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(54) Title: BACILLUS SP. I-52, A DETERGENT- AND OXIDANT-STABLE HALOALKALOPHILIC PROTEASE THEREFROM, A GENE ENCODING THE SAME AND A USE THEREOF

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(57) Abstract: The present invention is directed to an isolated *Bacillus calusii* I-52, a protease isolated therefrom, an isolated gene encoding the same and the use thereof. Particularly, the present invention is directed to an isolated *Bacillus calusii* I-52 which produces a protease retaining activity thereof under extreme conditions, a protease isolated therefrom and having such an activity, an isolated gene encoding the same, and industrial uses thereof such as laundry industry, leather industry, food industry, fee industry and waste treating industry. Since the pretease of the present invention retains enzymatic activity under extreme conditions such as alkine solution, presense of detergent and high temperature and has a broad substrate specificity capable of removing foods, blood or body fluids, it can be used as an effective ingredient of detergents for fabric, kitchen or contact lense as well as for processing of foods, feeds or leather and treating organic wastes.



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BACILLUS SP. I-52, A DETERGENT- AND OXIDANT-STABLE
HALOALKALOPHILIC PROTEASE THEREFROM, A GENE
ENCODING THE SAME AND A USE THEREOF

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Technical Field

Priority of Korean Patent Application No. 2005-13731 filed on February 18, 2005 is claimed. The disclosure of the co-pending application is incorporated herein in its entirety by reference.

10

The present invention relates to a *Bacillus* sp. strain, *Bacillus clausii* I-52, producing a protease, a protease mass-produced therefrom, a gene encoding the same and a use thereof, more precisely, *Bacillus clausii* I-52 producing a protease which was isolated from west coast mudflat of Korea, a protease therefrom which retains an enzyme activity under severe conditions, a gene encoding the same and a use thereof.

15

Background Art

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A protease has been widely used in variety of industrial fields, for example, for the treatment of human disease such as thrombosis, as a laundry detergent component, as a contact lens detergent component, for the modification of milk protein, for silk degumming, for improving feed, for leather soaking, for dehairing, for

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recovering silver from x-ray film and for regeneration of ultrafiltration membrane used for the concentration of protein solution. The availability of enzymes produced from a microorganism has been well known and the industrial enzyme market is now over a billion dollar. Among the industrial enzymes, proteases take more than 60%, which are the biggest parts of all, and thus the marketability of the protease is growing more and more. Proteases have a long history of industrial use in such fields as medicinal industry, dairy farming, leather industry, food industry, feed industry, film making, waste matter disposal, detergent industry, etc.

As detergent additives, proteases have been added to such detergents as laundry detergent, contact lens detergent, denture detergent, etc, and approximately 25% of industrial proteases are used as detergent additives. Since a bacterial protease was first used as a detergent additive in the name of BIO-40 in 1956, 13 billion tons of proteases have been annually produced as of today as detergent additives. Proteases for detergent have to retain wide substrate specificity enabling the elimination of food and blood or body fluid components and stability not to lose its enzyme activity under severe conditions such as alkali or surfactant environment and high temperature. In the past, proteases isolated from plants had been generally

used. But, recently, proteases isolated from microorganisms, in particular *Bacillus* sp., have been popular.

Proteases are divided into exoproteases and endoproteases according to operating properties thereof; serine proteases, aspartic proteases and cysteine proteases according to the functional group of the active site; alkaline, neutral and acidic proteases according to the optimum pH for the enzyme activity; or intracellular proteases and extracellular proteases according to their location. Among them, alkaline proteases are mainly used for detergent industry, which are generally isolated from a *Bacillus* sp. microorganism such as *Bacillus subtilis* and *B. licheniformis*, and fungi such as *Aspergillus oryzae* but preferably isolated from a *Bacillus* sp. microorganism.

For industrial use, a protease has to be very stable so as to keep its enzyme activity under severe external environmental conditions which are extremely variable, compared with those of internal conditions. For example, under the presence of surfactants, most proteases lose much or the entire of their activities, so proteases for the use as a detergent component are very limited. Under severe external environmental conditions such as exposure on severe pH, heavy metal or the level of oxidation-reduction, which are highly variable and strongly affect the enzyme

activity of a protease, the enzyme loses its activity as soon as those conditions are out of an acceptable range. Therefore, to use protease industrially, it is important to keep the enzyme activity of a protease under extremely
5 unstable physical and chemical conditions.

More precisely, to use a protease industrially, first, the protease activity should not be affected by surfactants; second, the protease activity should not be affected by an oxygen-bleaching agent; third, the protease
10 has to be an alkaline protease to keep its activity under high pH condition; forth, the protease activity should not be affected by low temperature (20~30°C); fifth, the protease activity should not be affected by a heavy metal; and sixth, the protease activity does not have to require
15 metal ions such as calcium. As an additive for detergent, a halophilic protease is more preferred since the protease can keep its enzyme activity under the high concentration of salt (more than 10% salt), which is because salt has to be added as a core material during the process for
20 preparing the protease in the form of granules.

The value of a halophilic protease is especially welcomed by the high salinity food industry. The unique taste and flavor of Geotgals (pickled fish) and fermented sauces which are Korean traditional foods prepared by
25 preserving with high salt and depends on the amino acids

produced from proteins therein by the action of a protease. And the fermented food includes salt more than 15% for long-term storage, thus enzyme activity of a protease has to be kept under the high concentration of salt.

5 U.S. Patent Nos. 534075 and 6136553 and Korean Patent No. 258740 describe that a novel protease having an excellent activity and stability has been produced to fulfill the above conditions from a conventional protease by inducing a specific mutation. And also, US Patent No.
10 6162635 and other recent reports describe the attempt to screen strains producing a protease suitable for the extreme conditions from the natural world such as soil and to use them industrially. However, most mutants have a problem of a low enzyme activity and a protease that can
15 meet the above conditions has not been successfully produced, yet. In particular, a protease which is stable in the presence of oxygen-bleaching agent and surfactant is extremely difficult to find.

Thus, the present inventors screened various strains
20 from the west coast mudflat of Korea, and among them, isolated a specific strain which can produce high amounts of a protease that retains its enzyme activity in the presence of heavy metals, anionic surfactants and oxidants used for oxygen-bleaching agents (ex; hydrogen peroxide,
25 sodium perborate, etc.) as well as under the condition of a

wide range of pH have been identified. Further, the inventors isolated a protease having those characteristics therefrom, examined conditions for mass-production of the protease, and finally completed the present invention by
5 establishing a simple and easy method for mass-production of an alkaline protease from the strain.

Disclosure

Technical Problem

10 It is an object of the present invention to provide a microorganism that is able to produce a protease retaining its activity under severe physical and chemical conditions, particularly, in the presence of high concentration of surfactants (more than 0.5%; ex; sodium dodecyl sulfate,
15 etc.) for over 3 days and in the presence of high concentration of an oxidant (more than 0.5%; ex; hydrogen peroxide, sodium perborate, etc.) for over 3 days.

It is another object of the present invention to provide a protease retaining its enzyme activity under
20 severe physical and chemical conditions, which is isolated from the microorganism and a gene encoding the same.

It is a further object of the present invention to provide a method for isolation and mass-production of the protease.

It is also an object of the present invention to provide industrial uses of the protease.

Technical Solution

5 1. Summary of the invention

To achieve the above objects, the present invention provides a *Bacillus clausii* I-52 strain (KCTC 10277BP) producing a protease retaining its enzyme activity under severe physical and chemical conditions.

10 The present invention also provides a culture supernatant of the strain harboring the protease activity.

 The present invention further provides a protease isolated from the strain, a gene encoding the same, an expression vector containing the gene and a transformant
15 transfected with the expression vector.

 The present invention also provides an expression vector for the insertion into *Bacillus* genome containing the above gene and a transformant transfected with the expression vector.

20 The present invention provides a method for isolation of the protease from the strain.

 The present invention provides a method for mass-production of the protease from the strain.

The present invention provides a detergent composition containing a culture supernatant of the strain and/or the protease of the invention.

5 The present invention provides a method for processing foods and animal feeds by using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant and/or the protease isolated therefrom.

10 The present invention provides a food or feed additive containing the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant and/or the protease as an effective ingredient.

15 The present invention provides a method for treating textiles, fibers and leather by using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant and/or the protease.

The present invention provides a method for treating paper and pulp by using the culture supernatant of the strain and/or the protease.

20 The present invention provides a method for decomposing organic waste by using the culture supernatant of the strain and/or the protease.

The terms used in the present invention are described in detail hereinafter.

25

2. Definitions

The term "protease (proteolytic enzyme)" includes all polypeptides having a protease activity, including a peptidase and/or a proteinase activity.

5 The phrase "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a structural gene (i.e., a protein coding sequence, such as a protease of the invention) in a host compatible with such sequences. Expression cassettes
10 include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, e.g., transcription termination signals. Additional elements necessary or helpful in effecting expression may also be used, e.g., enhancers. Thus,
15 expression cassettes also include plasmids, expression vectors, recombinant viruses, any form of recombinant "naked DNA" vector, and the like.

A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It
20 will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes (e.g., a cell membrane, a viral lipid envelope, etc.). Vectors include,
25 but are not limited to, replicons (e.g., RNA replicons,

bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA (e.g., plasmids, viruses, and the like, see, e.g., U.S. Patent No. 5,217,879), and include both the expression and non-expression plasmids. Where a recombinant microorganism or a cultured cell is described as a host of the "expression vector", this includes both extra-chromosomal circular and linear DNA and a DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

As used herein, the term "promoter" includes all sequences capable of driving transcription of a coding sequence in a cell, e.g., a bacterial cell. Thus, promoters used in the constructs of the invention include *cis*-acting transcriptional control elements and regulatory sequences that are involved in temporal or spatial transcriptional regulation and/or regulation or variation of transcription rate of a gene. For example, a promoter can be a *cis*-acting transcriptional control element, including an enhancer, a promoter, a transcription terminator, an origin of replication, a chromosomal

integration sequence, 5' and 3' untranslated regions, or an intronic sequence, which are involved in transcriptional regulation. These *cis*-acting sequences typically interact with proteins or other biomolecules to carry out (turn
5 on/off, regulate, modulate, etc.) transcription. "Constitutive" promoters are those that drive expression continuously under most environmental conditions and states of development or cell differentiation. "Inducible" or "regulatable" promoters direct expression of the nucleic
10 acid of the invention under the influence of environmental conditions or developmental conditions. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, drought, or the presence of light.

15 "Plasmid" herein indicates a circular single stranded or double stranded polynucleotide nucleic acid molecule which is capable of self-replication in prokaryotes like bacteria or in lower eukaryotes like yeast. "Plasmids" can be commercially available, publicly available on an
20 unrestricted basis, or can be constructed from available plasmids in accord with published procedures. Equivalent plasmids to those described herein are known in the art and will be apparent to the ordinarily skilled artisan.

The term "gene" includes a nucleic acid sequence
25 comprising a segment of DNA involved in producing a

transcription product (e.g., mRNA), which in turn is translated to produce a polypeptide chain, or regulates gene transcription, reproduction or stability. Genes can include regions preceding and following the coding region, such as leader and trailer, promoters and enhancers, as well as, where applicable, intervening sequences (introns) between individual coding segments (exons).

The phrase "nucleic acid" or "nucleic acid molecule" includes oligonucleotide, nucleotide, polynucleotide, or a fragment of any of these, DNA or RNA (e.g., mRNA, rRNA, tRNA, iRNA) of genomic or synthetic origin which may be single-stranded or double-stranded and may represent a sense or antisense strand, peptide nucleic acid (PNA), or any DNA-like or RNA-like material, natural or synthetic in origin, including, e. g., iRNA, ribonucleoproteins (e.g., double stranded iRNAs, e.g., iRNPs). The term encompasses nucleic acids, i.e., oligonucleotides, containing known analogues of natural nucleotides. The term also encompasses nucleic acid-like structures with synthetic backbones, see e.g., Mata (1997) *Toxicol. Appl. Pharmacol.* 144: 189-197; Strauss-Soukup (1997) *Biochemistry* 36: 8692-8698; Samstag (1996) *Antisense Nucleic Acid Drug Dev.* 6: 153-156.

"Amino acid" or "amino acid molecule" includes oligopeptide, peptide, polypeptide, or protein sequence, or

a fragment, portion, or subunit of any of these, and naturally occurring or synthetic molecules. The terms "polypeptide" and "protein" include amino acids joined to each other by peptide bonds or modified peptide bonds, i.e.,
5 peptide isosteres, and may contain modified amino acids other than the 20 gene-encoded amino acids. The term "polypeptide" also includes peptides and polypeptide fragments, motifs and the like. The term also includes glycosylated polypeptides. The peptides and polypeptides
10 of the invention also include all "mimetic" and "peptidomimetic" forms, as described in further detail, below.

The term "isolated" includes a material removed from its original environment, e.g., the natural environment if
15 it is naturally occurring. For example, a naturally occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such
20 polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. As used herein, an isolated material or composition can also
25 be a "purified" composition, i.e., it does not require

absolute purity; rather, it is intended as a relative definition. Individual nucleic acids obtained from a library can be conventionally purified to electrophoretic homogeneity. In alternative aspects, the invention
5 provides nucleic acids which have been purified from genomic DNA or from other sequences in a library or other environment by at least one, two, three, four, five or more orders of magnitude.

The term "transformant" indicates a transgenic cell
10 or organism with the insertion of a foreign gene. At this time, the cell or organism can be either prokaryotes or eukaryotes. In general, a foreign gene can be inserted episomally or integrated into a host genome by a vector such as plasmid, phagemid, cosmid, BAC and YAC. To
15 generate a transformant, various methods, including the method of Sambrook, known in the art can be used. For example, to prepare a prokaryotic transformant, heat shock and electroporation can be used. To prepare a eukaryotic transformant, gene gun, electroporation, DEAE-dextran,
20 calcium phosphate precipitation, lipofection and viral infection can be used. Further, to prepare a plant cell transformant, *Agrobacterium* can be used. The methods will be described in further detail, below.

As used herein, the term "recombinant" can include
25 nucleic acids adjacent to a "backbone" nucleic acid to

which it is not adjacent in its natural environment. In one aspect, nucleic acids represent 5% or more of the number of nucleic acid inserts in a population of nucleic acid "backbone molecules". "Backbone molecules" according to the invention include nucleic acids such as expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids, and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest. In one aspect, the enriched nucleic acids represent 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or more of the number of nucleic acid inserts in the population of recombinant backbone molecules. "Recombinant" polypeptides or proteins refer to polypeptides or proteins produced by recombinant DNA techniques; e.g., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide or protein. "Synthetic" polypeptides or proteins are those prepared by chemical synthesis, as described in further detail, below.

"Oligonucleotide" includes either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized.

"Variant" includes polynucleotides or polypeptides of the invention modified at one or more base pairs, codons,

introns, exons, or amino acid residues (respectively) yet still retain the biological activity of a protease of the invention (which can be assayed by, e.g., the hydrolysis of casein in zymograms, the release of fluorescence from gelatin, or the release of p-nitroanalide from various small peptide substrates). Variants can be produced by any number of means included methods such as, for example, error-prone PCR, DNA shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, in vivo mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, site-directed mutagenesis, gene reassembly, GSSM (Gene Site Saturation Mutagenesis) and any combination thereof. Techniques for producing variant protease having activity at a pH or temperature, for example, that is different from a wild-type protease, are included herein.

"Culture supernatant" or "microorganism culture supernatant" herein indicates a liquid component obtained by eliminating solid components including cells from centrifugation after liquid culture of *Bacillus clausii* I-52 of the invention or cells transfected with an expression vector containing an expression cassette harboring a gene encoding the protease of the invention or cells having a genome with an integrated expression cassette. The

fractions partially purified from the culture supernatant containing the protease of the invention are also included.

Hereinafter, the techniques known in the art for the
5 embodiment of the present invention are described.

3. Techniques used in the invention

General Techniques:

The nucleic acids used to practice this invention,
10 whether RNA, iRNA, antisense nucleic acid, cDNA, genomic DNA, vectors, viruses or hybrids thereof, may be isolated from a variety of sources, genetically engineered, amplified, and/or expressed/generated recombinantly. Recombinant polypeptides (e.g., proteases) generated from
15 these nucleic acids can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including bacterial, mammalian, yeast, insect or plant cell expression systems.

Alternatively, these nucleic acids can be synthesized
20 *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) *J. Am. Chem. Soc.* 105: 661; Belousov (1997) *Nucleic Acids Res.* 25: 3440-3444; Frenkel (1995) *Free Radic. Biol. Med.* 19: 373-380; Blommers (1994) *Biochemistry* 33: 7886-7896; Narang (1979) *Meth.*
25 *Enzymol.* 68: 90; Brown (1979) *Meth. Enzymol.* 68: 109;

Beaucage (1981) *Tetra. Lett.* 22: 1859; U.S. Patent No. 4,458,066.

Techniques for the manipulation of nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., *MOLECULAR CLONING: A LABORATORY MANUAL* (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); *LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES*, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N. Y. (1993).

Another useful means of obtaining and manipulating nucleic acids used to practice the methods of the invention is to clone from genomic samples, and, if desired, screen and re-clone inserts isolated or amplified from, e.g., genomic clones or cDNA clones.

In one aspect, a nucleic acid encoding a polypeptide of the invention is assembled in appropriate phase with a leader sequence capable of directing secretion of the translated polypeptide or fragment thereof.

The invention provides fusion proteins and nucleic acids encoding them. A polypeptide of the invention can be fused to a heterologous peptide or polypeptide, such as N-terminal identification peptides which impart desired characteristics, such as increased stability or simplified purification. Peptides and polypeptides of the invention can also be synthesized and expressed as fusion proteins with one or more additional domains linked thereto for, e.g., producing a more immunogenic peptide, to more readily isolate a recombinantly synthesized peptide, to identify and isolate antibodies and antibody-expressing B cells, and the like. Detection and purification facilitating domains include, e.g., metal chelating peptides such as polyhistidine tracts and histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, WA). The inclusion of a cleavable linker sequence such as Factor Xa or enterokinase (Invitrogen, San Diego, CA) between a purification domain and the motif-comprising peptide or polypeptide facilitates purification. For example, an expression vector can include an epitope-encoding nucleic acid sequence linked to six histidine residues followed by a thioredoxin and an enterokinase cleavage site (see e.g.,

Williams (1995) *Biochemistry* 34: 1787-1797; Dobeli (1998) *Protein Expr. Purif.* 12: 404-414). The histidine residues facilitate detection and purification while the enterokinase cleavage site provides a means for purifying the epitope from the remainder of the fusion protein. Technology pertaining to vectors encoding fusion proteins and application of fusion proteins are well described in the scientific and patent literature, see e.g., Kroll (1993) *DNA Cell. Biol.*, 12: 441-53.

Transcriptional and translational control sequences:

The invention provides nucleic acid (e.g., DNA) sequences operably linked to expression (e.g., transcriptional or translational) control sequence (s), e.g., promoters or enhancers, to direct or modulate RNA synthesis/expression. The expression control sequence can be in an expression vector. Exemplary bacterial promoters include *lacI*, *lacZ*, T3, T7, *gpt*, lambda PR, PL, *trp* and *aprE*. Exemplary eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein.

