, a dishwashing detergent, and a hard-surface cleaning detergent.

24. The composition of any of claims 20 to 22, wherein said composition further comprising a surfactant.

25. The composition of claim 24, wherein said surfactant is selected from the group consisting of an anionic surfactant, a cationic surfactant, a zwitterionic surfactant, a ampholytic surfactant, a semi-polar non-ionic surfactant, and a combination thereof.

26. The composition of claim 24, wherein said surfactant is an ionic surfactant.

27. The composition of claim 24, wherein said surfactant is a non-ionic surfactant.

28. The composition of any of claims 20-27, wherein said composition further comprises at least one calcium ion and/or zinc ion.

29. The composition of any of claims 20-28, wherein said composition further comprises at least one stabilizer.

30. The composition of any of claims 20-29, wherein said composition comprises from about 0.001 to about 0.1 weight % of said polypeptide.

31. The composition of any of claims 20-30, further comprising at least one bleaching agent.

32. The composition of any of claims 20-31, wherein said cleaning composition is phosphate-free.

33. The composition of any of claims 20-31, wherein said cleaning composition contains phosphate.

34. The composition of any of claims 20-33, further comprising at least one adjunct ingredient.

35. The composition of any of claims 20-34, wherein said composition is a granular, powder, solid, bar, liquid, tablet, gel, or paste composition.

36. The composition of any of claims 20-35, further comprising one or more additional enzymes or enzyme derivatives selected from the group consisting of acyl transferases, alpha-amylases, beta-amylases, alpha-galactosidases, arabinosidases, aryl esterases, beta-galactosidases, carrageenases, catalases, cellobiohydrolases, cellulases, chondroitinases, cutinases, endo-beta-1, 4-glucanases, endo-beta-mannanases, esterases, exo-mannanases, galactanases, glucoamylases, hemicellulases, hyaluronidases, keratinases, laccases, lactases, ligninases, lipases, lipoxygenases, mannanases, oxidases, pectate lyases, pectin acetyl esterases, pectinases, pentosanases, peroxidases, phenoloxidases, phosphatases, phospholipases, phytases, polygalacturonases, proteases, pullulanases, reductases, rhamnogalacturonases, beta-glucanases, tannases, transglutaminases, xylan acetyl-esterases, xylanases, xyloglucanases, and xylosidases, additional metallopotease enzymes and combinations thereof.

37. The composition of any of claims 20-36, wherein said composition is formulated at a pH of from about 5.5 to about 8.5.

38. A method for the pretreatment of animal feed comprising treating an animal feed pre-product with the polypeptide of any one of claims 1-19.

39. A method of cleaning, comprising contacting a surface or an item with a cleaning composition comprising the polypeptide of any one of claims 1-19.

40. A method of cleaning comprising contacting a surface or an item with the composition of any one of claims 20-37.

41. The method of claim 39 or 40, further comprising rinsing said surface or item after contacting said surface or item, respectively, with said composition.

42. The method of claims 39-41, wherein said item is dishware.

43. The method of any one of claims 39-41, wherein said item is fabric.

44. The method of any one of claims 39-43, further comprising the step of rinsing said surface or item after contacting said surface or item with said composition.

45. The method of claim 44, further comprising the step of drying said surface or item after said rinsing of said surface or item.

46. A method of cleaning a surface or item, comprising: providing the composition of any of claims 20-37 and a surface or item in need of cleaning; and contacting said composition with said surface or item in need of cleaning under conditions suitable for the cleansing of said surface of said surface or item, to produce a cleansed surface or item.

47. The method of claim 46, further comprising the step of rinsing said cleansed surface or item to produce a rinsed surface or item.

48. The method of any of claims 46 or 47, further comprising the step of drying said rinsed surface or item.

49. A method for producing the polypeptide of any of claims 1-19 comprising:

a. stably transforming a host cell with an expression vector comprising a polynucleotide encoding the polypeptide of any of claims 1-19;
b. cultivating said transformed host cell under conditions suitable for said host cell to produce said protease; and
c. recovering said protease.

50. The method of claim 49, wherein said host cell is a filamentous fungus or bacterial cell.

51. The method of claims 49 or 50, wherein said host cell is selected from *Bacillus spp., Streptomyces spp., Escherichia spp., Aspergillus spp., Trichoderma spp., Pseudomonas spp., Corynebacterium spp., Saccharomyces spp.,* or *Pichia spp.*

52. The method of any one of claims 49-51, wherein said expression vector comprises a polynucleotide sequence comprising:

a. at least 70% sequence identity to the polynucleotide sequence selected from the group consisting of SEQ ID NOs: 4, 9, 14, 19, 24, 29, 34 and 39; or
b. being capable of hybridizing to a probe derived from the polynucleotide sequence selected from the group consisting of SEQ ID NOs: 4, 9, 14, 19, 24, 29, 34 and 39 under conditions of intermediate to high stringency, or
c. a polynucleotide sequence complementary to a polynucleotide sequence having at least 70% sequence identity to the polynucleotide sequence selected from the group consisting of SEQ ID NOs: 4, 9, 14, 19, 24, 29, 34 and 39.

53. The method of any one of claims 49-52, wherein said vector comprises a DNA sequence coding for a native or non-naturally occurring signal peptide.

54. The method of any one of claims 49-53, wherein said vector comprises a heterologous promoter and/or DNA sequence coding for a signal peptide.

55. The method of any one of claims 49-53, wherein said vector comprises a homologous promoter and/or DNA sequence coding for a signal peptide.

56. The method of any one of claims 49-65, wherein said host cell is cultivated in a culture media or a fermentation broth.

57. A nucleic acid sequence comprising a nucleic acid sequence:

(i) having at least 70% identity to a sequence selected from the group consisting of SEQ ID NOs: 4, 9, 14, 19, 24, 29, 34 and 39, or

(ii) being capable of hybridizing to a probe derived from the polynucleotide sequence selected from the group consisting of SEQ ID NOs: 4, 9, 14, 19, 24, 29, 34 and 39, under conditions of intermediate to high stringency, or

(iii) being complementary to the polynucleotide sequence selected from the group consisting of SEQ ID NOs: 4, 9, 14, 19, 24, 29, 34 and 39.

58. A vector comprising the nucleic acid sequence of claim 57.

59. A host cell transformed with the vector of claim 58.

60. The host cell of claim 59 selected from *Bacillus spp., Streptomyces spp., Escherichia spp., Aspergillus spp., Trichoderma spp., Pseudomonas spp., Corynebacterium spp., Saccharomyces spp.,* or *Pichia spp.*

61. The host cell of claim 59 or 60, wherein said *Bacillus spp.* is *Bacillus subtilis.*

62. A textile processing composition comprising the polypeptide of any one of claims 1-19.

63. An animal feed composition comprising the polypeptide of any one of claims 1-19.

64. A leather processing composition comprising the polypeptide of any one of claims 1-19.

65. A feather processing composition comprising the polypeptide or recombinant polypeptide of any one of claims 1-19.

66. A feather processing composition comprising the polypeptide or recombinant polypeptide of any one of claims 1-19.

67. A corn soy protein processing composition comprising the polypeptide or recombinant polypeptide of any one of claims 1-19.

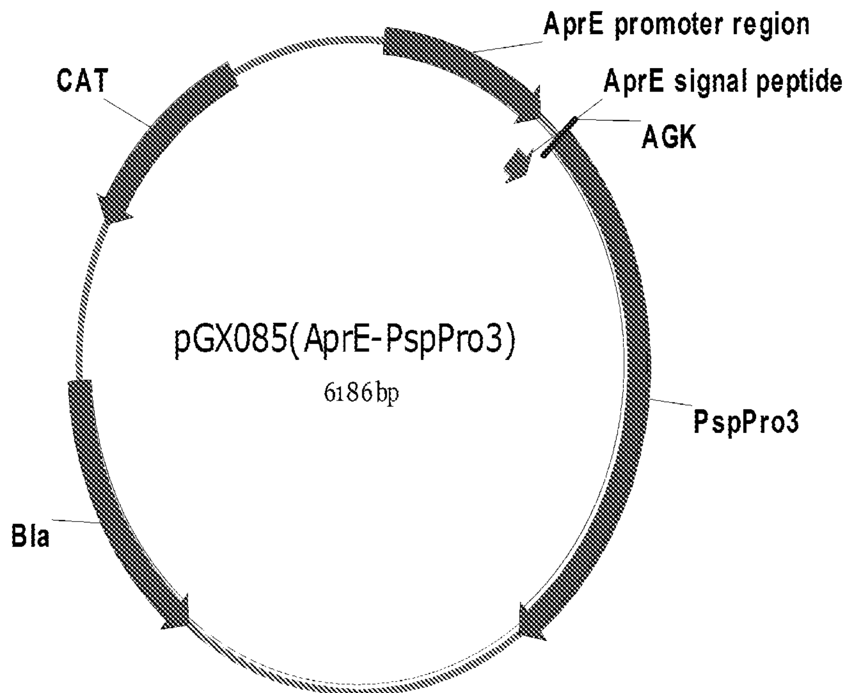


Figure 1.1. Plasmid map of pGX085(AprE-PspPro3).

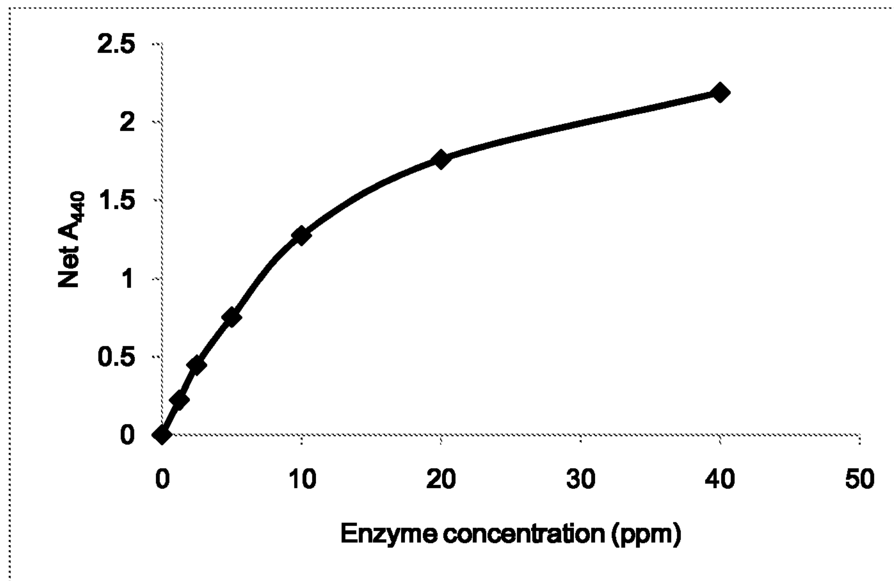


Figure 1.2. Dose response of PspPro3 in azo-casein assay at pH 7.

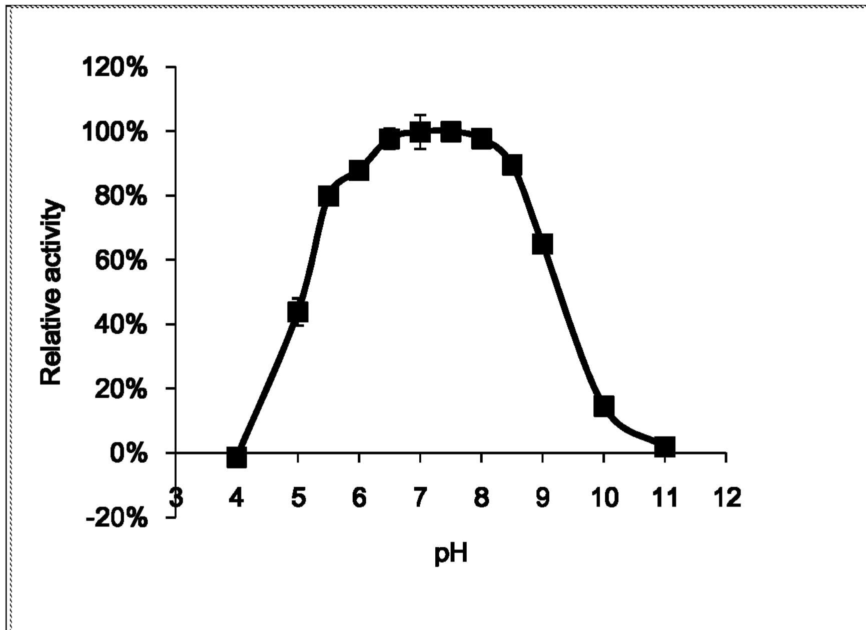


Figure 1.3. pH profile of PspPro3.

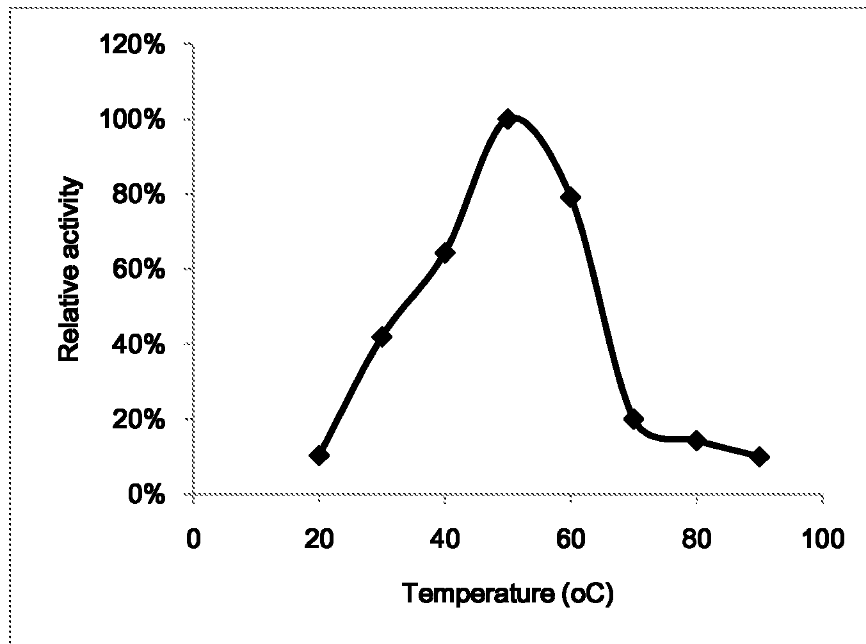


Figure 1.4. Temperature profile of PspPro3.

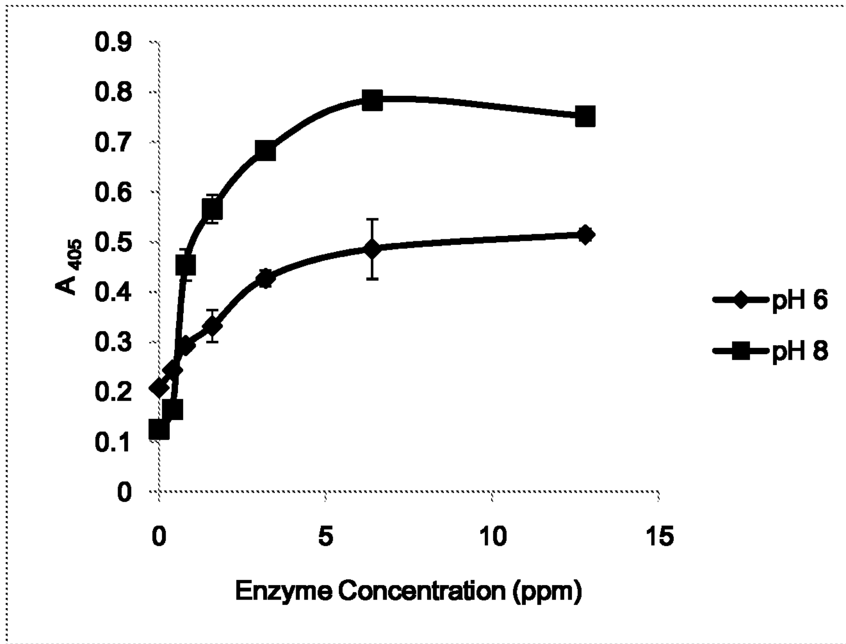


Figure 1.5A. Cleaning performance of PspPro3 at pH 6 and 8 in AT dish detergent.

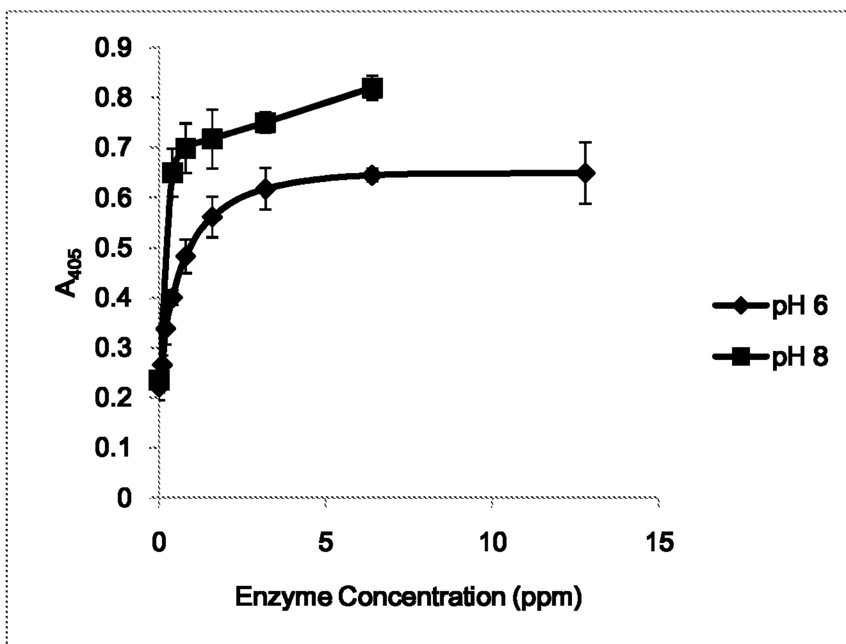


Figure 1.5B. Cleaning performance of PspPro3 at pH 6 and 8 in AT dish detergent with bleach

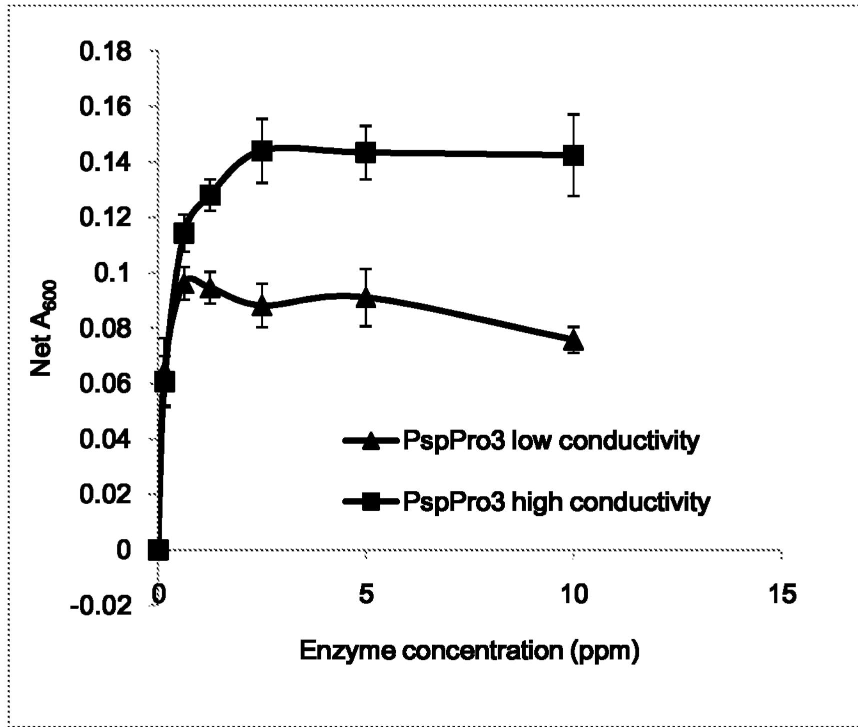


Figure 1.6. Cleaning performance of PspPro3 in liquid laundry detergent at pH 8

```
CLUSTAL W (1.83) multiple sequence alignment


PspPro3                             -----ATGTGKGVLGDTKTFNTTASGSSYQLRDTTRGNGIVTYTASNRQS
Paenibacillus_sp_Aloe-11            ---NEATGTGKGVLGDTKTFNTTASGSSYQLRDTTRGNGIVTYTASNRQS
B_thermoproteolyticus_P00800        ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                         :.*.*:***** *.:*** * : * *:*.******.** *. * :

PspPro3                             IPGTILTDADNVWN---DPAGVDAHAYAAKTYDYYKEKFNRNSIDGRGLQ
Paenibacillus_sp_Aloe-11            IPGTILTDADNVWN---DPAGVDAHAYAAKTYDYYKEKFNRNSIDGRGLQ
B_thermoproteolyticus_P00800        LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                    :**:: :**** :    *...**** **. ******: .** * **..

PspPro3                             LRSTVHYGNRYNNAFWNGSQMTYGDGDGTTFIAFSGDPDVVGHELTHGVT
Paenibacillus_sp_Aloe-11            LRSTVHYGNRYNNAFWNGSQMTYGDGDGTTFIAFSGDPDVVGHELTHGVT
B_thermoproteolyticus_P00800        IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                    :**:***.: ***********.****** ***.:**. ***.*****.**

PspPro3                             EYTSNLEYYGESGALNEAFSDIIGNDIQ-----RKNWLVGDDIYTPRIAG
Paenibacillus_sp_Aloe-11            EYTSNLEYYGESGALNEAFSDIIGNDIQ-----RKNWLVGDDIYTPRIAG
B_thermoproteolyticus_P00800        DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                    :**:.* * .****:***:***:*. ::     . :* :*:*:*** *:*

PspPro3                             DALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGT
Paenibacillus_sp_Aloe-11            DALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGT
B_thermoproteolyticus_P00800        DSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYLISQGGT
                                    *:*****:*: *.:*****: * *:.****** ******** **::****

PspPro3                             FHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAVVQAAKDLYGAS
Paenibacillus_sp_Aloe-11            FHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAVVQAAKDLYGAS
B_thermoproteolyticus_P00800        HYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYGST
                                    .:**:* *****   :*:* *:*:***.:*:**: * *.**:*.****::

PspPro3                             SAQATAAAKSFDAVGVN
Paenibacillus_sp_Aloe-11            SAQATAAAKSFDAVGVN
B_thermoproteolyticus_P00800        SQEVASVKQAFDAVGVK
                                    * :.::. ::******:
```

Figure 1.7. Alignment of PspPro3 with protease homologs

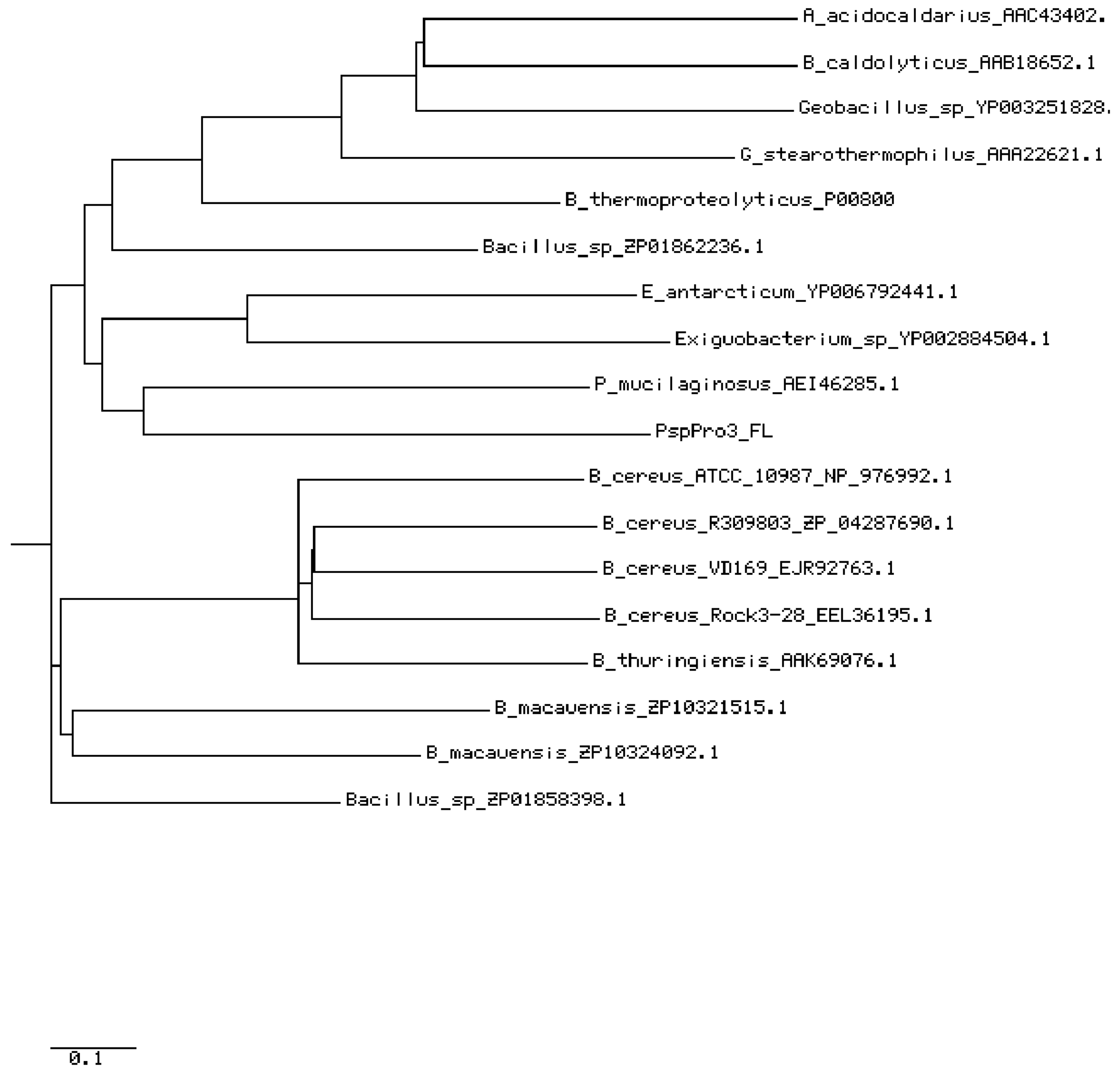


Figure 1.8. Phylogenetic tree of PspPro3 and homologs.

