ABSTRACT: A fusion gene encoding M. taiwanensis WR-220 keratinase is disclosed. The fusion comprises: (a) a first DNA sequence encoding a protein secretion signal peptide, located at the N-terminus of the fusion gene; (b) a second DNA sequence encoding an inhibitory domain of M. taiwanensis WR-220 keratinase, linked in translation frame with the first DNA sequence; and (c) a third DNA sequence encoding encoding a catalytic domain of M. taiwanensis WR-220 keratinase, linked in translation frame with the second DNA sequence, wherein the fusion gene is a non-naturally occurring chimeric DNA. Also disclosed are a method for preparation of the catalytic domain of M. taiwanensis WR-220 keratinase, and use of the M. taiwanensis WR-220 keratinase.

FIG. 1C

Weight of remaining feathers

Free amino group (µmol)

Ninhydrin assay
HEAT STABLE KERATINASE AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to proteases, and more specifically to microbial keratinases isolated from *Meiothermus Taiwanesis*.

BACKGROUND OF THE INVENTION

Keratins, a family of fibrous structure proteins, are the key structural components of skin, hair, wools, nails, scales and feathers. Keratin polypeptides are insoluble and resistant to most proteases. Accumulation of insoluble keratins in the environment, mainly in the form of feathers and hair, becomes an issue in the solid waste management.

Keratins can be efficiently degraded by keratinases. Hence, keratinases find applications in biowaste process and also in detergent and leather industries where they serve as specialty enzymes to remove proteinaceous stains and hair, respectively. The applications can also be extended to wool and silk cleaning and medicine. Recently, keratinase has been used extensively to increase digestibility of proteins in animal feed.

Beat is often required in industrial applications to speed up reactions. The spray drying process of keratinase powder also requires heating. Hence, a heat-stable keratinase can be very useful in the industries.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a fusion gene comprising: (a) a first DNA sequence encoding a protein secretion signal peptide, located at the N-terminus of the fusion gene; (b) a second DNA sequence encoding an inhibitory domain of *M. taiwanesis* WR-220 keratinase, linked in translation frame with the first DNA sequence; and (c) a third DNA sequence encoding a catalytic domain of *M. taiwanesis* WR-220 keratinase, linked in translation frame with the second DNA sequence, wherein the fusion gene is a non-naturally occurring chimeric DNA.

The protein secretion signal peptides may be selected from the group consisting of alpha-amylase signal peptide, gltcoamyliase signal peptide, serum albumin signal peptide, inulinase signal peptide, invertase signal peptide, kiiier virus signal peptide, Lvsozyme signal peptide, mating factor alpha-1 signal peptide, and mating factor alpha-2 signal peptide.

In one embodiment of the invention, the first DNA sequence encodes a yeast alpha-factor signal peptide.

In another aspect the invention relates to a protein expression vector comprising: (a) the fusion gene as aforementioned; and (b) a promoter, linked in translation frame with the fusion gene. The promoter may be selected from the group consisting of alcohol oxidase (AOX) promoter,
glyceraldehyde phosphate dehydrogenase promoter, translational elongation factor 1-α promoter, Na’-coupled phosphate symproier promoter, and formaldehyde dehydrogenase promoter.

In another aspect, the invention relates to a host cell comprising the expression vector as aforementioned.

In another aspect, the invention relates to a cell culture comprising the host cell as aforementioned and an artificial medium, the host cell secreting the catalytic domain of M faïwanensis WR-220 keratinase into the artificial medium.

In another embodiment of the invention, the second DNA sequence comprises a nucleotide sequence having at least 90% or 95% identity to SEQ ID NO: 11.

In another aspect, the invention relates to an isolated protease comprising a catalytic domain of M. faïwanensis WR-220 keratinase, the protease Sacking an inhibitory domain of M. faïwanensis WR-220 keratinase, and being in the form of a tablet, a caplet, a pellet, a capsule, a granule, a pill, a powder or a sachet, or in the form of a solution without containing a cell culture supplement. In one embodiment of the invention. The protease comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 14.

Further in another aspect, the invention relates to a method for degrading a proteinaceous material, comprising: exposing the proteinaceous material to an effective amount of the protease as aforementioned. Prior to the exposing step, the method may further comprise the step of preparation of the catalytic domain of M. faïwanensis WR-220 keratinase.

Alternative, the invention relates to use of the fusion gene for preparation of a protease as aforementioned for degrading a proteinaceous material.

The invention further relates to use of the fusion gene as aforementioned in the manufacture of a protease for degrading a proteinaceous material. Alternatively, the invention relates to use of a protease as aforementioned in manufacture of a composition for degrading a proteinaceous material.

The use may be performed at a temperature above 25°C. Prior to the use, the protease may be pretreated at a temperature above 40°C but below 95°C, or pretreated with a solution having a pH value ranging from 3 to 10, and still remains its activity. The proteinaceous material may be selected from the group consisting of animal feed, food, milk, casein, elastin, skin, hair, wool, silk, naijls, scales, fiber, leather, and feathers.