Promoters suitable for expressing a polypeptide in bacteria include the *E. coli lac* or *trp* promoters, the *lad* promoter, the *lacZ* promoter, the T3 promoter, the T7 promoter, the *gpt* promoter, the lambda PR promoter, the

lambda PL promoter, promoters from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), the acid phosphatase promoter and promoter of the alkaline protease from *Bacillus* sp. (*aprE*). Eukaryotic promoters
5 include the CMV immediate early promoter, the HSV thymidine kinase promoter, heat shock promoters, the early and late SV40 promoter, LTRs from retroviruses, and the mouse metallothionein-I promoter. Other promoters known to control expressions of genes in prokaryotic or eukaryotic
10 cells or their viruses may also be used.

Expression vectors and cloning vehicles:

The invention provides expression vectors and cloning vehicles comprising nucleic acids of the invention, e.g.,
15 sequences encoding the proteases of the invention. Expression vectors and cloning vehicles of the invention can comprise viral particles, baculovirus, phage, plasmids, phagemids, cosmids, fosmids, bacterial artificial chromosomes (BAC), viral DNA (e.g., vaccinia, adenovirus,
20 fowl pox virus, pseudorabies and derivatives of SV40), P1-based artificial chromosomes, yeast plasmids, yeast artificial chromosomes (YAC), and any other vectors specific for specific hosts of interest (such as *Bacillus*, *Aspergillus* and yeast). Vectors of the invention can
25 include chromosomal, non-chromosomal and synthetic DNA

sequences. Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Exemplary vectors are: bacterial: pQE vectors (Qiagen), pBluescript plasmids, pNH vectors, lambda-ZAP vectors (Stratagene); pET vectors (Novagen); ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene), pSVK3, pBPV, pMSG, pSVLSV40 (Pharmacia). However, any other plasmids or other vectors may be used so long as they are replicable and viable in the host. Low copy number or high copy number vectors may be employed with the present invention.

The expression vector can comprise a promoter, a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. Mammalian expression vectors can comprise an origin of replication, any necessary ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5'-flanking non-transcribed sequences. In some aspects, DNA sequences derived from the SV40 splice and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

In one aspect, the expression vectors contain one or more selectable marker genes to permit selection of host cells containing the vector. Such selectable markers

include genes encoding dihydrofolate reductase or genes conferring neomycin resistance for eukaryotic cell culture, genes conferring chloramphenicol, gentamycin, kanamycin, tetracycline, erythromycin or ampicillin resistance in *E. coli*, and the *S. cerevisiae* TRP1 gene. Promoter regions can be selected from any desired gene using chloramphenicol transferase (CAT) vectors or other vectors with selectable markers.

Vectors for expressing the polypeptide or a fragment thereof in eukaryotic cells can also contain enhancers to increase expression levels. Enhancers are cis-acting elements of DNA, usually from about 10 to about 300 bp in length that act on a promoter to increase its transcription. Examples include the SV40 enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer and the adenovirus enhancers.

A nucleic acid sequence can be inserted into a vector by a variety of procedures. In general, the sequence is ligated to the desired position in the vector following digestion of the insert and the vector with appropriate restriction endonucleases. Alternatively, blunt ends in both the insert and the vector may be ligated. A variety of cloning techniques are known in the art, e.g., as described in Ausubel and Sambrook. Such procedures and

others are deemed to be within the scope of those skilled in the art.

The vector can be in the form of a plasmid, a viral particle, or a phage. Other vectors include chromosomal, non-chromosomal and synthetic DNA sequences, derivatives of SV40; bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. A variety of cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by, e.g., Sambrook.

Particular bacterial vectors which can be used include the commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017), pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), GEM1 (Promega Biotec, Madison, WI, USA), pQE70, pQE60, pQE-9 (Qiagen), pD 10, psiX174 pBluescript II KS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene), ptrc99a, pKK223-3, pKK233-3, DR540, pRIT5 (Pharmacia), pKK232-8 and pCM7, pET vectors such as pET-3, pET-11 and pET-22 (Novagen). Particular eukaryotic vectors include pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, and pSVL (Pharmacia). However, any other vector may be used as long as it is replicable and viable in the host cell.

The nucleic acids of the invention can be expressed in expression cassettes, vectors or viruses and transiently or stably expressed in plant cells and seeds. One exemplary transient expression system uses episomal expression systems, e.g., cauliflower mosaic virus (CaMV) viral RNA generated in the nucleus by transcription of an episomal mini-chromosome containing supercoiled DNA, see, e.g., Covey (1990) *Proc. Natl. Acad. Sci. USA* 87: 1633-1637. Alternatively, coding sequences, i.e., all or sub-fragments of sequences of the invention can be inserted into a plant host cell genome becoming an integral part of the host chromosomal DNA. Sense or antisense transcripts can be expressed in this manner. A vector comprising the sequences (e.g., promoters or coding regions) from nucleic acids of the invention can comprise a marker gene that confers a selectable phenotype on a plant cell or a seed. For example, the marker may encode biocide resistance, particularly antibiotic resistance, such as resistance to kanamycin, G418, bleomycin, hygromycin, or herbicide resistance, such as resistance to chlorosulfuron or Basta.

Expression vectors capable of expressing nucleic acids and proteins in plants are well known in the art, and can include, e.g., vectors from *Agrobacterium* spp., potato virus X (see, e.g., Angell (1997) *EMBO J.* 16: 3675-3684), tobacco mosaic virus (see, e.g., Casper (1996) *Gene* 173:

69-73), tomato bushy stunt virus (see, e.g., Hillman (1989) *Virology* 169: 42-50), tobacco etch virus (see, e.g., Dolja (1997) *Virology* 234: 243-252), bean golden mosaic virus (see, e.g., Morinaga (1993) *Microbiol. Immunol.* 37: 471-476), cauliflower mosaic virus (see, e.g., Cecchini (1997) *Mol. Plant Microbe Interact.* 10:1094-1101), maize Ac/Ds transposable element (see, e.g., Rubin (1997) *Mol. Cell. Biol.* 17: 6294-6302; Kunze (1996) *Curr. Top. Microbiol. Immunol.* 204: 161-194), and the maize suppressor-mutator (Spm) transposable element (see, e.g., Schlappi (1996) *Plant Mol. Biol.* 32:717-725); and derivatives thereof. In one aspect, the expression vector can have two replication systems to allow it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector can contain at least one sequence homologous to the host cell genome. It can contain two homologous sequences which flank the expression construct. The integrating vector can be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

Expression vectors of the invention may also include a selectable marker gene to allow for the selection of

bacterial strains that have been transformed, e.g., genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers can also
5 include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways. The biosynthetic genes can be used for the selection of a transformant with the insertion of a foreign gene from a nutrient medium deficient in the essential nutrient which
10 is the product of the gene.

Host cells and transformed cells:

The invention also provides a transformed cell comprising a nucleic acid sequence of the invention, e.g.,
15 a sequence encoding a protease of the invention, or a vector of the invention. The host cell may be any of the host cells familiar to those skilled in the art, including prokaryotic cells, eukaryotic cells, such as bacterial cells, fungal cells, yeast cells, mammalian cells, insect
20 cells, or plant cells. Exemplary bacterial cells include *E. coli*, *Streptomyces*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. Exemplary insect cells include *Drosophila* S2 and *Spodoptera* Sf9.
25 Exemplary animal cells include, CHO, COS or Bowes melanoma

or any mouse or human cell line. The selection of an appropriate host is within the abilities of those skilled in the art. Techniques for transforming a wide variety of higher plant species are well known and described in the technical and scientific literature. See, e.g., Weising (1988) *Ann. Rev. Genet.* 22: 421-477; U.S. Patent No. 5,750,870. The vector can be introduced into the host cells using any of a variety of techniques, including transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Particular methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection, or electroporation (Davis, L., Dibner, M., Battey, I., *Basic Methods in Molecular Biology*, (1986)).

In one aspect, the nucleic acids or vectors of the invention are introduced into the cells for screening, thus, the nucleic acids enter the cells in a manner suitable for subsequent expression of the nucleic acid. The method of introduction is largely dictated by the targeted cell type. Exemplary methods include CaPO_4 precipitation, liposome fusion, lipofection (e.g., LIPOFECTINTM), electroporation, viral infection, etc. The candidate nucleic acids may stably integrate into the genome of the host cell (for example, with retroviral introduction) or may exist either transiently or stably in the cytoplasm (i.e. through the

use of traditional plasmids, utilizing standard regulatory sequences, selection markers, etc.). As many pharmaceutically important screens require human or model mammalian cell targets, retroviral vectors capable of
5 transfecting such targets are preferred.

Where appropriate, the engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the invention.
10 Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter may be induced by appropriate means (e.g., temperature shift or chemical induction) and the cells may be cultured for an additional period to allow
15 them to produce the desired polypeptide or fragment thereof.

Cells can be harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract is retained for further purification. Microbial cells employed for expression of proteins can be disrupted
20 by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from recombinant cell cultures by
25 methods including ammonium sulfate, ethanol or acetone

precipitation, acid extraction, anion or cation exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used,
5 as necessary, in completing configuration of the polypeptide. If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

Various mammalian cell culture systems can also be
10 employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts and other cell lines capable of expressing proteins from a compatible vector, such as the C127, 3T3, CHO, HeLa and BHK cell lines.

15 The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Depending upon the host employed in a recombinant production procedure, the polypeptides produced by host cells containing the vector may be
20 glycosylated or may be non-glycosylated. Polypeptides of the invention may or may not also include an initial methionine amino acid residue.

Cell-free translation systems can also be employed to produce a polypeptide of the invention. Cell-free
25 translation systems can use mRNAs transcribed from a DNA

construct comprising a promoter operably linked to a nucleic acid encoding the polypeptide or fragment thereof. In some aspects, the DNA construct may be linearized prior to conducting an *in vitro* transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

The expression vectors can contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

Amplification of Nucleic Acids:

In practicing the invention, nucleic acids of the invention and nucleic acids encoding the proteases of the invention, or modified nucleic acids of the invention, can be reproduced by amplification. Amplification can also be used to clone or modify the nucleic acids of the invention. Thus, the invention provides amplification primer sequence pairs for amplifying nucleic acids of the invention. One of skills in the art can design amplification primer

sequence pairs for any part of or the full length of these sequences.

Here in the present invention, forward primers F2 and F3 represented by SEQ. ID. NO: 6 and SEQ. ID. NO: 7 and
5 reverse primers R1, R2 and R3 represented by SEQ. ID. NO: 8, No 9 and No 10 were used for PCR but not always limited thereto.

Amplification reactions can also be used to quantify the amount of nucleic acid in a sample (such as the amount
10 of message in a cell sample), label the nucleic acid (e.g., to apply it to an array or a blot), detect the nucleic acid, or quantify the amount of a specific nucleic acid in a sample. In one aspect of the invention, message isolated from a cell or a cDNA library is amplified.

15 The skilled artisan can select and design suitable oligonucleotide amplification primers. Amplification methods are also well known in the art, and include, e.g., polymerase chain reaction, PCR (see, e.g., PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic
20 Press, N. Y. (1990) and PCR STRATEGIES (1995), ed. Innis, Academic Press, Inc., N. Y., ligase chain reaction (LCR) (see, e.g., Wu (1989) *Genomics* 4: 560; Landegren (1988) *Science* 241: 1077; Barringer (1990) *Gene* 89: 117); transcription amplification (see, e.g., Kwoh (1989) *Proc.*
25 *Natl. Acad. Sci. USA* 86: 1173); and, self-sustained

sequence replication (see, e. g. , Guatelli (1990) *Proc. Natl. Acad. Sci. USA* 87 : 1874); Q Beta replicase amplification (see, e.g., Smith (1997) *J. Clin. Microbiol.* 35: 1477-1491), automated Q-beta replicase amplification
5 assay (see, e.g., Burg (1996) *Mol. Cell. Probes* 10: 257-271) and other RNA polymerase mediated techniques (e.g., NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) *Methods Enzymol.* 152:307-316; Sambrook; Ausubel; U. S. Patent Nos. 4,683,195 and 4,683,202; Sooknanan (1995)
10 *Biotechnology* 13: 563-564.

4. Deposit of microorganisms

The present inventors deposited the *Bacillus clausii* I-52 strain isolated by the present inventors at Korean
15 Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB, #52, Oundong, Yusong-ku, Taejon, Korea), an international depository authority designated by the Budapest Treaty, on June 14, 2002 (Accession No: KCTC 10277BP), and deposited
20 the transformant *Escherichia coli* JM109/pHPS-BCAP on January 26, 2005 (Accession No: KCTC 10772BP). The deposit meets all the conditions asked by the Budapest Treaty in relation to the usages of the deposited microorganisms.

Under the Budapest Treaty (the provisions of 37 C.F.R. §1.14 and 35 U.S.C. §122), the present inventors certify
25

that (a) all restriction on the availability to the public of the deposited microorganisms will be irrevocably removed upon issuance of a United States patent of which any of such deposited microorganisms are subject matters; (b) the
5 deposited microorganisms will be maintained for the longest period among the followings; i) at least for 5 years after the most recent request for the furnishing of a sample of any of the deposited microorganisms were received by the KCTC, ii) at least for 30 years from the date of deposit
10 and iii) for the effective life of such patent; (c) vitality test for the microorganisms has to be performed on the date of deposit; (d) the original materials will be redeposited in case the microorganisms cannot survive any longer; and (e) access to the deposited microorganisms will
15 be available during the pendency of the patent application to be determined by the commissioner of USIPO to be entitled thereto.

【Description of Drawings】

20 The application of the preferred embodiments of the present invention is best understood with reference to the accompanying drawings, wherein:

Fig. 1 is an illustration of results of SDS-PAGE
25 examining the protease of the present invention:

M: standard protein, 1: Phenyl-Sepharose phase,
2: DEAE-Sepharose phase, 3: protease of the invention.

Fig. 2 is a graph showing the pH-dependent enzyme
5 activity of a protease of the invention.

Fig. 3 is a graph showing the temperature-dependent
enzyme activity of a protease of the invention.

10 Fig. 4 is a graph showing the enzyme activity of the
protease of the invention according to the concentration of
anionic surfactant, sodium dodecyl sulfate.

Fig. 5 is a graph showing the enzyme activity of the
15 protease of the invention according to the concentration of
a hydrogen peroxide solution.

Fig. 6 is a graph showing the enzyme activity of the
protease of the invention according to the concentration of
20 sodium perborate.

Fig. 7 is a graph showing the relative activity of
the protease of the invention according to the
concentration of salt.

25

Fig. 8 is a graph showing the result of the experiment examining whether a protease of the invention could retain the enzyme stability in the presence of a commercial detergent.

5

Fig. 9 is a graph showing the detergency of the protease of the invention produced by *Bacillus clausii* in the presence of the detergent composition linear alkylbenzene sulfonates (LAS, 15%).

10

Fig. 10 is a photograph showing the soybean cake decomposing capacity of a protease of the invention produced by *Bacillus clausii*:

Lane M: protein marker,

15

Lane 1: before treatment,

Lane 2: 2 hours after treatment,

Lane 3: 4 hours after treatment,

Lane 4: 8 hours after treatment, and

Lane 5: 16 hours after treatment.

20

Fig. 11 is a graph showing the decomposition of soybean trypsin inhibitor (SBTI) by a protease of the invention produced by *Bacillus clausii*.

Fig. 12 is a schematic diagram showing the locations of primers and amplified areas by PCR using the chromosomal DNA of *Bacillus clausii* I-52 as a template.

5 Fig. 13 is a photograph of gel electrophoresis showing the result of PCR using different combinations of primers:

Lane M: 100 bp DNA marker,

Lane 1: combination of primers F2/R1,

10 Lane 2: combination of primers F2/R2,

Lane 3: combination of primers F2/R3,

Lane 4: combination of primers F3/R1,

Lane 5: combination of primers F3/R2, and

Lane 6: combination of primers F3/R3.

15

Fig. 14 is a photograph of gel electrophoresis illustrating that the PCR product obtained from PCR using primers F3/R2 was introduced into pGEM T easy plasmid, which was digested with restriction enzyme to confirm the
20 insertion of a target DNA:

Lane M: lambda DNA marker, and

Lane 1: inserted DNA fragment after treated with *Eco* RI.

Fig. 15 is a schematic diagram illustrating the plasmid pT7-BCAP for the expression of a mature protease (BCAP).

Fig. 16 is a photograph of gel electrophoresis illustrating that a gene encoding the mature protease (BCAP) was introduced into the plasmid pT7-7, which was then digested with restriction enzyme to confirm the insertion of a target DNA,

Lane M: lambda DNA marker, and

Lane 1, 2 and 3: inserted DNA fragments after treated with *Nco* I and *Hind* III, clone 1, 2 and 3, respectively

Fig. 17 is a photograph showing the result of SDS-PAGE examining the mature protease induced by the culture of a transformant expressing the mature protease in the presence of IPTG:

Lane M: protein marker,

Lane 1: before induction with IPTG,

Lane 2: 1 hour after induction with IPTG,

Lane 3: 2 hours after induction with IPTG,

Lane 4: 3 hours after induction with IPTG, and

Arrow: band of a mature protease.

Fig. 18 is a schematic diagram illustrating the plasmid pHPS-BCAP harboring a gene represented by SEQ. ID. NO: 1 for the integration into *Bacillus clausii* genome.

5

Best Mode

The present invention provides a *Bacillus clausii* I-52 strain producing a protease.

The *Bacillus clausii* of the invention was isolated from the west coast mudflat of Korea, which is known to have bad growth conditions and produced a protease confirmed to keep its enzyme activity under severe physical and chemical conditions and in the presence of a heavy metal, and was proved to be stable in the wide range of pH and in the presence of an anionic surfactant and an oxidant (ex; hydrogen peroxide, sodium perborate, etc.) used as an additive to an oxygen-bleaching agent. The *Bacillus clausii* of the invention was confirmed to be a Gram-positive, to form a spore, to have catalase and oxidase activities but not to have urease activity. The *Bacillus clausii* uses sugar such as glucose, fructose, galactose, mannose, maltose, mannitol, sorbitol and starch, and proteins such as casein and gelatin to produce acid and gas, and other morphological and biochemical properties of the *Bacillus clausii* are presented in Table 1. And also, the

Bacillus clausii contains 16S ribosomal RNA represented by
SEQ. ID. NO: 1.

Table 1: Bacterial property of *Bacillus clausii*:
5 morphological and biochemical characteristics

property	<i>Bacillus clausii</i> I-52
Gram staining	+, Rod
Spore formation	+
Catalase	+
Oxidase	+
Urease	-
Reduction of nitrate	+
Acetate	+
Citrate	+
Arabinose	-
Glucose	+
Fructose	+
Galactose	+
Mannose	+
Lactose	-
Maltose	+
Sucrose	-
Inositol	-
Mannitol	+
Sorbitol	+
Glutamic acid	-
Starch	+
Casein	+
Gelatin	+

The present inventors named the strain producing the protease of the invention as '*Bacillus clausii* I-52' and deposited at Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB, #52, Oun-dong, Yusong-ku, Taejon, Korea), on Jun 14, 2002 (Accession No: KCTC 10277BP).

The present invention provides a protease produced by *Bacillus clausii* and a purification method of the protease.