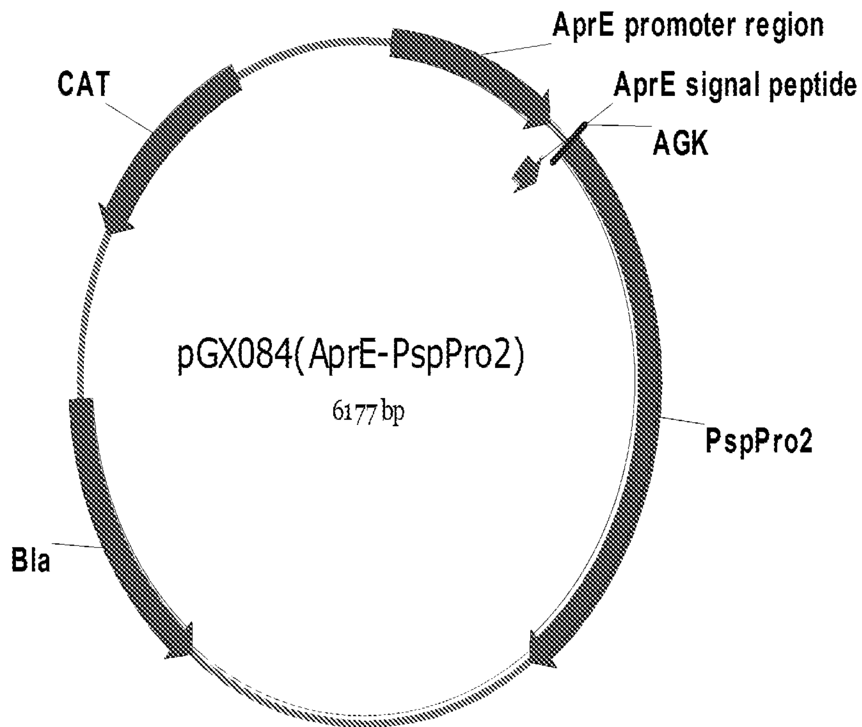


Figure 2.1. The map of plasmid pGX084(AprE-PspPro2).

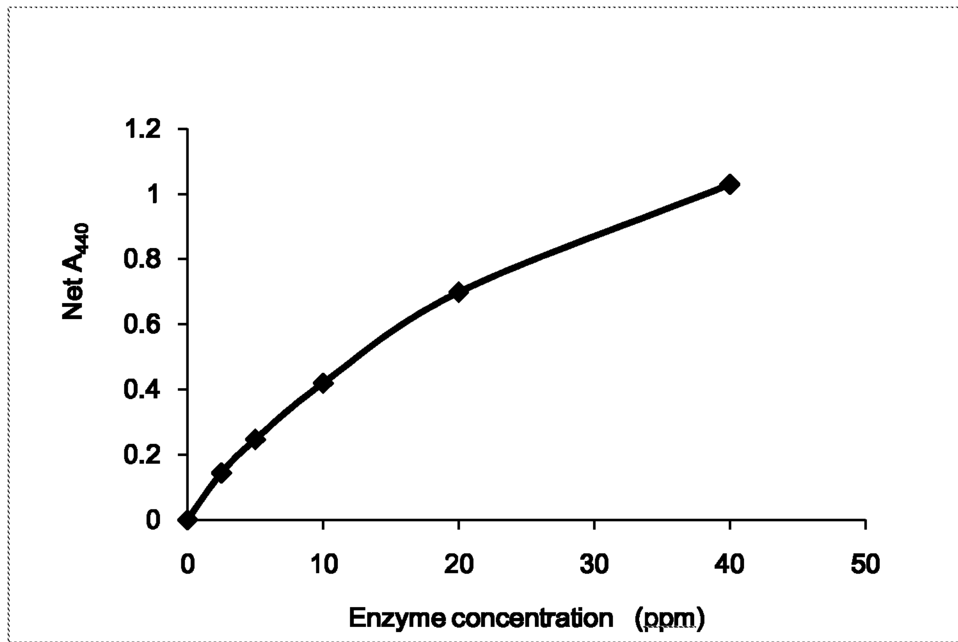


Figure 2.2. Dose response curve of PspPro2 in azo-casein assay at pH 7.

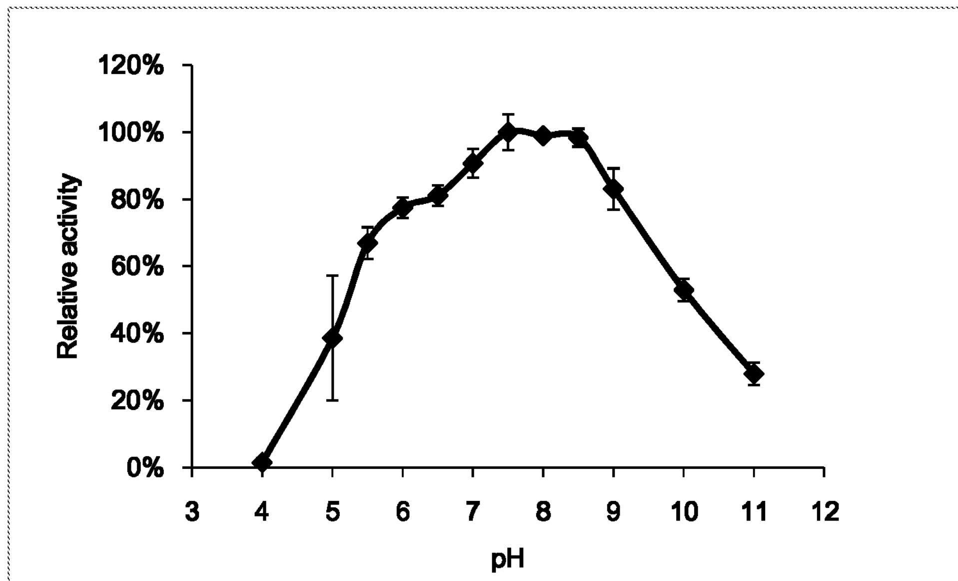


Figure 2.3. pH profile of purified PspPro2.

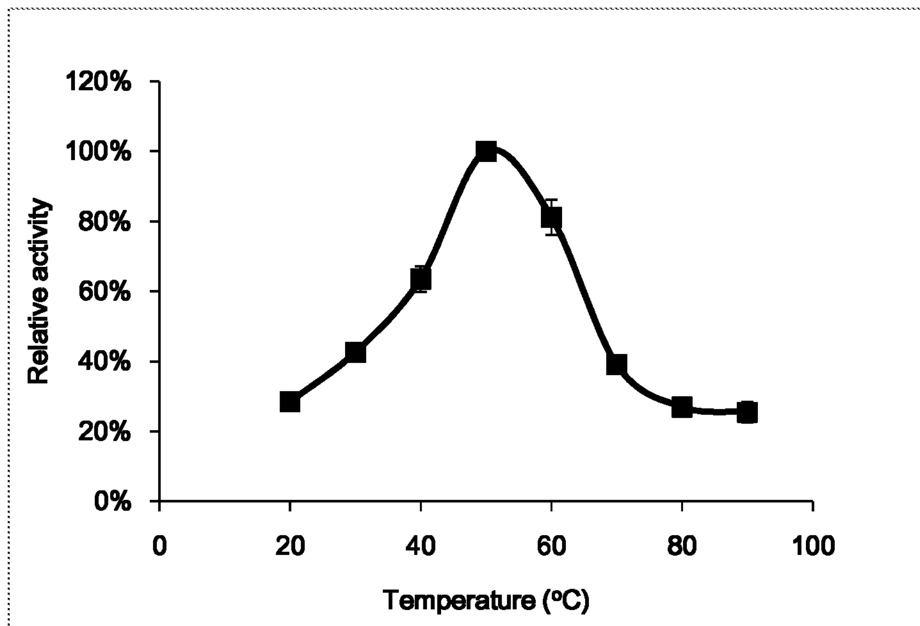


Figure 2.4. Temperature profile of purified PspPro2.

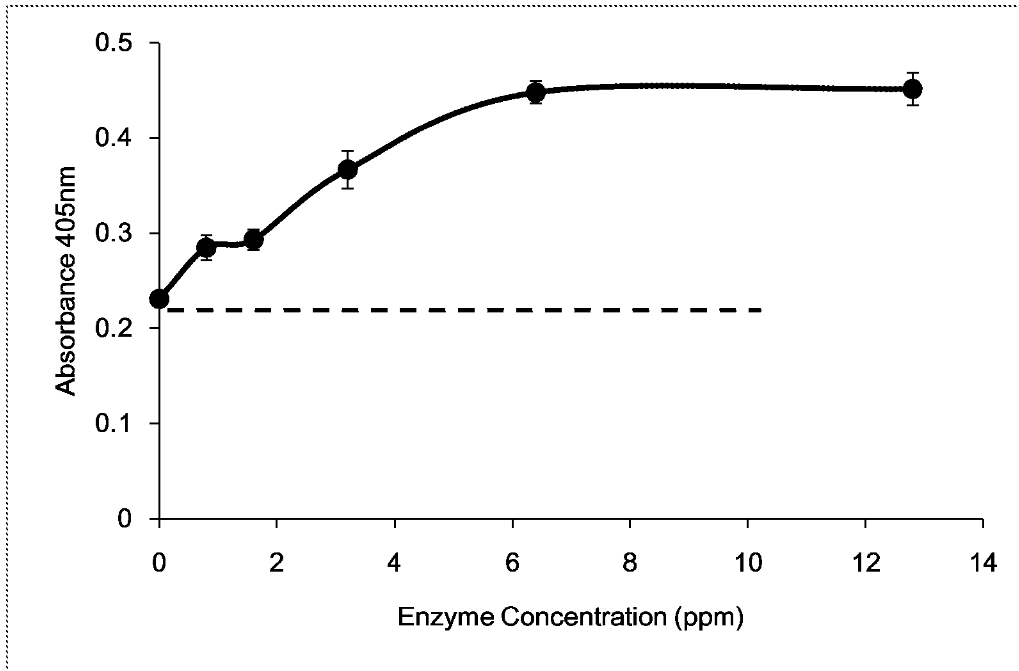


Figure 2.5A. Cleaning performance of PspPro2 protein at pH 6 in AT detergent with bleach.

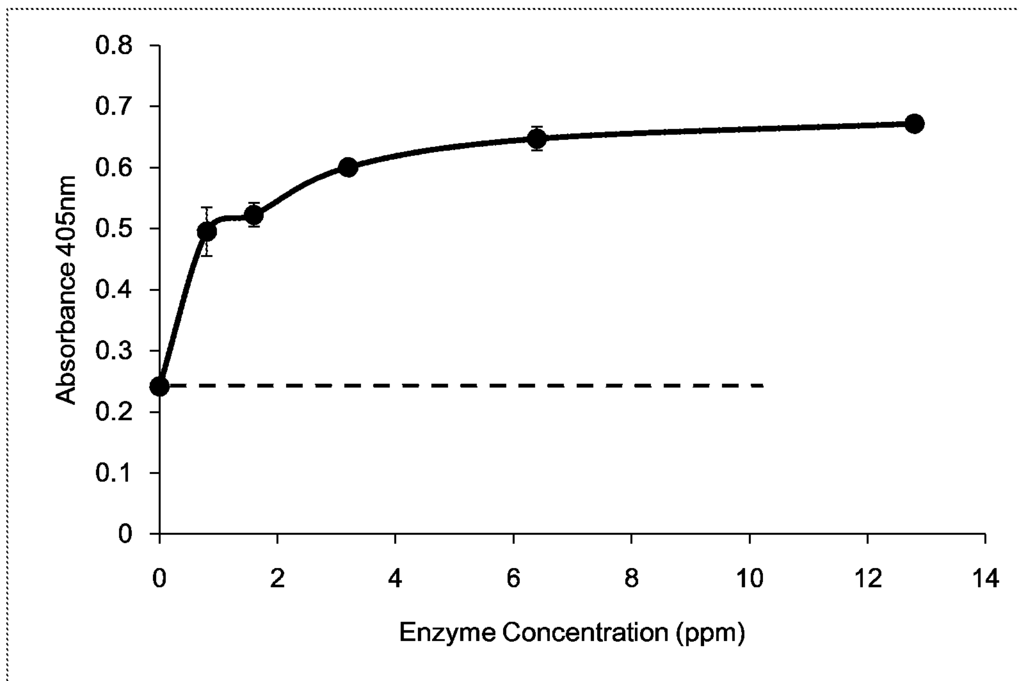


Figure 2.5B. Cleaning performance of PspPro2 protein at pH 8 in AT detergent with bleach.

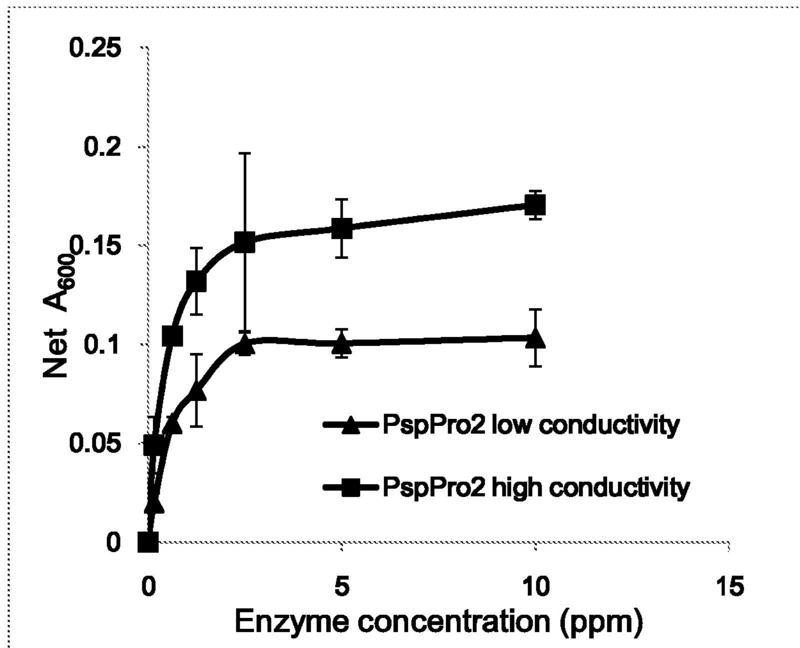


Figure 2.6A. Cleaning performance of PspPro2 protein in liquid laundry detergent.

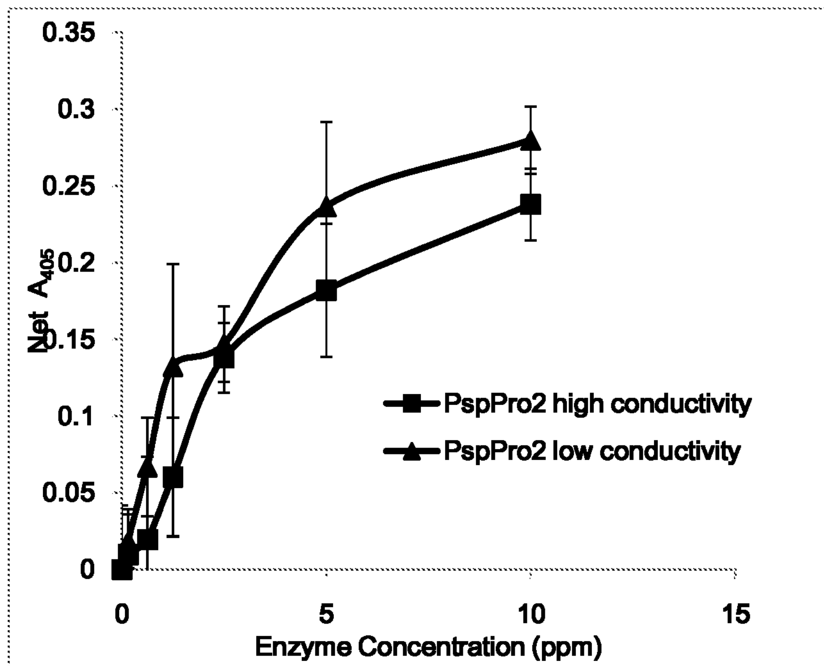


Figure 2.6B. Cleaning performance of PspPro2 protein in powder laundry detergent.

```
CLUSTAL W (1.83) multiple sequence alignment


PspPro2                             -----ATGTGRGVDGKTKSFTTTASGNRYQLKDTTRSNGIVTYTAGNRQT
ZP_09775365.1_P_sp_Aloe-11          -----ATGTGRGVDGKTKSFTTTASGNRYQLKDTTRSNGIVTYTAGNRQT
B_thermoproteolyticus_P00800        ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                         :.*.**** *. *.:.** * . * *:*.**.***.** *  * *

PspPro2                             TPGTILTDTDNVW---EDPAAVDAHAYAIKTYDYYKNKFGRDSIDGRGMQ
ZP_09775365.1_P_sp_Aloe-11          TPGTILTDTDNVW---EDPAAVDAHAYAIKTYDYYKNKFGRDSIDGRGMQ
B_thermoproteolyticus_P00800        LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                     **:: :*:** :    *..***** **  ******* ..* * **..

PspPro2                             IRSTVHYGKKYNNAFWNGSQMTYGDGDGSTFTFFSGDPDVVGHELTHGVT
ZP_09775365.1_P_sp_Aloe-11          IRSTVHYGKKYNNAFWNGSQMTYGDGDGSTFTFFSGDPDVVGHELTHGVT
B_thermoproteolyticus_P00800        IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                    ***:***.: ***********.******.**  :**. ***.*****.**

PspPro2                             EFTSNLEYYGESGALNEAFSDIIGNDID-----GTSWLLGDGIYTPNIPG
ZP_09775365.1_P_sp_Aloe-11          EFTSNLEYYGESGALNEAFSDIIGNDID-----GTSWLLGDGIYTPNIPG
B_thermoproteolyticus_P00800        DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                    ::*:.* * .****:***:***:*. ::     ...* :*:.:***.*.*

PspPro2                             DALRSLSDPTRFGQPDHYSNFYPDPNNDDEGGVHTNSGIINKAYYLLAQG
ZP_09775365.1_P_sp_Aloe-11          DALRSLSDPTRFGQPDHYSNFYPDPNNDDEGGVHTNSGIINKAYYLLAQG
B_thermoproteolyticus_P00800        DSLRSMSDPAKYGDPDHYSKRYT--GTQDNGGVHINSGIINKAAYLISQG
                                    *:***:***:::*:*****: *.  ..:*:**** ******** **::**

PspPro2                             GTSHGVTVTGIGREAAVFIYYNAFTNYLTSTSNFSNARAAVIQAAKDFYG
ZP_09775365.1_P_sp_Aloe-11          GTSHGVTVTGIGREAAVFIYYNAFTNYLTSTSNFSNARAAVIQAAKDFYG
B_thermoproteolyticus_P00800        GTHYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYG
                                    ** :**:*.****:    *:*.*:*:***.*****: ***.:*:*.*:**

PspPro2                             ADSLAVTSAIQSFDAVGIK
ZP_09775365.1_P_sp_Aloe-11          ADSLAVTSAIQSFDAVGIK
B_thermoproteolyticus_P00800        STSQEVASVKQAFDAVGVK
                                    : *  *:*. *:*****:*
```

Figure 2.7: Alignment of PspPro2 protein with homologous protease sequences.

