Chemicals

Culture media and analytical chemicals were purchased from HiMedia, Mumbai, India. Casein and other fine chemicals were purchased from Sisco laboratory, India. Acrylamide, Bis-acrylamide, TEMED, β-mercaptoethanol, SDS and Ammonium persulfate were purchased from Amersham Biosciences, Sweden. The standard molecular weight protein marker and protease inhibitor kit was purchased from Sigma chemical Co., CA, USA.

Bacterial strain

Marine sediment samples were collected by using a core sampler at four different depths (1.5 m, 2.4 m, 3 m and 4.5 m) in the East coastal region of Thondi, Palk Bay, India (Latitude: 9°45’ N and Longitude: 79°13’ E). The bacterial isolates were screened on skim milk agar plates incubated at 37°C for 24 h and depending on the zone of clearance, isolates showing maximum activity in plates and liquid culture were further selected for dehairing studies. Among 46 isolates tested, TMS55 showed profound dehairing activity and hence this isolate was identified by biochemical methods and 16S rRNA gene sequencing.

Identification and taxonomical studies

The isolate TMS55 was biochemically characterized according to the method described in “Bergey’s manual of determinative bacteriology” (Holt et al. 1994) and identified based on 16S rRNA gene sequence. The 16S rRNA gene amplification, cloning and sequencing of 16S rRNA gene was done and the sequence data were analyzed by comparison with 16S rRNA genes in the GenBank database.

Isolate TMS55 was characterized as *B. pumilus* by physiological and biochemical characteristics and also via 16S rRNA gene sequence analysis. Similarity searching using BLAST at NCBI showed a high similarity with *B. pumilus* strains. 16S rRNA gene sequence of the strain TMS55 has been deposited in the GenBank database under accession number **DQ988522**. The strain *B. pumilus* TMS55 has been deposited in Microbial Type Culture Collection & Gene Bank (MTCC), India with accession number **MTCC 5357**.
Polymerase Chain Reaction (PCR) amplification of 16S rRNA gene

Bacterial 16S rRNA gene was amplified from the genomic DNA using the following universal eubacterial 16S rRNA primers: Forward primer 5’ AGAGTTTGATCCTGGCTCAG 3’ (E. coli positions 8 to 27) and Reverse primer 5' ACGGCTACCTTGTTACGACTT 3' (E. coli positions 1494 to 1513) (Weisburg et al. 1991).

PCR was performed with a 50 μl reaction mixture containing 2 μl (10 ng) of bacterial genomic DNA as the template, each primer at a concentration of 0.5 μM, 1.5 mM MgCl₂, and each deoxynucleotide triphosphate at a concentration of 50 μM, as well as 1 U of Taq polymerase and buffer used as recommended by the Manufacturer (MBI Fermentas). After the initial denaturation for 4.5 min at 95°C, there were 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min and then a final extension step consisting of 5 min at 72°C; Mastercycler Personal (Eppendorf, Germany) was used. The amplification of 16S rRNA gene was confirmed by running the amplification product in 1% agarose gel electrophoresis in 1X TAE.

Bacterial identification by 16S rRNA gene sequence analysis

The amplified product (~1500 bp) was purified using HiYield™ Gel/PCR DNA Extraction Kit (RBC Biotech Corporation) according to manufacturer’s instruction. The 16S rRNA amplicon was cloned in pTZ57R/T vector according to the manufacturer’s instruction (InstAclone™ PCR Product Cloning Kit #K1214, MBI Fermentas). Sequencing of the 16S rRNA gene (about 1500 bp) of the marine isolate TMS55 was carried out using M13F (-20) primer 5’GTAAAACGACGGCCAGT3’, M13RpUC (-40) primer 5’ CAGGAAACAGCTATGAC 3’ and internal primer 5’GCCACATTGGGACTGAGACA 3’ in Macrogen (Seoul, Korea). The sequences obtained were assembled, analyzed, and manually edited using CAP3 software package (Huang and Madan 1999) and compared to sequences within the NCBI database (http://www/ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997). Multiple sequence alignment and NJ plot were carried out using CLUSTALX (Thompson et al. 1997).
Media
Zobell Marine Broth (HiMedia, India)
Agar Powder, bacteriological (HiMedia, India)

Optimization of culture conditions

Effect of culture conditions on enzyme production

Potent strain was studied in detail by changing the media conditions along with the control. To optimize the culture conditions for maximum production of protease, the effect of following parameters were recorded.