The purification method of the protease produced by *Bacillus clausii* of the invention comprises the following steps:

1) recovering culture supernatant by centrifugation after culturing *Bacillus clausii*;

2) eluting active fractions after absorbing a protease by adding a synthetic absorbent;

3) eluting active fractions by loading the eluent onto chromatography; and

4) isolating a 28 kDa protein having a protease activity.

In step 2), the synthetic absorbent can be any of aromatic synthetic absorbents, and Diaion HP absorbents are preferred. After absorbing a protease, an absorbent is recovered by filtering and then washed briefly with tap water. Then, an active fraction is eluted.

In step 3), the chromatography is either hydrophobic chromatography or ion exchange chromatography, and/or ion exchange chromatography and hydrophobic chromatography are used serially in that order. In a preferred embodiment of the present invention, the eluent was loaded to Phenyl-Sephadex column, and then an active fraction was eluted. Then, the fraction obtained by Phenyl-Sephadex column chromatography was loaded onto a DEAE-sephadex column in order to elute an active fraction. And a CM-Sephadex column was used to elute an active fraction and the activity of the fraction was measured. To confirm whether a target protein was purely isolated and to determine the molecular weight of the protein, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the fraction. As a result, it was confirmed that a 28 kDa protein having a protease activity was isolated and purified (see Fig. 1).

A protease produced by *Bacillus clausii* of the invention has the titer of 120,000 U per 1 ml of culture supernatant, indicating that the *Bacillus clausii* produces a protease much more than any other strain (see Korean Patent No. 258740).

From the amino acid sequencing of the isolated protease, the amino acid sequence of N-terminal of the protease was confirmed to be a sequence represented by SEQ. ID. NO: 2 and the entire amino acid sequence was proved to

be a sequence represented by SEQ. ID. NO: 4 when it was a precursor and to be a sequence represented by SEQ. ID. NO: 5 when it was an active protease produced with the digestion of a pro-pre sequence.

5 The protease of the invention consists of 381 amino acids, and 32 amino acids of N-terminal are a signal sequence and the 107th amino acid alanine (Ala) is the N-terminal amino acid of an active protein. The active protease region consists of 275 amino acid residues
10 represented by SEQ. ID. NO: 5 and particularly contains three activation-related amino acids (catalytic triad), Asp32, His64 and Ser221, which are commonly found in serine proteases, suggesting that the protease of the invention is one of typical serine proteases.

15 The protease of the invention is a very stable enzyme retaining its activity under various physical and chemical conditions including high pH and the presence of a detergent, an organic solvent and a heavy metal. The protease of the invention shows its protease activity in
20 the wide range of pH (pH 7.0 - pH 12.0), but preferable pH range to show the maximum activity is 9.0 - 12.0, more preferable pH range is 10.0 - 11.0, and the most preferable pH is 11.0 (see Fig. 2). And also the protease of the invention retains its activity in the temperature range of
25 10 - 70°C but to show the maximum protease activity, 40 -

65°C is preferred, 55 - 65°C is more preferred and 60 - 65°C is most preferred (see Fig. 3). The protease of the invention can keep its enzyme activity in the presence of not only non-ionic surfactants such as Tween 20 and Triton X-100 but also strong anionic surfactants such as sodium dodecyl sulfate (SDS) and linear alkylbenzene sulfonates (LAS), in particular the protease retained its activity for three days even by the treatment of 5% SDS (see Fig. 4) and so did in the presence of a strong oxidant like hydrogen peroxide and sodium perborate, proving the protease as a very stable enzyme (see Fig. 5 and 6). Besides, the protease of the invention shows the maximum activity in the presence of 2 - 5% salt, and keeps its activity further in the high concentration of salt (20%), confirming that the protease of the invention is a halophilic protease (see Fig. 7). Based on such enzymatic characteristics, it was confirmed that the protease derived from the *Bacillus clausii* I-52 fits a variety of industrial uses.

The present invention also provides a gene encoding the protease.

The sequence of a gene encoding the protease of the invention having the above properties was analyzed. Precisely, chromosomal DNA was isolated from the *Bacillus clausii*, and PCR was performed using the DNA as a template to obtain DNA fragments of the protease (see Fig. 12 and

13), leading to the determination of nucleotide sequence thereof (SEQ. ID. NO: 3).

The present invention further provides an expression vector containing the gene.

5 To produce the protease of the invention, which retains its enzyme activity under severe conditions, as an active form, a gene (SEQ. ID. NO: 3) of the protease having the amino acid sequence represented by SEQ. ID. NO: 5 was introduced into an expression vector. The expression
10 vector constructed thereby was named "pT7-BCAP" (BCAP: *Bacillus clausii* Alkaline Protease) (see Fig. 15).

The present invention also provides a transformant transfected with the expression vector.

15 The constructed expression vector above was introduced into a host cell, resulting in a transformant expressing the protease of the invention as an active form. In the present invention, the vector pT7-BCAP was introduced into *E. coli* to produce a transformant producing the protease of the invention as a stable active form (see
20 Fig. 16).

The present invention also provides a recombinant expression vector harboring the above gene for the insertion into *Bacillus* genome.

25 A recombinant expression vector was constructed to integrate an expression cassette represented by SEQ. ID.

NO: 2 containing the gene encoding the protease of the invention linked operably to the promoter thereof into *Bacillus clauui* genome, and the constructed vector was named "pHPS-BCAP" (see Fig. 18). The recombinant
5 expression vector for genomic insertion was introduced into the chromosome of *Bacillus clausii* to increase the copy number of BCAP in the chromosome.

The present invention also provides a transformant transfected with the recombinant expression vector for
10 genomic insertion.

The expression vector pHPS-BCAP was introduced into *E. coli* JM109, which was deposited at Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology on January 26, 2005 (Accession
15 No: KCTC 10772BP).

The present invention also provides the culture supernatant of the *Bacillus clausii* I-52 or the transformant.

As described above, the culture supernatant herein
20 means the liquid composition obtained by eliminating solid components containing cells from centrifugation after liquid-culture of the *Bacillus clausii* I-52 or the transformant. If necessary, a partial purification is allowed, and endotoxin such as lipopolysaccharide can be
25 eliminated by methods known in the art. For example, the

methods described in Japanese Patent Publication No. 2000-1897921 A 1, Japanese Patent No. 8193031 and U.S. Patent No. 5,760,177 can be used.

5 The present invention provides a method for purifying the protease of the invention isolated from the transformant or the culture supernatant thereof. The purification method of the invention is basically same as the purification method for the protease of the invention from the *Bacillus clauui* I-52, and modifications are
10 allowed as long as they are accepted to those in the art.

The invention provides detergent compositions comprising one or more proteases of the invention isolated from the *Bacillus clausii* I-52, the transformant or the culture supernatant thereof (referred conveniently as 'microorganism culture supernatant of the invention'
15 hereinafter), and methods of making and using these compositions. The invention incorporates all methods of making and using detergent compositions, see, e.g., U.S. Patent Nos. 6,413,928; 6,399,561; 6,365,561 and 6,380,147.
20 The detergent compositions can be a one and two part aqueous composition, a non-aqueous liquid composition, a cast solid, a granular form, a particulate form, a compressed tablet, a gel and/or a paste and a slurry form. The proteases of the invention can also be used as a
25 detergent additive product in a solid or a liquid form.

Such additive products are intended to supplement or boost the performance of conventional detergent compositions and can be added at any stage of the cleaning process.

5 The invention also provides methods capable of removing gross food wastes, films of food waste sludge and other minor food compositions using these detergent compositions. Proteases of the invention can facilitate the removal of stains by means of catalytic hydrolysis of proteins. The protease of the invention can be used in
10 dishwashing detergents and/or in textile laundering detergents. The actual active enzyme content depends upon the method for manufacturing of a detergent composition and is not critical, assuming the detergent solution has the desired enzymatic activity. In one aspect, the amount of a
15 protease present in the final solution ranges from about 0.001 mg to 0.5 mg per gram of the detergent composition. The particular enzyme chosen for use in the process and products of the present invention depends upon the conditions of final utility, including the physical product
20 form, pH and temperature to be used, and type of wastes to be degraded or altered. The enzyme can be chosen to provide optimum activity and stability for any given set of utility conditions. In one aspect, the proteases of the present invention are active in the pH ranges of from about
25 7.0 to about 12.0 and in the temperature range of from

about 10°C to about 75°C The detergents of the invention can comprise cationic, semi-polar nonionic or zwitterionic surfactants; or, mixtures thereof.

Proteases of the invention can be formulated into powdered and liquid detergents having pH between 7.0 and 12.0 at levels of about 0.01% to about 5% (preferably 0.1% to 0.5%) by weight. The microorganism culture supernatant of the invention is dried and powdered by freeze-drying or spray-drying, and then formulated into a powdered detergent having pH between 7.0 and 12.0 at levels of about 0.1% to about 10% by weight. Or the supernatant can be formulated into a liquid detergent having pH between 7.0 and 12.0 at levels of about 0.1% to about 20% by volume as a liquid form without being dried or concentrated. These detergent compositions can also include other enzymes such as proteases, cellulases, lipases or endoglycosidases, endo-beta-1,4-glucanases, beta-glucanases, endo-beta-1,3(4)-glucanases, cutinases, peroxidases, laccases, amylases, glucoamylases, pectinases, reductases, oxidases, phenoloxidases, ligninases, pullulanases, arabinanases, hemicellulases, mannanases, xyloglucanases, xylanases, pectin acetyl esterases, rhamnogalacturonan acetyl esterases, polygalacturonases, rhamnogalacturonases, galactanases, pectin lyases, pectin methylesterases, cellobiohydrolases and/or transglutaminases. These

detergent compositions can also include builders and stabilizers.

5 The addition of proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the compositions of the invention as long as the enzyme is active at or tolerant of the pH and/or temperature of the intended use. In addition, the proteases of the invention
10 can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The present invention provides cleaning compositions including detergent compositions for cleaning hard surfaces,
15 detergent compositions for cleaning fabrics, dishwashing compositions, oral cleaning compositions, denture cleaning compositions, and contact lens cleaning solutions.

In one aspect, the invention provides a method for washing an object comprising contacting the object with the
20 culture supernatant and/or the protease of the invention under conditions sufficient for washing. The culture supernatant and/or the protease of the invention may be included as a detergent additive. The detergent composition of the invention may, for example, be
25 formulated as a hand or machine laundry detergent

composition comprising the culture supernatant and/or the protease of the invention. A laundry additive suitable for pre-treatment of stained fabrics can comprise the culture supernatant and/or the protease of the invention. A fabric
5 softener composition can comprise the culture supernatant and/or the protease of the invention. Alternatively, the culture supernatant and/or the protease of the invention can be formulated as a detergent composition for use in general household hard surface cleaning operations. In
10 alternative aspects, detergent additives and detergent compositions of the invention may comprise one or more other enzymes such as a protease, a lipase, a cutinase, another protease, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an
15 oxidase, e.g., a lactase, and/or a peroxidase (see also, above). The properties of the culture supernatant and/or the protease of the invention are chosen to be compatible with the selected detergent (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.)
20 and the culture supernatant and/or the protease is present in effective amounts. In one aspect, the culture supernatant and/or the protease of the invention is used to remove malodorous materials from fabrics. Various detergent compositions and methods for making them that can
25 be used in practicing the invention are described in, e.g.,

U.S. Patent Nos. 6,333,301; 6,329,333; 6,326,341; 6,297,038; 6,309,871; 6,204,232; 6,197,070 and 5,856,164.

When formulated as compositions suitable for use in a method for washing laundry machines, the culture
5 supernatant and/or the protease of the invention can comprise both a surfactant and a builder compound. They can additionally comprise one or more detergent components, e.g., organic polymeric compounds, bleaching agents, additional enzymes, suds suppressors, dispersants, lime-
10 soap dispersants, soil suspension and anti-redeposition agents and corrosion inhibitors. Laundry compositions of the invention can also contain softening agents, as additional detergent components. Such compositions containing carbohydrase can provide fabric cleaning, stain
15 removal, whiteness maintenance, softening, color appearance, dye transfer inhibition and sanitization when formulated as laundry detergent compositions.

The density of the laundry detergent compositions of the invention can range from about 200 to 1500 g/l, or,
20 about 400 to 1200 g/l, or, about 500 to 950 g/l, or, 600 to 800 g/l, of composition; this can be measured at about 20°C

The "compact" form of laundry detergent compositions of the invention is best reflected by density and, in terms of composition, by the amount of inorganic filler salt.
25 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 17% to 35% by weight of the total composition. The inorganic filler salts can be
5 selected from the alkali and alkaline-earth-metal salts of sulphates and chlorides, e.g., sodium sulphate.

Liquid detergent compositions of the invention can also be in a "concentrated" form. In one aspect, the liquid detergent compositions can contain a lower amount of
10 water, compared to conventional liquid detergents. In alternative aspects, the water content of the concentrated liquid detergent is less than 40%, or, less than 30%, or, less than 20% by weight of the detergent composition. Detergent compounds of the invention can comprise
15 formulations as described in WO 97/01629.

The culture supernatant and/or the protease of the invention can be useful in formulating various cleaning compositions. A number of known compounds, suitable surfactants including nonionic, anionic, cationic, or
20 zwitterionic detergents, can be used, e.g., as disclosed in U.S. Patent Nos. 4,404,128; 4,261,868; 5,204,015. In addition, proteases can be used, for example, in bar or liquid soap applications, dish care formulations, contact lens cleaning solutions or products, peptide hydrolysis,
25 waste treatment, textile applications, as fusion-cleavage

enzymes in protein production, and the like. Proteases may provide enhanced performance in a detergent composition as compared to another detergent protease, that is, the enzyme group may increase cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle. The protease of the invention can be formulated into known powdered and liquid detergents having pH between 7.0 and 12.0 at levels of about 0.01 to about 5% (for example, about 0.1% to 0.5%) by weight. These cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The culture supernatant and/or the protease of the invention have numerous applications in food processing industry. For example, in one aspect, the culture supernatant and/or the protease of the invention are used to improve the extraction of oil from oil-rich plant material, e.g., oil-rich seeds, for example, soybean oil from soybeans, olive oil from olives, rapeseed oil from rapeseed and/or sunflower oil from sunflower seeds.

The culture supernatant and/or the protease of the invention can be used for separation of components of plant cell materials. For example, the culture supernatant and/or the protease of the invention can be used in the

separation of protein-rich material (e.g., plant cells) into components, e.g., sucrose from sugar beet or starch or sugars from potato, pulp or hull fractions. In one aspect, the culture supernatant and/or the protease of the invention can be used to separate protein-rich or oil-rich crops into valuable protein and oil and hull fractions. The separation process may be performed by use of methods known in the art.

The culture supernatant and/or the protease of the invention can be used in the preparation of fruit or vegetable juices, syrups, extracts and the like to increase yield. The culture supernatant and/or the protease of the invention can be used in the enzymatic treatment (e.g., hydrolysis of proteins) of various plant cell wall-derived materials or waste materials, e.g., from wine or juice production, or agricultural residues such as vegetable hulls, bean hulls, sugar beet pulp, olive pulp, potato pulp, and the like. The culture supernatant and/or the protease of the invention can be used to modify the consistency and appearance of processed fruit or vegetables. The culture supernatant and/or the protease of the invention can be used to treat plant materials to facilitate processing of plant materials, including foods, facilitate purification or extraction of plant components. The culture supernatant and/or the protease of the invention can be used to improve

feed value, decrease the water binding capacity, improve the degradability in waste water plants and/or improve the conversion of a plant material to ensilage, and the like.

The invention provides methods for treating animal feeds and foods and food or feed additives using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention. The animals include mammals (e.g., humans), birds, fish and the like.

The invention provides animal feeds, foods, and additives comprising the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention.

In one aspect, treating animal feeds, foods and additives using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can help in the availability of nutrients, e.g., starch, in the animal feed or additive. By breaking down difficult to digest proteins or indirectly or directly unmasking starch (or other nutrients), the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention make nutrients more accessible to other endogenous or exogenous enzymes.

The *Bacillus clausii* I-52 strain, the transformant, the

culture supernatant thereof and/or the protease isolated therefrom of the invention can also simply cause the release of readily digestible and easily absorbed nutrients and sugars.

5 The *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention, in the modification of animal feed or a food, can process the food or feed either *in vitro* (by modifying components of the feed or food) or
10 *in vivo*. The *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can be added to animal feed or food compositions containing high amounts of proteins, e.g., feed or food containing plant proteinous
15 materials from soy bean, rape seed, lupin and the like. When added the protease isolated therefrom of the invention to the feed or food, it significantly improves the *in vivo* break-down of plant proteinous materials, whereby a better utilization of the plant nutrients by the animal (e.g.,
20 human) is achieved. In one aspect, the growth rate and/or feed conversion ratio (i.e. the weight of ingested feed relative to weight gain) of the animal is improved. For example a partially or indigestible galactan-comprising protein is fully or partially degraded by the *Bacillus*
25 *clausii* I-52 strain, the transformant, the culture

supernatant thereof and/or the protease isolated therefrom of the invention, e.g. in combination with another enzyme, e.g., beta-galactosidase, to peptides and galactose and/or galactooligomers. These enzyme digestion products are more digestible by the animal. Thus, the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can contribute to the available energy of the feed or food. Also, by contributing to the degradation of galactan-comprising proteins, the protease of the invention can improve the digestibility and uptake of proteins from the feed or food constituents containing carbohydrate and non-carbohydrate.

In another aspect, the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can be supplied by expressing the enzymes directly in transgenic feed crops (as, e.g., transgenic plants, seeds and the like), such as corn, soy bean, rape seed, lupin and the like.

In one aspect, the nucleic acid is expressed such that the protease of the invention is produced in recoverable quantities. The protease can be recovered from any plant or plant part. Alternatively, the plant or plant part containing the recombinant polypeptide can be used as

such for improving the quality of a food or feed, e.g., improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

5 The invention provides methods of treating fibers and fabrics using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention.

10 The culture supernatant and/or the protease of the invention can be used in any fiber- or fabric-treating method, which are well known in the art, see, e.g., U.S. Patent Nos. 6,261,828; 6,077,316; 6,024,766; 6,021,536; 6,017,751; 5,980,581; U.S. Patent Publication No. 2002/0142438A1. For example, proteases of the invention can be used in fiber and/or fabric desizing. In one aspect,
15 the feel and appearance of a fabric is improved by a method comprising contacting the fabric with the culture supernatant and/or the protease of the invention in a solution. In one aspect, the fabric is treated with the solution under pressure. For example, the culture
20 supernatant and/or the protease of the invention can be used in the removal of stains.

In one aspect, the culture supernatant and/or the protease of the invention is applied during or after the weaving of textiles, or during the desizing stage, or one
25 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 yams are often coated with sizing starch or starch derivatives in order to increase their tensile strength and to prevent breaking. The culture supernatant and/or the protease of the invention can be applied to remove these sizing starch or starch derivatives. After the textiles have been woven, a fabric can proceed to a desizing stage. This can be followed by one or more additional fabric processing steps. Desizing is the act of removing "size" from textiles.

After weaving, the size coating must be removed before further processing the fabric in order to ensure a homogeneous and wash-proof result. The invention provides a method of desizing comprising enzymatic treatment of the "size" by the action of the culture supernatant and/or the protease of the invention.