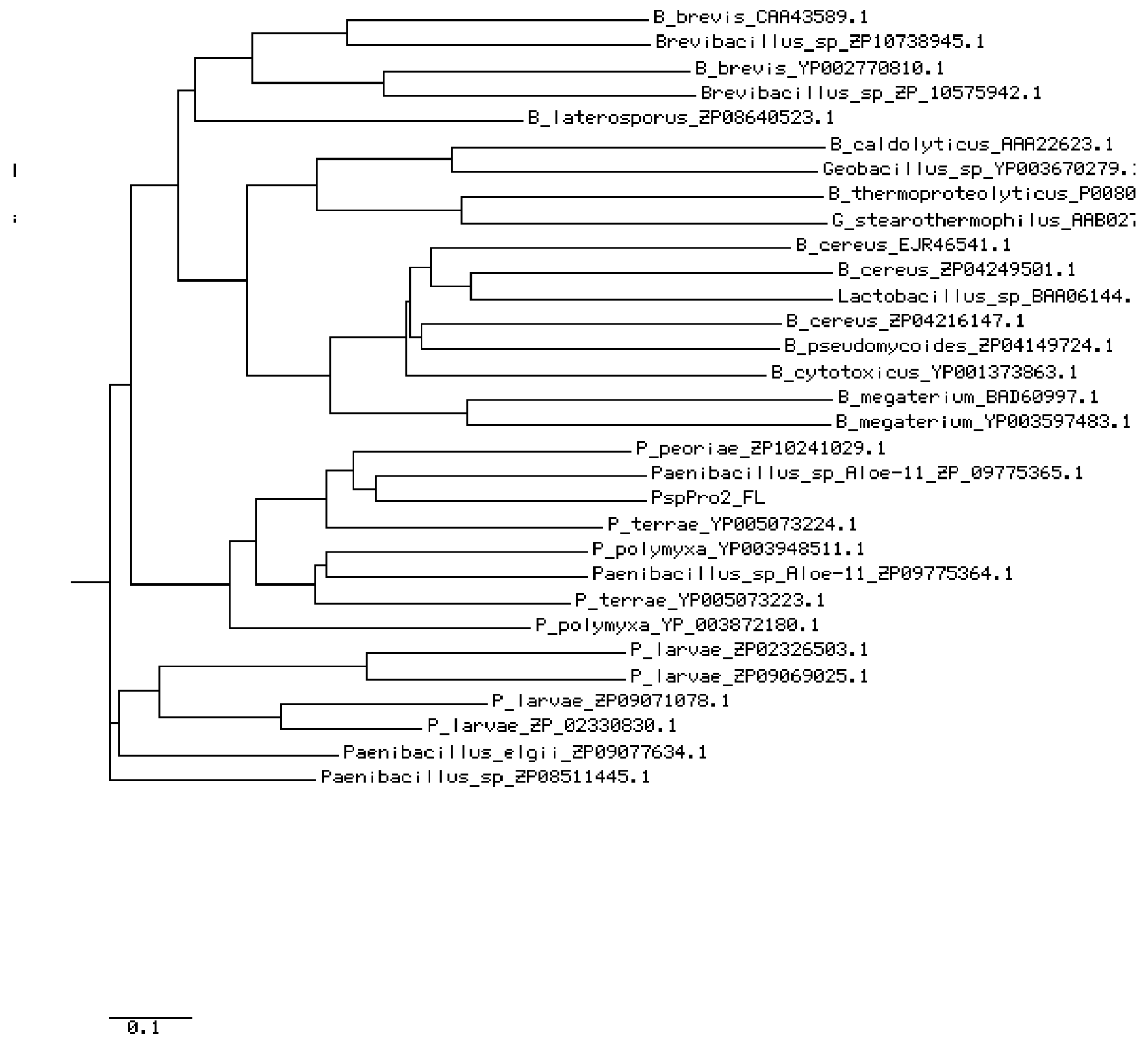


Figure 2.8: Phylogenetic tree for PspPro2 and its homologs

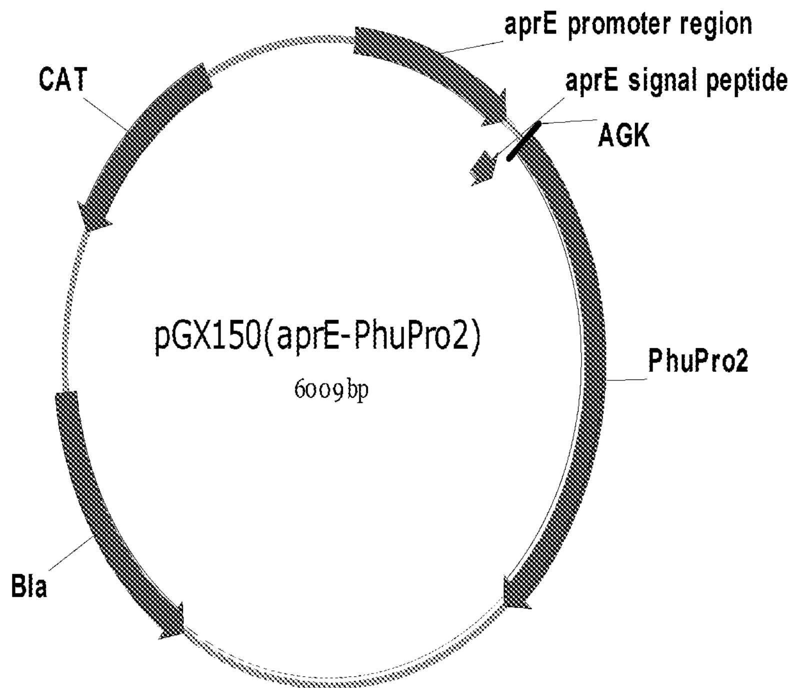


Figure 3.1. The plasmid map of pGX150 (AprE-PhuPro2).

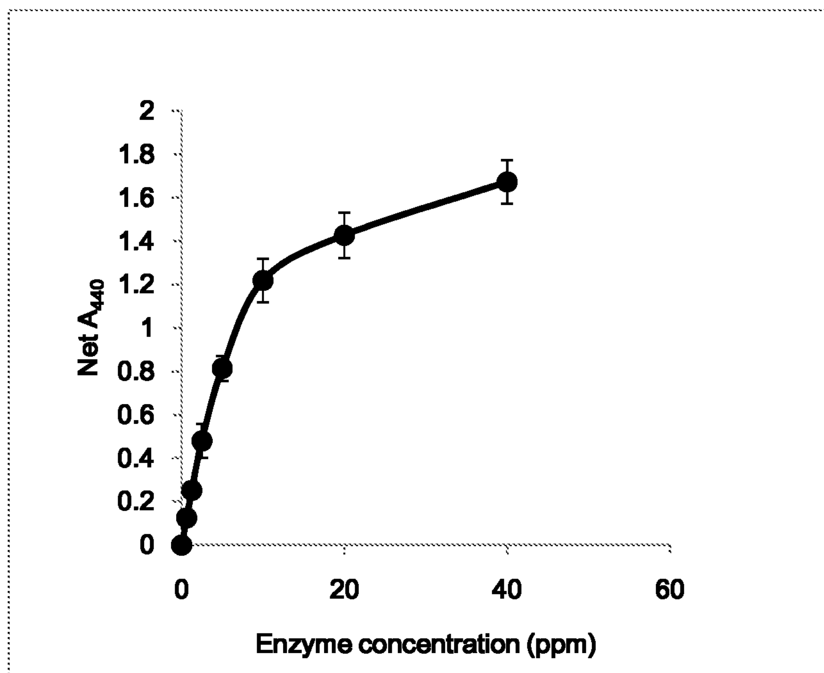


Figure 3.2. Dose response curve of PhuPro2 in azo-casein assay at pH 7.

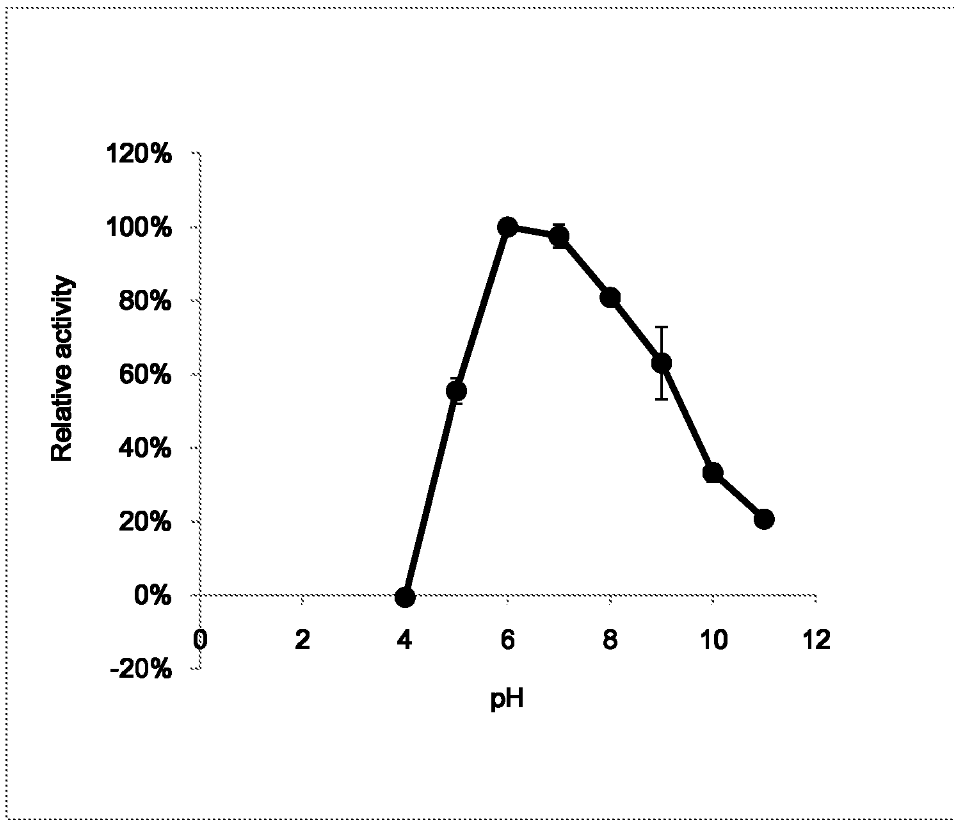


Figure 3.3. pH profile of PhuPro2.

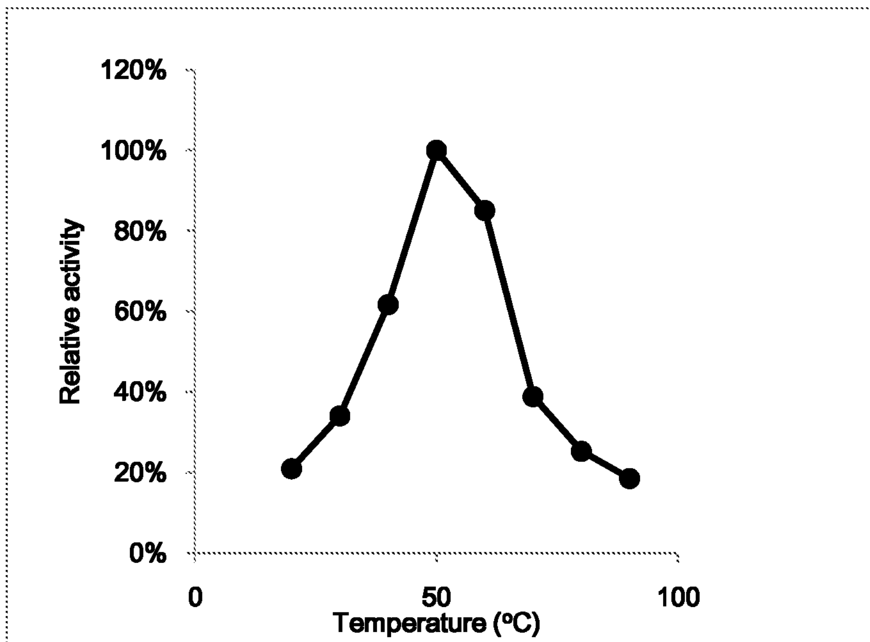


Figure 3.4. Temperature profile of PhuPro2.

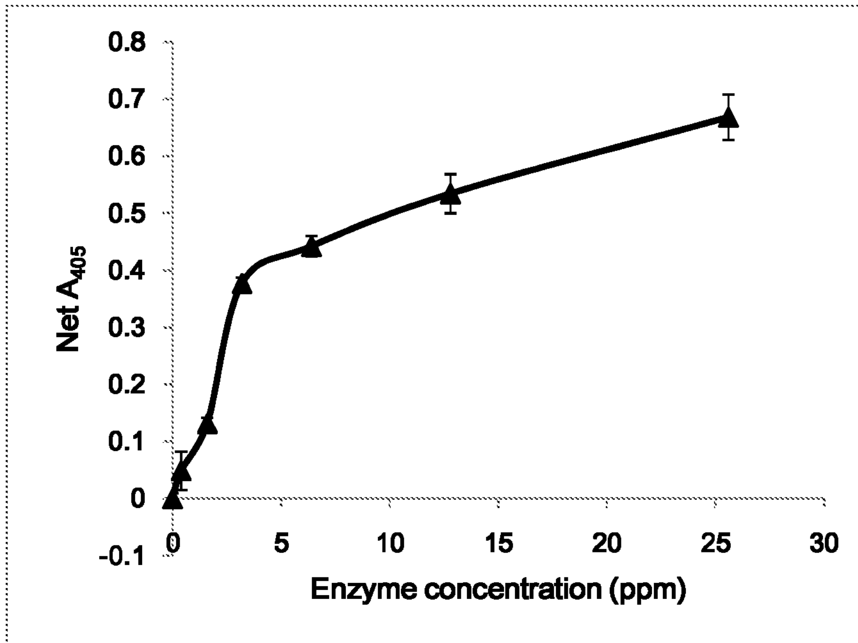


Figure 3.5A. Cleaning performance of PhuPro2 in AT dish detergent at pH 6.

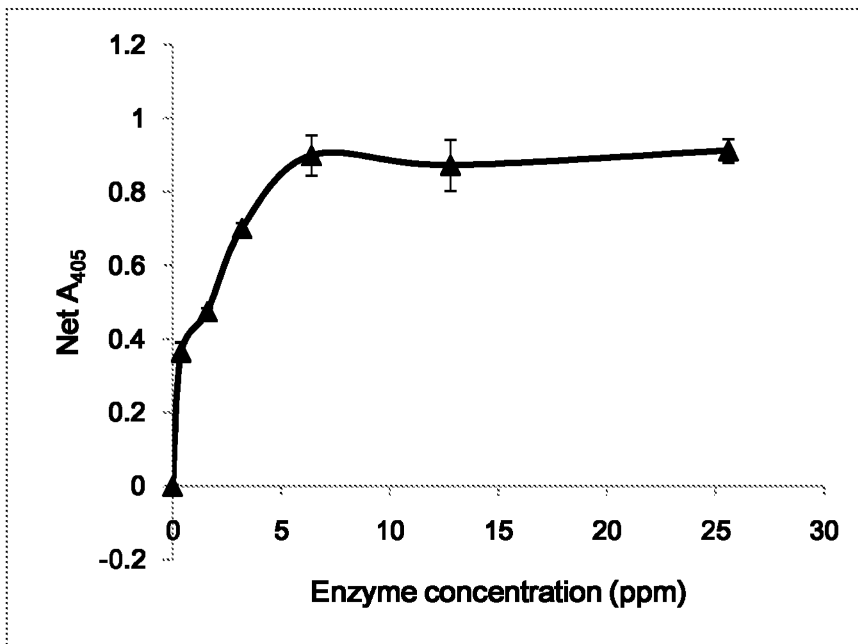


Figure 3.5B. Cleaning performance of PhuPro2 in AT dish detergent at pH 8.

```
CLUSTAL W (1.83) multiple sequence alignment


PhuPro2                             -----ATGSGTGVLGDNKTFQTTLSGSTYQLKDTTRGNGIYTYTASNRTT
P_terrae_HPL-003_YP_005073223.      -----ATGTGKGVLGDTKSFNTTQSGSSYQLKDTTRGNGIVTYTASNRQT
B_thermoproteolyticus_P00800        ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                         :.* * ***** *.::** * : * *:*.****** ** *. * *

PhuPro2                             IPGTLLTDADNVWT---DGAAVDAHTYAGKVYDFYKTKFGRNSLDGNGLL
P_terrae_HPL-003_YP_005073223.      IPGTLLTDADNVWN---DPAGVDAHAYAAKTYDYYKDKFGRNSIDGRGLQ
B_thermoproteolyticus_P00800        LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                    :**:* :**** :    * ..**** **. .**:**  ..* * **..

PhuPro2                             IRSSVHYSSRYNNAFWNGTQIVFGDGDGSTFIPLSGDLDVVGHELSHGVI
P_terrae_HPL-003_YP_005073223.      LRSTVHYGSRYNNAFWNGSQMTYGDGDGTTFIAFSGDPDVVGHELTHGVT
B_thermoproteolyticus_P00800        IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                    :**:***.. ********:*:.:***** ***.:**. ***.***:*.*

PhuPro2                             EYTSNLQYLNESGALNESYADVLGNSIQ-----AKNWLIGDDVYTPGISG
P_terrae_HPL-003_YP_005073223.      EYTSNLDYYGESGALNESFSDIIGNDIQ-----RKNWLVGDDIYTPSIAG
B_thermoproteolyticus_P00800        DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                    :**:.* * .****:**: :*::*. ::       :* :*:*:***.*:*

PhuPro2                             DALRSMSNPTLYGQPDNYANRYTGSSDNGGVHTNSGITNKAFYLLAQGGT
P_terrae_HPL-003_YP_005073223.      DALRSMSNPTLYDQPDHYSNLYKGSSDNGGVHTNSGIINKAYYLLAQGGT
B_thermoproteolyticus_P00800        DSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYLISQGGT
                                    *:*****:*: *.:**:*:: *.*:.****** **** *** **::****

PhuPro2                             QNGVTVAGIGRDAAVNIFYNTVAYYLTSTSNFAAAKNASIQAAKDLYGTG
P_terrae_HPL-003_YP_005073223.      FHNVTVSGIGRDAAVQIYYSAFTNYLTSTSNFSNTRAAVVQAAKDLYGAN
B_thermoproteolyticus_P00800        HYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYGST
                                      .*:* *****   :*:* :.: ***.****:  : * :*:*.****:

PhuPro2                             SSYVTSVTNAFRAVGL-
P_terrae_HPL-003_YP_005073223.      SAQATAAAKSFDAVGVN
B_thermoproteolyticus_P00800        SQEVASVKQAFDAVGVK
                                    *  .::. ::* ***:
```

Figure 3.6: Alignment of PhuPro2 with homologous protease sequences.

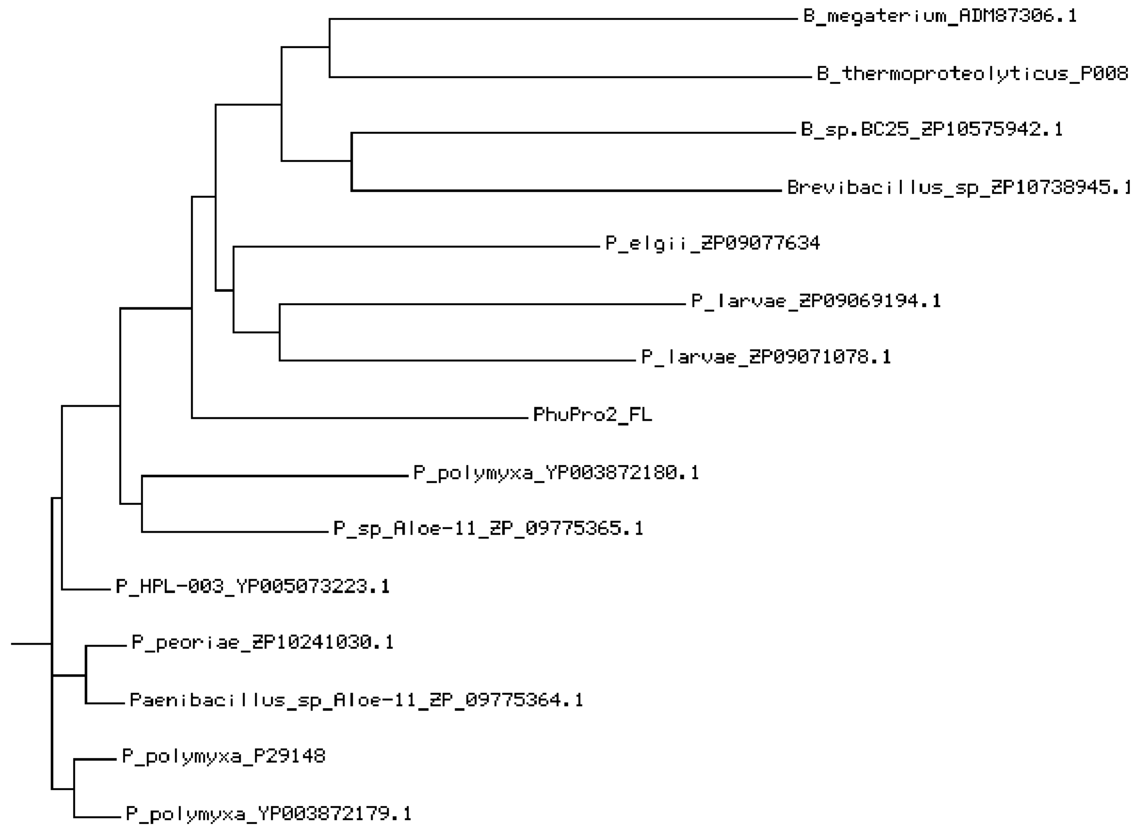


Figure 3.7: Phylogenetic tree for PhuPro2 and homologs.

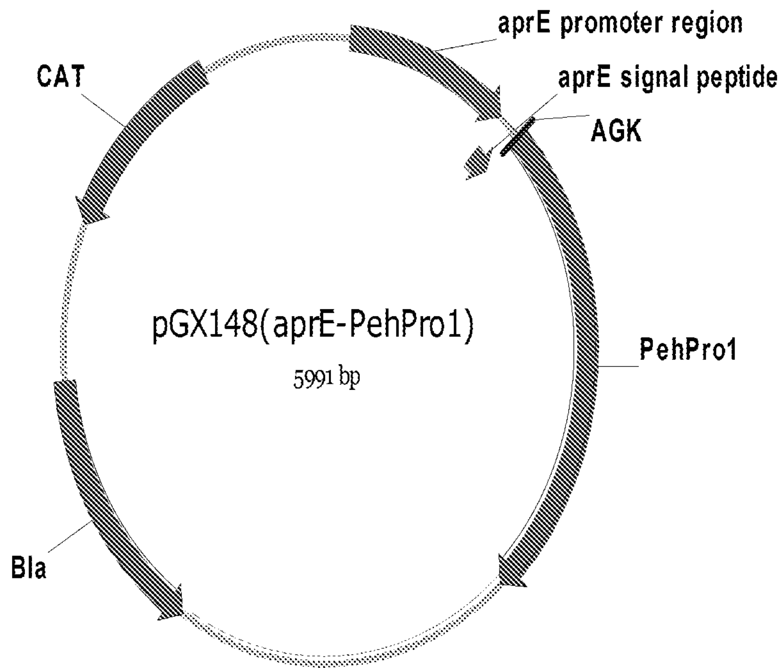


Figure 4.1. The plasmid map of pGX148 (AprE-PehPro1).