Further in another aspect, the invention relates to a method for preparation of the catalytic domain of M. faïwanensis WR-220 keratinase, comprising; (ai) growing the host cell as aforementioned in a culture medium under conditions that permits expression of M. faïwanensis WR-220 keratinase and secretion of the catalytic domain thereof into the medium; or (aii) growing a host cell transformed with an expression plasmid comprising a DNA insert encoding an inhibitor'd domain
and a catalytic domain of *M. taiwanensis* WR-220 keratinase under conditions that permits expression of *M. taiwanensis* WR-220 keratinase and secretion of the catalytic domain thereof into the medium; and (b) removing the host cell to obtain a supernatant containing the catalytic domain of the *M. taiwanensis* WR-220 keratinase; and (c) isolating the catalytic domain from the supernatant or removing liquid from the supernatant by a spray drying method or others to obtain the catalytic domain in a solid form.

Yet in another aspect, the invention relates to an isolated protease comprising a catalytic domain and lacking an inhibitory domain of *M. taiwanensis* WR-220 keratinase, the protease being prepared from the method as aforementioned.

In another embodiment of the invention, the inhibitory domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 13; and the catalytic domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 14. Alternatively, the inhibitory domain comprises an amino acid sequence of SEQ ID NO: 13; and the catalytic domain comprises an amino acid sequence of SEQ ID NO: 14.

These and other aspects will become apparent from the following description of the preferred embodiment taken in conjunction with the following drawings, although variations and modifications therein may be affected without departing from the spirit and scope of the novel concepts of the disclosure.

The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like elements of an embodiment.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**FIGs. 1A-C** show degradation ability of intact chicken feathers by *M. taiwanensis* WR-220. (A) The culture medium containing feathers as carbon and nitrogen sources is clear at Day 0. The cloudy medium at Day 2 indicates the degradation of feathers and the growth of microbes. (B) Photographs showing the remaining feathers in the medium. (C) Graphs showing the weight of the remaining feathers (left panel) or insoluble residues in the culture medium and the amount of soluble free-amino acid (right panel) from decayed feathers in the culture supernatant with or without the presence of *M. taiwanensis*.

**FIGs. 2A-B** are photographs showing protein electrophoresis for the fractions of extracellular proteases secreted by *M. taiwanensis* WR-220, and an urography assay where areas of keratinase activity appear as clear zones of the stained agarose replica, respectively. (A) 4-20% gradient SDS-PAGE stained by SYPRORUBY™. (B) 1% agarose within keratin/casein powders.
FIG. 3 is a photograph of protein electrophoresis, indicating the molecular weight of the recombinant keratinase is less than 30KDa.

FIG. 4 is a series of photographs showing that the recombinant keratinase exhibits a broad range of proteolytic activities against proteins in milk, casein, elastin and feathers. The Qμg, 1μg, 2μg, 4μg, 8μg and 16μg of keratinases are labeled as a, b, c, d, and e, respectively.

FIGS. 5A-B are photographs showing the heat and pH tolerance of keratinase. (A) A clear zone around the disc-shaped filter paper indicates protease activities. The corresponding heating temperatures are indicated near the filter papers. (B) The corresponding pH values are indicated near the rectangular filter papers.

FIG. 6 shows the results of a paper disk-agar diffusion assay for protease activity. The culture supernatants of 8 elected yeast clones (Day 5) were collected for protease activities.

FIG. 7 shows the results of SDS-PAGE electrophoreses of the culture supernatants of 8 elected yeast clones (No. 2, 3, 5, 7, 8, 9, 10 and 12). D1, D2, D3 and D5 denote day 1, 2, 3, and 5 respectively.

FIG. 8A shows the nucleotide sequence of the SPR2261 gene (SEQ ID NO: 3). The part cloned is shown in bold. The predicted signal peptide for protein secretion is shown in italic.

FIG. 8B shows the amino acid sequence of SPR2261 (SEQ ID NO: 4). The predicted signal peptide for protein secretion is labeled in italic, the predicted inhibitory domain labeled as underlined, and the active form of keratinase labeled in bold only.

FIG. 9A shows the cloned DNA sequence of keratinase (SEQ ID NO: 5). The first methionine (starting codon) is labeled as underlined, the inhibitory domain labeled in non-bold, the active form of truncated keratinase labeled in bold only, and a fusion tag for purification labeled in italic.

FIG. 9B shows the amino acid sequence of cloned keratinase (SEQ ID NO: 6) from *Meiothermus taranensis* WR-220. The first methionine (starting codon) is labeled as underline, the inhibitory domain labeled in non-bold, the active form of truncated keratinase labeled in bold only, and a fusion tag for protein purification labeled in italic.

FIGs. 10A-B show the nucleotide sequence of the plasmid construct pHTRY2 spr2261ic (SEQ ID NO: 7) and its corresponding keratinase protein sequence (SEQ ID NO: 8). (A) The genes of alpha factor is labeled as underline, LIC cloning sites labeled in italic, inserted spr2261ic (containing inhibitory and catalytic domains) labeled in bold with italic, and 8xHIS tag with a stop codon labeled in bold only. (B) The corresponding expressed protein sequence (SEQ ID NO: 8) comprises the alpha factor (SEQ ID NO: 15) labeled as underline, the inhibitory domain labeled in italic only, and catalytic domain labeled in bold with italic, and 8xHIS tag labeled in bold only.
F)Gs. 11A-B show the nucleotide sequence of the plasmid construct of pHTPY2_spr2261c (SEQ ID NO: 9) and its corresponding keratinase protein sequence (SEQ ID NO: 10). (A) The alpha factor labeled as underline, LIC cloning sites labeled in italic, inserted spr2261c (containing only the catalytic domain but without the inhibitory domain) labeled in bold with italic, and SxHis tag (with a stop codon) labeled in bold. (B) The corresponding expressed protein sequence comprises the alpha factor labeled as underline, the catalytic domain labeled in bold with italic, and SxHis tag labeled in bold only, but does not comprise the inhibitory domain.