The culture supernatant and/or the protease of the invention can be used to desize fabrics, including cotton-containing fabrics, as detergent additives, e.g., in aqueous compositions. The invention provides 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. These can be finished before or after the treatment. 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 amylolytic enzymes in order to provide softness to the fabric and make the cotton more accessible to the subsequent enzymatic finishing steps. The invention provides methods of finishing denim garments (e.g., a "bio-stoning process"), enzymatic desizing and providing softness to fabrics using the culture supernatant and/or the protease of the invention. The invention provides methods for quickly softening denim garments in a desizing and/or finishing process.

Other enzymes can be also be used in these desizing processes. For example, an alkaline and thermostable amylase and a protease can be combined in a single bath for desizing and bioscouring. Among advantages of combining desizing and scouring in one step are cost reduction and lower environmental impact due to savings in energy and water usage and lower waste production. Exemplary application conditions for desizing and bioscouring are about pH 8.5 to 10.0 and temperatures of about 40°C and up. Using a protease of the invention, low enzyme dosages, e.g., about 100 grams (g) per ton of cotton, and short reaction

times, e.g., about 15 minutes, can be used to obtain efficient desizing and scouring without added calcium.

In one aspect, an alkaline and thermostable amylase and a protease are combined in a single bath desizing and bioscouring. Among advantages of combining desizing and scouring in one step are cost reduction and lower environmental impact due to savings in energy and water usage and lower waste production. Application conditions for desizing and bioscouring can be between about pH 8.5 to pH 10.0 and temperatures at about 40°C and up. Low enzyme dosages (e.g., about 100 g per a ton of cotton) and short reaction times (e.g., about 15 minutes) can be used to obtain efficient desizing and scouring without added calcium.

The culture supernatant and/or the protease of the invention can be used in combination with other carbohydrate degrading enzymes, e.g., cellulase, arabinanase, xyloglucanase, pectinase, and the like, for the preparation of fibers or for cleaning of fibers. These can be used in combination with detergents. In one aspect, the culture supernatant and/or the protease of the invention can be used in treatments to prevent the graying of a textile.

The culture supernatant and/or the protease of the invention can be used to treat any cellulosic material,

including fibers (e.g., fibers from cotton, hemp, flax or linen), sewn and unsewn fabrics, e.g., knits, wovens, denims, yarns, and toweling, made from cotton, cotton blends or natural or manmade cellulosics (e.g. originating
5 from xylan-containing cellulose fibers such as from wood pulp) or blends thereof. Examples of blends are blends of cotton or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g. polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers,
10 polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g. rayon/viscose, ramie, hemp, flax/linen, jute, cellulose acetate fibers, lyocell).

The textile treating processes of the invention
15 (using the culture supernatant and/or the protease of the invention) can be used in conjunction with other textile treatments, e.g., scouring and bleaching. Scouring is the removal of non-cellulosic material from the cotton fiber, e.g., the cuticle (mainly consisting of waxes) and primary
20 cell wall (mainly consisting of pectin, protein and xyloglucan). A proper wax removal is necessary for obtaining a high wettability. This is needed for dyeing. Removal of the primary cell walls by the processes of the invention improves wax removal and ensures a more even
25 dyeing. Treating textiles with the processes of the

invention can improve whiteness in the bleaching process. The main chemical used in scouring is sodium, hydroxide in high concentrations and at high temperatures. Bleaching comprises oxidizing the textile. Bleaching typically involves use of hydrogen peroxide as the oxidizing agent in order to obtain either a fully bleached (white) fabric or to ensure a clean shade of the dye.

The invention also provides methods of treating leather using the culture supernatant and/or the protease of the invention.

The culture supernatant and/or the protease of the invention can be used in any leather-treating method, which are well known in the art, see, e.g., International Patent Publication No. WO03/008650; Australian Patent No. 696441; U.S. Patent Publication No. 2003/061666.

The conventional leather processing is accomplished by the procedure of soaking, liming & deliming and bating. Herein, the microorganism culture supernatant and/or the protease of the invention can be treated independently or added to an additive to increase the stability of an enzyme or to be mixed with other materials added to each process. It is preferred to add the protease of the invention for each process, soaking, liming & deliming and bating, at levels of about 0.1% - 15% by weight. And the microorganism culture supernatant of the invention is

preferably added to each process, soaking, liming & deliming and bating, at levels of about 0.1% - 50% by volumn.

5 It is preferred to carry out the whole procedure of soaking, liming & deliming and bating at 60 - 65°C which is the optimum temperature for the protease of the invention, but not always limited thereto. It is also preferred to carry out the processes under pH 8 - pH 12, but not always limited thereto. In general, during the processes of
10 soaking, liming & deliming and bating, huge amount of salt is added, and most proteases lose their enzyme activities under such high concentration of slat. However, the protease of the invention showed the optimum activity in the presence of 2 - 5% salt, and maintained its activity
15 under high salt condition (20%), thus the protease of the invention can be used for each process.

In addition, the use of the microorganism culture supernatant and/or the protease of the invention enable pro-environmental leather processing because organic and
20 inorganic leather reagents can be significantly reduced thereby.

Besides, the microorganism culture supernatant and/or the protease of the invention can be used not only for the leather processing but also for the elimination of the

outer skin, hair and soluble protein left over during the procedure.

Soaking is the process that a rawhide salt-treated right after slaughter is transported to a leather processing company where the rawhide is soaked in water in
5 a paddle or a drum for a long while to absorb water enough to eliminate impurities and to recover the protein tissues as soft as before slaughter, and at this time the treatment of the microorganism culture supernatant and/or the
10 protease of the invention helps the elimination of impurities, soluble protein, hair, etc, from the rawhide. Besides, the treatment of the culture supernatant and/or the protease of the invention makes the protein tissues of the leather more soft and the liming & deliming process
15 easy.

During leather processing, the waste water produced by liming & deliming affects COD most. But, the microorganism culture supernatant and/or the protease of the invention decompose cells of stratum germinativum or
20 base cells of hair root, which is based on the principal that the protease or the supernatant decomposes medulla but not cortex of a hair. Besides, the supernatant or the protease can eliminate hair roots and follicles, leading to swelling hair poaches to pluck the hairs, so that lime or

other reagents can be easily invaded and as a result the doses of those reagents are reduced.

In the bating process, the microorganism culture supernatant and/or the protease of the invention eliminates
5 unnecessary proteins included in the rawhide and opens the tissues of the leather, increasing the penetration and coherence of provided reagents after bating. So, the required amount of reagents can be reduced and as a result, the increase of COD or BOD owing to the excessive reagents
10 can be moderated. The treatment of the microorganism culture supernatant and/or the protease of the invention also contribute to the production of very soft leather and thus improve the quality of the final product as well as decrease the waste disposal problem by reducing the dose of
15 dye by eliminating impurities and pollutant and thereby improves the leveling of color.

The present invention provides methods of treating waste produced during the leather processing using the microorganism culture supernatant and/or the protease of
20 the invention.

The microorganism culture supernatant and/or the protease of the invention treat waste produced during the procedure of soaking, liming & deliming, bating and finishing.

Herein, the waste can be waste water or a solid waste, and the waste can be re-cycled.

The microorganism culture supernatant and/or the protease of the invention used for the leather processing
5 can be treated independently or added to an additive to increase the stability of an enzyme or to another material added to each process.

It is also possible to use the microorganism culture supernatant and/or the protease of the invention together
10 with one of lipases and amylases to treat the waste produced during leather processing.

To treat waste of leather processing, the microorganism culture supernatant and/or the protease of the invention can be treated independently to the waste
15 water and a solid waste produced during soaking and liming & deliming, a solid waste resulted from bating and another solid waste produced from the finishing touches, or added to an additive to increase the stability of an enzyme or mixed with a material added to each process.

20 The waste produced during the leather processing takes 40 - 80% of the whole weight of the primary rawhide. Most of the solid waste are classified as an industrial waste to be burned up or simply reclaimed. And the treatment cost of such solid waste takes a huge part of the
25 production cost.

The microorganism culture supernatant and/or the protease of the invention can be applied to such solid waste as fleshing scrap, trimming scrap and fur produced during soaking and liming and pelt scrap produced during
5 bating as well as leather pieces produced during the finishing after drying.

In fleshing scrap and trimming scrap, protein and lipid are mixed together and lipid takes 30 - 50% of the total. To decompose those fleshing scrap and trimming
10 scrap, one or more enzymes containing a lipase or a glucosidase can be added in addition to the microorganism culture supernatant and/or the protease of the invention.

The solid waste of pelt scrap resulted from the bating takes approximately 40% of total waste materials
15 from leather processing. The pelt scrap is composed of 4.0% lipid, 1.5% calcium, 5.5% ash, 50 - 55% water and 35 - 40% protein. The protease of the invention retains its activity even in the presence of a metal ion. In the pelt scrap, numbers of metal ions such as calcium ion are
20 included, so the protease of the invention can be very effective to decompose the protein of the pelt scrap.

Many chemicals are also used in the processes of producing a leather product from a rawhide. Precisely, a huge amount of water, activators, limes, sulfides, salts,
25 acids, chromes, synthetic tannins, dyes, fatliquors,

binders, adjuvants, brightners and solvents are used to produce a leather product. In particular, the excessive use of sulfides and bases during the liming process to get rid of various non-structural proteins included in a raw hide produce a lot of pollutants, causing pollution by waste water. The waste water produced from the leather processing contains a lot of organic substances, inorganic substances and suspended solids as well as highly toxic heavy metals like chrome, increasing BOD and COD. The microorganism culture supernatant and/or the protease of the invention reduce the required amounts of activators, limes, sulfides, salts, acids and chrome.

In the meantime, there is a report on the re-cycling leather waste using a protease (Korean Registered Patent Publication No. 1994-0007333), but not specifically on the method for recycling leather waste by decomposing the protein therein with a microorganism producing a protease. The microorganism of the invention is highly capable of being growing in the presence of even small amount of carbon source and nitrogen source, and can produce a protease during growth, by which solid leather waste can be decomposed. If the solid waste is a protein, the microorganism culture supernatant and/or the protease of the invention hydrolyze the protein waste into peptides, which will be recycled to produce food, cosmetics and

industrial products. The microorganism culture supernatant and/or the protease of the invention is also effective to decompose proteins included in the waste water containing salt produced during soaking and liming & deliming, by which the proteins can also be recycled.

Considering that collagen of waste leather can be recycled as feed or eatable gelatin after cutting the polypeptide chains therein, the microorganism culture supernatant and/or the protease of the invention can prevent environmental pollution and enables the re-use of solid waste produced from the processing as a secondary product.

The invention provides methods of treating paper and paper pulp using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention.

The *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can be used in any paper-or pulp-treating method, which are well known in the art, see, e.g., U.S. Patent Nos. 6,241,849; 6,066,233; 5,582,681. For example, in one aspect, the invention provides a method for deinking and decolorizing a printed paper containing a dye, comprising pulping a printed paper to obtain a pulp slurry, and dislodging an ink from the

pulp slurry in the presence of the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention (other enzymes can also be added). In another aspect, the invention provides a method for enhancing the freeness of pulp, e.g., pulp made from secondary fiber, by adding an enzymatic mixture comprising the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention (can also include other enzymes, e.g., pectate lyase, cellulase, amylase or glucoamylase enzymes) to the pulp and treating under conditions to cause a reaction to produce an enzymatically treated pulp. The freeness of the enzymatically treated pulp is increased from the initial freeness of the secondary fiber pulp without a loss in brightness.

The invention provides chemical and enzymatic deinking processes using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention. In one aspect, the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can be used in combination with cellulases, pectate lyases or other enzymes. The paper can be treated by the following three

processes: 1) disintegration in the presence of the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention, 2) disintegration with a deinking chemical and the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention, and/or 3) disintegration after soaking with the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention. The recycled paper treated with the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can have a higher brightness due to removal of toner particles as compared to the paper treated with just cellulase. While the invention is not limited by any particular mechanism, the effect of the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention may be due to its behavior as surface-active agents in pulp suspension.

The invention provides methods of decomposing organic wastes using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention.

To use a protease industrially, it is required for the protease to be very stable to maintain its activity under severe environmental conditions which are very vulnerable, compared with those *in vivo*. That is, the severe environmental conditions herein include severe exposure on pH and heavy metals and frequent changes of oxidation-reduction, which are generally believed to affect the enzyme activity significantly. Thus, when an enzyme is out of proper ranges of those conditions, it loses its enzyme activity. Therefore, in order to use a protease industrially, the protease needs to maintain its activity under extremely unstable physical and chemical conditions.

The *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can be used in a variety of other industrial applications, e.g., in waste treatment. For example, in one aspect, the invention provides a solid waste digestion process using the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention. The methods can comprise reducing the mass and volume of substantially untreated solid waste. The *Bacillus clausii* I-52 strain and the transformant can be sterilized by autoclaving. Solid waste can be treated with an enzymatic digestive process in the presence of an

enzymatic solution (including proteases of the invention) at a controlled temperature. This results in a reaction without appreciable bacterial fermentation from added microorganisms. The solid waste is converted into a liquefied waste and any residual solid waste. The resulting liquefied waste can be separated from said any residual solidified waste. See e.g., U.S. Patent No. 5,709,796. In addition, the *Bacillus clausii* I-52 strain, the transformant, the culture supernatant thereof and/or the protease isolated therefrom of the invention can be used in the animal rendering industry, to e.g., get rid of feathers, e.g., as described by Yamamura (2002) *Biochem. Biophys. Res. Com.* 294: 1138-1143.

The invention provides oral care products comprising the culture supernatant and/or the protease of the invention. Exemplary oral care products include toothpastes, dental creams, gels or tooth powders, odontics, mouth washes, pre- or post brushing rinse formulations, chewing gums, lozenges, or candy. See, e.g., U.S. Patent No. 6,264,925.

The invention provides methods of brewing (e.g., fermenting) beer comprising the culture supernatant and/or the protease of the invention. In one exemplary process, starch-containing raw materials are disintegrated and processed to form malt. The culture supernatant and/or the

protease of the invention are used at any point in the fermentation process. For example, the culture supernatant and/or the protease of the invention can be used in the processing of barley malt. The major raw material of beer
5 brewing is barley malt. This can be a three-stage process. First, the barley grain can be steeped to increase water content, e.g., to around about 40%. Second, the grain can be germinated by incubation at 15 to 25°C for 3 to 6 days when enzyme synthesis is stimulated under the control of
10 gibberellins. In one aspect, a culture supernatant and/or a protease of the invention are added at this (or any other) stage of the process. The action of proteases results in an increase in fermentable reducing sugars. This can be expressed as the diastatic power, DP, which can
15 rise from around 80 to 190 degree in 5 days at 12°C. The culture supernatant and/or the protease of the invention can be used in any beer or alcoholic beverage producing process, as described, e.g., in U.S. Patent Nos. 5,762,991; 5,536,650; 5,405,624; 5,021,246; 4,788,066.

20 Proteases of the invention can be used for cell isolation from tissue for cellular therapies in the same manner that collagenases. Additionally, proteases of the invention can be used as antimicrobial agents, due to their bacteriolytic properties, as described, e.g., in Li, S. et.

al. Bacteriolytic Activity and Specificity of *Achromobacter* b-Lytic Protease, *J. Biochem.* 124, 332-339 (1998).

Proteases of the invention can also be used therapeutically to cleave and destroy specific proteins.

5 Potential targets include toxin proteins, such as Anthrax, *Clostridium botulinum*, Ricin, and essential viral or cancer cell proteins.

Proteases of the invention can also be used in disinfectants, as described, e.g., in *J. Gen Microbiol*
10 (1991) 137 (5): 1145-1153; *Science* (2001) 249: 2170-2172.

Additional medical uses of the proteases of the invention include lipoma removal, wound debridement and scar prevention (collagenases), debriding chronic dermal ulcers and severely burned areas.

15 Proteases of the invention can be used to in sterile enzymatic debriding compositions, e.g., ointments, in one aspect, containing about 250 collagenase units per gram. White petrolatum USP (United States Pharmacopoeia) grade can be a carrier. In one aspect, proteases of the
20 invention can be used in indications similar to Santyl Ointment (BTC, Lynbrook, NY). Proteases of the invention can also be used in alginate dressings, antimicrobial barrier dressings, burn dressings, compression bandages, diagnostic tools, gel dressings, hydro-selective dressings,
25 hydrocellular (foam) dressings, hydrocolloid dressings, I.V

dressings, incise drapes, low adherent dressings, odor absorbing dressings, paste bandages, post operative dressings, scar management, skin care, transparent film dressings and/or wound closure. Proteases of the invention
5 can be used in wound cleansing, wound bed preparation, to treat pressure ulcers, leg ulcers, burns, diabetic foot ulcers, scars, I.V. fixation, surgical wounds and minor wounds.

Additionally, proteases of the invention can be used
10 in proteomics and lab work in general. For instance, proteases can be used in the same manner as protein restriction enzymes.

The culture supernatant and/or the protease of the invention can be used for peptide synthesis, in the leather
15 industry, e.g., for hide processing, e.g., in hair removal and/or bating, for waste management, e.g., removal of hair from drains, in the photography industry, e.g., for silver recovery from the used X-ray film, in the medical industry, e.g., as discussed above, e.g., for treatment of burns,
20 wounds, carbuncles, furuncles and deep abscesses or to dissolve blood clots by dissolving fibrin, for silk degumming.

In other aspects, the culture supernatant and/or the protease of the invention can be used as flavor enhancers
25 in, for example, cheese and pet food, as described, e.g.,

in Pommer, K., Investigating the impact of enzymes on pet food palatability, *Petfood Industry*, May 2002, 10-11.

In yet another embodiment of the invention, the culture supernatant and/or the protease of the invention
5 can be used to increase starch yield from corn wet milling, as described, e.g., in Johnston, D. B., and Singh, V. Use of proteases to Reduce Steep Time and SO₂ requirements in a corn wet-milling process, *Cereal Chem.* 78 (4): 405-411.

In other aspects, the culture supernatant and/or the
10 protease of the invention can be used in biodefense (e.g., destruction of spores or bacteria). Use of proteases in biodefense applications offers a significant benefit, in that they can be very rapidly developed against any currently unknown biological warfare agents of the future.
15 In addition, the culture supernatant or the protease of the invention can be used for decontamination of affected environments.

Additionally, the culture supernatant and/or the protease of the invention can be used in biofilm
20 degradation, in biomass conversion to ethanol, and/or in the personal care and cosmetics industry. The culture supernatant and/or the protease of the invention can also be used to enhance enantioselectivity, as described, e.g., in Arisawa, A. et. al. *Streptomyces Serine Protease (DHP-A)*
25 as a New Biocatalyst Capable of Forming Chiral

Intermediates of 1,4-Dihydropyridine Calcium Antagonists.
Appl. Environ. Microbiol. 2002 Jun; 68 (6): 2716-2725;
Haring, D. et. al. Semisynthetic Enzymes in Asymmetric
Synthesis: Enantioselective Reduction of Racemic
5 Hydroperoxides Catalyzed by Seleno-Subtilisin. *J. Org. Chem.*
1999, 64: 832-835.