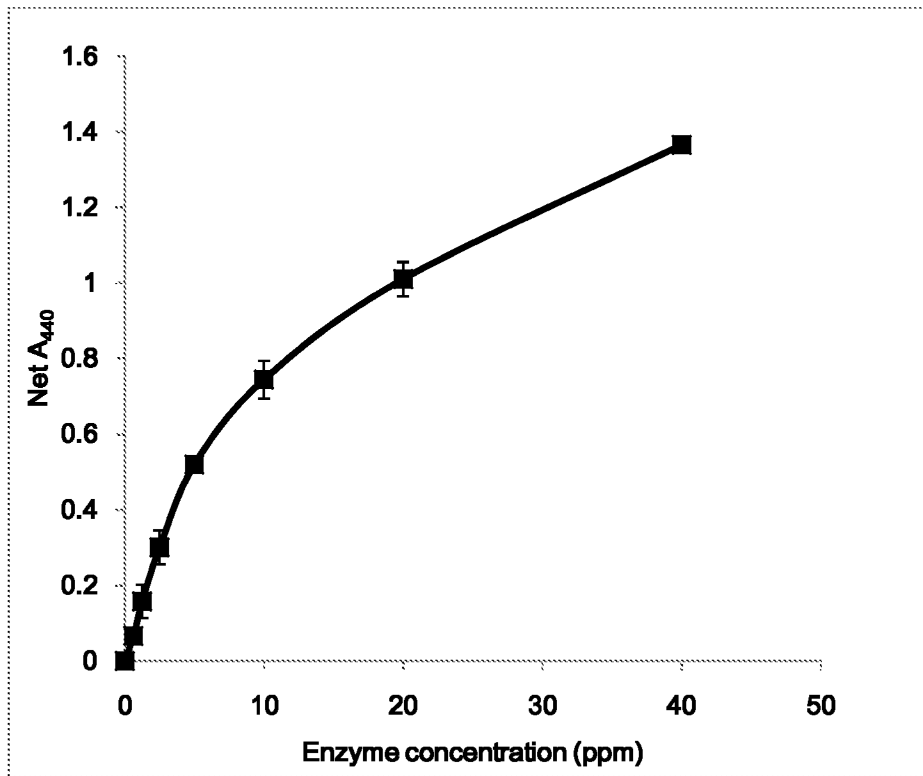


Figure 4.2. Dose response curve of PehPro1 in azo-casein assay at pH 7.

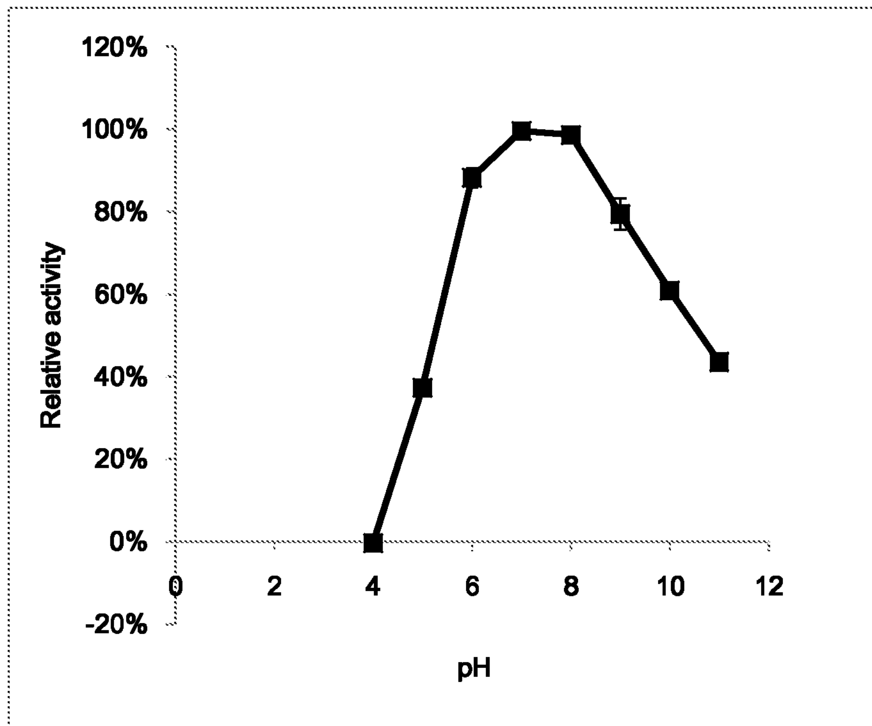


Figure 4.3. pH profile of PehPro1.

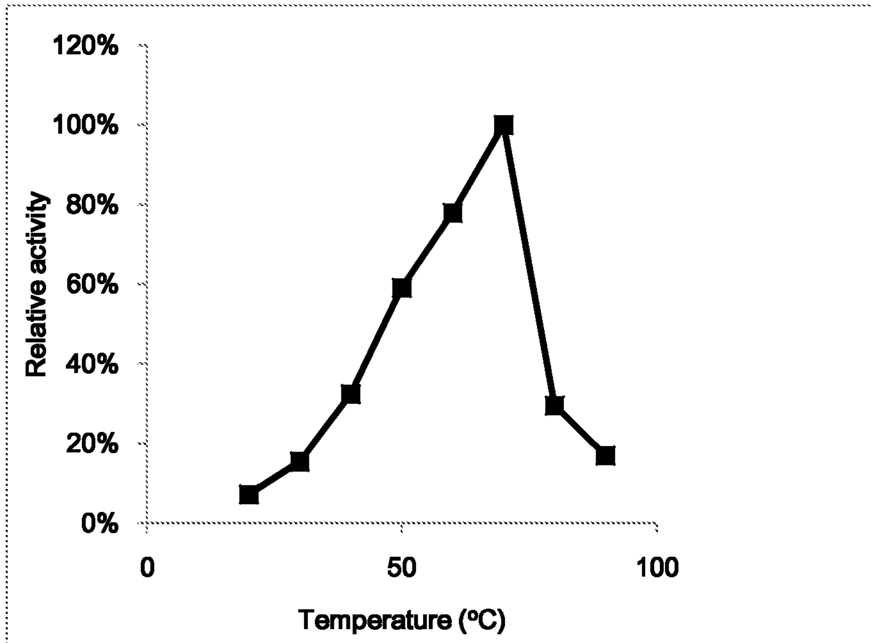


Figure 4.4. Temperature profile of PehPro1.

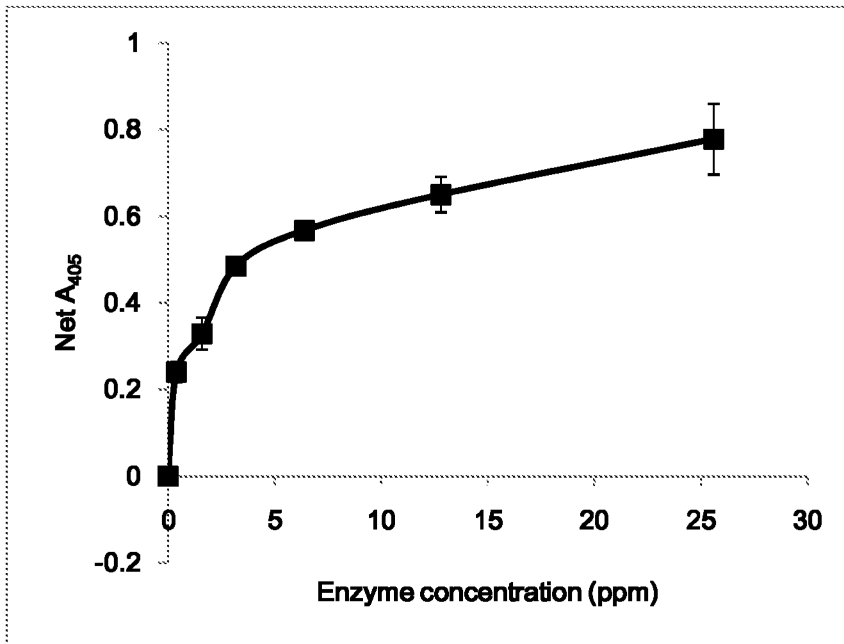


Figure 4.5A: Cleaning performance of PehPro1 in AT detergent at pH 6.

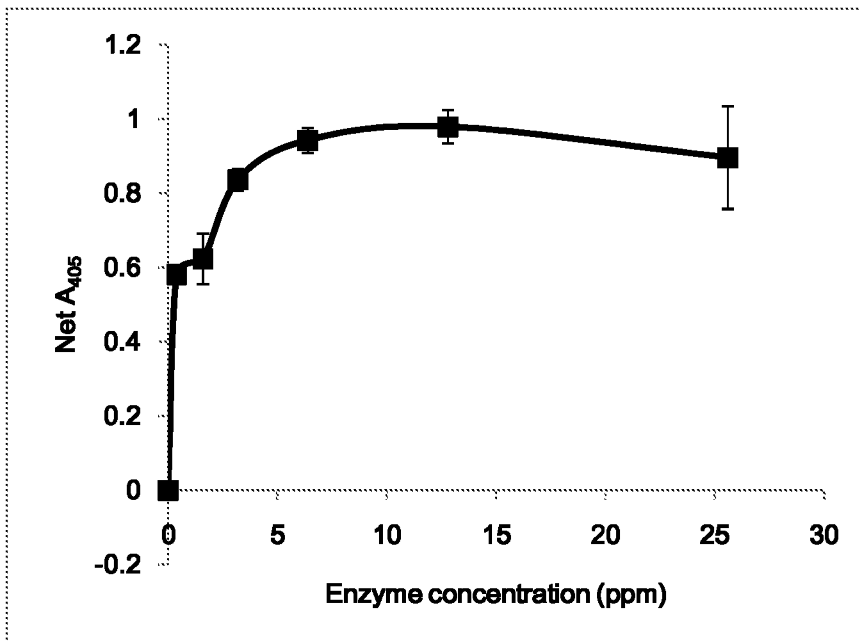


Figure 4.5B: Cleaning performance of PehPro1 in AT detergent at pH 8.

```
CLUSTAL W (1.83) multiple sequence alignment

PehPro1_mature                      -----ATGTGKGVLGDTKSFTTTQSGSTYQLKDTTRGQGIVTYSAGNRSS
Paenibacillus_elgii_B69_ZP_090      -----ATGTGKGVLGDTKSFTTTQSGSSYQLKDTTRGQGIVTYSAGNRTS
B_thermoproteolyticus_P00800        ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                         :.*.*:***** *.:.** * : * *:*.***:**.**.*  *::

PehPro1_mature                      LPGTLLTSSSNIWN---DGAAVDAHAYTAKVYDYYKNKFGRNSIDGNGFQ
Paenibacillus_elgii_B69_ZP_090      LPGSLLTSTNNIWN---DGSAVDAHAYTGKVYDYYKNKFGRNSIDGNGLQ
B_thermoproteolyticus_P00800        LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                    ***:* :.:.* :    *..***** *:. .****** ..* * ***.

PehPro1_mature                      LKSTVHYSSRYNNAFWNGVQMVYGDGDGVTFIPFSADPDVIGHELTHGVT
Paenibacillus_elgii_B69_ZP_090      LKSTVHYSTRYNNAFWNGVQMVYGDGDGVTFRSFPADPDVIGHELTHGVT
B_thermoproteolyticus_P00800        IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                    ::*:****  ******** ********* ** .:... **:.*****.**

PehPro1_mature                      EHTAGLEYYGESGALNESISDIIGNAID-----GKNWLIGDLIYTPNTPG
Paenibacillus_elgii_B69_ZP_090      ESTAGLEYYGESGALNESISDIFGNAIE-----GKNWLIGDLI---TLNA
B_thermoproteolyticus_P00800        DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                    : **** * .****:**:****:*. ::     . :* **: :      .

PehPro1_mature                      DALRSMENPKLYNQPDRYQDRYTGPSDNGGVHINSGINNKAFYLIAQGGT
Paenibacillus_elgii_B69_ZP_090      GALRSMENPKLYRQPDRYQDRYTGPSDNGGVHTNSGINNKAFHLIAQGGT
B_thermoproteolyticus_P00800        DSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYLISQGGT
                                    .:****.:*  * :**:*..****..****** **** *** :**:****

PehPro1_mature                      HYGVTVNGIGRDAAVQIFYDALINYLTPTSNFSAMRAAAIQAATDLYGAN
Paenibacillus_elgii_B69_ZP_090      HYGVTVNGIGRSAAEQIFYDALTHYLTPTSNFSAIRAAAIQAATDSFGAN
B_thermoproteolyticus_P00800        HYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYGST
                                    ****:* ****.   :*** ** :********* :****:*:*** :*:.

PehPro1_mature                      SSQVNAVKKAYTAVGVN
Paenibacillus_elgii_B69_ZP_090      SSQVDAVKKAYNAVGVN
B_thermoproteolyticus_P00800        SQEVASVKQAFDAVGVK
                                    *.:* :**:*: ****:
```

Figure 4.6: Alignment of PehPro1 protein with homologous protease sequences.

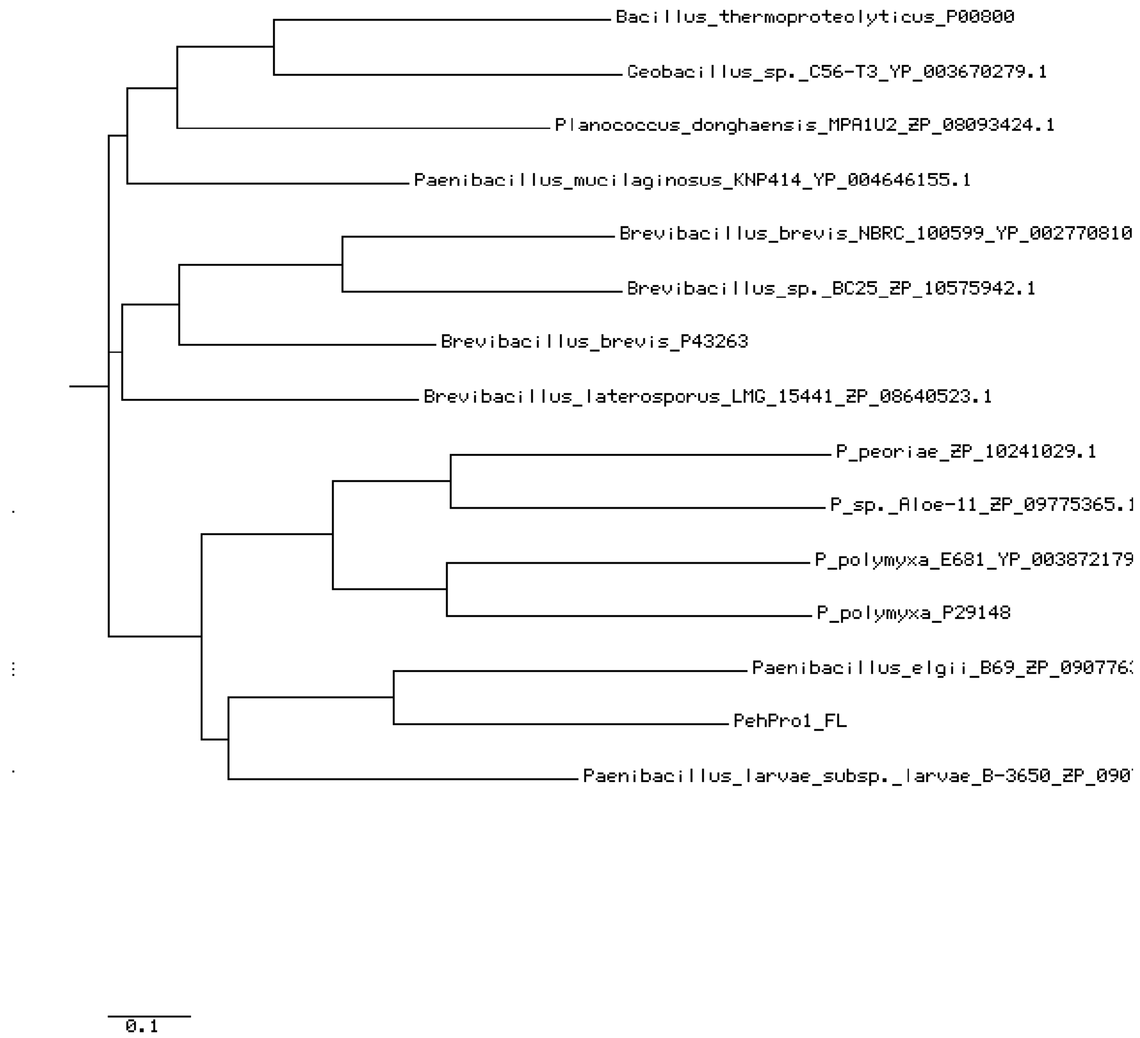


Figure 4.7: Phylogenetic tree for PehPro1 and its homologs.

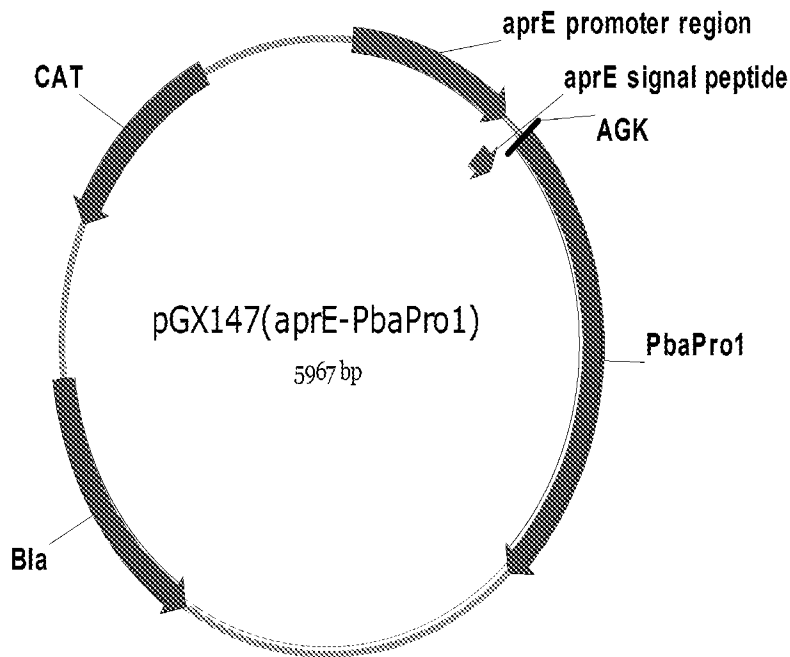


Figure 5.1. The plasmid map of pGX147(AprE-PbaPro1).

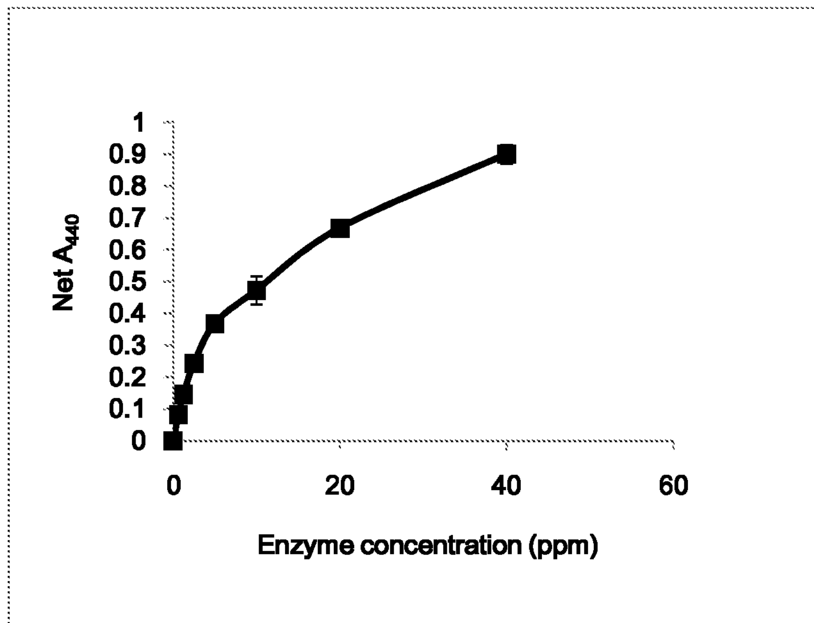


Figure 5.2. Dose response curve of PbaPro1 in azo-casein assay at pH 7.

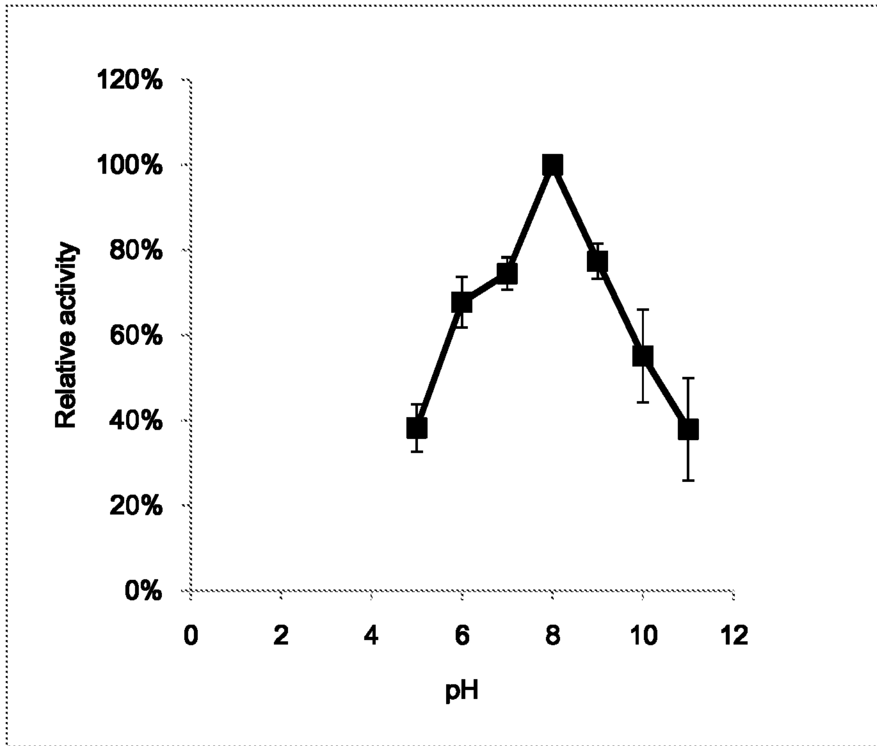


Figure 5.3. pH profile of PbaPro1.

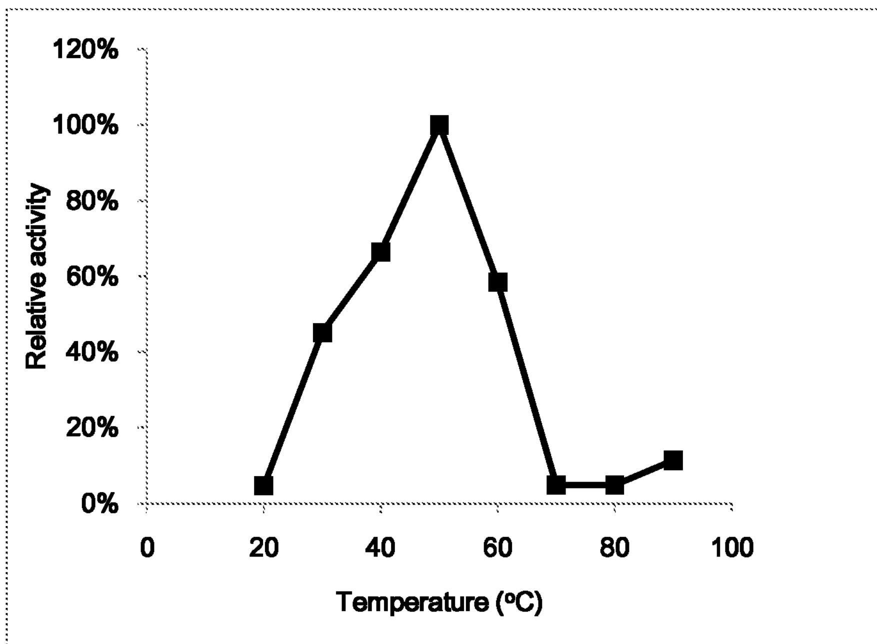


Figure 5.4. Temperature profile of PbaPro1.