Effect of carbon and nitrogen sources

The effect of carbon and nitrogen sources for protease production were determined using different carbon sources (0.5%) consisting of glucose, sucrose and maltose. Nitrogen sources (0.5%) such as peptone, beef extract, yeast extract, soybean meal, casein and gelatine were used for supplementation individually in the production medium for protease production from *Bacillus* sp. Protease yield was determined after 24 h of incubation at 37°C under shaking condition of 180 rpm.

Effect of media pH

The effect of initial media pH was determined adjusting the media to different initial pH using 5N NaOH. Inoculation was performed with 1% (v/v) inoculum and incubated at 37°C and 180 rpm for 24h.

Effect of the incubation temperature

The effect of the incubation temperature on the proteolytic activity was determined by inoculating 50 ml of medium (in 250 ml of Erlenmeyer flask) with 1% inoculum of culture and incubated at 22, 28, 37, 45 and 50°C at 180 rpm for 24 h.
Effect of agitation speed

The effect of the agitation speed on the protease enzyme synthesis was determined by inoculating 50 ml of medium (in 250 ml of Erlenmeyer flask) with 1% inoculum of culture and incubated at 28°C and 120, 140, 160, 180 and 200 rpm under the same fermentation conditions.

Effect of inoculation ratio

To determine the effect of inoculation ratio, 50 ml of the medium was inoculated, with 1%, 2%, 3% and 5% (v/v) and incubated at 37°C.

Effect of incubation time

In order to determine the optimum incubation time for maximum enzyme production, 50 ml of medium inoculated with 1% (v/v) was incubated at the predetermined optimum conditions (28°C and 160 rpm) for 24, 48 and 72 h.

Characterization of the protease enzyme

The crude protease obtained from the B. pumillus TMS55, which showed the highest potential for proteolytic activity, was further subjected to partial purification with ammonium sulphate and preliminary characterization study was made. Therefore, the effect of pH and temperature on activity and stability were studied.

Partial purification and characterization of enzyme

The cell free culture supernatant was subjected to 50% saturation with ammonium sulphate by slow continuous stirring. The saturated solution was centrifuged at 13000 × g for 20 min at 4°C and the pellet was suspended in a minimum amount of 50 mM phosphate buffer (pH 7.5). Pellets and supernatants were checked for protease activity with the azocasein assay. Any insoluble material present after suspension was removed by centrifugation at 13,000 × g for 20 min at 4°C and the supernatant was then collected. Enzyme assay was carried out at different pH (7.5 - 12) and temperature (35 - 75°C) and relative activity was determined by assay
described earlier. Various activity buffers such as phosphate buffer (pH 7.5 - 8), glycine/NaOH buffer (pH 9 - 11) and NaCl/NaOH buffer (pH 12) were used. Temperature stability of the enzyme was studied by incubating the enzyme at different temperatures (55°C, 60°C and 65°C) for 1 h prior to assay. The effect of various protease inhibitors (Sigma, USA) was also determined by pre-incubating the enzyme with the inhibitors at 1 mM concentration for 30 min and the relative activity was determined as mentioned earlier.

**Protease production medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose (HiMedia, India)</td>
<td>0.5 %</td>
</tr>
<tr>
<td>Beef Extract (HiMedia, India)</td>
<td>0.5%</td>
</tr>
<tr>
<td>Magnesium Sulphate (HiMedia, India)</td>
<td>0.5%</td>
</tr>
<tr>
<td>Sodium Chloride (HiMedia, India)</td>
<td>0.5%</td>
</tr>
<tr>
<td>Dipotassium Hydrogen Phosphate (HiMedia, India)</td>
<td>0.5%</td>
</tr>
<tr>
<td>Soyabean Meal (HiMedia, India)</td>
<td>1%</td>
</tr>
</tbody>
</table>

**Enzyme production**

The culture medium (pH 7.5) used in this work for protease production contained MgSO₄ (0.2%), K₂HPO₄ (0.5%), Maltose (0.5%), NaCl (0.5%), Beef extract (0.5%) and Soya bean meal (1%). The culture medium (50 ml in 250 ml Erlenmeyer flasks) was inoculated at 1% with 24 h seed culture and incubated at 28°C for 48 h. The cells were then separated by centrifugation and the cell free supernatant was used as crude enzyme preparation.