FIGs 12A-B are cartoon models showing the results of structural studies of M. taiwanensis WR-220 keratinase. (A) The structures of two keratinases are colored in light gray and dark gray, respectively. The active site is highlighted in the black box and the key active site residue, Ser224 (referring to SEQ ID NO: 14) is shown as a stick model. The linker region from one of keratinases (light gray) is located in the active site of the other keratinase (dark gray). (B) A close look of the active site of the keratinase. The active site residues (Asp39, His2 and Ser224) and the residues involved in substrate interaction are labeled.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. For convenience, certain terms may be highlighted, for example using italics and/or quotation marks. The use of highlighting has no influence on the scope and meaning of a term; the scope and meaning of a term is the same, in the same context, whether or not it is highlighted. It will be appreciated that same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification including examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to various embodiments given in this specification.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including definitions will control.
As used herein, "around", "about" or "approximately" shall generally mean within 20 percent, preferably within 10 percent, and more preferably within 5 percent of a given value or range. Numerical quantities given herein are approximate, meaning that the term "around", "about" or "approximately" can be inferred if not expressly stated.

Protein secretion signal peptides determines the efficiency of protein secretion. They include, but not limited to, alpha-amylase signal peptide (SEQ ID NOs: 17, 18), glucoamylase signal peptide (SEQ ID NOs: 19, 20), serum albumin signal peptide (SEQ ID NOs: 21, 22), inulinase signal peptide (SEQ ID NOs: 23, 24), invertase signal peptide (SEQ ID NOs: 25, 26), killer virus signal peptide (SEQ ID NOs: 27, 28), Lysozyme signal peptide (SEQ ID NOs: 29, 30), mating factor alpha-1 signal peptide (SEQ ID NOs: 31, 32), and mating factor alpha-2 signal peptide (SEQ ID NOs: 33, 34).

As used herein, "an inhibitory domain of M. taiwanensis WR-220 keratinase" refers to an amino acid sequence having at least 90% identity to SEQ ID NO: 13. Thus, as used herein “an inhibitory domain of M. taiwanensis WR-220 keratinase” includes a wild-type or a mutant inhibitory domain of M. taiwanensis WR-220 keratinase, wherein the mutant inhibitory domain comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 13.

As used herein, "a catalytic domain of M. taiwanensis WR-220 keratinase" refers to a protease that comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 14. Thus, as used herein “a catalytic domain of M. taiwanensis WR-220 keratinase” includes a wild-type or a mutant catalytic domain of M. tarwanemisi WR-220 keratinase, wherein the mutant catalytic domain comprises an amino acid sequence having at least 90% identity to SEQ ID NO: 14.

Meiothermus taiwanensis WR-220, a thermophilic species found in Taiwan, habits in the temperature ranging from 55 to 65°C. The proteins produced in M. taiwanensis must remain stable at temperature over 55°C, an ability that renders the proteins suitable for industrial use.

The invention relates to the discovery of M. taiwanensis keratinase that not only possesses a high stability against heat, acidic and basic environment but also exhibits a wide range proteolytic activity. The heat and acid tolerance is useful for industrial applications.

The invention further relates to an expression plasmid for the production of recombinant M. taiwanensis WR-220 keratinase as secreted protein using methylotrophic Pichia pastoris expression systems, which is under the control of the promoter of alcohol oxidase gene. P. pastoris itself does not secrete any endogenous keratinase into the cultivation medium. In addition, P. pastoris is generally regarded as safe (GRAS) and has an added advantage for recombinant keratinase production.

It was discovered that M. taiwanensis WR-220 was able to degrade feathers. The corresponding keratinase was then isolated and its DNA and amino acid sequences were identified. The keratinase
with a fusion tag was cloned to the vector for recombinant protein production using E. coli
expression system. The recombinant and truncated keratmase showed a wide range protease activity
that could hydrolyze proteins in skim milk, casein, elastin and feathers. Moreover, the recombinant
keratmase not only possessed a high stability against heat, acidic and basic environment but also
exhibited a wide range of proteolytic activity, which may be useful for industrial applications.

Inhibitory domain DNA sequence: SEQ ID NO: 11; Catalytic domain DNA sequence: SEQ ID
NO: 12. Inhibitory domain protein sequence: SEQ ID NO: 13; Catalytic domain protein sequence:
SEQ ID NO: 14.

EXAMPLES

Without intent to limit the scope of the invention, exemplary instalments, apparatus, methods and
their related results according to the embodiments of the present invention are given below. Note
that titles or subtitles may be used in the examples for convenience of a reader, which in no way
should limit the scope of the invention. Moreover, certain theories are proposed and disclosed
herein; however, in no way they, whether they are right or wrong, should limit the scope of the
invention so long as the invention is practiced according to the invention without regard for any
particular theory or scheme of action.