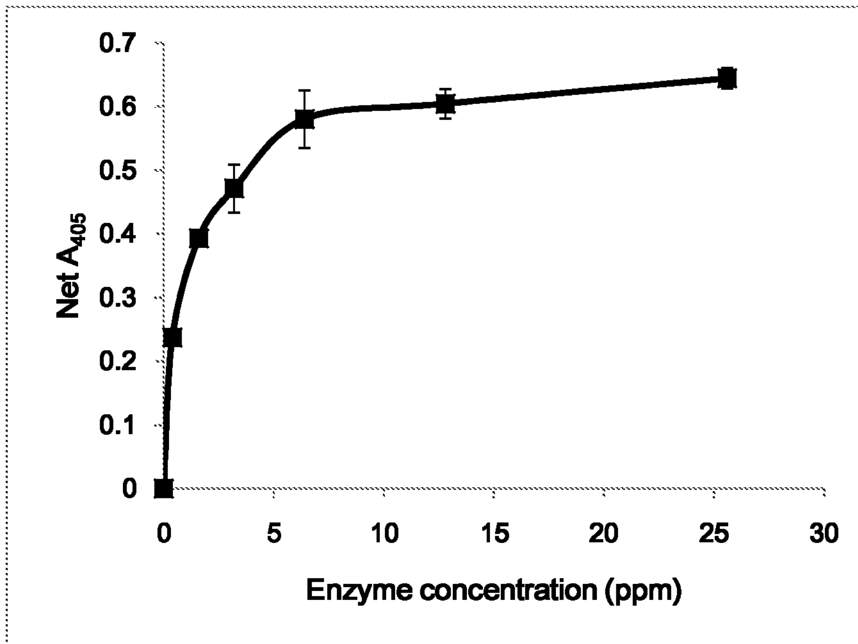


Figure 5.5A: Cleaning performance of PbaPro1 in AT dish detergent at pH 6.

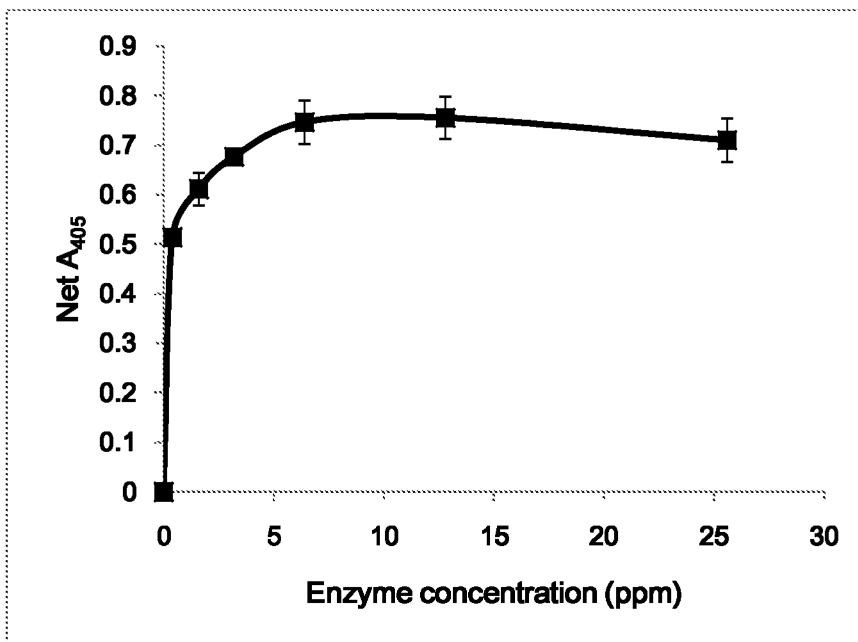


Figure 5.5B: Cleaning performance of PbaPro1 in AT dish detergent at pH 8.

```
CLUSTAL W (1.83) multiple sequence alignment


PbaPro1                             -----ATGTGTGVHGDTKTLTTTQSGSTYQLKDTTRGKGIQTYTANNRSS
P_polymyxa_SC2                      ---NEATGTGKGVLGDSKSFTTTASGSSYQLKDTTRGNGIVTYTASNRQS
B_thermoproteolyticus_P00800        ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                         :.*.* ** ** *.:.** * : * *:*.***:** ** *. * :

PbaPro1                             LPGSLSTSSNNVWT---DRAAVDAHAYAAATYDFYKNKFNRNGIDGNGLL
P_polymyxa_SC2                      IPGTILTDADNVWN---DPAGVDAHAYAAKTYDYYKAKFGRNSIDGRGLQ
B_thermoproteolyticus_P00800        LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                    :**:: :.::* :    * ..**** **. ***:**  ..* . **..

PbaPro1                             IRSTVHYGSNYKNAFWNGAQIVYGDGDGIEFGPFSGDLDVVGHELTHGVI
P_polymyxa_SC2                      LRSTVHYGSRYNNAFWNGSQMTYGDGDGSTFIAFSGDPDVVGHELTHGVT
B_thermoproteolyticus_P00800        IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                    :**:***.. *:******:*:.******  * .:**. ***.*****.*

PbaPro1                             EYTANLEYRNEPGALNEAFADIMGNTIE-----SKNWLLGDGIYTPNIPG
P_polymyxa_SC2                      EYTSNLEYYGESGALNEAFSDVIGNDIQ-----RKNWLVGDDIYTPNIAG
B_thermoproteolyticus_P00800        DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                    :**:.* * .*.**:***::*::*. ::       :* :*:.:***.*.*

PbaPro1                             DALRSLSDPTLYNQPDKYSDRYTGSQDNGGVHINSGIINKAYYLAAQGGT
P_polymyxa_SC2                      DALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGN
B_thermoproteolyticus_P00800        DSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYLISQGGT
                                    *:***:*:*: *.:**:**. * *:.****** ******** ** :***.

PbaPro1                             HNGVTVSGIGRDKAVRIFYSTLVNYLTPTSKFAAAKTATIQAAKDLYGAN
P_polymyxa_SC2                      FHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAVIQAAKDLYGAN
B_thermoproteolyticus_P00800        HYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYGST
                                    . **:* *****   :*:* ::.:***.:*.*:  ::*.:*:*.****:.

PbaPro1                             SAEATAITKAYQAVGL-
P_polymyxa_SC2                      SAEATAAAKSFDAVGVN
B_thermoproteolyticus_P00800        SQEVASVKQAFDAVGVK
                                    * *.::  ::::***:
```

Figure 5.6: Alignment of PbaPro1 protein with homologous protease sequences.

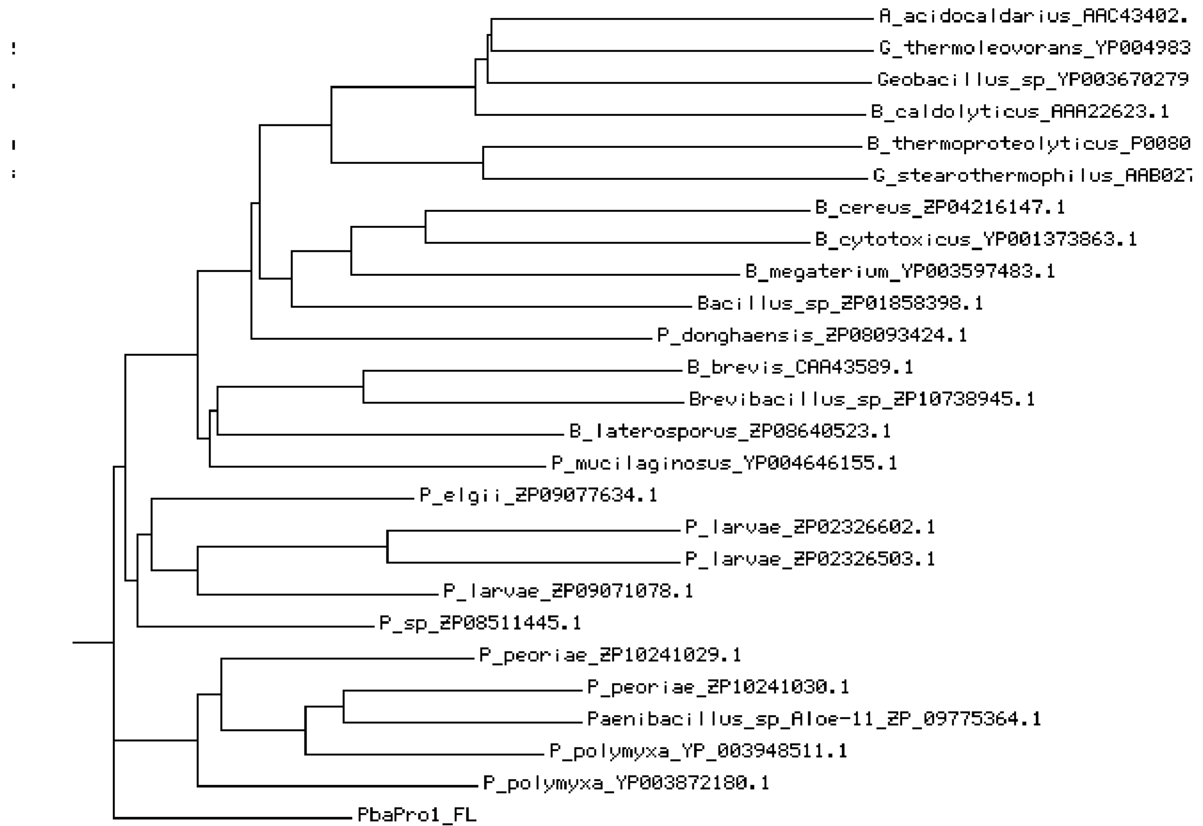


Figure 5.7: Phylogenetic tree for PbaPro1 and homologs.

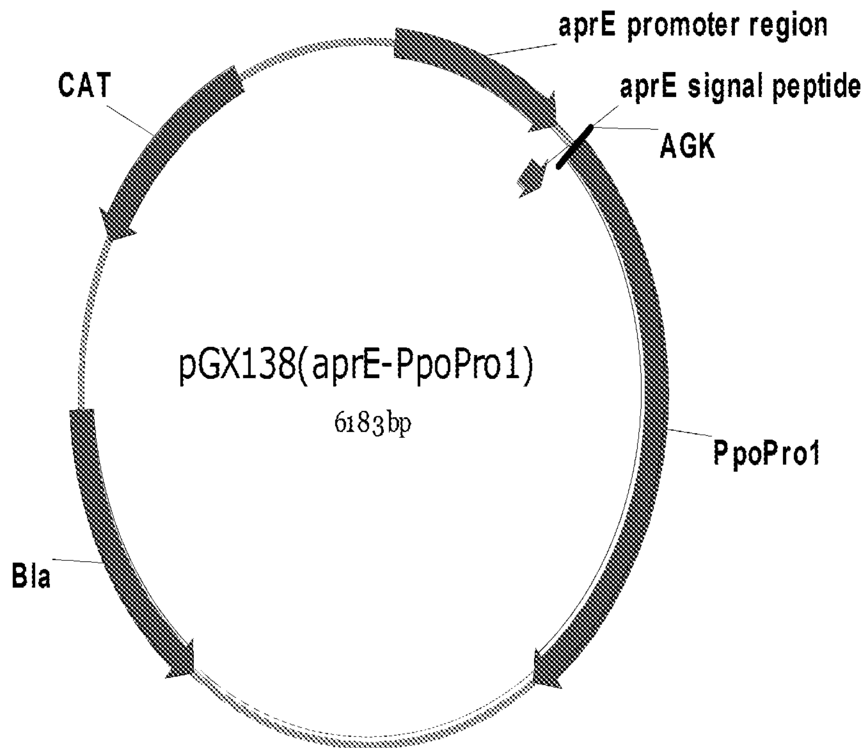


Figure 6.1. The plasmid map of pGX138 (AprE-PpoPro1).

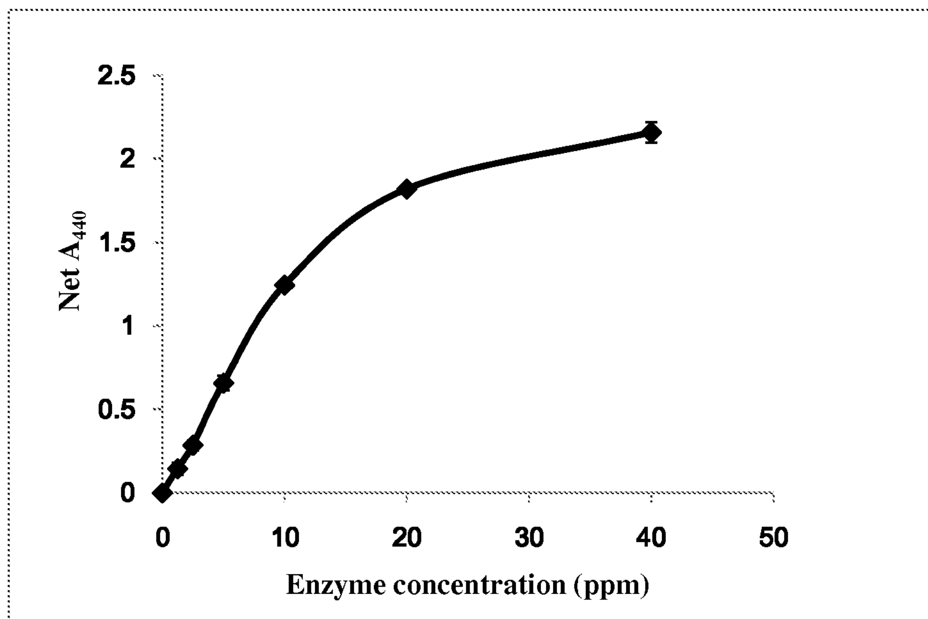


Figure 6.2. Dose response of PpoPro1 in azo-casein assay at pH 7.

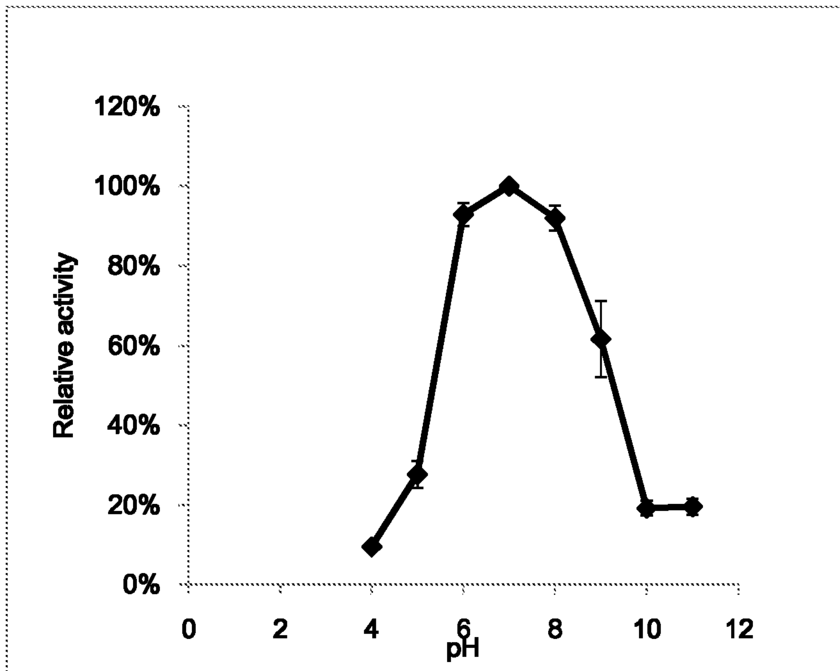


Figure 6.3. pH profile of purified PpoPro1.

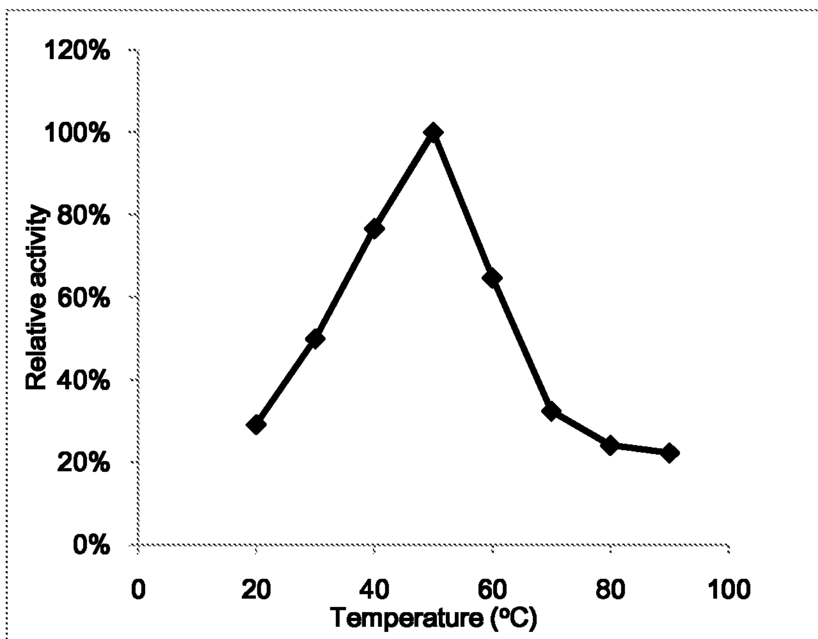


Figure 6.4. Temperature profile of purified PpoPro1.

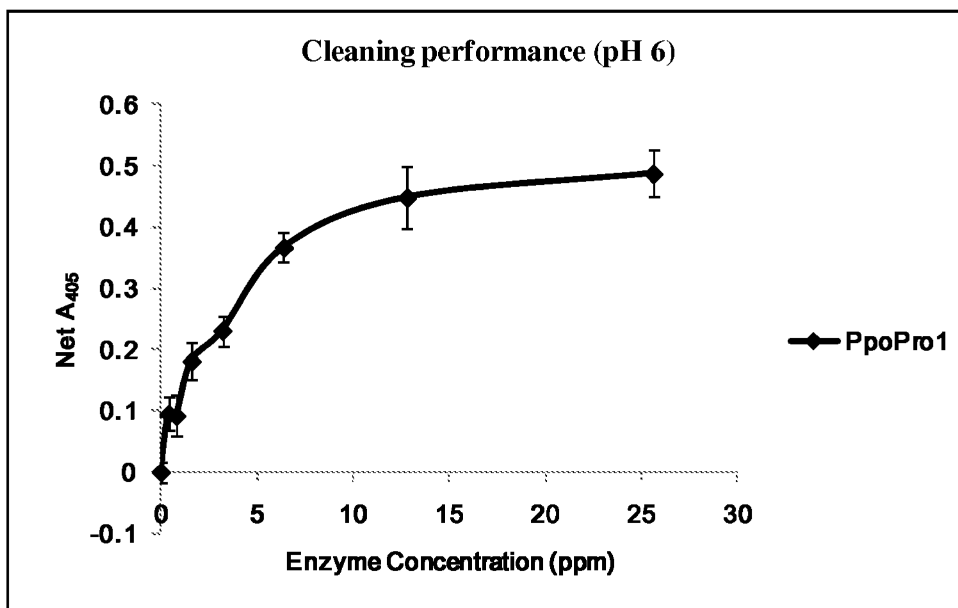


Figure 6.5A: Cleaning performance of PpoPro1 at pH 6 in AT detergent with PAP.

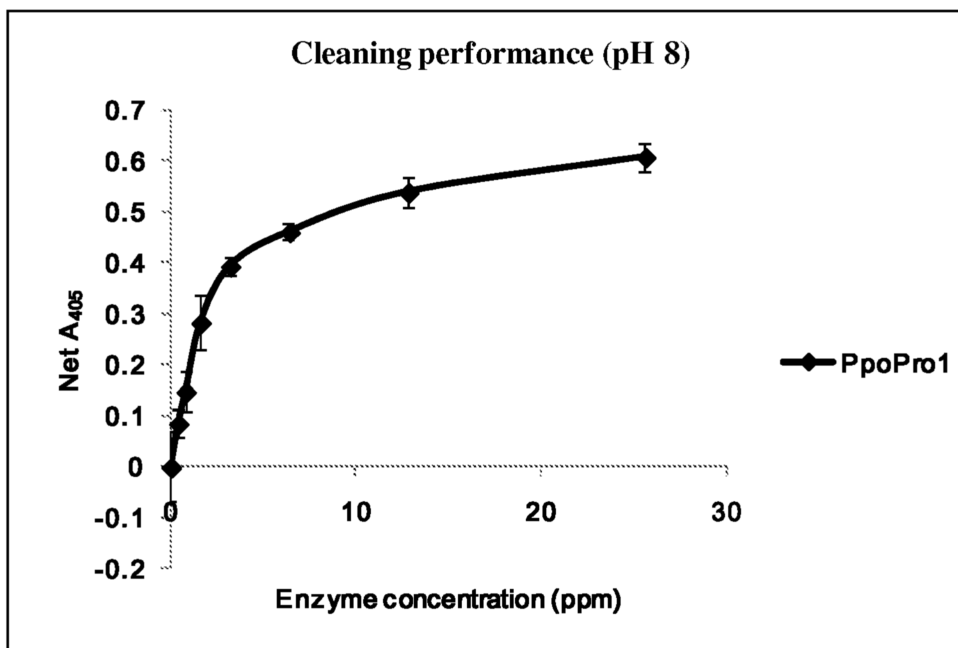


Figure 6.5B: Cleaning performance of PpoPro1 at pH 8 in AT detergent with PAP.

```
CLUSTAL W (1.83) multiple sequence alignment


PpoPro1                            -----ATGTGKGVLGDSKSFTTTASGSSYQLKDTTRGNGIVTYTASNRQS
P_polymyxa_SC2_YP_003948511.1      ---NEATGTGKGVLGDSKSFTTTASGSSYQLKDTTRGNGIVTYTASNRQS
B_thermoproteolyticus_P00800       ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                        :.*.*:*****.*.:.** * : * *:*.******.** *. * :

PpoPro1                            IPGTILTDADNVWN---DPAGVDAHAYAAKTYDYYKAKFGRNSIDGRGLQ
P_polymyxa_SC2_YP_003948511.1      IPGTILTDADNVWN---DPAGVDAHAYAAKTYDYYKAKFGRNSIDGRGLQ
B_thermoproteolyticus_P00800       LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                   :**:: :**** :    *...**** **. ******  ..* * **..

PpoPro1                            LRSTVHYGSRYNNAFWNGSQMTYGDGDGSTFIAFSGDPDVVGHELTHGVT
P_polymyxa_SC2_YP_003948511.1      LRSTVHYGSRYNNAFWNGSQMTYGDGDGSTFIAFSGDPDVVGHELTHGVT
B_thermoproteolyticus_P00800       IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                   :**:***.. ***********.******.***.:**. ***.*****.**

PpoPro1                            EYTSNLEYYGESGALNEAFSDVIGNDIQ-----RKNWLVGDDIYTPNIAG
P_polymyxa_SC2_YP_003948511.1      EYTSNLEYYGESGALNEAFSDVIGNDIQ-----RKNWLVGDDIYTPNIAG
B_thermoproteolyticus_P00800       DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                   :**:.* * .****:***:**::*. ::     . :* :*:*:***.*:*

PpoPro1                            DALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGN
P_polymyxa_SC2_YP_003948511.1      DALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGN
B_thermoproteolyticus_P00800       DSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYLISQGGT
                                   *:*****:*: *.:*****: * *:.****** ******** **::***.

PpoPro1                            FHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAVIQAAKDLYGAN
P_polymyxa_SC2_YP_003948511.1      FHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAVIQAAKDLYGAN
B_thermoproteolyticus_P00800       HYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYGST
                                   .:**:* *****   :*:* *:*:***.:*:**: ***.:*:*.****:.

PpoPro1                            SAEATAAAKSFDAVGVN
P_polymyxa_SC2_YP_003948511.1      SAEATAAAKSFDAVGVN
B_thermoproteolyticus_P00800       SQEVASVKQAFDAVGVK
                                   * *.::. ::******:
```

Figure 6.6. Alignment of PpoPro1 with protease homologs.