As explained hereinbefore, the protease of the
invention has a stability not to lose its enzyme activity
under severe conditions such as alkaline environment, a
10 surfactant and high temperature, and has a wide substrate
specificity enabling the elimination of food and blood or
body fluid. Therefore, the protease, the *Bacillus clausii*
I-52 strain and the transformant producing the protease,
and the microorganism culture supernatant containing the
15 same can be used as a detergent component with generally
known synthetic surfactants including SDS, octylglucoside,
Brij-35, Tritons, Tweens, LAS, AOS (α -orephin sulfonate),
etc, or with a natural surfactant. Besides, the protease
of the invention retains its activity under wide range of
20 pH and oxidation-reduction condition, so that it can be
widely used in various fields of processing food, feed,
fabrics, natural leather processing, brewing, paper and
pulp, and in the production of oral care products and
medical applications, in research fields, and in waste

treatment because it retains a high activity in the presence of a heavy metal.

The detergent composition containing the protease of the invention may be formulated by the conventional method for a laundry detergent or a contact lens detergent. And a processing method or a decomposing method using the protease is not limited. In fact, every method including the step of treating the protease of the invention, according to general manners well known to those in the art, can be included in the criteria of the present invention.

Mode for Invention

Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Example 1: Isolation of a strain producing a protease from the west coast mudflat

The present inventors isolated a strain producing a protease from the west coast mudflat of Korea as follows:

Firstly, 1 g of the mudflat sample was suspended in 10 ml of

sterilized distilled water, which was then left at room temperature for 5 minutes to precipitate shore components. 0.2 ml of the supernatant was obtained and loaded on a agar plate containing 0.5% sodium carbonate and powdered skim milk, followed by observation on a clear zone formed around a colony formed by hydrolysis of the skim milk. Then, those colonies forming a clear zone in more than 2 mm diameter were selected. From the selected colonies, a strain producing a protease having an excellent activity was selected by using a liquid medium.

The cell culture supernatant was recovered to examine the protease activity. Particularly, 0.5 ml of a substrate solution prepared by dissolving Hammerstan casein (final concentration: 0.5%) in 0.1 M glycine-NaOH buffer (pH 11.0) was mixed well with 0.1 ml of a 100 - 1000 fold diluted enzyme solution, followed by reaction at 60°C for 10 minutes. Then, 0.5 ml of 10% trichloro acetic acid (TCA) was added thereto in order to terminate the reaction. The reaction solution was put in ice for 10 minutes to precipitate proteins remaining unreacted. Supernatant was obtained by centrifugation and tyrosine was quantified at 275 nm. At this time, 1 unit of a protease activity was defined as the amount of enzyme necessary for the production of 1 μ g of tyrosine for one minute.

As a result, a strain showing the best protease activity was selected, which was confirmed to be a Gram-positive by the Gram-test. The morphological and biochemical characteristics of the selected strain were also investigated. The strain formed a spore and showed catalase and oxidase activities, but did not show urease activity. The strain also produced acid and gas by using a sugar such as glucose, fructose, galactose, mannose, maltose, mannitol, sorbitol and starch, and proteins such as casein and gelatin. The morphological and biochemical characteristics of the strain are presented in Table 1. From a nucleotide sequencing, it was confirmed that the strain has 16S ribosomal RNA represented by SEQ. ID. NO: 1, and thereby the strain was further confirmed to be a *Bacillus* sp. *Bacillus clausii* strain.

The present inventors named the strain selected above as "*Bacillus clausii* I-52" and deposited at Korean Collection for Type Cultures (KCTC) of Korea Research Institute of Bioscience and Biotechnology (KRIBB, #52, Oundong, Yusong-ku, Taejon, Korea), on Jun 14, 2002 (Accession No: KCTC 10277BP).

Example 2: Investigation on factors affecting the protease activity

<2-1> The effect of pH on the protease activity

The present inventors investigated whether pH of a medium could affect the protease activity during the culture of *Bacillus clausii* I-52. Particularly, a basal medium was composed of the components listed in Table 2, to which sodium carbonate was added (final concentration: 0-1%). Seed culture was performed for 20 hours. After inoculating the inoculum by 2%, shaking culture was performed for 40 hours at 37°C. The culture solution was centrifuged to obtain supernatant. The protease activity of the supernatant was measured by the same manner as described in Example 1.

Table 2: Components of the Basal Medium

Component	Concentration
Soybean meal	2%
Potassium phosphate	0.5%
Magnesium sulfate	0.5 mM

As a result, the level of the protease was increased in a weak alkaline medium containing sodium carbonate more than 0.3%, and the highest production of the enzyme was detected when sodium carbonate was added by 0.5% (pH 10.12). Thus, the optimum concentration of sodium carbonate in a culture medium was determined as 0.5% (Table 3).

Table 3: Enzyme activities according to concentration of sodium carbonate

Conc. of sodium carbonate (%)	pH	Enzyme activity (U/ml, x 10 ⁻³)
-	6.96	14.32
0.3	8.61	18.80
0.4	9.84	20.55
0.5	10.12	21.25
0.6	10.35	20.99
0.7	10.55	19.87
0.8	10.62	19.39
0.9	10.71	19.28
1.0	10.77	18.64

<2-2> The effect of an organic nitrogen source on the protease activity

The present inventors investigated the effect of an organic nitrogen source on the production of the protease in *Bacillus clausii* I-52. A basal medium having the composition of Table 2 was supplemented with each nitrogen source (1%) and sodium carbonate (0.5%), to which the inoculum pre-cultured for 20 hours was inoculated, followed by shaking-culture for 40 hours at 37°C. The culture solution was centrifuged to obtain a supernatant, and the

protease activity of the supernatant was measured by the same manner as described in Example 1.

As a result, the addition of an organic nitrogen source increased the production of the protease. In particular, when casein was added, the production of the protease was approximately 50% increased (Table 4). From the result, it was confirmed that the optimum production of the protease in *Bacillus clausii* I-52 is induced by the addition of one or more organic nitrogen sources selected from the group consisting of casein, soybean meal, peptone, yeast extract, powdered skim milk and whey. At this time, soybean meal or casein is preferred and the preferable concentration of it is 0.2 - 5%.

Table 4: Enzyme activities according to organic nitrogen sources

Organic nitrogen source	Enzyme activity (U/ml, x 10 ⁻³)
-	16.75
Casein	24.63
Powdered skim milk	22.66
Whey	18.05
Yeast extract	19.29
Peptone	18.04

<2-3> The effect of an inorganic nitrogen source on the protease activity

The present inventors investigated the effect of an inorganic nitrogen source on the production of the protease in the *Bacillus clausii* I-52. A medium was prepared by adding each inorganic nitrogen source by 0.5% to the basal medium of Table 3 containing casein (1%) and sodium carbonate (0.5%). The inoculum cultured for 20 hours was inoculated in the medium by 2%, followed by shaking culture for 40 hours at 37°C. The culture solution was centrifuged to obtain a supernatant, and the protease activity of the supernatant was measured by the same manner as described in Example 1.

As a result, the protease production was increased by the addition of an inorganic nitrogen source except a nitrate. When ammonium sulfate was added, the production of the protease was increased most (approximately up to 20%). From the results, it was confirmed that the optimum production of the protease in the *Bacillus clausii* I-52 was induced by the addition of one or more inorganic ammonium salts selected from a group consisting of ammonium sulfate, ammonium phosphate, etc. And at this time, the concentration of an inorganic ammonium salt is preferably in the range between 0.1 and 5% (Table 5).

Table 5: Enzyme activities according to inorganic nitrogen sources

Inorganic nitrogen source	Enzyme activity (U/ml, $\times 10^3$)
-	15.36
Ammonium sulfate	19.21
Ammonium phosphate	18.75
Ammonium chloride	16.43
Ammonium acetate	15.29
Sodium nitrate	11.18

<2-4> The effect of a carbon source on the protease activity

The present inventors investigated the effect of a carbon source on the protease activity in *Bacillus clausii* I-52. A medium was prepared by adding each carbohydrate by 0.5% to the basal medium containing casein (1%) and sodium carbonate (0.5%), to which the inoculum cultured for 20 hours was inoculated by 2%, followed by shaking culture for 40 hours at 37°C. The culture solution was centrifuged to obtain supernatant, and the protease activity of the supernatant was measured by the same manner as described in Example 1.

As a result, the highest production of the protease was observed when wheat flour and liquid maltose were added to the culture of *Bacillus clausii* I-52. Even with the

addition of a carbon source having a low molecular weight selected from a group consisting of sodium pyruvate, sodium citrate, sodium acetate and sodium succinate, the protease production was increased. From the result, it was confirmed that the optimum production of the protease in the *Bacillus clausii* I-52 is induced by the addition of one or more carbon sources selected from a group consisting of liquid maltose, maltose, wheat flour, sodium pyruvate, sodium citrate and sodium succinate, and the preferable concentration of a carbon source is 0.1 - 5% (Table 6).

Table 6: Enzyme activities according to carbon sources

Carbon source	Enzyme activity (U/ml, x 10 ³)
-	18.28
Glucose	15.42
Lactose	8.33
Maltose	23.16
Sweet potato starch	16.58
Corn starch	17.51
Liquid maltose	29.19
Sodium pyruvate	23.17
Sodium citrate	20.11
Sodium acetate	19.14
Sodium succinate	19.27
Sodium glutamate	18.12

Example 3: Protease production<3-1> Protease production 1

The present inventors produced a protease from the
5 *Bacillus clausii* I-52 strain selected in Example 1.
Particularly, a medium for the culture of the *Bacillus*
clausii I-52 was prepared as a liquid medium composed of
1.5% soybean meal, 1% casein, 0.4% potassium phosphate,
0.4% sodium citrate, 0.5 mM magnesium sulfate, 0.5% wheat
10 flour, and autoclaved for 20 minutes at 121°C. In addition,
40% sodium carbonate solution and 50% liquid maltose
solution were autoclaved separately for 20 minutes at 121°C,
and the sodium carbonate solution and the liquid maltose
solution were added to the medium at a concentration of
15 0.5% and 1.5% respectively, before a culture. And then, an
inoculum from a seed culture using a medium having the same
composition as the above medium, which was cultured in a
flask for 24 hours at 37°C was inoculated by 2%, followed
by culture for 48 hours at 37°C in a 5 L fermentor (NBS,
20 USA). At that time, the airflow speed was 1 vvm and the
stirring speed was 400 rpm. Centrifugation was performed
at 12,000 g for 20 minutes and supernatant was recovered to
measure the protease activity by the same manner as
described in Example 1. The culture solution was
25 centrifuged at 12,000 g for 20 minutes to obtain

supernatant, and the protease activity of the supernatant was measured by the same manner as described in Example 1.

As a result, the protease activity was 36,560 U per 1 ml of the *Bacillus clausii* I-52 culture supernatant.

5

<3-2> Protease production 2

Culture of the microorganism was performed with increased concentration of soybean meal (2%) for 48 hours and the other culture conditions and compositions were the same as in Example 3-1. A supernatant was obtained to examine the protease activity.

As a result, the protease activity was 52,760 U per 1 ml of the *Bacillus clausii* I-52 culture supernatant.

15

<3-3> Protease production 3

Among culture conditions and compositions of Example 3-2, the concentration of wheat flour was adjusted to 1% and the concentration of liquid maltose was adjusted to 2%, followed by culture for 48 hours. A supernatant was obtained to examine the protease activity.

As a result, the protease activity was 80,295 U per 1 ml of the *Bacillus clausii* I-52 culture supernatant.

<3-4> Protease production 4

Culture of the microorganism was performed for 48 hours in a medium having the same composition as that in Example 3-3. At that time, the airflow speed was 1.5 vvm and the stirring speed was 600 rpm. A supernatant was
5 obtained to examine the protease activity.

As a result, the protease activity was 123,520 U per 1 ml of the *Bacillus clausii* I-52 culture supernatant.

Example 4: Separation and purification of the protease from
10 *Bacillus clausii* I-52

(Step 1) Absorption

The microorganism was cultured for 48 hours in a medium having the same composition as that of Example 3-4 and the obtained supernatant was used as a crude enzyme
15 solution. Diaion HP20 synthetic absorbent was added to the crude enzyme solution, followed by absorption for 16 hours with stirring. The absorbent was recovered by filtering. The absorbent was then washed with distilled water and active fractions were eluted by sodium phosphate buffer (pH
20 7.5) containing 30% acetone.

(Step 2) Phenyl-Sepharose hydrophobic chromatography

Phenyl-Sepharose column (5x8 cm, Amersham Pharmacia) was equilibrated with 0.1 M sodium phosphate buffer (pH
25 7.5) containing 1 M ammonium sulfate, and then the eluent

was loaded to the column. The column was washed until OD₂₈₀ reached almost 0, and active fractions were eluted by 10 mM sodium phosphate buffer (pH 7.5).

5 (Step 3) DEAE-Sepharose ion exchange chromatography

DEAE-Sepharose column (2.5x10 cm, Amersham Pharmacia) was equilibrated with 10 mM sodium phosphate buffer (pH 7.5), then the fraction obtained from the Phenyl-Sepharose column chromatography was loaded onto the
10 DEAE-Sepharose column. The column was washed with the same buffer until OD₂₈₀ reached almost 0.

(Step 4) CM-Sepharose ion exchange chromatography

CM-Sepharose column (2.5x5 cm, Amersham Pharmacia)
15 was equilibrated with 50 mM sodium phosphate buffer (pH 7.0), then the fraction obtained from the DEAE-Ssepharose column chromatography was loaded onto the CM-Ssepharose column. The column was washed with the same buffer until OD₂₈₀ reached almost 0. Active fractions were eluted by
20 linear gradient using 100 ml of 50 mM sodium phosphate buffer (pH 7.0) and 100 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl. And, the activity of the fraction was measured.

(Step 5) Protease purification by SDS-PAGE and molecular weight determination

To confirm whether the protease was successfully separated and purified and to determine the molecular weight of the same, 15% SDS-PAGE was performed using the ion-exchange chromatography fractions separated above. 97 kDa phosphorylase b, 66 kDa BSA, 45 kDa ovalbumin, 30 kDa carbonic anhydrase, 20.1 kDa trypsin inhibitor and 14.4 kDa α -lactalbumin were used as standard proteins to measure the molecular weight. To calculate relative Rf value for the molecular weight, semi-log coordinates were used to produce a linear equation, from which the molecular weight of the purified protease subunit was calculated.

As a result, a 28 kDa pure band was detected, indicating that a protein having the protease activity was purified (Fig. 1).

(Step 6) N-terminal analysis

To analyze the amino acid sequence of N-terminal of the purified protease, the enzyme was purified and 5 μ g (20 μ l) of the purified enzyme was electrophoresed in SDS-PAGE composed of 15% separating gel and 4% stacking gel. After electrophoresis, the separating gel was loaded on a cassette which was covered with a fiber pad and a filter paper soaked in protein transfer buffer (10 mM CAPS/10%

methanol, pH 11.0), and then, PVDF (polyvinylidene difluoride) membrane soaked for one hour in 100% methanol was loaded onto the cassette. Finally, a filter paper and a fiber pad were loaded over again for the close adhesion of the gel and the membrane. 300 mA of constant current was given to the protein on the gel using S cell Blot module (Novex, Inc., USA) for one hour, resulting in the transfer to PVDF membrane. After finishing the transfer, PVDF membrane was stained with Coomassie brilliant blue R-250 for one minute, and then destained with 50% methanol solution briefly. The stained proteins on PVDF membrane were selectively cut out, whose N-terminal amino acid sequence was analyzed at Korea Basic Science Institute. And the amino acid sequence is as follows:

Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln Ile Lys Ala
Pro Ala Leu (SEQ. ID. NO: 2).

Example 5: Optimum conditions for the protease activity

<5-1> Optimum pH for the protease activity

The present inventors investigated optimum pH for the protease activity. Particularly, casein, a substrate, was prepared with buffers having different pH. Each protease activity under different pH conditions was measured as follows: the activity was measured using 0.1 M sodium phosphate buffer under pH 7.0 - 7.5, using 0.1 M Tris-HCl

buffer under pH 8.0 - 9.5 and using 0.1 M glycine-NaOH
buffer under pH 10.0 - pH 12.0.

As a result, the protease showed high enzyme activity
in pH range between 8.0 and 12.0 and particularly, pH 11.0
5 was the optimum pH of the protease activity (Fig. 2).

<5-2> Optimum temperature for the protease activity

The present inventors investigated the optimum
temperature for the protease activity. Particularly,
10 casein, a substrate, was prepared (0.5%) with 0.1 M
glycine-NaOH buffer (pH 11.0) and the protease activity was
measured at the temperature range between 10 - 70°C

As a result, the optimum temperature for the protease
activity was confirmed to be 60 - 65°C (Fig. 3).

15

Example 6: Analysis of the protease stability

<6-1> Stability of the protease against a surfactant

To investigate the stability of the protease
separated and purified in the Example 4 against a
20 surfactant, the inventors measured the enzyme activity
after treating the enzyme with an anionic surfactant.
Particularly, the protease activity was measured after
treating SDS (sodium dodecyl sulfate) with different
concentrations such as 1%, 2.5%, 5% and 10%.

As a result, the protease activity was maintained when 5% SDS was treated thereto for three days, indicating that the enzyme is very stable against SDS (Fig. 4).

5 <6-2> Stability of the protease against oxidants

To investigate the stability of the protease against an oxidant, the present inventors measured the time-dependent enzyme activity after treating the enzyme with oxidants such as hydrogen peroxide and sodium perborate. 10 Particularly, the protease activity was measured after treating with hydrogen peroxide and sodium perborate, known as strong oxidants, with different concentrations such as 0.1%, 0.5%, 1% and 2.5%.

As a result, the protease of the invention did not 15 lose the activity after being treated with 2.5% of hydrogen peroxide and sodium perborate for three days, proving the protease to be a very stable enzyme (Fig. 5 and 6).

<6-3> Stability of the protease against an organic solvent

20 To investigate the stability of the protease against an organic solvent, the present inventors measured the enzyme activity after treating the protein with various organic solvents with 10% or 20%. Particularly, the protease was treated with 10% or 20% of acetone, 25 acetonitrile, ethanol, isopropanol, methanol,

dimethylformamide or dimethylsulfoxide, followed by measuring the time-dependent enzyme activity.

As a result, the protease retained the activity after treating with 20% of various organic solvents for three days, proving the protease to be a very stable enzyme against an organic solvent (Table 7). The result also suggests that the protease of the invention can be effectively used for the biosynthesis of an oligopeptide.