METHODS AND MATERIALS

Cell culture and feather degraded on assay. M. taiwanensis WR-220, a gift from Dr. San-San
Tsay, was incubated in a modified Thermus medium (TM medium) at 55°C. The modified TM
medium contained 0.3% of peptone, 0.1% of yeast extract, 0.1% of glutamic acid, and 1X
Castenholtz salts (pH 7.8). For the feather degradation, 1% of the overnight culture was added to the
medium supplemented with 3% (w/v) intact chicken feathers and 1X Castenholtz salts at 55°C and
65°C. The change in the weight of remaining feathers in the culture was examined by weighting the
insoluble residues along the cultivation. The insoluble residues were obtained and weighted after the
filtration of the culture using filter papers. The amount of the released amino acids from decayed
feathers was quantified by the ninhydrin colorimetric method (Rosen, H A "Modified ninhydrin

Isolation of keratmase from M. taiwaneNSIS WR-220. M. taiwaneNSIS was cultured in 4 liters of
TM medium. The cultivated medium was centrifuged at 6,000 x g for 20 minutes and the supernatant
was filtered with a 0.22 μm filter to remove microbes and insoluble residues. The collected
supernatant was concentrated and buffer exchanged to 50mL PBS buffer (pH 7.4) using a
LABSCALE™ TFF system (Millipore, USA). The pigments were removed by ultracentrifugation at
137,24 x g and the supernatant was further concentrated to 2 mL using Amicon-Ultra 15 centrifugal
filter devices. The concentrated sample was loaded into a 1-mL resource S column (GE Healthcare,
USA) pre-equilibrated with 50mM sodium acetate buffer (pH 5.0). The protein was eluted with a linear gradient of NaQ from 0 to 1M in 50mM sodium acetate buffer (pH 5.0). Each fraction was collected and examined for feather degradation ability using zymography assay.

Zymography by assay and identification of amino acid sequence of keratinase. Proteins in each chromatographic fraction were further separated by 4 to 20% SDS-PAGE using Laemmli method (Laemmli, U. K. “Cleavage of structural proteins during the assembly of the head of bacteriophage T4” Nature (1970) 227, 680-685). The SDS-PAGE was washed with a 1.5% Triton X-100 solution twice, which allowed the target extracellular proteases in SDS-PAGE to retain their activities for further zymography assay. The renatured gel was then put on 1% agarose with keratin/casein powder in PBS buffer. Areas of proteolytic activities appeared as clear zones of lysis in the stained agarose replica. The proteins in the areas of proteolytic activities were identified by a standard proteomic analysis using M. laiwanensis genomic sequence as the reference (our unpublished result, NCBI bioproject submission ID: SUB251796 and bioproject ID: PRJNA20S607). The proteomics analyses indicated that the annotated SPR2261 was most likely to be keratinase.

Molecular Cloning of Keratinase gene to protein expression vector. The chromosomal DNA extracted using standard phenol extraction method was used to amplify keratinase gene using phusion flash high-fidelity PGR master mix (Thermo Scientific, USA) with forward (5'TTA AGG AGA TAT AGC ATG CTA GCC CCG GTG CTA GOA; SEQ ID NO: 1) and reverse (5'GAT TGG AAG TAG AGG TTC TCT GC G TAA TTG CTG TAG AGC AGC AGG TTG; SEQ ID NO: 2) primers. The sites for cloning purpose are underlined. The PGR products were purified by electrophoresis and treated with T4 DNA polymerase in the presence of dGTP. The modified pET9 vector that contained the corresponding ligation independent cloning site, TEV protease site, and two affinity tags (6xHis and Strep) was amplified by phusion flash high-fidelity PGR master mix, followed by dpnI treatment (Thermo Scientific, USA) and purified by PGR clean up kit (Geneaid, Taiwan). The linearized vector was then treated with T4 DNA polymerase (Thermo Scientific, USA) in the presence of dGTP. The keratinase gene and the vector were annealed and transformed into E. coli DH5a. The plasmid encoding keratinase extracted from successful clones was sent for DNA sequence analysis and the translated protein sequence was listed in FIGs. SB (SEQ ID NO: 4).

Enzyme Purification and Preparation The plasmid containing keratinase was transformed into E. coli (DE3) ArticExpress cell and grown overnight at 37 °C in 20 ml, of TB medium containing 50μg/mL of kanamycin and 10 μg/mL of tetracycline. The cultures were transferred into 1L of TB containing the antibiotics, 5% lactose and 0.5% glucose at 37 °C for 4 h and then were cooled to 20 °C for overnight expression. The cells were harvested by centrifugation at 6000 x g for 30 min and resuspended by lysis buffer (20 mM imidazole, 250 mM NaCl, and 50 mM HEPES, pH 8.0) in the
presence of DNase I (5ug/mL) and lysozyme (1mg/mL) on ice for 30min. The cells were disrupted by sonicaiion, followed by centrifugation at 20000 x g for 30 min. The supernatant was loaded onto a 2 mL Ni Sepharose (GE healthcare) column that was pre-equilibrated with 20 mL lysis buffer. The column was washed by 25 mL wash buffer (50 mM imidazole, 250 mM NaCl, and 50 mM HEPES, pH 8.0). The keratinase was eluted with 10 mL elution buffer (250 mM imidazole, 250 mM NaCl, and 50 mM HEPES, pH 8.0). The eluted keratinase was dialyzed against the solution containing 1OmM CaCl₂ 150mM NaCl and 50mM HEPES, pH8.0 and concentrated to ~10 mg/mL. The molecular weight of recombinant keratinase was determined by protein electrophoresis and MS spectrometry.