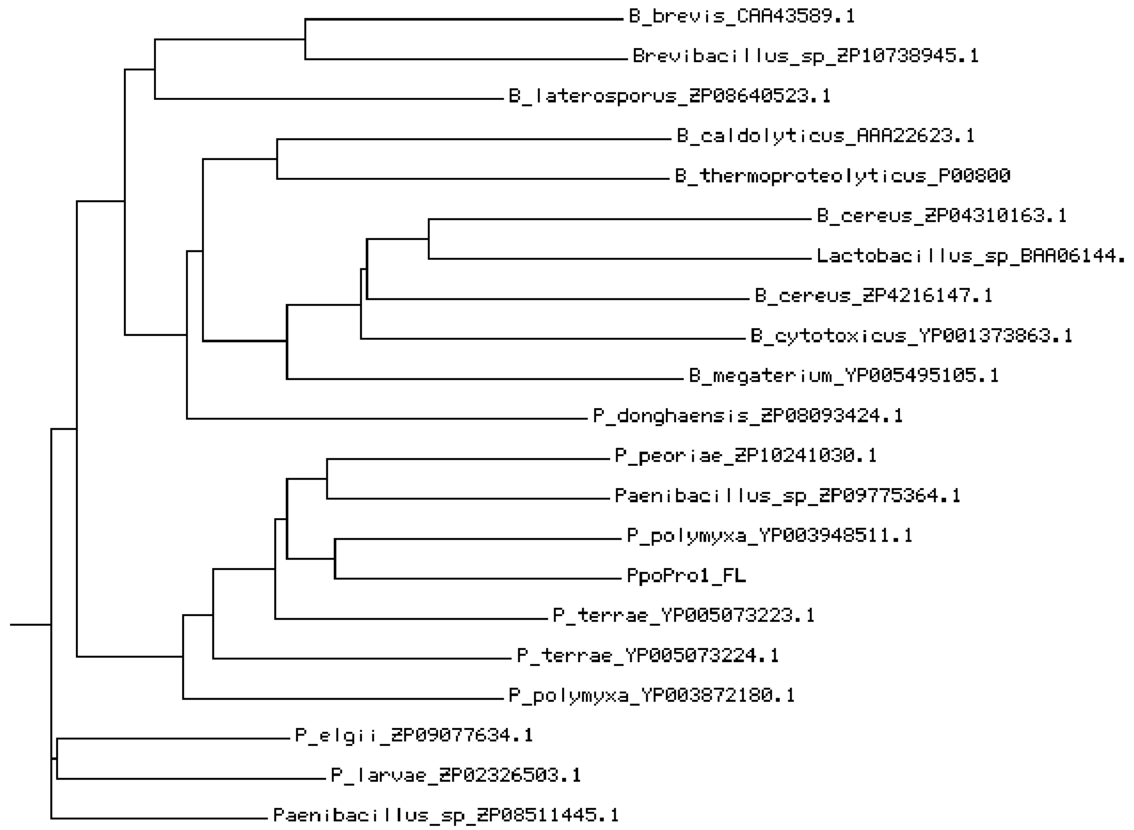


Figure 6.7. Phylogenetic tree of PpoPro1 and homologs.

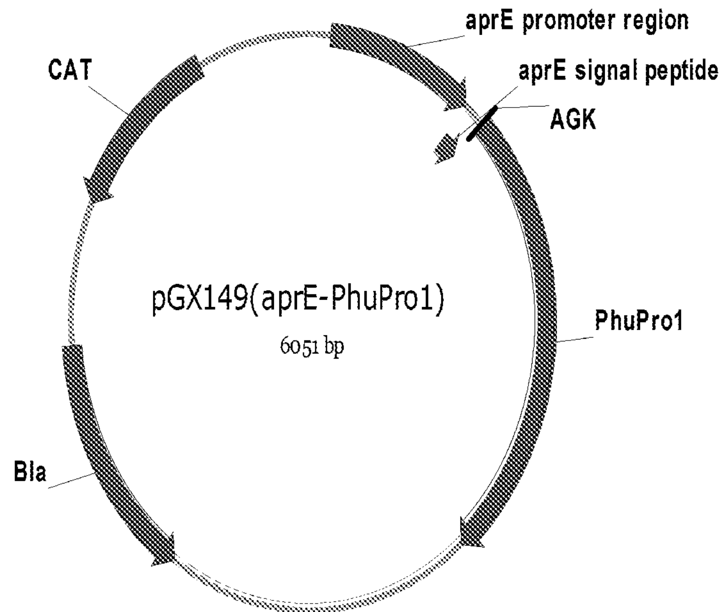


Figure 7.1. The plasmid map of pGX149(AprE-PhuPro1).

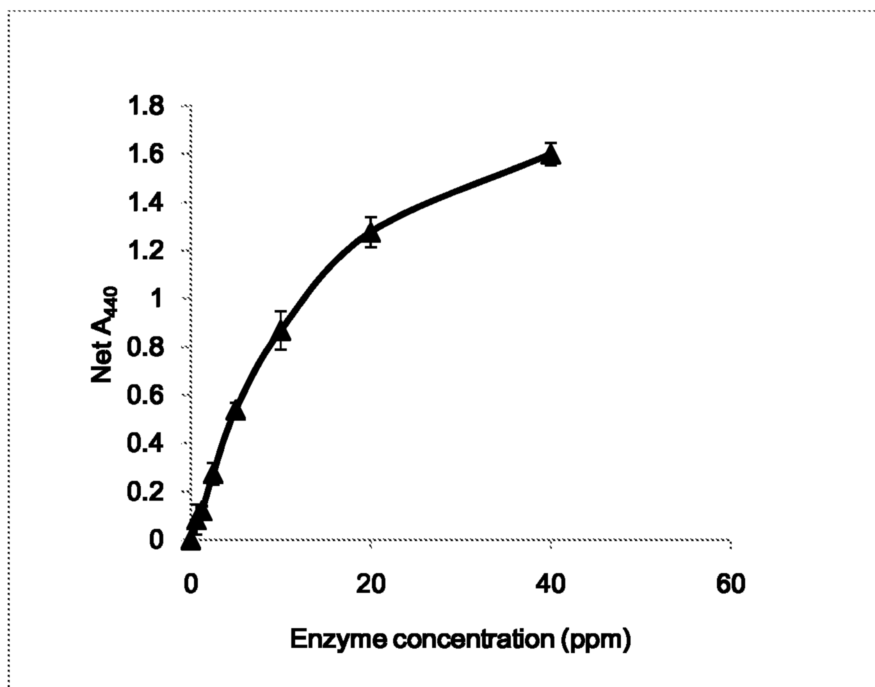


Figure 7.2. Dose response curve of PhuPro1 in azo-casein assay at pH 7.

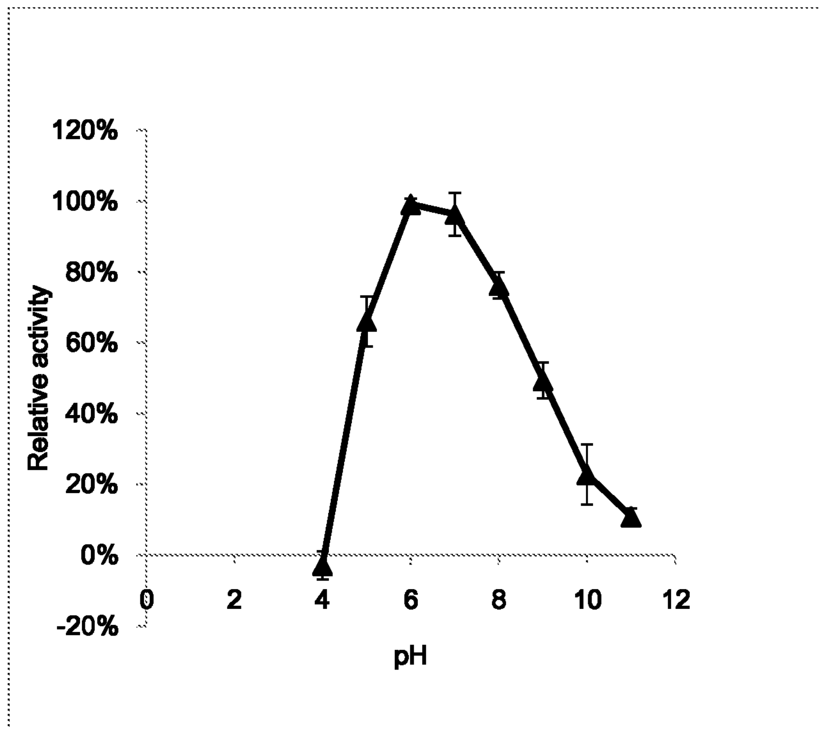


Figure 7.3. pH profile of PhuPro1.

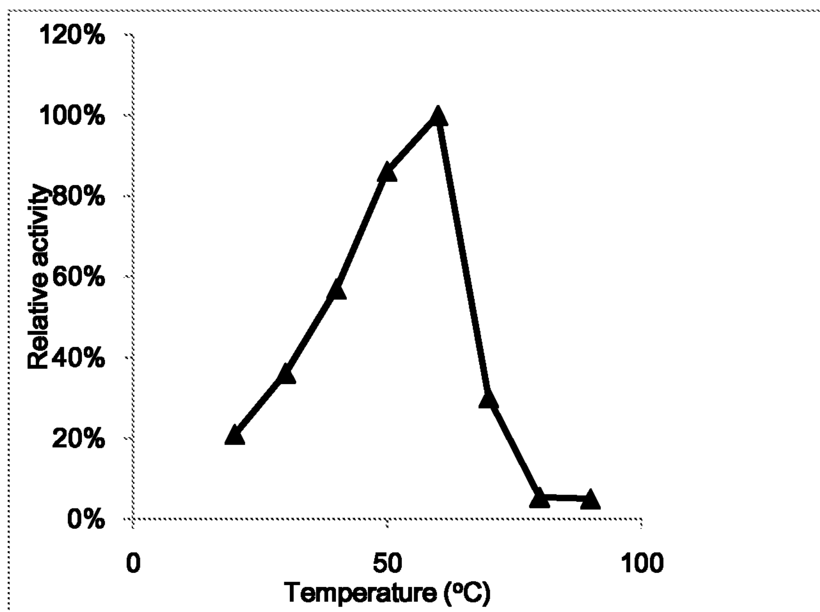


Figure 7.4. Temperature profile of PhuPro1.

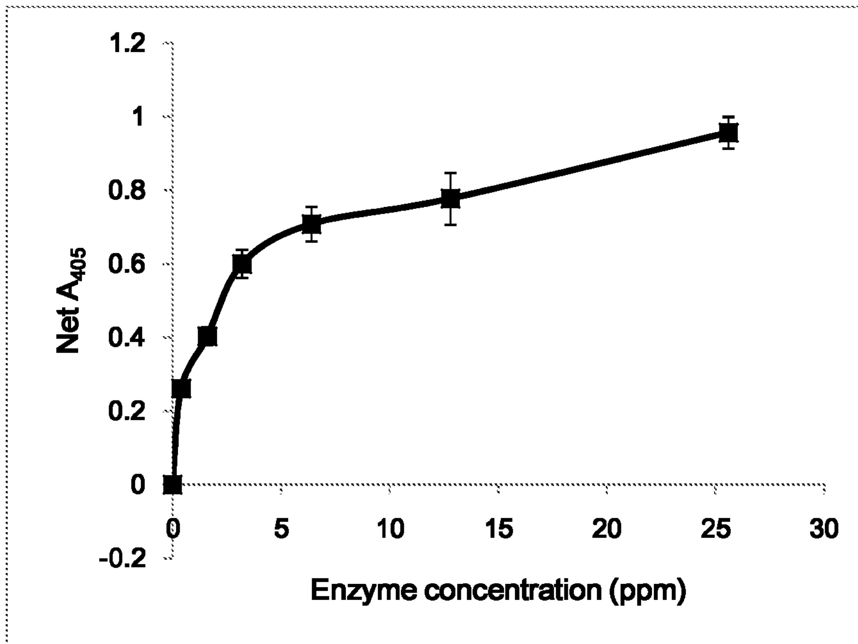


Figure 7.5A. Cleaning performance of PhuPro1 in dish detergent at pH 6.

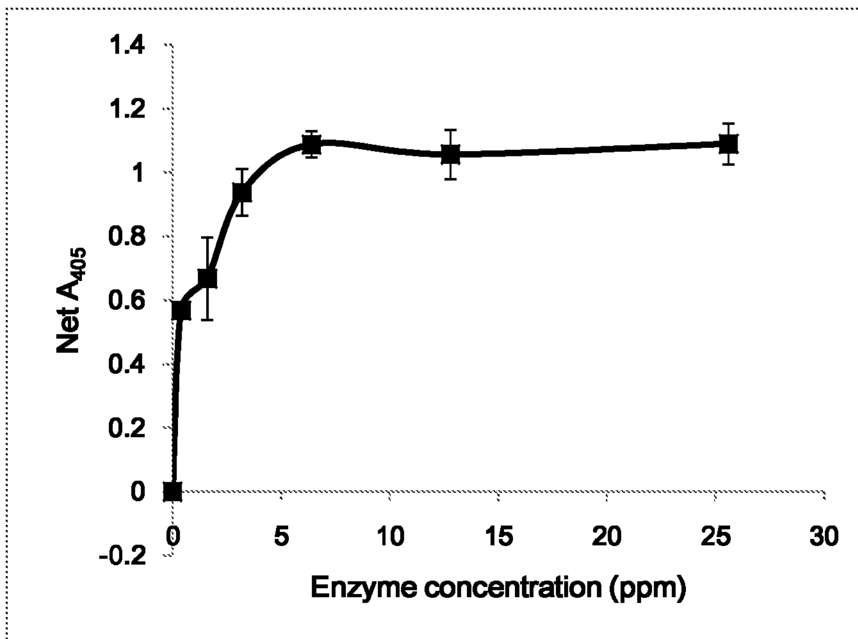


Figure 7.5B. Cleaning performance of PhuPro1 in dish detergent at pH 8.

```
CLUSTAL W (1.83) multiple sequence alignment

PhuPro1                             -----ATGTGKGVLGDTKSFTVGTSGSSYVMTDSTRGKGIQTYTASNRTS
P_terrae_HPL-003_YP_005073223.      -----ATGTGKGVLGDTKSFNTTQSGSSYQLKDTTRGNGIVTYTASNRQT
B_thermoproteolyticus_P00800        ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                         :.*.*:***** *.:..  * : * : *.***:** ** *. * :

PhuPro1                             LPGSTVTSSSSTFN---DPASVDAHAYAQKVYDFYKSNFNRNSIDGNGLA
P_terrae_HPL-003_YP_005073223.      IPGTLLTDADNVWN---DPAGVDAHAYAAKTYDYYKDKFGRNSIDGRGLQ
B_thermoproteolyticus_P00800        LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                    :**:  :.:.. :    *...**** **  .**:**. ..* * **..

PhuPro1                             IRSTTHYSTRYNNAFWNGSQMVYGDGDGSQFIAFSGDLDVVGHELTHGVT
P_terrae_HPL-003_YP_005073223.      LRSTVHYGSRYNNAFWNGSQMTYGDGDGTTFIAFSGDPDVVGHELTHGVT
B_thermoproteolyticus_P00800        IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                    :**:.**.  ***********.******  **.:**. ***.*****.**

PhuPro1                             EYTANLEYYGQSGALNESISDIFGNTIE-----GKNWMVGDAIYTPGVSG
P_terrae_HPL-003_YP_005073223.      EYTSNLDYYGESGALNESFSDIIGNDIQ-----RKNWLVGDDIYTPSIAG
B_thermoproteolyticus_P00800        DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                    :**:.* * .:***:**::***:*. ::       :* :*: :***.::*

PhuPro1                             DALRYMDDPTKGGQPARMADYNNTSADNGGVHTNSGIPNKAYYLLAQGGT
P_terrae_HPL-003_YP_005073223.      DALRSMSNPTLYDQPDHYSNLYKGSSDNGGVHTNSGIINKAYYLLAQGGT
B_thermoproteolyticus_P00800        DSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYLISQGGT
                                    *:** *.:*:  .:* : :.  . : ****** **** *** **::****

PhuPro1                             FGGVNVTGIGRSQAIQIVYRALTYYLTSTSNFSNYRSAMVQASTDLYGAN
P_terrae_HPL-003_YP_005073223.      FHNVTVSGIGRDAAVQIYYSAFTNYLTSTSNFSNTRAAVVQAAKDLYGAN
B_thermoproteolyticus_P00800        HYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYGST
                                    . .*.* ****.   :* * *:* ***.*****: *:* **::.****:.

PhuPro1                             STQTTAVKNSLSAVGIN
P_terrae_HPL-003_YP_005073223.      SAQATAAAKSFDAVGVN
B_thermoproteolyticus_P00800        SQEVASVKQAFDAVGVK
                                    * :.::. :::.***::
```

Figure 7.6: Alignment of PhuPro1 with homologous protease sequences.

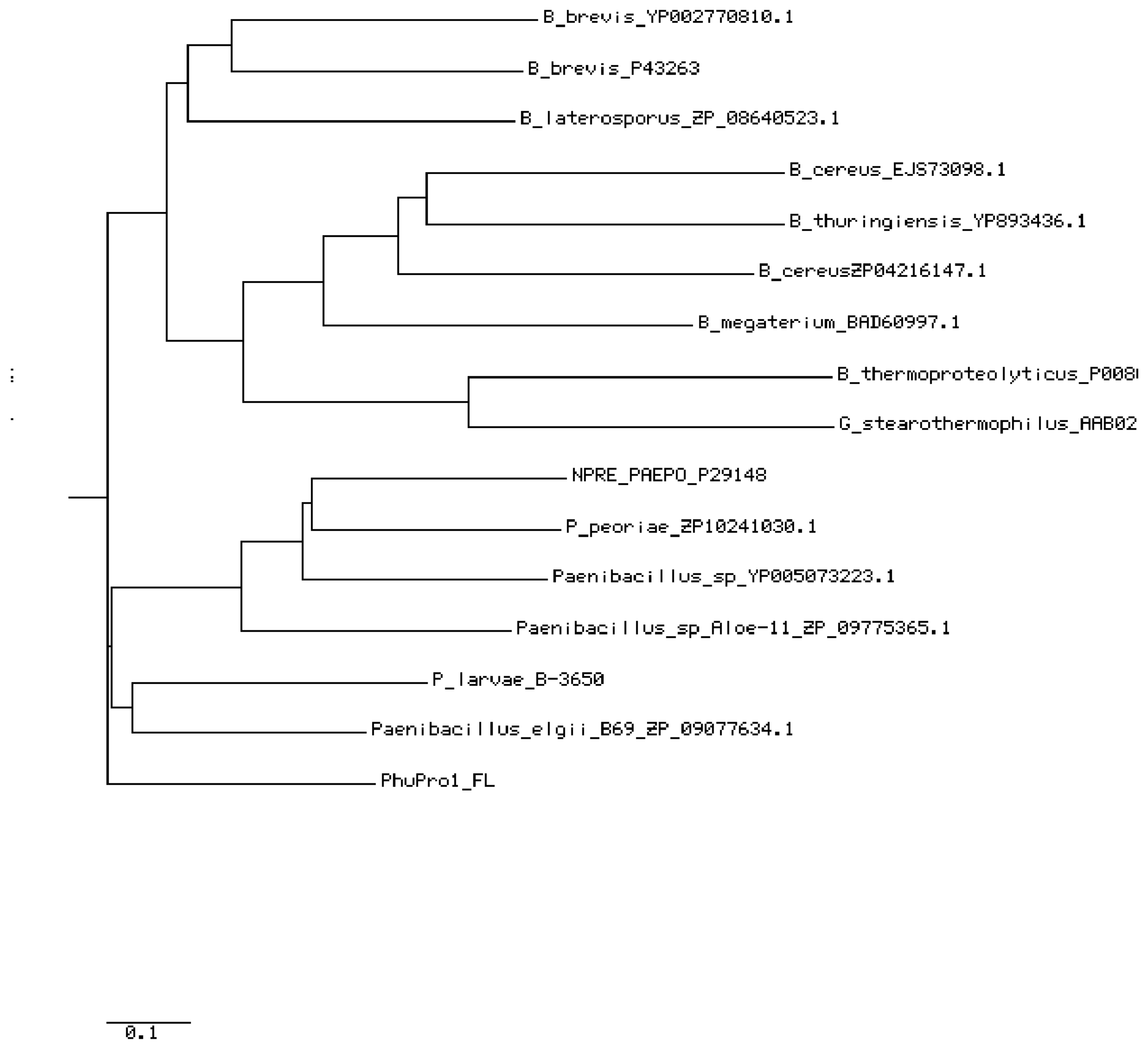


Figure7.7: Phylogenetic tree for PhuPro1 and homologs.

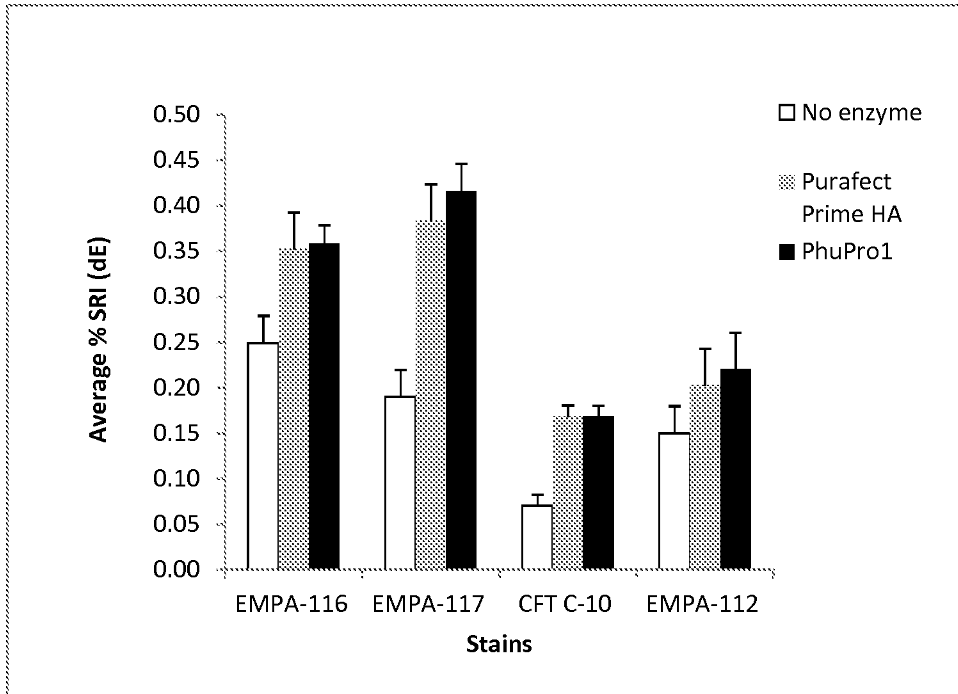


Figure 7.8A: Cleaning performance in Terg-o-Tometer assay at 32°C, on four technical stains.