Table 7: Relative activity of the protease of the present invention after treating various organic solvents

Solvent	Relative Activity (%)			
	4 h	24 h	48 h	72 h
Acetone	92.9	97.6	99.2	97.1
	102.0	95.3	105.8	101.0
Acetonitrile	90.9	94.5	94.2	93.5
	93.5	86.4	97.6	92.5
Ethanol	98.2	94.6	97.2	96.5
	91.8	87.6	95.5	91.7
Isopropanol	103.3	104.9	109.3	106.2
	108.1	109.8	112.2	110.3
Methanol	93.4	96.1	94.2	94.7
	104.0	95.2	95.8	97.6
Dimethylformamide	107.3	107.0	109.1	107.9
	110.8	107.7	111.4	109.9
Dimethylsulfoxide	91.4	88.6	94.3	94.8
	94.1	93.1	97.4	95.0

<6-4> Optimum salinity for the protease activity

To investigate the optimum salinity for the protease activity, the concentration of NaCl in the reaction solution containing the protease was adjusted differently and then the protease activity was measured. Inoculum precultured for 24 hours was inoculated on the culture medium (soybean meal 2%, wheat flour 1%, liquid maltose 2%, sodium citrate 0.5%, potassium phosphate 0.5%, magnesium sulfate 0.5 mM, sodium carbonate 0.6%), followed by shaking culture for 48 hours at 37°C. The culture solution was centrifuged to obtain a supernatant. NaCl was added to the supernatant by 0 - 20%, and the enzyme activity was measured by the same manner as described in Example 5. As a result, when NaCl was added by 0.1 - 10%, the enzyme activity was increased, compared with when NaCl was not added. And 0.1 - 5% was more preferred and 1% concentration of NaCl was proved to be the optimum concentration (Fig. 7).

As a result, the *Bacillus clausii* derived protease did not lose the enzyme activity under the high concentration of salt (more than 10%), in particular, the protease retained more than 75% of the activity even fewer than 20% concentration of salt. The above results indicate that the *Bacillus clausii* derived protease is a halophilic

protease, which can be effectively used as a detergent additive and for the food processing.

<6-5> Effect of a metal ion on the protease activity

5 To investigate the effect of a metal ion on the protease activity, the present inventors treated various metal ions to the reaction solution and measured the enzyme activity. Particularly, the protease was treated with 1 mM of calcium, cadmium, cobalt, chrome, copper, magnesium or
10 manganese and measured the enzyme activity thereafter.

As a result, the protease of the invention was not affected by the metal ions, even by the heavy metals such as cadmium, cobalt, chrome, etc (Fig. 8).

15 Table 8: Enzyme activity according to metal ions

Metal Ion	Relative Activity (%)
-	100.0
Ca ²⁺	116.7
Cd ²⁺	75.4
Co ²⁺	130.0
Cr ³⁺	136.0
Cu ²⁺	79.7
Hg ²⁺	48.2
Mg ²⁺	107.9
Mn ²⁺	110.9
Zn ²⁺	83.5

<6-7> Stability of the protease against a commercial detergent

To investigate the stability of the protease against an oxidant, the present inventors examined the stability of the protease against a commercial detergent.

The protease was treated with 4 different commercial detergents (BrightTM, Mukunahwa, Korea; One ScoopTM, LG Household & Health Care Ltd., Korea; SparkTM, Aekyung Ind., Korea; Super TiTM, LG Household & Health Care Ltd., Korea) on market by the concentration of 2.5% each and stood for 4 weeks at room temperature. As a result, the protease retained the enzyme activity, suggesting that the protease is a very stable enzyme and thus can be used as a detergent additive (Fig. 8).

Example 7: Detergency test of the protease

To confirm whether the protease produced from the *Bacillus clausii* of the invention is appropriate to be used as a detergent additive, the present inventors performed detergency test of the protease of the invention, which was separated and purified in Example 4. As a soiled cloth, cotton test cloth (EMPA 116) contaminated with blood and milk, and mixed fiber test cloth (EMPA 117) contaminated

with blood and milk were used to examine the detergency of the protease.

As a result, the protease showed excellent detergency in the presence of the commercial surfactant LAS (linear alkylbenzene sulfonates) 15%, indicating that the protease
5 can be used as a detergent additive (Fig. 9).

Example 8: Investigation on the soybean meal decomposing capacity of the protease

10 <8-1> Decomposition of soybean meal

To confirm whether the protease produced from the *Bacillus clausii* of the invention is appropriate for the use as a feed additive, the present invention investigated soybean meal decomposing capacity of the protease separated
15 and purified in Example 4. Soybean meal was added to distilled water at a concentration of 10%, to which the protease was added by 0.5% for the total soybean meal volume, followed by stirring. Soybean meal decomposition was measured by SDS-PAGE.

20 As a result, the protease of the invention was proved to decompose soybean meal efficiently within 2 hours (Fig. 10).

<8-2> Investigation on the decomposition of a trypsin
25 inhibitor for the decomposition of soybean meal

To confirm whether the protease produced from the *Bacillus clausii* of the invention is appropriate for the use as a feed additive, the present inventors performed soybean meal decomposition test using the protease produced in Example 4. Soybean meal contains soybean trypsin inhibitor (SBTI), known as an antinutritional factor, so when soybean meal is provided as a major protein supplier, the digestibility of protein decreases. Therefore, the protease of the invention needs to inactivate the soybean trypsin inhibitor in order to be used as a feed additive.

As a result, the protease of the invention was proved to inactivate the soybean trypsin inhibitor approximately 80% within 2 hours and when the protease was treated for 16 hours, the soybean trypsin inhibitor was completely inactivated (Fig. 11).

Example 9: Nucleotide sequence and amino acid sequence analysis of the protease

Following experiments were performed to investigate the amino acid sequence of the protease separated and purified in the above and the nucleotide sequence of a gene encoding the same.

<9-1> Separation of genomic DNA

The microorganism of the invention, *Bacillus clausii* I-52, was inoculated into 3 ml of tryptic soy broth (TSB), followed by shaking culture at 37°C with 250 rpm. The microorganism was inoculated again into a 500 ml Erlenmeyer flask containing 100 ml of TSB, followed by further culture for 24 hours. The culture solution was centrifuged at 5,000 x g, and lysozyme and proteinase K were added to the resultant *Bacillus*, followed by reaction for 18 hours at room temperature. Same volume of phenol was added thereto and well mixed for 20 minutes at room temperature. Centrifugation was performed at 13,000 x g for 20 minutes to obtain supernatant. The experiments were repeated and same volume of chloroform/isoamyl alcohol mixture (24:1) was added thereto, which was also mixed for 20 minutes at room temperature. Centrifugation was performed again at 13,000 x g for 20 minutes to obtain a supernatant. Same amount of chloroform was added thereto, followed by stirring for 20 minutes at room temperature. Centrifugation was performed at 13,000 x g for 20 minutes to obtain a supernatant, to which 2X volume of ethanol was added, followed by slow stirring at room temperature until DNA precipitate was obtained. The genomic DNA in the reaction solution was recovered by using a sterilized Pasteur pipette and the DNA was washed with 70% ethanol to remove the remaining salts. The DNA was dried in a clean

bench and then resuspended in 1 ml TE buffer (10 mM Tris-HCl, pH 8.2/1 mM EDTA). The resultant DNA was examined by 1% agarose gel electrophoresis.

5 <9-2> Analysis of nucleotide sequence

For the cloning of a gene encoding the protease of the invention, the present inventors prepared primers with well-preserved regions of the sequence of alkaline serine protease by Blast searching. And the primers were designed
10 to include the entire amino acid sequence of the alkaline serine protease.

Particularly, forward primers F2 and F3, represented by SEQ. ID. NO: 6 and 7, respectively, and reverse primers R1, R2 and R3, represented by SEQ. ID. NO: 8, 9 and 10,
15 respectively were synthesized (Fig. 12).

PCR was performed by using the *Bacillus clausii* genomic DNA extracted in Example <9-1> as a template and various combinations of the primers as follows: pre-denaturation at 94°C for 2 minutes with *Taq* DNA polymerase,
20 denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, polymerization at 72°C for 1 minute, 30 cycles from denaturation to polymerization. As a result, PCR products according to different primer combinations were obtained (Fig. 13). The PCR product obtained by the primer
25 combination F3/R2 was cloned into a PCR cloning vector,

pGEM T easy (Promega, USA) (Fig. 14), followed by nucleotide sequencing. As a result, the whole DNA size was 2280 bp (SEQ. ID. NO: 3), TCTACT (872-877) was -35 sequence and TACAAT (897-902) was -10 sequence, which contained a promoter region. The presence of N-terminal amino acid sequence (SEQ. ID. NO: 2) of the protease purified in Example 4 (step 6) was confirmed, so the separated gene was confirmed to be the whole gene of the protease separated from the *Bacillus clausii* of the invention.

<9-3> Analysis of amino acid sequence

The whole DNA nucleotide sequence (SEQ. ID. NO: 3) obtained in Example <9-2> was analyzed by BLAST searching. As a result, the DNA was proved to be composed of 1143 bp open reading frames encoding 381 amino acids represented by SEQ. ID. NO: 4, and 32 amino acid residues in N-terminal thereof were signal sequence, and the 107th amino acid alanine (Ala) was the N-terminal amino acid of an active protein. In particular, the active protease region was composed of 275 amino acid residues represented by SEQ. ID. NO: 5 and three active catalytic triads Asp32, His64 and Ser221, which are common in serine proteases, were also included therein. Thus, the protease was confirmed to be one of typical serine proteases. The active protease

region represented by SEQ. ID. NO: 5 was named *Bacillus clausii* alkaline protease (BCAP).

Example 10: Construction of an expression vector for an active protease and transformation

To construct an expression vector containing a gene of the active protease derived from *Bacillus clausii* of the invention, PCR was performed by using pGEM T easy-BCAP as a template with primers represented by SEQ. ID. NO: 11 and 12 as follows: pre-denaturation at 94°C for 2 minutes with *Taq* DNA polymerase, denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, polymerization at 72°C for 1 minute, 30 cycles from denaturation to polymerization. The amplified approximately 870 bp DNA fragment, which includes 825 bp DNA sequence encoding 275 amino acid residues represented by SEQ. ID. NO: 5, was electrophoresed, followed by separation of DNA by using an agarose gel elution kit (Qiagen, USA). The DNA fragment and pT7-7 plasmid (Roche Applied Science) were double digested with *Nde* I and *Hind* III, and then ligated, resulting in the construction of vector pT7-BCAP. *E. coli* BL21 (DE3) was transformed with the vector pT7-BCAP (Fig. 15). Particularly, 0.1 - 0.5 µg of the expression vector pT7-BCAP was added into 100 µl of competent cell *E. coli* BL21 (DE3), which was put in ice for 30 minutes. Then, heat-shock was

given for 1.5 minute at 42°C 1 ml of LB medium was added thereto, followed by culture for one hour at 37°C The cells were smeared on LB agar plate containing ampicillin (50 µg/ml), IPTG and X-gal, followed by further culture for 5 16 hours at 37°C to obtain a transformant. The transformed *E. coli* was inoculated into the LB liquid medium containing ampicillin, and then some of the culture solution was taken to extract a plasmid by alkaline dissolution. The plasmid was digested with a restriction enzyme of the cloned site 10 to confirm the insertion of the gene (Fig. 16).

Example 11: Inducement of the expression of a recombinant protein

E. coli BL21 (DE3) transformed with the expression 15 vector pT7-BCAP constructed in Example 8 was inoculated into a LB medium containing ampicillin (50 µg/µl), followed by culture for overnight at 37°C which was used as an inoculum. 1 ml of the inoculum was inoculated into 100 ml LB medium (500 ml Erlenmeyer flask) containing ampicillin, 20 followed by further culture until OD₆₀₀ reached 0.6 - 0.8. To induce protein synthesis, IPTG was added (final concentration, 1 mM), and then the culture solution was taken every hour by 1 ml per hour. After collection of the cell by centrifugation for 5 min at 15,000 rpm, the cell 25 pellet was suspended in 20 µl of 1X SDS-PAGE sample buffer

0.05 M Tris-HCl, pH 6.8, 0.1 M DTT, 2% SDS, 10% glycerol and 0.1% bromophenol blue), followed by heating at 100°C for 5 minutes to lysate the cells. Electrophoresis was performed according to the method of Laemmli, et al
5 (Laemmli, U.K., 1970, *Nature*, 227, 680-685). The gel was stained with Coomassie brilliant blue R-250 to confirm the protein produced in the *E. coli* transformed with pT7-BCAP.

As a result, the mature protease of the invention was produced by IPTG induction as time went by (Fig. 17).

10

Example 12: Construction of a plasmid for the insertion of a protease gene containing a promoter

pGEM-T easy plasmid with the DNA sequence represented by SEQ. ID. NO: 3 which contains the protease promoter
15 derived from the *Bacillus clausii* of the invention and pHPS9 (ATCC) plasmid for the insertion of *Bacillus* genome were treated with *EcoR* I, followed by electrophoresis. Then, DNAs were extracted by using DNA elution kit. After ligation, transformation of *E. coli* JM109 was performed
20 according to the same manner as described in Example 9. Chloramphenicol was used as a selection marker instead of ampicillin. A plasmid was extracted from the transformant, which was digested with a restriction enzyme corresponding to the cloning site to confirm the insertion of DNA
25 fragment represented by SEQ. ID. NO: 3. The transformation

vector with the insertion of DNA fragment represented by
SEQ. ID. NO: 3 was named "pHPS-BCAP" (Fig. 18). The
expression vector was introduced into *E. coli* JM109,
resulting in the transformant *Escherichia coli* JM109/pHPS-
5 BCAP, which was deposited at Korean Collection for Type
Cultures (KCTC) of Korea Research Institute of Bioscience
and Biotechnology (KRIBB, #52, Oun-dong, Yusong-ku, Taejon,
Korea), on Jun 14, 2002 (Accession No: KCTC 10772BP).

10 Example 13: Measurement of the enzyme activity of the
recombinant protease

To investigate the activity of the recombinant
protease produced from the transformant of Example 10, cell
culture supernatant of the transformant was recovered and
15 the enzyme activity was measured. Particularly, Hammerstan
casein was dissolved in 0.1 M glycine-NaOH buffer (pH 11.0)
(final concentration of the hammerstan casein therein was
0.5%) to prepare a substrate solution. Then, 0.1 ml of 100
- 1000 fold diluted enzyme solution was added to 0.5 ml of
20 the substrate solution, followed by reaction for 10 minutes
at 60°C 0.5 ml of 10% trichloroacetic acid (TCA) was added
to terminate the reaction. The reaction solution was put
in ice for 10 minutes to precipitate unreacted proteins.
Centrifugation was performed to obtain supernatant,
25 followed by measuring the amount of tyrosine produced at

275 nm. Herein, 1 unit of the protease means the amount of enzyme necessary for the production of 1 μ g of tyrosine for one minute. As a result, the titer was 160,000 U per 1 ml of the transformant culture solution.

5

Industrial Applicability

As described hereinbefore, the protease of the invention is so much stable that it retains the enzyme activity under alkaline condition, in the presence of a
10 surfactant and at the high temperature. Besides, the protease of the invention has a broad substrate specificity, so that it can eliminate food, blood or body fluid. Thus, the protease, the *Bacillus clausii* I-52 strain and the transformant producing the protease, and the microorganism
15 culture supernatant containing the same can be used as a detergent component with generally known synthetic surfactants including SDS, octylglucoside, Brij-35, Tritons, Tweens, LAS, AOS or with a natural surfactant. The protease of the invention retains the enzyme activity even
20 under wide range of pH and oxidation-reduction condition, so that it can be widely used in various fields of processing food, feed, fabrics, natural leather, brewing, paper and pulp, and in the production of oral care products and medical applications, in research fields, and in waste

treatment because it retains a high activity in the presence of a heavy metal.

Sequence List Text

5 The SEQ. ID. NO: 1 is a nucleotide sequence of 16S rDNA of the *Bacillus clausii* I-52 strain of the invention.

 The SEQ. ID. NO: 2 is an amino acid sequence of N-terminal of the protease of the invention.

10 The SEQ. ID. NO: 3 is a nucleotide sequence of a polynucleotide fragment containing a gene encoding the protease of the invention.

 The SEQ. ID. NO: 4 is an amino acid sequence corresponding to the full length of the protease of the invention.

15 The SEQ. ID. NO: 5 is an amino acid sequence of the active form of the protease of the invention generated by the digestion of the leader sequence and the pre-pro sequence of the protease represented by SEQ. ID. NO: 3.

20 The SEQ. ID. NO: 6 - 10 are promoter sequences used for the cloning of a gene encoding the protease of the invention.

 The SEQ. ID. NO: 11 and 12 are primer sequences used for the construction of an expression vector for the active form of the protease of the invention.

25

Those skilled in the art will appreciate that the conceptions and specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying
5 out the same purposes of the present invention. Those skilled in the art will also appreciate that such equivalent embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.

What is claimed is:

1. A *Bacillus clausii* I-52 strain producing a protease deposited under Accession NO: KCTC 10277BP.

5 2. A protease separated from the strain of claim 1.

3. The protease according to claim 2, which is characterized by the followings:

10 (a) optimum temperature: 55°C- 65°C

 (b) optimum pH: 10.0 - 11.0;

 (c) stable for more than 72 hours in the presence of SDS surfactants, oxidants such as hydrogen peroxide and sodium perborate, organic solvents and heavy metal
15 ions;

 (d) retaining enzyme activity more than 75% under the concentration of 0.1 - 20% NaCl; and

 (e) molecular weight by SDS-PAGE: 28 kDa.

20 4. The protease according to claim 2, which characteristically includes the amino acid sequence represented by SEQ. ID. NO: 5.

 5. The protease according to claim 4, which
25 characteristically contains the amino acid sequence represented either by SEQ. ID. NO: 4 or 5.

6. The protease according to claim 5, which is characteristically encoded by the nucleotide sequence represented by SEQ. ID. NO: 3.

5

7. A gene encoding the protease of claim 2.

8. The gene according to claim 7, which is represented by SEQ. ID. NO: 3.

10

9. An expression vector containing the gene of claim 7.

10. The expression vector according to claim 9,
15 wherein the vector is pT7-BCAP shown in Fig. 14.

11. A transformant transfected with the expression vector of claim 9.

20 12. An expression vector containing the gene of claim 7 for the insertion into *Bacillus* genome.

13. The expression vector according to claim 12,
wherein the vector is pHPS-BCAP.

25

14. A transformant transfected with the expression vector of claim 12.

15. The transformant according to claim 14, which is *Escherichia coli* JM109/pHPS-BCAP deposited under Accession No: KCTC 10772BP.

16. A culture supernatant obtained from the culture of the strain of claim 1, the transformant of claim 11 or the transformant of claim 14.

17. A method for purification of the protease of claim 2, comprising the following steps:

1) recovering a culture supernatant by centrifugation after culturing the strain of claim 1, the transformant of claim 11 or the transformant of claim 14;

2) eluting active fractions after absorbing a protease by adding a synthetic absorbent;

3) eluting active fractions by loading the eluent onto chromatography; and

4) isolating a 28 kDa protein having a protease activity.

18. The method according to claim 17, wherein the synthetic absorbant of step 2) is a diaion HP synthetic absorbant.

5 19. The method according to claim 17, wherein the chromatography of step 3) is hydrophobic chromatography and ion exchange chromatography.

10 20. The method according to claim 19, wherein the hydrophobic chromatography is performed on Phenyl-Sepharose column and the ion exchange chromatography is performed on DEAE-Sepharose column and CM-Sepharose column.

15 21. A detergent composition containing the protease of claim 2 or the culture supernatant of claim 16.

20 22. The detergent composition according to claim 22, which is characteristically used for a laundry detergent, a dish-washing detergent or a contact lens detergent.

25 23. A method for treating food or feed which contains the step of treating food or feed with the strain of claim 1, the protease of claim 2, the

transformant of claim 11, the transformant of claim 14
or the culture supernatant of claim 16.