Crystallization, Data Collection and Structure Determination. The keratinase was crystallized in 0.2M lithium sulfate, 0.1M sodium acetate and 50% PEG400 at 19 °C using a sitting drop vapor diffusion method. Crystals were flash-cooled in liquid nitrogen prior to data collection. X-ray diffraction data were collected at 1.5A beamline of National Synchrotron Radiation Resource Center on an ADSC Q3i.5 detector at 100K. Data were processed using HKL2000 program suite. The structure of the keratinase was determined by molecular replacement using the crystal structure of Aqualysin I (PDB ID: 4DZT). Models were iteratively rebuilt in COOT and refined in ReftmacS.

Paper disk-agar assay for keratinase and protease activity. Protease or keratinase activities were measured by 1% agarose supplemented with 1% skim milk, 1% casein, 1% elastin, or 1% feather powder in 150 mM NaCl* 10 mM CaCl₂ and 50 mM CHES buffer at pH 6. Disc-shaped filter papers soaked with keratinase were lightly pressed onto the agar surface at 55 °C. A clear zone around each disc indicated protease or keratinase activities.

The heat and pH tolerance tests. For the heat tolerance test, keratinase solutions (1.6mg/mL) in the solution containing 1OmM CaCl₂ 150mM NaCl and 50mM HEPES at pH 8.0) were heated to corresponding temperatures for 2 mins. For the pH tolerance test, keratinase solutions (L6mg/mL) were prepared in the solutions containing 1OmM CaCl₂ and 100mM corresponding buffers (phosphate, pH 2.0; citric acid, pH 3.0; citric acid, pH 4.0; phosphate, pH 5.0; phosphate, pH 6.0; phosphate, pH 7.0; HEPES, pH 8.0; TAPS, pH 8.0; borate, pH 9.0; CAPS, pH 10.0; CAPS, pH 11.0). The remaining protease activities were examined by a paper disk-agar diffusion assay using a 5% skim milk agar plate.

Construct of the recombinant plasmids. The pITPY2 vector was modified by removing its cloning, TEV and part of 6x His tag sites and incorporating the designed ligation independent site, TEV and 8xHis tag sites right after the alpha factor signal sequence, Kex2 signal cleavage and Ste3 signal cleavage sites (Wang et al, "Parallel Gene Cloning and Protein Production in Multiple Expression Systems" Biotechnol Progr (2009) 25, 1582-1586). Two different recombinant M.
The WR-220 keratinase gene constructs were amplified by PGR and ligated with the modified pHTYP2, one pHTYP2spr2261c, contained the inhibitory domain and the catalytic domain (FIG. 10A-B) and the other, pHTYP2 spr2261c, only contained the catalytic domain (FIG. 11A-B). The recombinant plasmids, pHTYP2spr2261c and pHTYP2spr2261c were transformed into *E. coli* DH5α on low salt LB agar plates with 25 μg/ml zeocin. The positive clones were identified by colony PCR and the sequences were confirmed by DNA sequencing.

**Transformation.** About 35 μg plasmids were linearized using pmeI and purified by alcohol precipitation prior to transformation into *P. pastoris* X33 strain by electroporation. The positive colonies were chosen from YPDS plates with 100 μg/ml zeocin at 30°C and validated by the MD/MM plate method.

Small scale keratinase production. The positive colonies were cultivated in 5ml of YPD medium with 100 μg/ml zeocin at 29°C under agitation at 300rpm in dark. The cells were collected by centrifugation until OD600 of 2-6 was reached and diluted to BMM medium (1.34% YNB, 4 E-5% biotin, 0.5% methanol and 100 mM potassium phosphate, pH 5.0) with 100 μg/ml ampicilln until OD600 value was reached to 2. Cells were cultured at 30°C under agitation at 300rpm. After one day incubation, the culture medium was supplemented with methanol to a final concentration of 0.5% every day. The culture supernatant was collected every 24 hour until day 5 and stored at 4°C.

**SDS-PAGE and Mass spectrometry analyses.** The proteins in 480 μl of the culture supernatant were precipitated by addition of 120 μl of 100% TCA for 1.0 mins on ice, followed by centrifugation. The pellets were washed by 200 μl of cold acetone for three time and incubated at 95°C for 5-10 mins to remove remaining acetone prior to the SDS-PAGE electrophoresis. The bands observed in the SDS-PAGE was treated by in-gel tryptic digestion prior to Mass spectrometric characterization.

**Paper disk-agar diffusion assay for protease activity after heat pH pretreatment.** Protease activities were detected by 1% agarose supplemented with 1% skim milk powder in 150 mM NaCl, 10 mM CaCl₂ and 50 mM HEPES buffer at pH 5.0. Disc-shaped filter papers soaked with culture supernatant were lightly pressed onto the agar surface at 55°C. A clear zone around each disc indicated protease or keratinase activities.