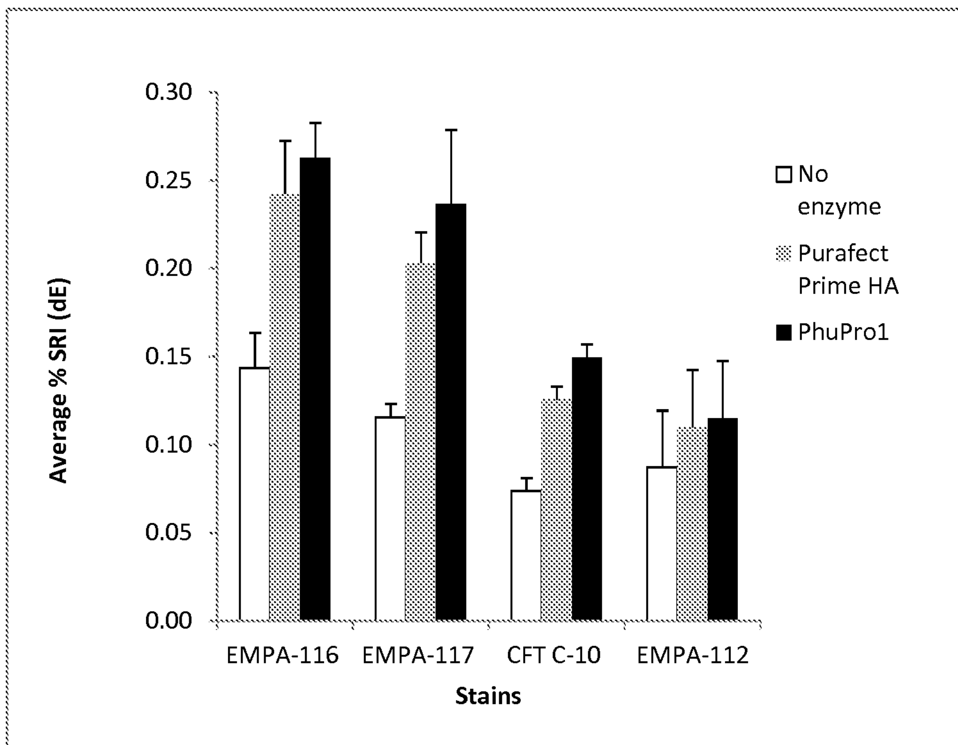


Figure 7.8B: Cleaning performance in Terg-o-Tometer assay at 16°C, on four technical stains.

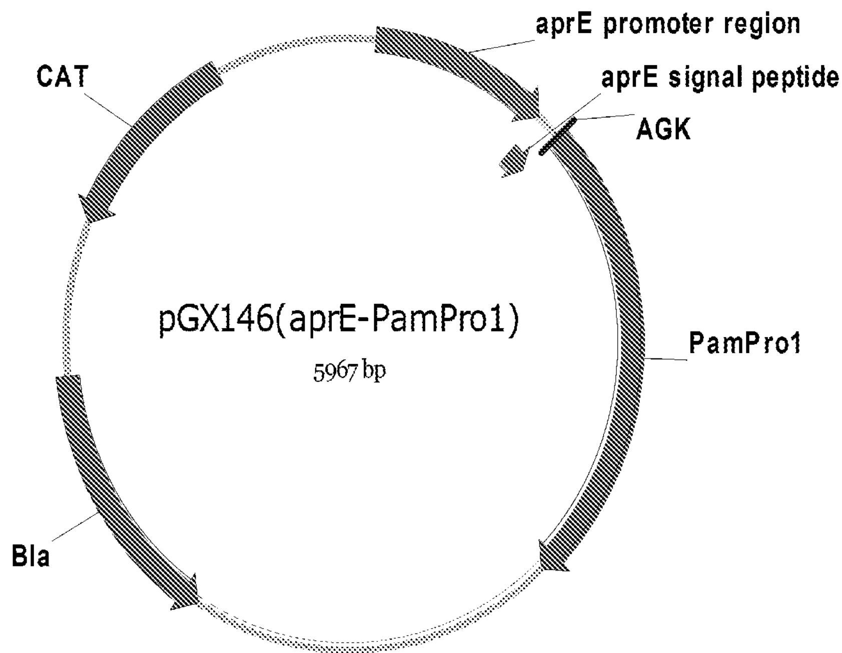


Figure 8.1. The plasmid map of pGX146(AprE-PamPro1).

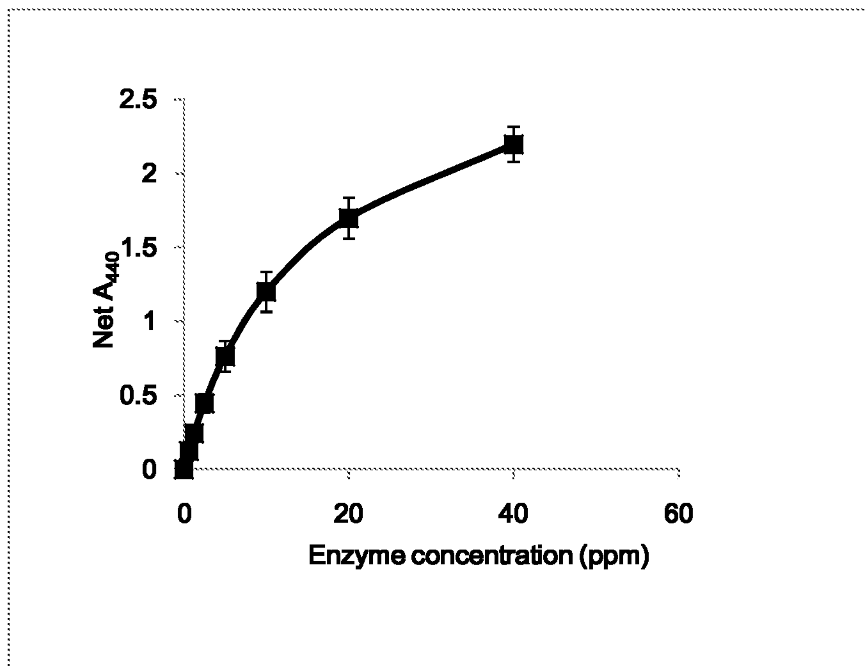


Figure 8.2. Dose response curve of PamPro1 the azo-casein assay at pH 7.

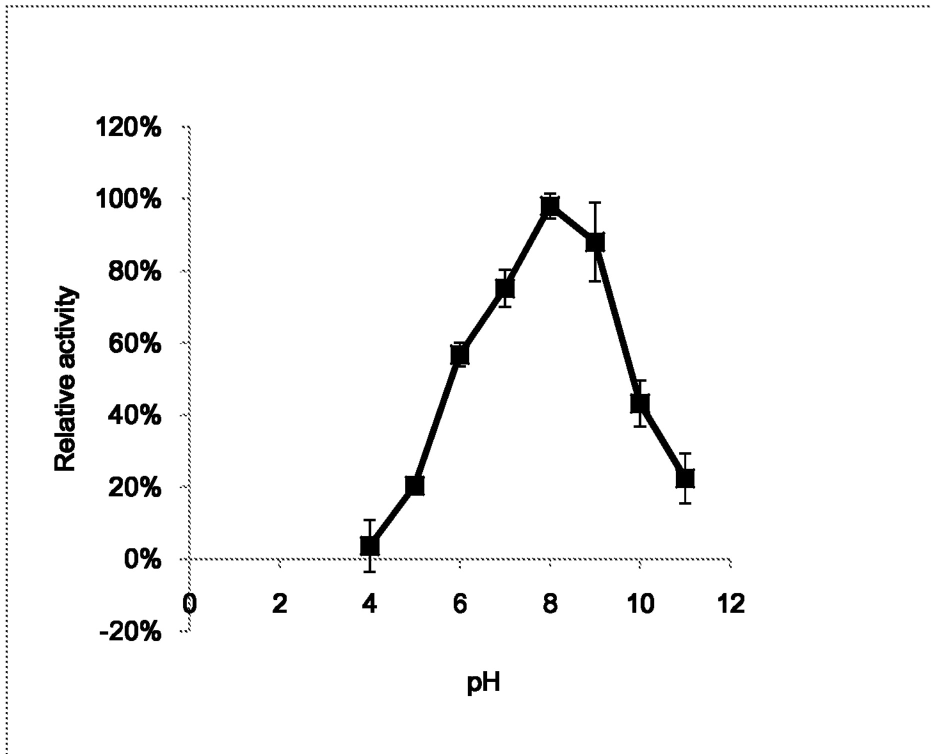


Figure 8.3. pH profile of PamPro1.

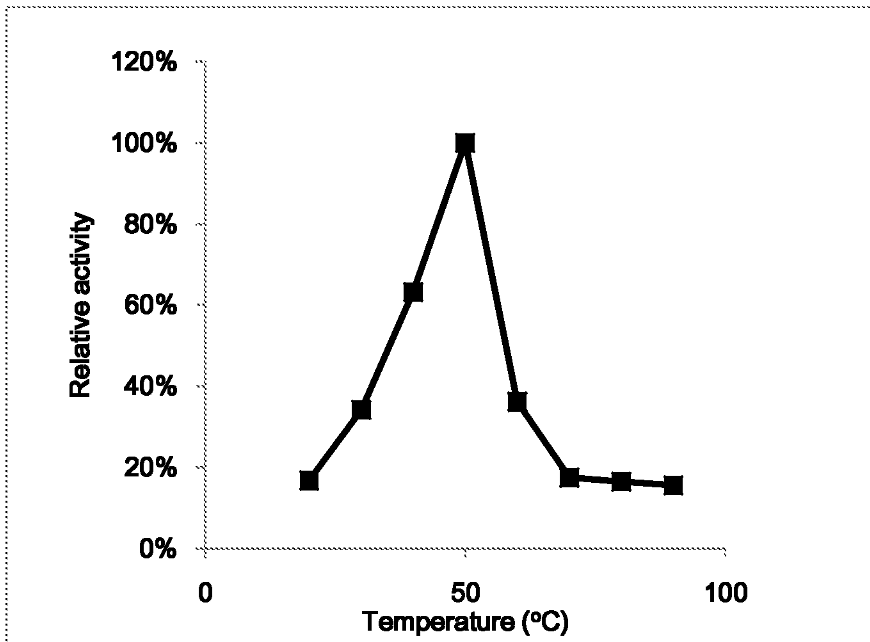


Figure 8.4. Temperature profile of PamPro1.

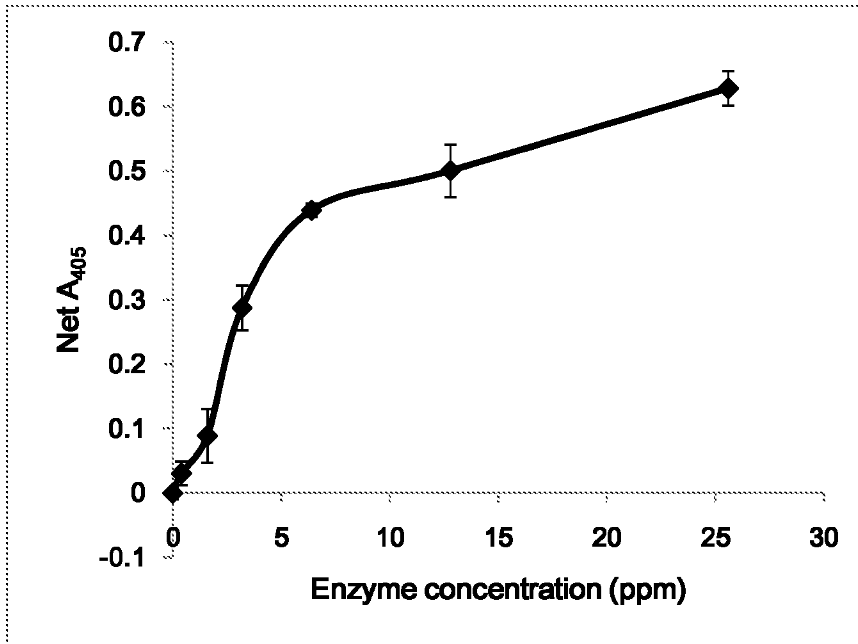


Figure 8.5A: Cleaning performance of PamPro1 in AT dish detergent at pH 6.

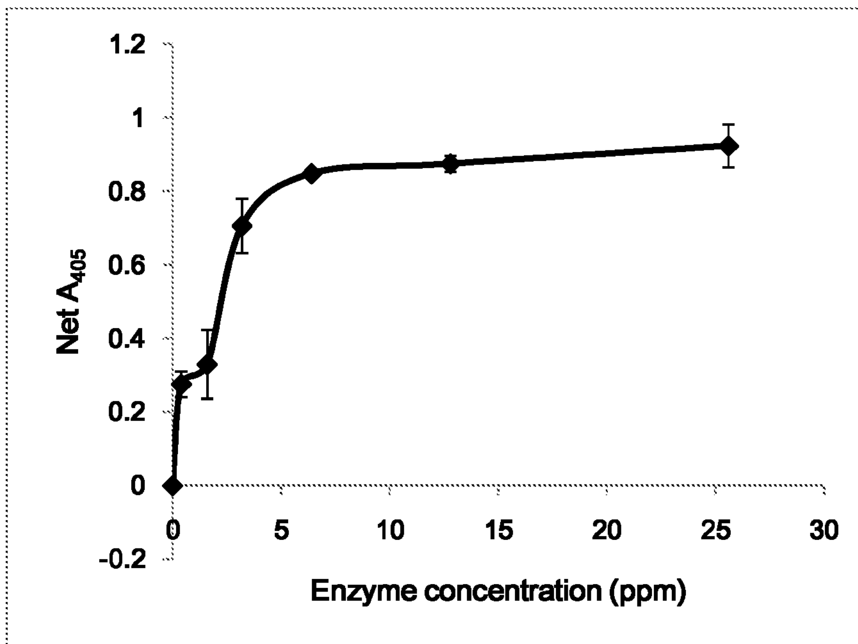


Figure 8.5B: Cleaning performance of PamPro1 in AT dish detergent at pH 8.

```
CLUSTAL W (1.83) multiple sequence alignment


PamPro1                             -----ATGTGTGVLGDTKTLTTTQSGSTFQLKDTTRGNGIQTYTANNGSS
P_peoriae_KCTC                      DIINEATGTGKGVLGDTKSFTTTASGSSYQLRDTTRGNGIVTYTASNRQS
B_thermoproteolyticus_P00800        ITGTSTVGVGRGVLGDQKNINTTYS-TYYYLQDNTRGNGIFTYDAKYRTT
                                         :.*.* ***** *.:.** * : : *:*.****** ** *.   :

PamPro1                             LPGSLLTDSDNVWT---DRAGVDAHAHAAATYDFYKNKFNRNGINGNGLL
P_peoriae_KCTC                      IPGTILTDADNVWN---DPAGVDAHAYAAKTYDYYKEKFNRNSIDGRGLQ
B_thermoproteolyticus_P00800        LPGSLWADADNQFFASYDAPAVDAHYYAGVTYDYYKNVHNRLSYDGNNAA
                                    :**:: :*:** :    * ..**** :*. ***:**: .** . :*..

PamPro1                             IRSTVHYGSNYNNAFWNGAQIVFGDGDGTMFRSLSGDLDVVGHELTHGVI
P_peoriae_KCTC                      LRSTVHYGNRYNNAFWNGSQMTYGDGDGTTFIAFSGDPDVVGHELTHGVT
B_thermoproteolyticus_P00800        IRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAHELTHAVT
                                    :**:***.. ********:*:.:*****  * .:**. ***.*****.*

PamPro1                             EYTANLEYRNEPGALNEAFADIFGNTIQ-----SKNWLLGDDIYTPNTPG
P_peoriae_KCTC                      EYTSNLEYYGESGALNESFSDIIGNDIQ-----RKNWLVGDDIYTPRIAG
B_thermoproteolyticus_P00800        DYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDVYTPGISG
                                    :**:.* * .*.**:**:::**:*. ::       :* :*:*:***  .*

PamPro1                             DALRSLSNPTLYGQPDKYSDRYTGSQDNGGVHINSGIINKAYFLAAQGGT
P_peoriae_KCTC                      DALRSMSNPTLYDQPDHYSNLYRGSSDNGGVHTNSGIINKAYYLLAQGGT
B_thermoproteolyticus_P00800        DSLRSMSDPAKYGDPDHYSKRYTGTQDNGGVHINSGIINKAAYLISQGGT
                                    *:***:*:*: *.:**:**. * *:.****** ******** :* :****

PamPro1                             HNGVTVTGIGRDKAIQIFYSTLVNYLTPTSKFAAAKTATIQAAKDLYGAT
P_peoriae_KCTC                      FHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAVVQAAKDLYGAS
B_thermoproteolyticus_P00800        HYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAAVQSATDLYGST
                                    . **:* *****   :*:* ::.:***.:*.*:  : *.:*:*.****::

PamPro1                             SAEATAITKAYQAVGL-
P_peoriae_KCTC                      SAQATAAAKAFDAVGVN
B_thermoproteolyticus_P00800        SQEVASVKQAFDAVGVK
                                    * :.::  :*::***:
```