24. A food or feed additive containing the strain
5 of claim 1, the protease of claim 2, the transformant
of claim 11, the transformant of claim 14 or the
culture supernatant of claim 16.

25. A method for treating fibers or fabrics
10 containing the step of treating fibers or fabrics with
the protease of claim 2 or the culture supernatant of
claim 16.

26. A composition for fibers or fabrics
15 processing, which contains the protease of claim 2 or
the culture supernatant of claim 16.

27. A method for leather processing containing
the step of treating leather with the protease of claim
20 2 or the culture supernatant of claim 16.

28. A composition for leather processing which
contains the protease of claim 2 or the culture
supernatant of claim 16.

25

29. A method for decomposing organic wastes containing the step of treating organic wastes with the strain of claim 1, the protease of claim 2, the transformant of claim 11, the transformant of claim 14
5 or the culture supernatant of claim 16.

30. A composition for organic waste decomposition containing the strain of claim 1, the protease of claim 2, the transformant of claim 11, the transformant of
10 claim 14 or the culture supernatant of claim 16.

31. A method for treating paper or pulp containing the step of treating paper or pulp with the strain of claim 1, the protease of claim 2, the
15 transformant of claim 11, the transformant of claim 14 or the culture supernatant of claim 16.

32. A composition for paper or pulp processing containing the strain of claim 1, the protease of claim
20 2, the transformant of claim 11, the transformant of claim 14 or the culture supernatant of claim 16.

33. A method for mass-producing the protease of claim 2 which includes the step of culturing the strain
25 of claim 1, the transformant of claim 11 or the transformant of claim 14 in a medium with pH 7.0 - pH

12.0 containing one or more organic nitrogen sources
(0.1 - 5%) selected from a group consisting of casein,
powdered skim milk, whey, soybean meal and peptone, one
or more inorganic nitrogen sources (0.1 - 5%) selected
5 from a group consisting of ammonium sulfate and
ammonium phosphate, and one or more carbon sources (0.1
- 5%) selected from a group consisting of liquid
maltose, maltose, honey potato starch, corn starch,
wheat flour, sodium pyruvate, sodium citrate, sodium
10 acetate and sodium succinate, at 25 - 45°C with 150 -
1000 rpm for 20 - 96 hours under the air flow condition
of 0.1 - 5 vvm.

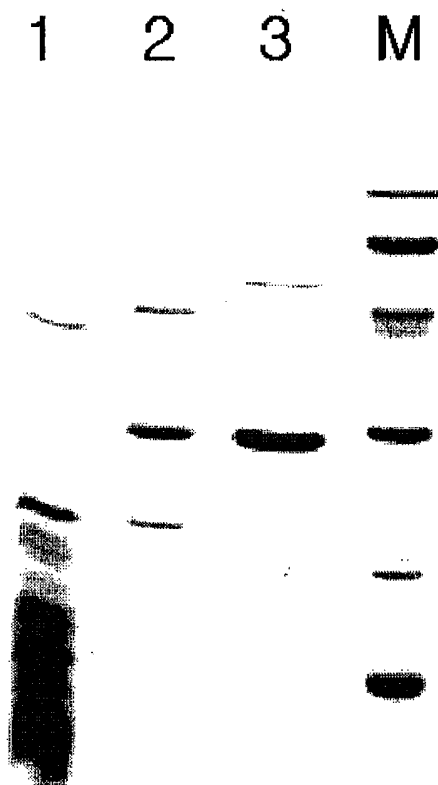
34. The method according to claim 33, wherein the
15 medium includes 0.2 - 2.5% of soybean meal or casein as
an organic nitrogen source, 0.2 - 2.5% of wheat flour
or liquid maltose as a carbon source, and 0.2 - 2.5% of
a low molecular weight carbon source selected from a
group consisting of sodium pyruvate, sodium citrate and
20 sodium succinate with pH 8.0 - pH 11.0 and the culture
is performed at 30 - 40°C with 200 - 800 rpm for 24 - 72
hours.

35. The method according to claim 33, wherein the
25 airflow condition is 0.5 - 1.5 vvm.

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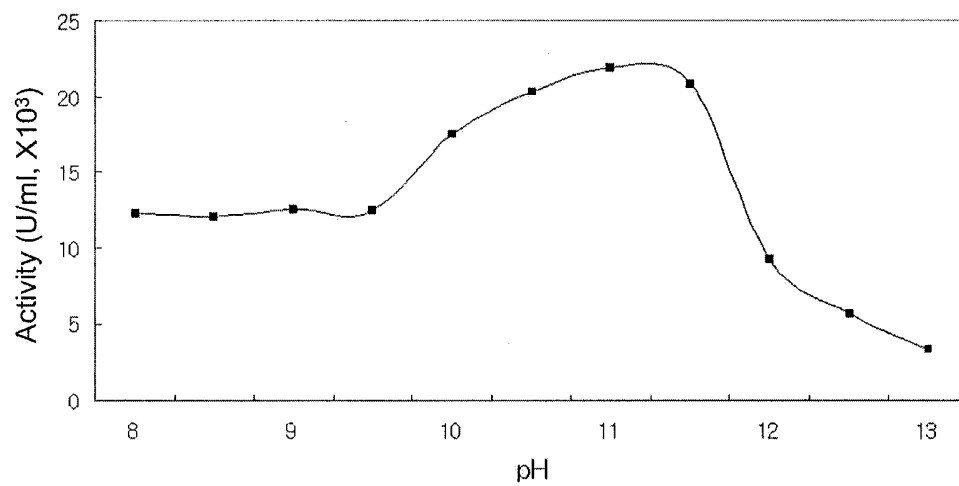
FIGURES

Fig. 1



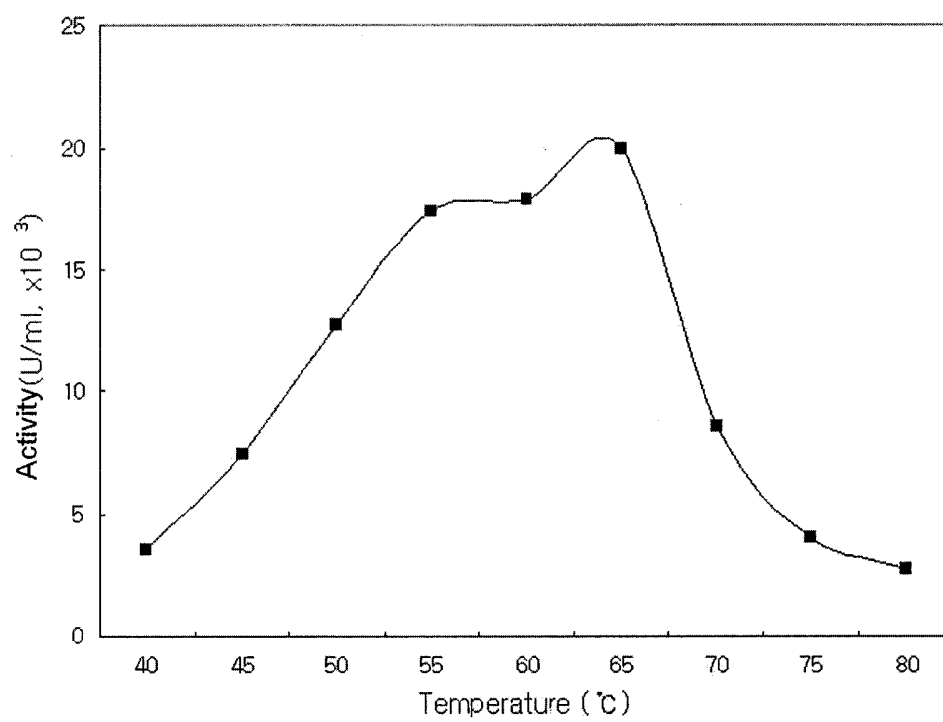
2/18

Fig. 2



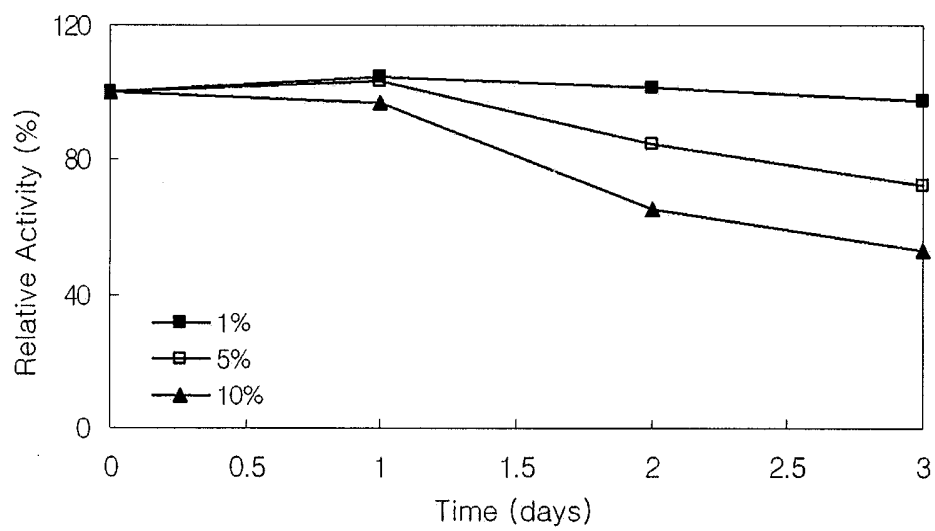
3/18

Fig. 3



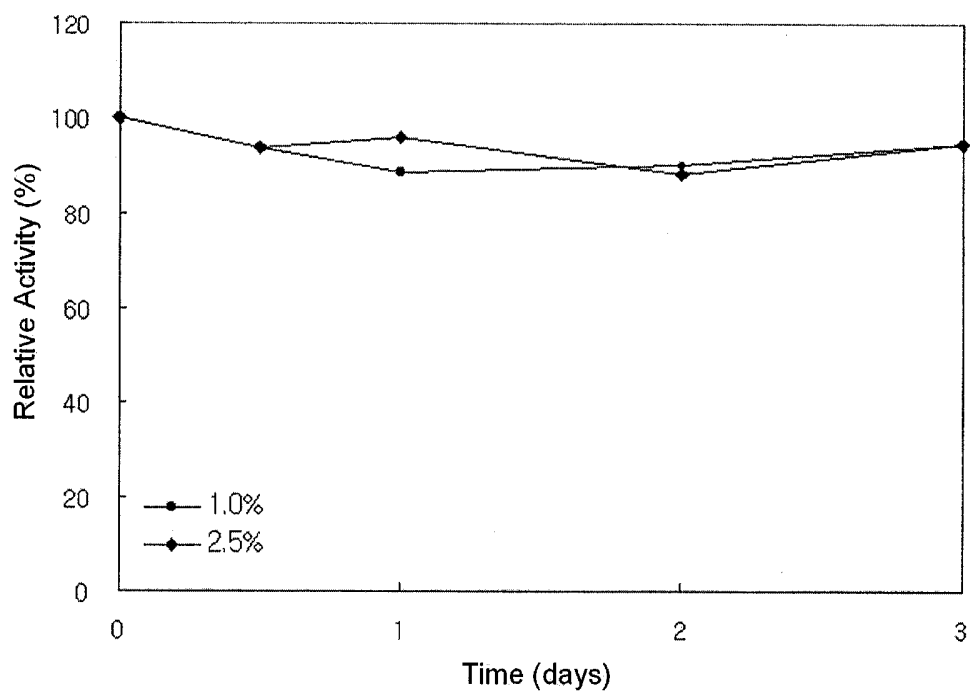
4/18

Fig. 4



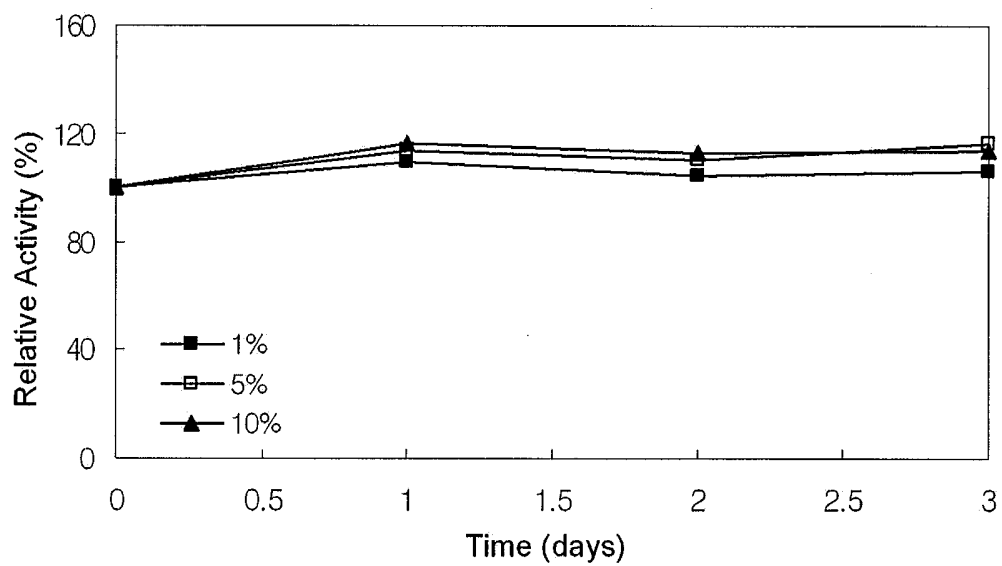
5/18

Fig. 5



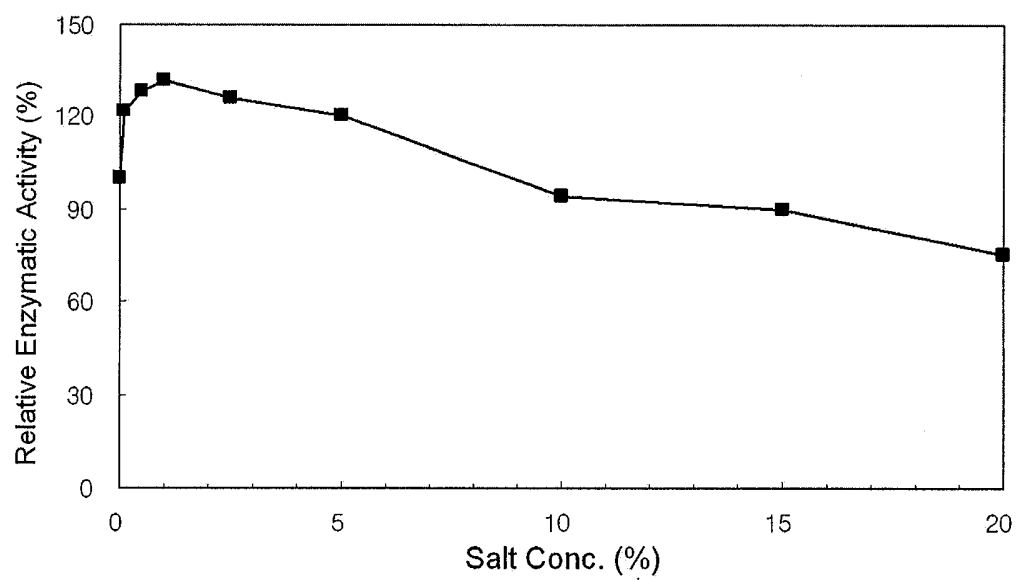
6/18

Fig. 6



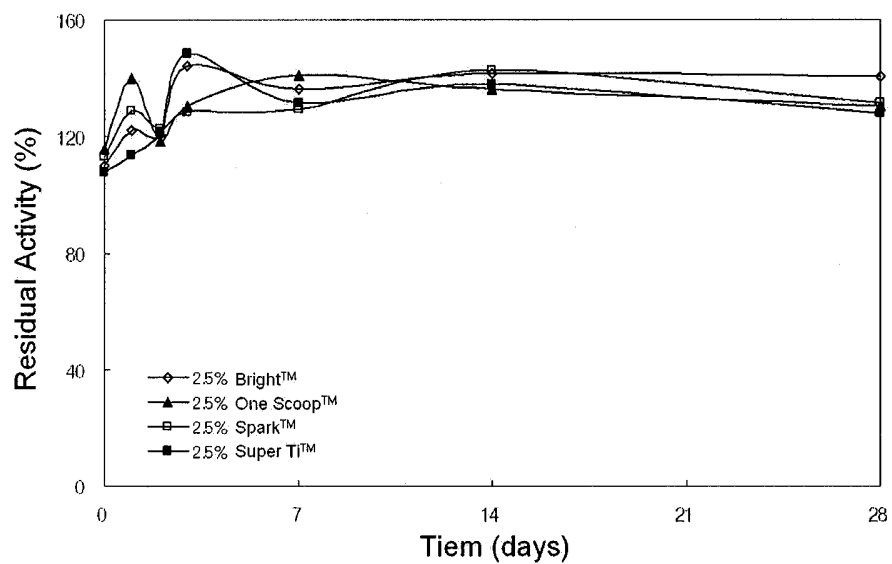
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Fig. 7



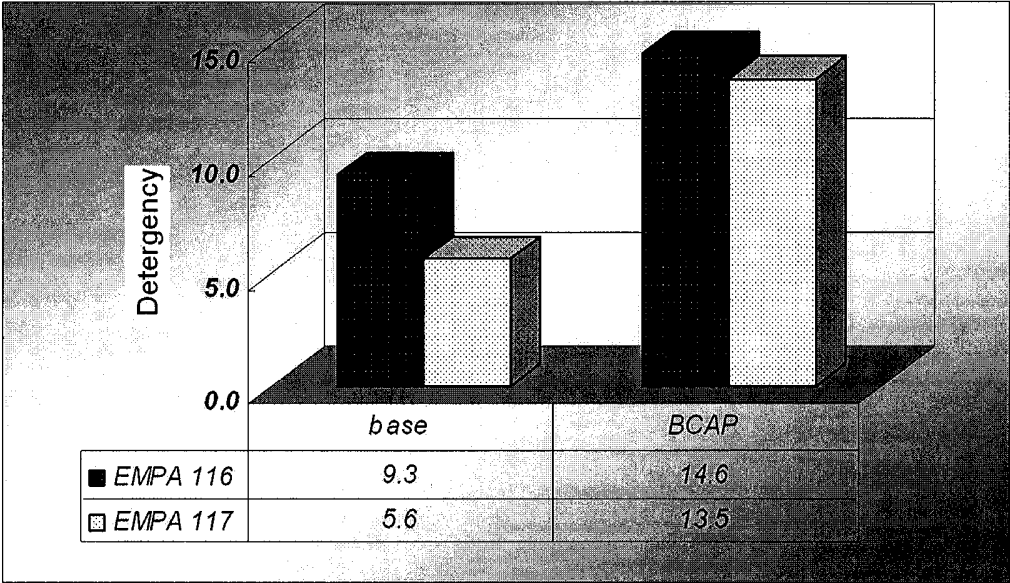
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Fig. 8