**RESULTS**

Keratinase ability of *Meiothermus tawanensis* WR-220

*M. tawanensis* WR-220 was incubated in the medium with insoluble feathers as the only source of nutrients. The cloudy medium and reduction of feathers indicated that degradation occurred (FIG. 1A), suggesting *M. tawanensis* could produce enzymes to consume feathers (FIG. 1). It was further found that *M. tawanensis* WR-220 was able to degrade half of 3% feathers and release free amino acids from feathers in 2 days at 55°C and 65°C (FIGs. 1A-C).
Isolation and Identification Keratinase

*M. taiwanensis* was removed from the growth medium and it was further found that the keratinase activity was kept in the medium, suggesting keratinase was produced as a secreted form. The total proteins were fractionated from the medium using ion exchange chromatography. The fractionated proteins were further separated by protein electrophoresis, following by zymography assay (FIGs. 2A-B). The zymography assay clearly showed that proteins in multiple areas of gel could digest keratin/casein. We then applied proteomics methods to identify the proteins in those areas using our genomic sequencing result (NCBI bioproject submission ID: SUB251796) as the reference. The translated amino acid sequence consisted of a predicted signal peptide, the predicted inhibitor domain and the predicted catalytic domain as shown in FIG. 5B (SEQ ID NO: 4). The cross comparison indicated that the gene SPR2261 is the putative keratinase of *M. taiwanensis* WR-220 (FIG. 8A; SEQ ID NO: 3), however, the sequence of the catalytic core shares only 39% sequence identity to the known keratinase from *Bacillus tifeniformis*.

Production of Recombinant keratinase by *E. coli* expression system

The keratinase gene (SPR2261) was amplified by PCR and cloned into an expression plasmid containing a starting codon and a fusion tag for *E. coli* expression system (FIG. 9A, SEQ ID NO: 5). The recombinant keratinase with an expected molecular weight of ~41.5 kDa was expressed in *E. coli* BL21 (DE3) Arctic cells and purified by affinity chromatography. The results of protein electrophoresis indicated that the molecular weight of the purified protein was 28.5 kDa, suggesting a truncated form of cloned keratinase (FIG. 3), which was consistent with the 28,468 Dalton found by ESI-MS spectrometry. Our further structural analysis of keratinase by protein crystallography showed that the truncated form of the recombinant keratinase started from 102th residue of the cloned amino acid sequence (FIGs. 9B, SEQ ID NO: 6).

Broad protease activity of recombinant keratinase and heat/pH pretreatment

The truncated form of the recombinant keratinase revealed a broad range of proteolytic activities that degraded not only proteins in feathers but also proteins in milk, casein and elastin (FIG. 4). The recombinant keratinase retained activities after being heated up to 95°C or pre-incubation in an acidic or basic solution (FIGs. 5A-B).

Cloning of *M. tamanensis* WR-220 keratinase gene for yeast expression system

The gene of the inhibitory and the catalytic domain of keratinase (spr2261ic) and the gene of the catalytic domain of keratinase (spr2261c) were, respectively, fused with the yeast alpha-factor signal peptide (SEQ ID NO: 15), which allows secretion of keratinase into the culture medium. The constructed plasmids containing spr2261c and spr2261c were named pHTPY2_spr2261c and
pHTPY2__spr2261c, respectively (FIGs. 10 and 11). The fused gene transcription was under the control of alcohol oxidase 1 promoter (AOX1). The inserted gene were verified by DMA sequencing. Recombinant production by P. pastoris X-33 strain

Twelve positive colonies containing pHTPY2__spr2261c were chosen for recombinant keratinase production. Eight of 12 colonies were cultured and 5 out of 8 culture supernatants showed protease activities in a paper disk-agar diffusion assay, indicating that the keratinase was produced (FIG. 6). The SDS-PAGE electrophorograms of the culture supernatants showed two major protein bands (FIG. 7). A subsequent Mass spectrometrical characterization revealed that both protein bands contained the catalytic domain of M. taiwanemis keratinase. Based on our experience and crystal structure, we believe that protein in the upper band was the catalytic domain with the linker and 8xHis tag and the protein in the lower band was the catalytic domain only. The estimated molecular weight of the protein, which was based on the band position in the SDS-PAGE, indicated that the inhibitory domain was removed during protein production. This result was similar to keratinase production using the E. coli expression system. The data suggested that the major secreted protein from the P. pastoris with our constructed plasmid was the catalytic domain of M. taiwanemis keratinase.

The requirement of the inhibitory domain for recombinant keratinase production

Although the constructed pHTPY2__spr2261c plasmid contained both the inhibitory domain and the catalytic domain, the secreted protein showed no attachment ofN-terminal inhibitory domain. This phenomenon has been observed in M. taiwanemis keratinase production using E. coli expression system. Furthermore, the constructed pHTPY2__spr2261c plasmid containing no inhibitory domain failed to produce keratinase, suggesting the requirement of the inhibitory domain for protein production even though the final product did not contain the inhibitory domain.

Structural analysis of M. taiwanemis WR-220 keratinase

Our crystal structure of M. taiwanemis WR-220 keratinase has shown that the part of the linker region connecting the catalytic domain and the His fusion tag is located in the active site of the catalytic domain of the adjacent keratinase (FIG. 12A). This data shows that the Ser221, His72 and Asp39 are the catalytic residues (FIG. 12B; only the catalytic domain residue numbering is used; the first residue is Ala and the second one is Thr; SEQ ID NO: 14). Moreover, the structural analysis showed that Gly103, Ser104, Gly105, Ser106, Leu! 31, Gly132, Gly133, Gly134, Ala156, Gly158, Asn159, Ser22L, Gly222, Thr223 and Met225 are involved in substrate interaction (FIG 12B). The sequences shown in FIGs. 10B, 11B are produced from yeast system. The structural analyses were performed using keratinase produced by E. coli. expression system. The linker sequence used in yeast system expression system was GNLYFQS (SEQ ID NO: 16). In E. coli system, the residue G was replaced by E.
The foregoing description of the exemplary embodiments of the invention has been presented only for the purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Many modifications and variations are possible in light of the above teaching.