Figure 8.6: Alignment of PamPro1 with homologous protease sequences

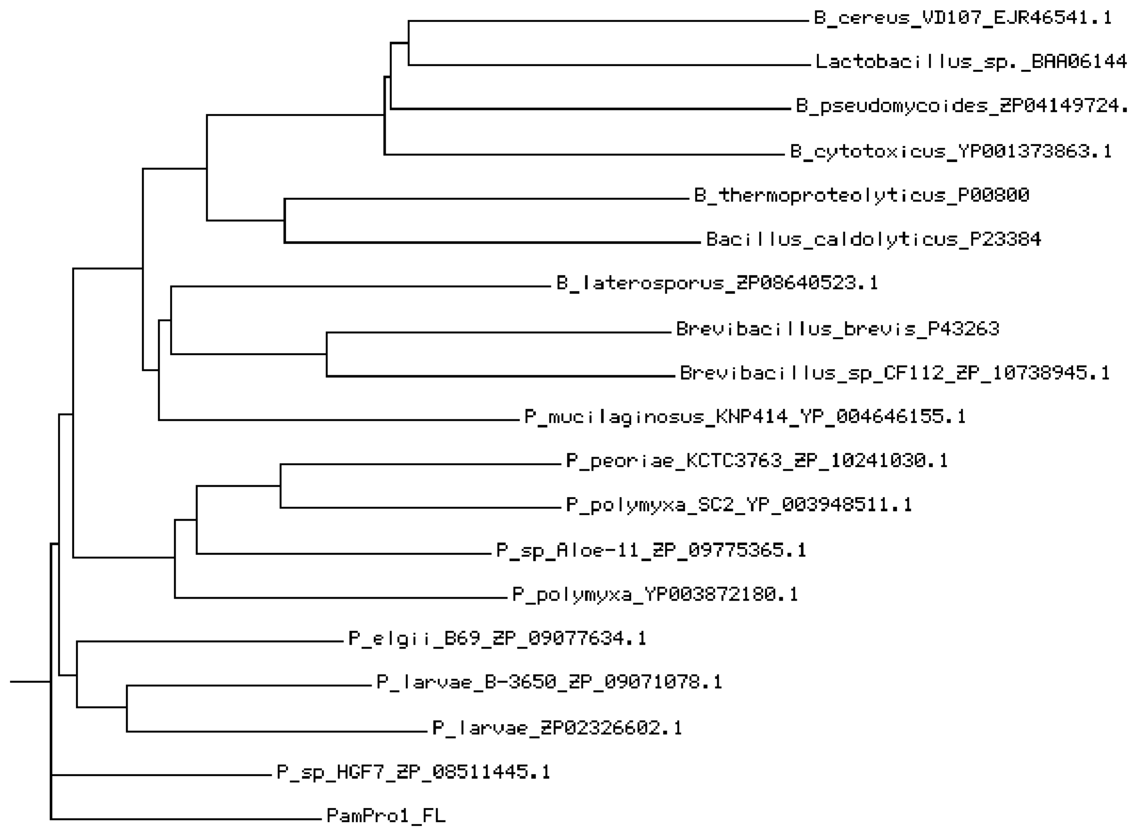


Figure 8.7: Phylogenetic tree for PamPro1 and homologs

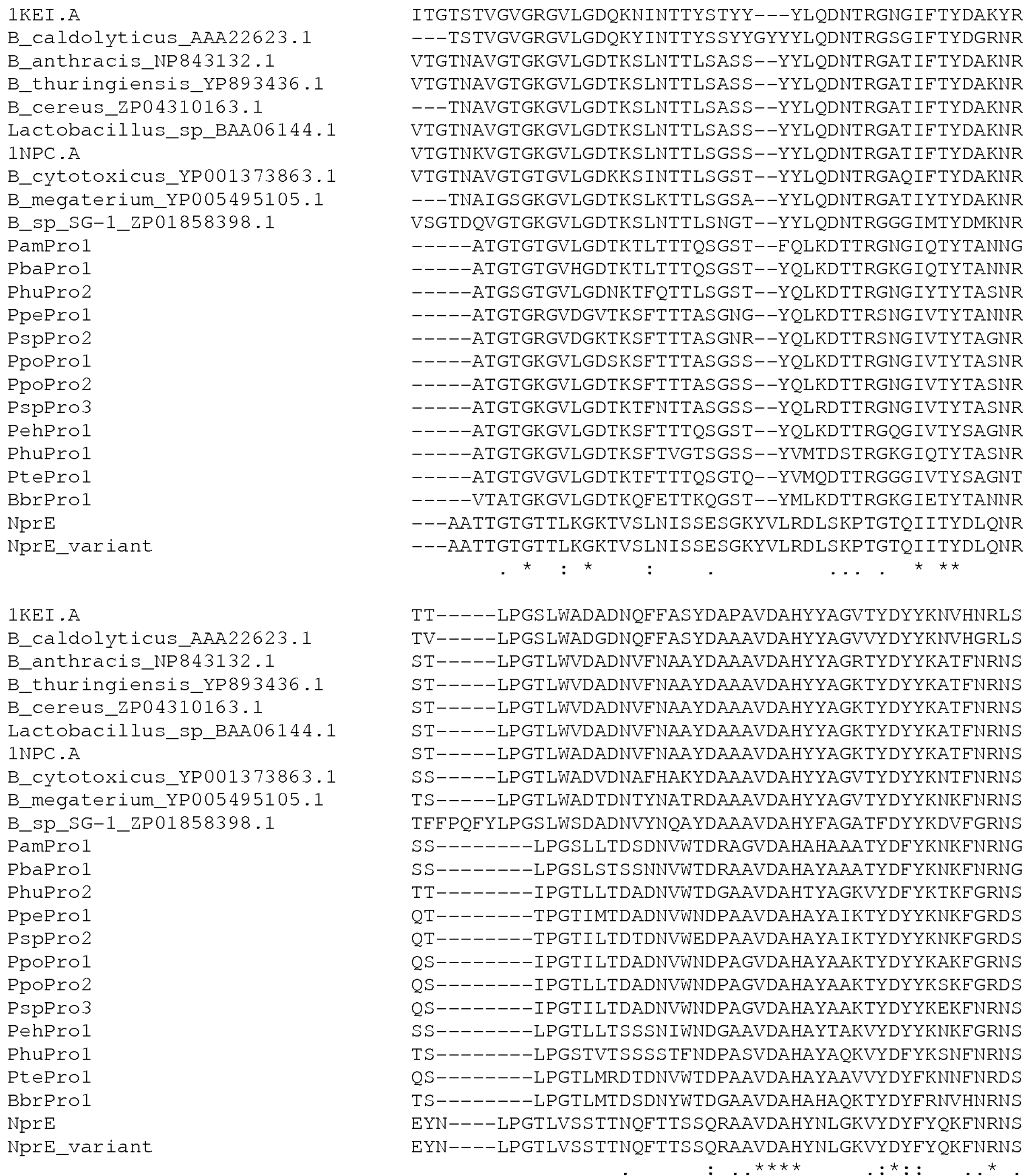
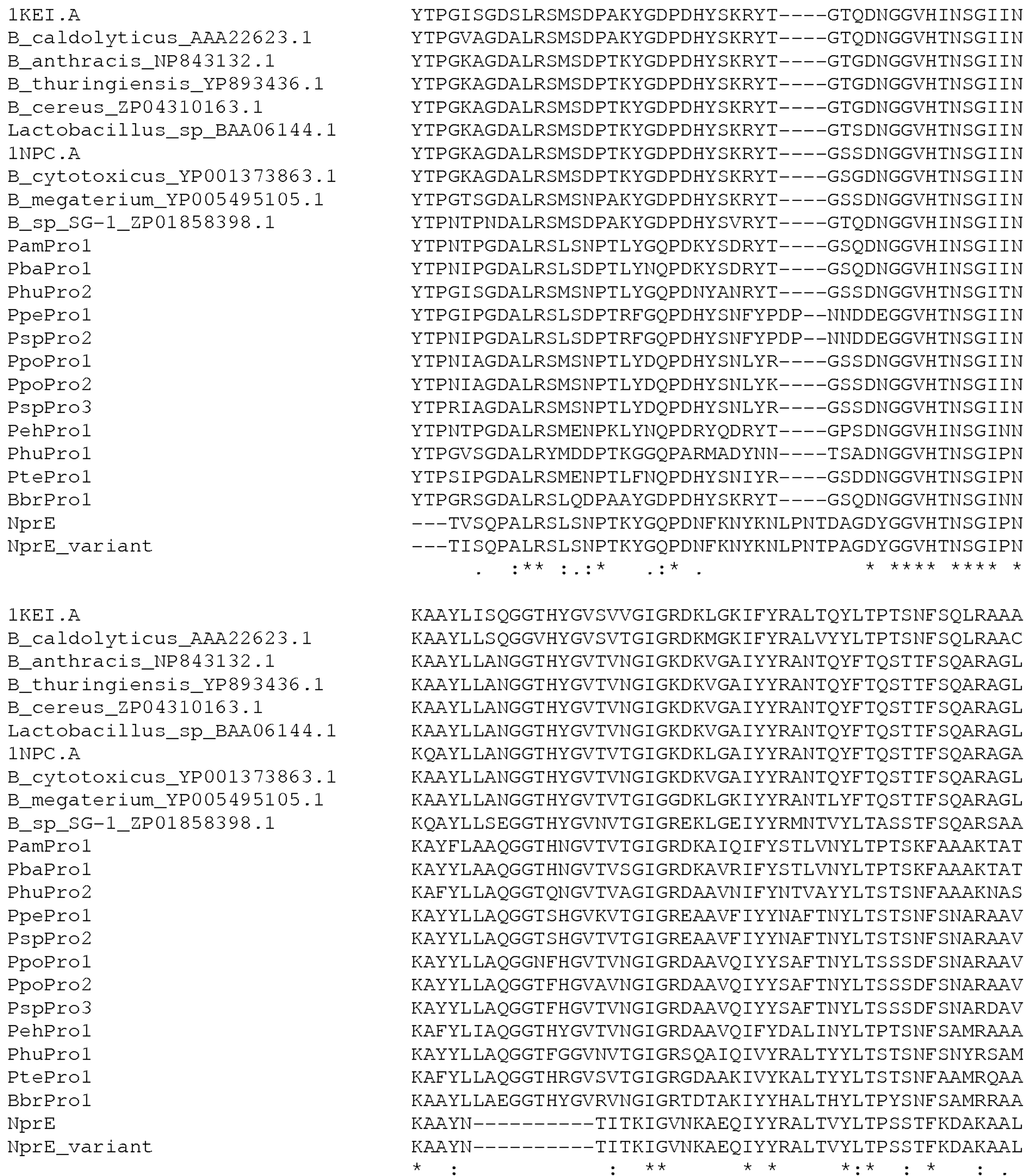


Figure 9.1A CLUSTAL 2.0.10 multiple sequence alignment of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.

```
1KEI.A                          YDGNNAAIRSSVHYSQGYNNAFWNGSQMVYGDGDGQTFIPLSGGIDVVAH
B_caldolyticus_AAA22623.1       YDGSNAAIRSTVHYGRGYNNAFWNGSQMVYGDGDGQTFLPFSGGIDVVGH
B_anthracis_NP843132.1          INDAGAPLKSTVHYGSRYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGH
B_thuringiensis_YP893436.1      INDAGAPLKSTVHYGSRYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGH
B_cereus_ZP04310163.1           INDAGAPLKSTVHYGSRYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGH
Lactobacillus_sp_BAA06144.1     INDAGAPLKSTVHYGSKYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGH
1NPC.A                          INDAGAPLKSTVHYGSNYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGH
B_cytotoxicus_YP001373863.1     INDAGAALKSTVHYGSNYNNAFWNGSQMVYGDGDGVTFTSLSGGIDVIGH
B_megaterium_YP005495105.1      YDNAGAPLKSTVHYSSGYNNAFWNGSQMVYGDGDGTTFVPLSGGLDVIGH
B_sp_SG-1_ZP01858398.1          YDNKGTTIQSSVHYSKNYNNAFWNGSQMVYGDGDGTTFIPLSGGLDVVAH
PamPro1                         INGNGLLIRSTVHYGSNYNNAFWNGAQIVFGDGDGTMFRSLSGDLDVVGH
PbaPro1                         IDGNGLLIRSTVHYGSNYKNAFWNGAQIVYGDGDGIEFGPFSGDLDVVGH
PhuPro2                         LDGNGLLIRSSVHYSSRYNNAFWNGTQIVFGDGDGSTFIPLSGDLDVVGH
PpePro1                         IDGRGMQIRSTVHYGKKYVNAFWNGSQMTYGDGDGSTFTFFSGDPDVVGH
PspPro2                         IDGRGMQIRSTVHYGKKYNNAFWNGSQMTYGDGDGSTFTFFSGDPDVVGH
PpoPro1                         IDGRGLQLRSTVHYGSRYNNAFWNGSQMTYGDGDGSTFIAFSGDPDVVGH
PpoPro2                         VDGRGLQLRSTVHYGSRYNNAFWNGSQMTYGDGDGSTFIAFSGDPDVVGH
PspPro3                         IDGRGLQLRSTVHYGNRYNNAFWNGSQMTYGDGDGTTFIAFSGDPDVVGH
PehPro1                         IDGNGFQLKSTVHYSSRYNNAFWNGVQMVYGDGDGVTFIPFSADPDVIGH
PhuPro1                         IDGNGLAIRSTTHYSTRYNNAFWNGSQMVYGDGDGSQFIAFSGDLDVVGH
PtePro1                         LDGRGMAIKSTVHYGSRYNNAFWNGTQIAYGDGDGTTFRAFSGDLDVIGH
BbrPro1                         YDGNGAVIRSTVHYSTRYNNAFWNGSQMVYGDGDGTTFLPLSGGLDVVAH
NprE                            YDNKGGKIVSSVHYGSRYNNAAWIGDQMIYGDGDGSFFSPLSGSMDVTAH
NprE_variant                    YDNKGGKIVSSVHYGSRYNNAAWIGDQMIYGDGDGILFSPLSGSLDVTAH
                                 :. .  : *:.**.  * ** * * *: :*****  *  :*.. ** .*

1KEI.A                          ELTHAVTDYTAGLIYQNESGAINEAISDIFGTLVEFYANKNPDWEIGEDV
B_caldolyticus_AAA22623.1       ELTHAVTDYTAGLVYQNESGAINEAMSDIFGTLVEFYANRNPDWEIGEDI
B_anthracis_NP843132.1          ELTHAVTEYSSDLIYQNESGALNEAISDVFGTLVEYYDNRNPDWEIGEDI
B_thuringiensis_YP893436.1      ELTHAVTEYSSDLIYQNESGALNEAISDVFGTLVEFYDNRNPDWEIGEDI
B_cereus_ZP04310163.1           ELTHAVTEYSSDLIYQNESGALNEAISDVFGTLVEFYDNRNPDWEIGEDI
Lactobacillus_sp_BAA06144.1     ELTHAVTEYSSDLIYQNESGALNEAISDVFGTLVEYYDNRNPDWEIGEDI
1NPC.A                          ELTHAVTENSSNLIYQNESGALNEAISDIFGTLVEFYDNRNPDWEIGEDI
B_cytotoxicus_YP001373863.1     ELTHAVTEYSSNLIYQYESGALNEAISDIFGTLVEYYDNRNPDWEIGEDI
B_megaterium_YP005495105.1      ELTHAVTERSSNLIYQYESGALNEAISDIFGTLVEYYDNRNPDWEIGEDI
B_sp_SG-1_ZP01858398.1          ELTHAVTDTSSDLVYQNESGALNEAISDIFGTLVEYHENHNPDFEIGEDI
PamPro1                         ELTHGVIEYTANLEYRNEPGALNEAFADIFGNTIQ-----SKNWLLGDDI
PbaPro1                         ELTHGVIEYTANLEYRNEPGALNEAFADIMGNTIE-----SKNWLLGDGI
PhuPro2                         ELSHGVIEYTSNLQYLNESGALNESYADVLGNSIQ-----AKNWLIGDDV
PpePro1                         ELTHGVTEFTSNLEYYGESGALNEAFSDIIGNDID-----GANWLLGDGI
PspPro2                         ELTHGVTEFTSNLEYYGESGALNEAFSDIIGNDID-----GTSWLLGDGI
PpoPro1                         ELTHGVTEYTSNLEYYGESGALNEAFSDVIGNDIQ-----RKNWLVGDDI
PpoPro2                         ELTHGVTEYTSNLEYYGESGALNEAFSDVIGNDIQ-----RKNWLVGDDI
PspPro3                         ELTHGVTEYTSNLEYYGESGALNEAFSDIIGNDIQ-----RKNWLVGDDI
PehPro1                         ELTHGVTEHTAGLEYYGESGALNESISDIIGNAID-----GKNWLIGDLI
PhuPro1                         ELTHGVTEYTANLEYYGQSGALNESISDIFGNTIE-----GKNWMVGDAI
PtePro1                         ELTHGITEKTAGLIYQGESGALNESISDVFGNTIQ-----GKNWLIGDDI
BbrPro1                         ELTHAVTERTAGLVYQNESGALNESMSDIFGAMVD-----NDDWLMGEDI
NprE                            EMTHGVTQETANLNYENQPGALNESFSDVFGYFND-----TEDWDIGEDI
NprE_variant                    EMTHGVTQETANLNYENQPGALNESFSDVFGYFND-----TEDWDIGEDI
                                *::*.: : ::.* *  :.**:**: :*::*   :       .: :*: :
```

Figure 9.1B CLUSTAL 2.0.10 multiple sequence alignment of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.



```
1KEI.A                          YTPGISGDSLRSMSDPAKYGDPDHYSKRYT----GTQDNGGVHINSGIIN
B_caldolyticus_AAA22623.1       YTPGVAGDALRSMSDPAKYGDPDHYSKRYT----GTQDNGGVHTNSGIIN
B_anthracis_NP843132.1          YTPGKAGDALRSMSDPTKYGDPDHYSKRYT----GTGDNGGVHTNSGIIN
B_thuringiensis_YP893436.1      YTPGKAGDALRSMSDPTKYGDPDHYSKRYT----GTGDNGGVHTNSGIIN
B_cereus_ZP04310163.1           YTPGKAGDALRSMSDPTKYGDPDHYSKRYT----GTGDNGGVHTNSGIIN
Lactobacillus_sp_BAA06144.1     YTPGKAGDALRSMSDPTKYGDPDHYSKRYT----GTSDNGGVHTNSGIIN
1NPC.A                          YTPGKAGDALRSMSDPTKYGDPDHYSKRYT----GSSDNGGVHTNSGIIN
B_cytotoxicus_YP001373863.1     YTPGKAGDALRSMSDPTKYGDPDHYSKRYT----GSGDNGGVHTNSGIIN
B_megaterium_YP005495105.1      YTPGTSGDALRSMSNPAKYGDPDHYSKRYT----GSSDNGGVHTNSGIIN
B_sp_SG-1_ZP01858398.1          YTPNTPNDALRSMSDPAKYGDPDHYSVRYT----GTQDNGGVHINSGIIN
PamPro1                         YTPNTPGDALRSLSNPTLYGQPDKYSDRYT----GSQDNGGVHINSGIIN
PbaPro1                         YTPNIPGDALRSLSDPTLYNQPDKYSDRYT----GSQDNGGVHINSGIIN
PhuPro2                         YTPGISGDALRSMSNPTLYGQPDNYANRYT----GSSDNGGVHTNSGITN
PpePro1                         YTPGIPGDALRSLSDPTRFGQPDHYSNFYPDP--NNDDEGGVHTNSGIIN
PspPro2                         YTPNIPGDALRSLSDPTRFGQPDHYSNFYPDP--NNDDEGGVHTNSGIIN
PpoPro1                         YTPNIAGDALRSMSNPTLYDQPDHYSNLYR----GSSDNGGVHTNSGIIN
PpoPro2                         YTPNIAGDALRSMSNPTLYDQPDHYSNLYK----GSSDNGGVHTNSGIIN
PspPro3                         YTPRIAGDALRSMSNPTLYDQPDHYSNLYR----GSSDNGGVHTNSGIIN
PehPro1                         YTPNTPGDALRSMENPKLYNQPDRYQDRYT----GPSDNGGVHINSGINN
PhuPro1                         YTPGVSGDALRYMDDPTKGGQPARMADYNN----TSADNGGVHTNSGIPN
PtePro1                         YTPSIPGDALRSMENPTLFNQPDHYSNIYR----GSDDNGGVHTNSGIPN
BbrPro1                         YTPGRSGDALRSLQDPAAYGDPDHYSKRYT----GSQDNGGVHTNSGINN
NprE                            ---TVSQPALRSLSNPTKYGQPDNFKNYKNLPNTDAGDYGGVHTNSGIPN
NprE_variant                    ---TISQPALRSLSNPTKYGQPDNFKNYKNLPNTPAGDYGGVHTNSGIPN
                                      .  :** :.:*    .:* .             * **** **** *

1KEI.A                          KAAYLISQGGTHYGVSVVGIGRDKLGKIFYRALTQYLTPTSNFSQLRAAA
B_caldolyticus_AAA22623.1       KAAYLLSQGGVHYGVSVTGIGRDKMGKIFYRALVYYLTPTSNFSQLRAAC
B_anthracis_NP843132.1          KAAYLLANGGTHYGVTVNGIGKDKVGAIYYRANTQYFTQSTTFSQARAGL
B_thuringiensis_YP893436.1      KAAYLLANGGTHYGVTVNGIGKDKVGAIYYRANTQYFTQSTTFSQARAGL
B_cereus_ZP04310163.1           KAAYLLANGGTHYGVTVNGIGKDKVGAIYYRANTQYFTQSTTFSQARAGL
Lactobacillus_sp_BAA06144.1     KAAYLLANGGTHYGVTVNGIGKDKVGAIYYRANTQYFTQSTTFSQARAGL
1NPC.A                          KQAYLLANGGTHYGVTVTGIGKDKLGAIYYRANTQYFTQSTTFSQARAGA
B_cytotoxicus_YP001373863.1     KAAYLLANGGTHYGVTVNGIGKDKVGAIYYRANTQYFTQSTTFSQARAGL
B_megaterium_YP005495105.1      KAAYLLANGGTHYGVTVTGIGGDKLGKIYYRANTLYFTQSTTFSQARAGL
B_sp_SG-1_ZP01858398.1          KQAYLLSEGGTHYGVNVTGIGREKLGEIYYRMNTVYLTASSTFSQARSAA
PamPro1                         KAYFLAAQGGTHNGVTVTGIGRDKAIQIFYSTLVNYLTPTSKFAAAKTAT
PbaPro1                         KAYYLAAQGGTHNGVTVSGIGRDKAVRIFYSTLVNYLTPTSKFAAAKTAT
PhuPro2                         KAFYLLAQGGTQNGVTVAGIGRDAAVNIFYNTVAYYLTSTSNFAAAKNAS
PpePro1                         KAYYLLAQGGTSHGVKVTGIGREAAVFIYYNAFTNYLTSTSNFSNARAAV
PspPro2                         KAYYLLAQGGTSHGVTVTGIGREAAVFIYYNAFTNYLTSTSNFSNARAAV
PpoPro1                         KAYYLLAQGGNFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAV
PpoPro2                         KAYYLLAQGGTFHGVAVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARAAV
PspPro3                         KAYYLLAQGGTFHGVTVNGIGRDAAVQIYYSAFTNYLTSSSDFSNARDAV
PehPro1                         KAFYLIAQGGTHYGVTVNGIGRDAAVQIFYDALINYLTPTSNFSAMRAAA
PhuPro1                         KAYYLLAQGGTFGGVNVTGIGRSQAIQIVYRALTYYLTSTSNFSNYRSAM
PtePro1                         KAFYLLAQGGTHRGVSVTGIGRGDAAKIVYKALTYYLTSTSNFAAMRQAA
BbrPro1                         KAAYLLAEGGTHYGVRVNGIGRTDTAKIYYHALTHYLTPYSNFSAMRRAA
NprE                            KAAYN----------TITKIGVNKAEQIYYRALTVYLTPSSTFKDAKAAL
NprE_variant                    KAAYN----------TITKIGVNKAEQIYYRALTVYLTPSSTFKDAKAAL
                                *   :              :  **       * *     *:*  : *   : .
```

Figure 9.1C CLUSTAL 2.0.10 multiple sequence alignment of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.

```
1KEI.A                              VQSATDLYGSTSQEVASVKQAFDAVGVK
B_caldolyticus_AAA22623.1           VQAAADLYGSTSQEVNSVKQAFNAVGVY
B_anthracis_NP843132.1              VQA-------------------------
B_thuringiensis_YP893436.1          VQAATDLYGASSAEVAAVKQSYSAVGVN
B_cereus_ZP04310163.1               VQAAADLYGASSAEVAAVKQSYSAVGVN
Lactobacillus_sp_BAA06144.1         VQAAADLYGASSAEVAAVKQSYSAVGVN
1NPC.A                              VQAAADLYGANSAEVAAVKQSFSAVGVN
B_cytotoxicus_YP001373863.1         VQAAADLYGANSAEVTAVKQSYDAVGVK
B_megaterium_YP005495105.1          VQAAADLYGSGSQEVISVGKSFDAVGVQ
B_sp_SG-1_ZP01858398.1              VQAASDLYGSNSPEVQSVNQSFDAVGIN
PamPro1                             IQAAKDLYGATSAEATAITKAYQAVGL-
PbaPro1                             IQAAKDLYGANSAEATAITKAYQAVGL-
PhuPro2                             IQAAKDLYGTGSSYVTSVTNAFRAVGL-
PpePro1                             IQAAKDFYGADSLAVTSAIKSFDAVGIK
PspPro2                             IQAAKDFYGADSLAVTSAIQSFDAVGIK
PpoPro1                             IQAAKDLYGANSAEATAAAKSFDAVGVN
PpoPro2                             IQAAKDLYGANSAEATAAAKSFDAVGVN
PspPro3                             VQAAKDLYGASSAQATAAAKSFDAVGVN
PehPro1                             IQAATDLYGANSSQVNAVKKAYTAVGVN
PhuPro1                             VQASTDLYGANSTQTTAVKNSLSAVGIN
PtePro1                             ISSATDLFGANSAQVNSVKAAYAAVGI-
BbrPro1                             VLSATDLFGANSRQVQAVNAAYDAVGVK
NprE                                IQSARDLYGSQDAASVEAAWNAVGL---
NprE_variant                        IQSARDLYGSQDAASVEAAWNAVGL---
                                    : :
```

Figure 9.1D CLUSTAL 2.0.10 multiple sequence alignment of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.

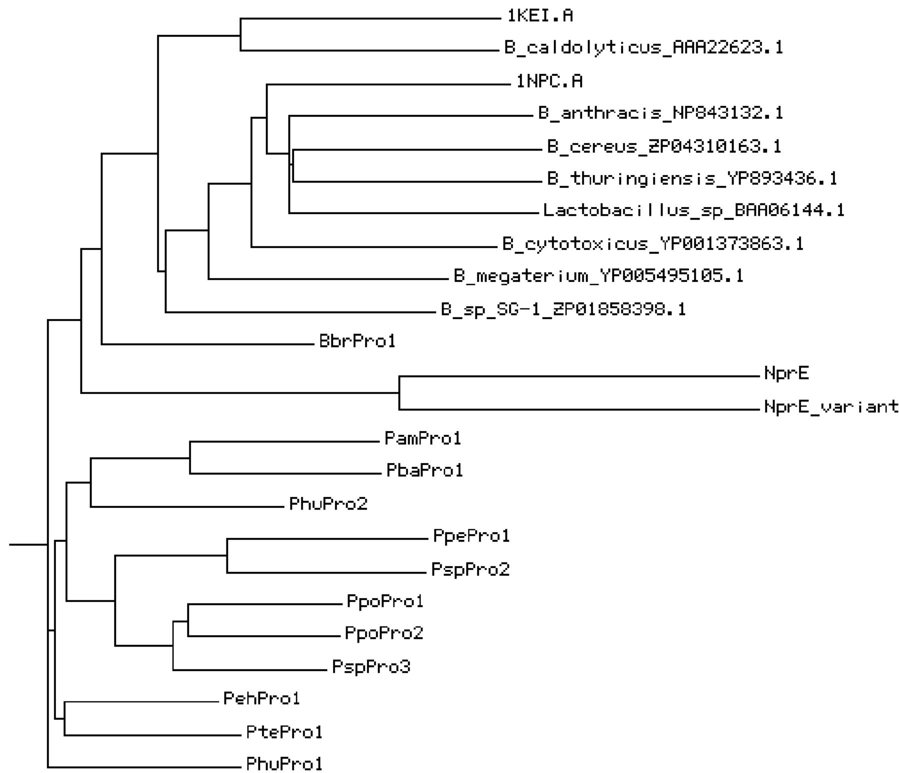


Figure 9.2 The phylogenetic tree of the various Paenibacillus metalloproteases with other bacterial metalloprotease homologs.