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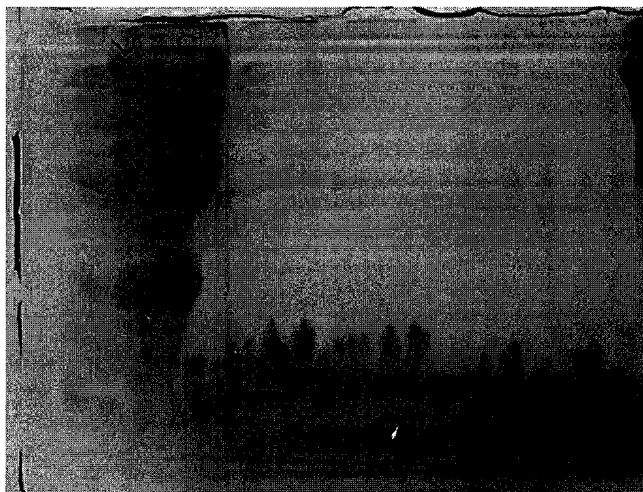
Fig. 9



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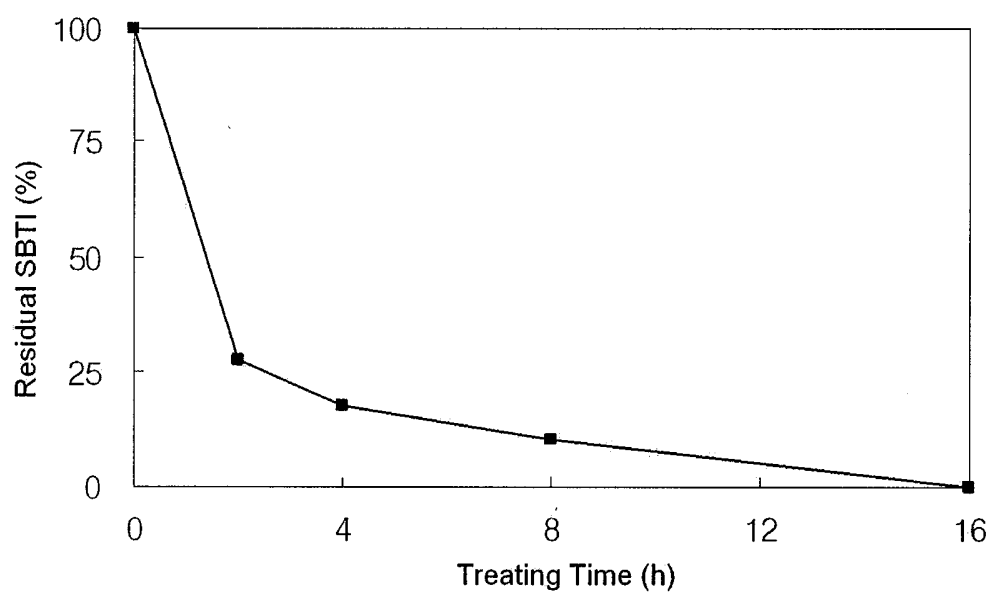
Fig. 10

M 1 2 3 4 5

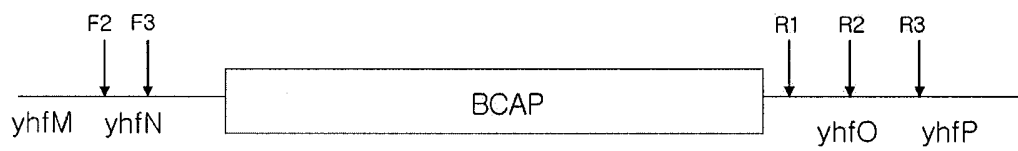


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Fig. 11

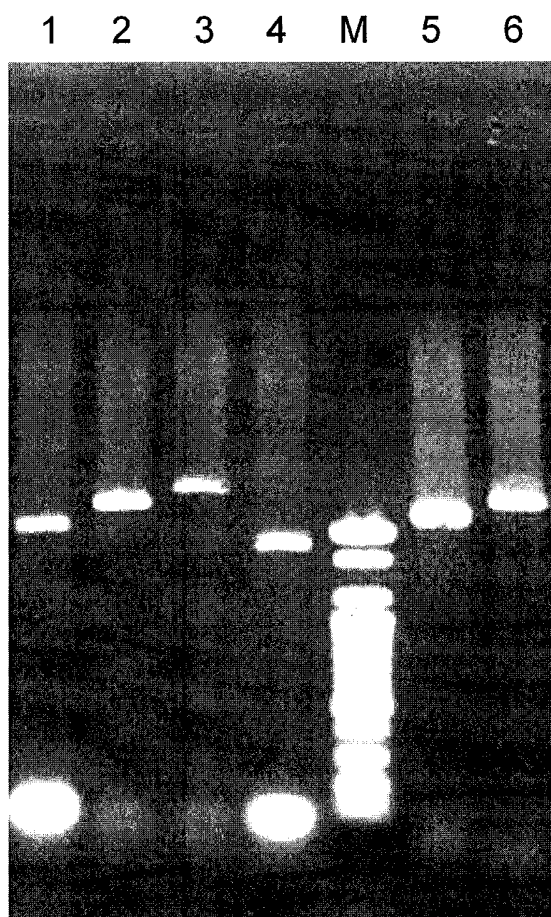


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Fig. 12

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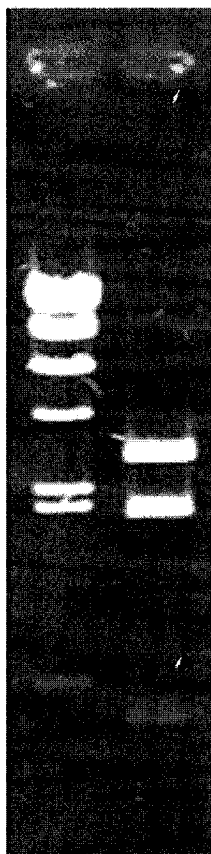
Fig. 13



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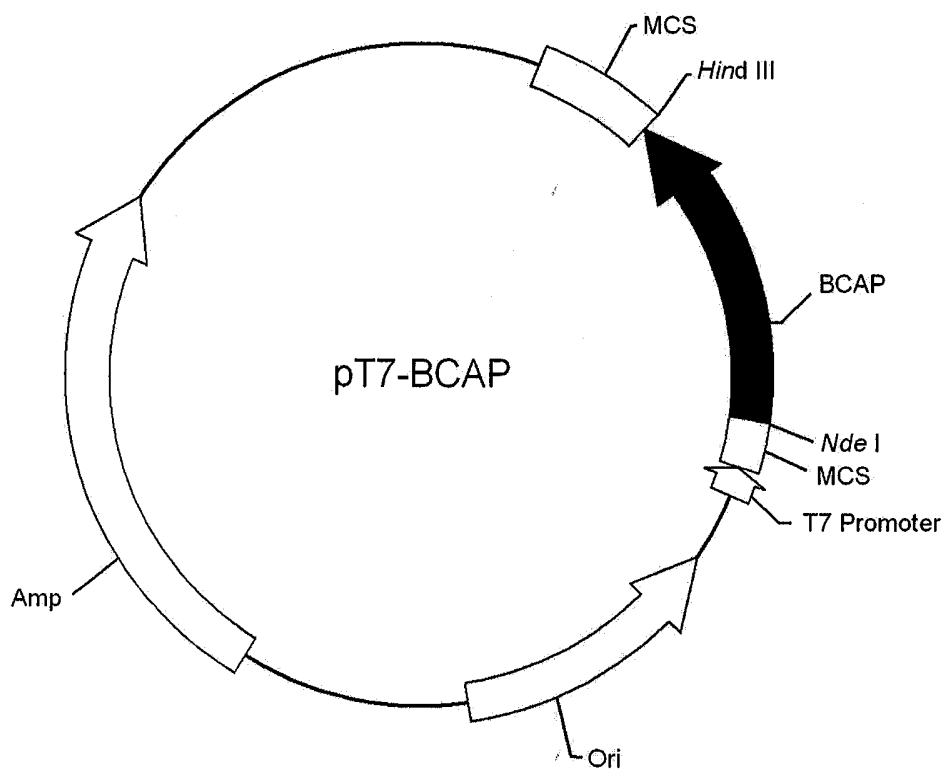
Fig. 14

M 1



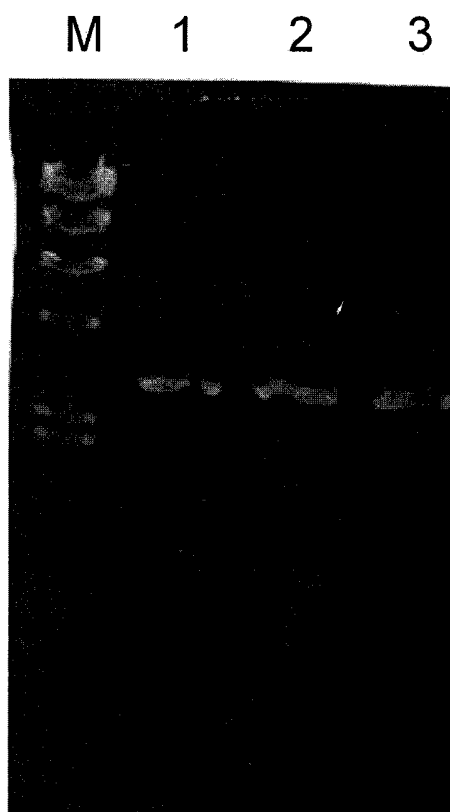
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Fig. 15



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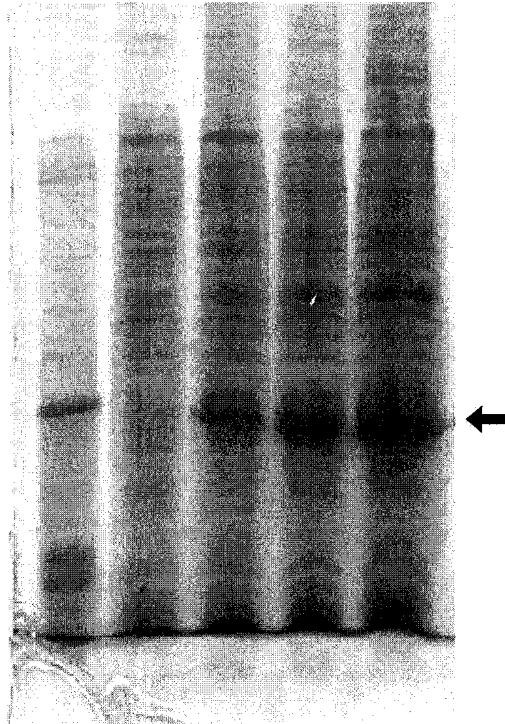
Fig. 16



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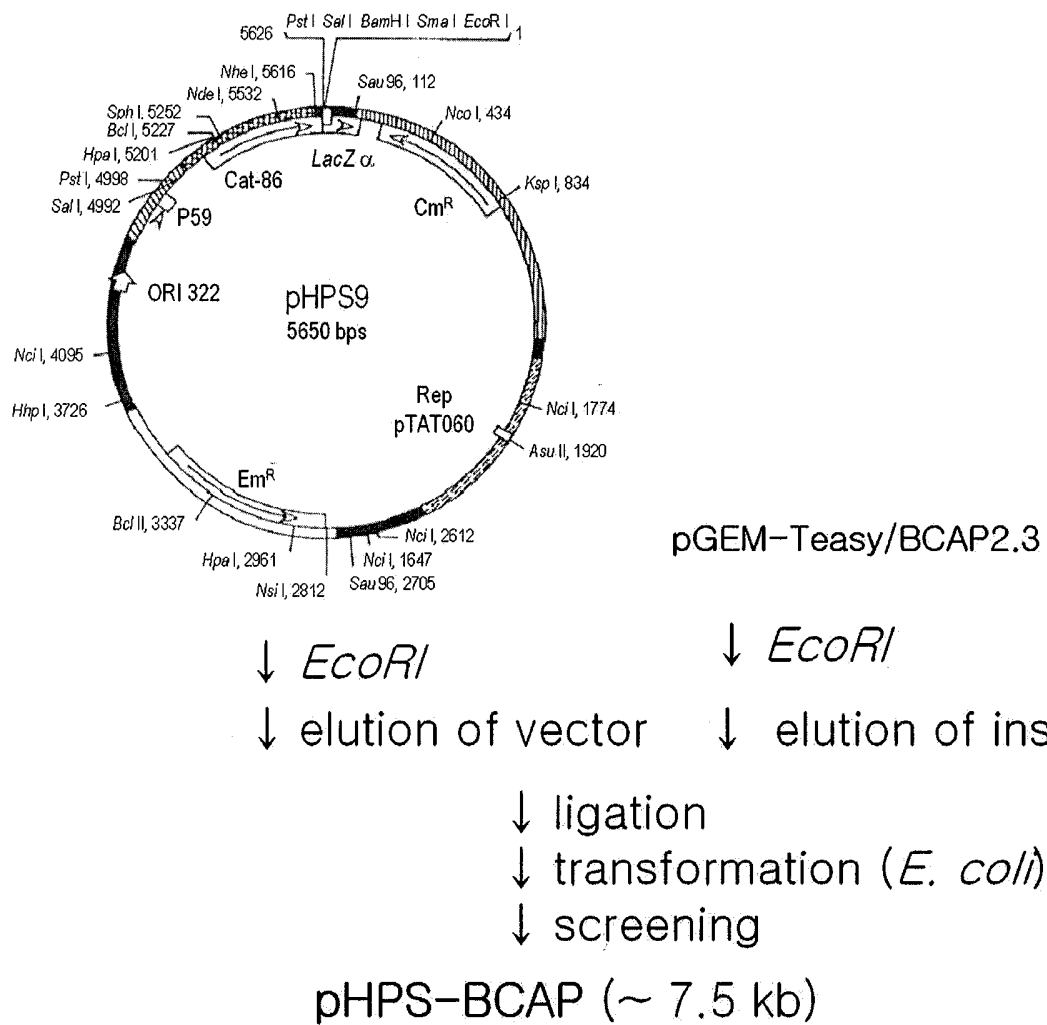
Fig. 17

M	1	2	3	4
---	---	---	---	---



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Fig. 18



<110> INHA-INDUSTRY PARTNERSHIP INSTITUTE

<120> BACILLUS SP. I-52, A DETERGENT- AND OXIDANT-STABLE HALOALKALOPHILIC
PROTEASE THEREFROM, A GENE ENCODING THE SAME AND A USE THEREOF

<130> 5FPO-12-01

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<220>

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5

10

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	2280

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 Ser Ser Thr Glu Lys Lys Tyr Ile Val Gly Phe Lys Gln Thr Met Ser
 35 40 45
 Ala Met Ser Ser Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly
 50 55 60
 Lys Val Gln Lys Gln Phe Lys Tyr Val Asn Ala Ala Ala Ala Thr Leu
 65 70 75 80
 Asp Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tyr
 85 90 95
 Val Glu Glu Asp His Ile Ala His Glu Tyr Ala Gln Ser Val Pro Tyr
 100 105 110
 Gly Ile Ser Gln Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr
 115 120 125
 Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser
 130 135 140
 His Pro Asp Leu Asn Val Arg Gly Gly Ala Ser Phe Val Pro Ser Glu
 145 150 155 160
 Thr Asn Pro Tyr Gln Asp Gly Ser Ser His Gly Thr His Val Ala Gly
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 Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro
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 Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ser Asn Asn
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Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Thr Gly Ser Thr Ala
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Leu Lys Thr Val Val Asp Lys Ala Val Ser Ser Gly Ile Val Val Ala
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Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly Ser Thr Ser Thr Val Gly
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Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala Val Gly Ala Val Asn Ser
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Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala Gly Ser Glu Leu Asp Val
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Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Gly Gly Thr Tyr
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Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly Ser Ser His	50	55	60
Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly	65	70	75
Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu	85	90	95
Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu	100	105	110
Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly	115	120	125
Pro Thr Gly Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser	130	135	140
Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly	145	150	155
Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala	165	170	175
Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala	180	185	190
Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr	195	200	205
Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr	210	215	220

Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr
 225 230 235 240

Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr
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5fpo-12-01

1/1

PCT

Print Out (Original in Electronic Form)

0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT-SAFE Version 3.51.001.176 MT/FOP 20060101/0.20.4rc.2.7
0-2	International Application No.	PCT/KR2006/000545
0-3	Applicant's or agent's file reference	5fpo-12-01

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	paragraph number	61
1-3	Identification of deposit	
1-3-1	Name of depositary institution	KCTC Korean Collection for Type Cultures
1-3-2	Address of depositary institution	52, Oun-dong, Yusong-Ku, Taejon 305-333, Republic of Korea
1-3-3	Date of deposit	14 June 2002 (14.06.2002)
1-3-4	Accession Number	KCTC 10277BP
1-5	Designated States for Which Indications are Made	all designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	paragraph number	62
2-3	Identification of deposit	
2-3-1	Name of depositary institution	KCTC Korean Collection for Type Cultures
2-3-2	Address of depositary institution	52, Oun-dong, Yusong-Ku, Taejon 305-333, Republic of Korea
2-3-3	Date of deposit	26 January 2005 (26.01.2005)
2-3-4	Accession Number	KCTC 10772BP
2-5	Designated States for Which Indications are Made	all designations

FOR RECEIVING OFFICE USE ONLY

0-4	This form was received with the international application: (yes or no)	
0-4-1	Authorized officer	

FOR INTERNATIONAL BUREAU USE ONLY

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2006/000545

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000545**A. CLASSIFICATION OF SUBJECT MATTER***C12N 9/54(2006.01)i, C12N 1/00(2006.01)i, C12N 15/00(2006.01)i, C12N 15/57(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC8 C12N 9/54, C12N 1/00, C12N 1/20, C12N 15/00, C12N 15/57

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NCBI PubMed, NCBI GenBank, Esp@cenet, CA, e-KIPASS "Bacillus clausii, protease, detergent, oxidant"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KR 2005/0465852 B1 (INHA-INDUSTRY PARTNERSHIP INTSTITUTE) 13 JANUARY 2005 (13.01.2005) See the whole document	1-3, 16-35
X	JOO H.S. et al., 'Oxidant and SDS-stable alkaline protease from Bacillus clausii I-52: production and some properties', In: J. Appl. Microbiol., 2003, Vol. 95(2), pp. 267-272 See the whole document	1-3
X	JOO H.S. & CHANG C.S., 'Oxidant and SDS-stable alkaline protease from a halo-tolerant Bacillus clausii I-52: enhanced production and simple purification', In: J. Appl. Microbiol., December 2004, Vol. 98(2), pp. 491-497 See the whole document	1-3, 16-20
X	US 4914031 A (AMGEN INC.) 03 APRIL 1990 (03.04.1990) See the whole document	4-15
X	WO 1987/004461 A1 (AMGEN INC.) 30 JULY 1987 (30.07.1987) See the whole document	4-15

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

23 MAY 2006 (23.05.2006)

Date of mailing of the international search report

24 MAY 2006 (24.05.2006)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHO, YOUNG GYUN

Telephone No. 82-42-481-8132



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2006/000545

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 2005/0465852 B1	13.01.2005	None	
US 4914031 A	03.04.1990	WO 1988/08033 A1 EP 0309565 B1 JP 1502957 T	20.10.1988 15.03.1995 12.10.1989
WO 1987/004461 A1	30.07.1987	US 5399283 A EP 0254735 B1 JP 63502396 T	21.03.1995 19.06.1991 14.09.1988