The embodiments and examples were chosen and described in order to explain the principles of the invention and their practical application so as to enable others skilled in the art to utilize the invention and various embodiments and with various modifications as are suited to the particular use contemplated. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its spirit and scope. Accordingly, the scope of the present invention is defined by the appended claims rather than the foregoing description and the exemplary embodiments described therein.

Some references, which may include patents, patent applications and various publications, are cited and discussed in the description of this invention. The citation and/or discussion of such references is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the invention described herein. All references cited and discussed in this specification are incorporated herein by reference in their entireties and to the same extent as if each reference was individually incorporated by reference.
CLAIMS

What is claimed is:

1. A fusion gene comprising:
   (a) a first DNA sequence encoding a protein secretion signal peptide, located at the N-terminus of the fusion gene;
   (b) a second DNA sequence encoding an inhibitory domain of M. taiwanensis WR-220 keratinase, linked in translation frame with the first DNA sequence; and
   (c) a third DNA sequence encoding a catalytic domain of M. taiwanensis WR-220 keratinase, linked in translation frame with the second DNA sequence, wherein the fusion gene is a non-naturally occurring chimeric DNA.

2. The fusion gene of claim 1, wherein the first DNA sequence encodes a yeast alpha-factor signal peptide.

3. A protein expression vector comprising:
   (a) the fusion gene of claim 1 or 2; and
   (b) a promoter, linked in translation frame with the fusion gene.

4. The protein expression vector of claim 3, wherein the promoter is selected from the group consisting of alcohol oxidase (AOX) promoter, glyeeraldehyde de phosphate dehydrogenase promoter, translational elongation factor J-α promoter, Na⁺-coupled phosphate symproier promoter, and formaldehyde dehydrogenase promoter.

5. A host cell comprising the expression vector of claim 3.

6. A cell culture comprising:
   (a) an artificial medium;
   (b) the host cell of claim 5, the host cell secreting the catalytic domain of M. taiwanensis WR-220 keratinase into the artificial medium.

7. An isolated protease comprising a catalytic domain of M. taiwanensis WR-220 keratinase, the protease lacking an inhibitory domain of M. taiwanensis WR-220 keratinase, and being in the form of a tablet, a caplet, a pellet, a capsule, a granule, a pill, a powder or a sachet, or in the form of a solution without containing a cell culture supplement.

8. The protease of claim 7, which comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 14.
9. Use of a protease as claimed in claim 7 or 8 in manufacture of a composition for degrading a proteinaceous material.

10. A use as claimed in claim 9, wherein the protease is pretreated at a temperature above 40° C but below 95° C and remains its activity.

11. A use as claimed in any one of claims 9, wherein the proteinaceous material is selected from the group consisting of animal feed, food, milk, casein, elastin, skin, hair, wool, silk, nails, scales, fiber, leather, and feathers,

12. A use as claimed in claim 9, wherein the protease is pretreated with a solution having a pH value ranging from 3 to 10 and remains its activity.

13. A method for preparation of a catalytic domain of *M. taiwmemis* WR-220 keratinase, comprising:
   (ai) growing the host cell of claim 5 in a culture medium under conditions that permits expression of *M. taiwmemis* WR-220 keratinase and secretion of the catalytic domain thereof into the medium; or
   (a)ii) growing a host cell transformed with an expression plasmid comprising a DNA insert encoding an inhibitory domain and a catalytic domain of *M taiwamnsis* WR-220 keratinase under conditions that permits expression of *M. taiwam m* is WR-220 keratinase and secretion of the catalytic domain thereof into the medium; and
   (b) removing the host cell to obtain a supernatant containing the catalytic domain of the *M. taiwamensis* WR-220 keratinase; and
   (c) isolating the catalytic domain from the supernatant or drying the supernatant to obtain the catalytic domain in solid form.

14. The method of claim 13, wherein the inhibitory domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 13; and the catalytic domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 14.

15. The fusion gene of claim 1, wherein the inhibitory domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 13; and the catalytic domain comprises an amino acid sequence having at least 95% identity to SEQ ID NO: 14.
FIG. 1A

Day 0  Day 1  Day 2

FIG. 1B

Before cultivation  55°C  65°C  Cultivation with WR-220

FIG. 1C

Weight of remaining feathers

Ninhydrin assay

Free amino group (µM)

- WR-220  + WR-220

- WR-220  + WR-220

55°C  65°C

55°C  65°C
### FIG. 8A

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### FIG. 8B

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<td>70 YLQSNPSASP</td>
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**NOTE:** The substitute sheet (Rule 26) indicates that this page contains additional sequence information that is not fully visible due to the page format or print quality. The full sequence data should be reviewed for accurate representation.
FIG. 9A

SEQ ID NO: 5

0001 ATGCTAGCCC CGGTGCTAGG ACTGGATAAC CCAACGCTTA TCCAGGGGCA
0051 GTACATTGGT GTCTACAAAGG ATGATGCCCA GTGCTGCCAC ACCCTCGCAA
0101 GCCGAAAGCC GGCTTCTAGAT GGGGCTGAA CACCTGACCA GGAACCTGGGAA
0151 AGCCTGCGGAC TGGCACCAGCA GCAAGAGGTT GACGAGGTTT ACGACCGCTTC
0201 TTCGCTGGGG CTCGACCGCA GGCATCACC CCCGAATTTA GCCGCGCGCTG
0251 GCCAAGCACG CCGGCTGCGG TACATCGAGG CGAGGAGATT CATGAGCCTTC
0301 AGGAGGAACG AGACCAGTGGC GACCTGGGGC CTGGATCGCA TAGACCAGCG
0351 CACCCCTACCC CTGACCGGTA CTTTGCACCTA CAGCAACAGC GGCAGCGCCG
0401 TGAAAGCACTA CATCATGATG ACCGGAATCC GGGTGAGGCA CAGCGAGGTTT
0451 GCCGAGTCGG GCAAAGCGGTT TTCTGAGACT ATGGAGAACG CCCAGAATGGG
0501 CAAGCAACTGC CACGGCGCAG GCACCCATGT GGGTGCGAGC ATGCAGCGGGC
0551 CGGTCTACGG CTAGCCAAA AGCCTGCGGT GTGACGCGGA GCGCGTGGCTT
0601 AAATGAGCGG GTCCGGCGCA CAATCGGGGC GATATGCGGC GGGTGCACTG
0651 GGTTCGCGGAG AAATCGCCGCA GCCACCGGATG AGCCACATG AGCCTGGGTTT
0701 CGGCTGCGGTC GAGCGCCGCG CATACCCGGG CAAATGGGGG CTACAAAGGC
0751 GGTTACACTT TTGCGCTGGG CGCAAGTAAAC AGCAGCGGCG AGCCTGCGCCA
0801 GTGCTCGCAG CGACGGGGTTA CTGCAGGCTA ATGGAGACGG GCAGCCCCGCC
0851 CCACCAGGCC CGGAGCGCTCC TATACCAACT AGCTAGACTG CTTGCAGCCTC
0901 TTGGCGCCCC GTCTTTCATC AACCCTGCGC TGGAGTACAG GCAAGACCTCC
0951 GACCAACACC ATCAGCGGGA CCTGCGATGG CCACCCCATG TGGCGCGGGG
1001 TAGCGGCTTTG ATACCTCGCA AAGACCCCCA GTCGACGGCC GCGACCCCTG
1051 CGCAACGCCA TTGTCGGGCA CGGACACCTG GTGCTGGGTA GCAACCGGCGG
1101 GCGCGGTTCG CGCAAGTGTTG CTGCTGTACG CAAATGAGACG AAGCTCTGACT
1151 TCAATCGCCA CCATCTACAC CACCAATGGAG GCCATCGCGCA GTTGGAABAA
1201 TAG

FIG. 9B

SEQ ID NO: 6

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MLAPVGLDNP PNVIQGQYIV VYKDDANVLP TLQSLKAALBD GGVTQRELE SLGAPDARY
70  80  90 100 110 120
EQQYTAALLG LAARLEPNLE AALRODPRVAA YIEADQYMSI SATQTGATWG LDRIQRTILP
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LSGTFTYSNT GSGVNAIYID TGIRVSHSEF GGRATAVEFA IGDQGNMDC NGHOTHVAGT
190 200 210 220 230 240
VGGTVYGVAR SRVLAYVRVL NCSSGSSNSG VIAVGDVRVRQ NARRPAVANM SLGGGASSAL
250 260 270 280 290 300
DTAVNNAINA GITFALAAGN SNRDACQFSP ARVTAGITVG ATITSTDRAR YSNYGMLDL
310 320 330 340 350 360
FAPGSSITSA WISSDTSTNT ISGTSMAFTH VAGVAALYLQ SNPSASAPTV RNAIVGNATS
370 380 390 400
GVVSNAGRRS PNLLLYSYNE NLHFQSHHHH HHWSHPFEK

SUBSTITUTE SHEET (RULE 26)
FIG. 10A (Part 2 of 2)  SEQ ID NO: 7 (Continued)

[Sequences shown but not transcribed]

FIG. 10B  SEQ ID NO: 8

[Sequences shown but not transcribed]
INTERNATIONAL SEARCH REPORT

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 9/50 (2014.01)
CPC - C07K 231/900 (2014.09)

B. DOCUMENTS CONSIDERED TO BE RELEVANT

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<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim</th>
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<tr>
<td>A</td>
<td>YANG et al. 'Feather Keratin Hydrolysis by an Aquatic Bacterium Meiothermus I40 from Hot Spring Water,' International Journal of Food Engineering, 01 March 2011 (01.03.2011), Vol. 7, Iss. 2, Pgs. 1-21, entire document</td>
<td>1-15</td>
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<tr>
<td>A</td>
<td>KNO et al. Purification and characterization of a thermostable keratinase from Meiothermus sp. I40/ International Biodegradation &amp; Biodegradation, 01 May 2010 (01.05.2010), Vol. 70, Pgs. 111-116, entire document</td>
<td>1-15</td>
</tr>
<tr>
<td>A</td>
<td>CHIN et al. 'Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data,' Nature Methods, 31 January 2013 (31.01.2013), Vol 10, No. 06, Pgs. 563-569, entire document</td>
<td>1-15</td>
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</tbody>
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Further documents are listed in the continuation of Box C.

Date of the actual completion of the international search
04 November 2014

Date of mailing of the international search report
26 NOV 2014

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PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)