**Immobilization of protease and its dehairing efficacy**

Immobilization of the protease was performed by entrapment method using polyacrylamide gel. Culture supernatant (8 ml) containing 64 U of the protease was added to 2 ml of 30% polyacrylamide (Amersham Biosciences, USA) to make 6% (w/v) polyacrylamide for immobilization. Before adding TEMED and APS, the mixture of acrylamide and protease was mixed for 20 min gently. After polymerization, the resulting gel was cut into approximately of 1 cubic cm pieces. The gel pieces were washed with 50 mM phosphate buffer (pH 7.5) for 5 min,
twice, to remove the soluble enzyme and the gel pieces were stored in 50 mM phosphate buffer (pH 7.5) at 4°C for further use. The efficiency of the immobilized enzyme was determined by assaying with azocasein as mentioned earlier. Effect of number of assay cycles on the dehairing activity of immobilized protease was determined by subjecting the immobilized enzyme for 5 cycles with goatskin samples of 4 cm by 4 cm for overnight incubation at 37°C for each cycle. For control, uninoculated culture medium was used. After every cycle, the immobilized enzyme was washed in buffer and used for a fresh assay. Before every assay the buffer in which the beads are stored was tested for protease activity to check for any leaching of enzyme.

Stock solutions

Buffers

1 M Potassium dihydrogen phosphate

Potassium dihydrogen phosphate (13.6 g) was initially dissolved in 50 ml distilled water and the volume was made up to 100 ml with distilled water.

1 M Dipotassium hydrogen phosphate

Dipotassium hydrogen phosphate (17.41 g) was initially dissolved in 50 ml distilled water and the volume was made up to 100 ml with distilled water.

1M Glycine

Glycine (7.5 g) was initially dissolved in 50 ml distilled water and the volume was made up to 100 ml with distilled water.

1M Sodium hydroxide

Sodium hydroxide (4 g) was initially dissolved in 50 ml distilled water and the volume was made up to 100 ml with distilled water.
1 M Potassium phosphate buffer (pH 7.8)

Potassium phosphate buffer (1 M, pH 7.8) was made by adding appropriate volume of 1 M potassium dihydrogen phosphate with 1 M dipotassium hydrogen Phosphate, until pH 7.8 is reached and it was monitored through pH meter (Eutech, USA).

1 M Potassium phosphate buffer (pH 9.5)

Potassium phosphate buffer (1 M, pH 9.5) was made by adding appropriate volume of 1 M potassium dihydrogen phosphate with 1 M dipotassium hydrogen phosphate, until pH 9.5 is reached and it was monitored through pH meter (Eutech, USA).

1 M Potassium phosphate buffer (pH 7.0 to 9.5)

Potassium phosphate buffer (1 M, pH 7.0 to 9.5) was made by adding appropriate volume of 1 M potassium dihydrogen phosphate with 1 M dipotassium hydrogen phosphate, until desired pH is reached and it was monitored through pH meter (Eutech, USA).

1 M Glycine-sodium hydroxide buffer (pH 10 to 12)

Glycine - sodium hydroxide buffer (1 M, pH 10 to 12) was made by adding appropriate volumes of 1 M Glycine with 1 M Sodium hydroxide, until desired pH is reached and it was monitored through pH meter (Eutech, USA).

5 M Sodium chloride

Sodium chloride (29.2 g) was initially dissolved in 70 ml distilled water and the volume was made up to 100 ml with distilled water.

Protein substrate

Azocasein (2%) (Sigma, USA) was prepared in 50 mM phosphate buffer.
Reagents for total protein estimation (Lowry’s method)

Reagent A: 2% Sodium carbonate in 0.1N sodium hydroxide.
Sodium hydroxide (4 g) was dissolved in 800 ml distilled water and followed by the addition of
20 g of sodium carbonate (anhydrous). The volume was made up to 1000 ml with distilled
water.

Reagent B: 2% Copper sulphate (CuSO₄·5H₂O)
Copper sulphate (2 g) was initially dissolved in 50 ml of distilled water. The volume was made
up to 100 ml with distilled water.

Reagent C: (2 %) Sodium potassium tartarate
Sodium potassium tartarate (2 g) was initially dissolved in 50 ml of distilled water. The volume
was made up to 100 ml with distilled water.

Folin - Ciocalteau reagent (Sisco Research Laboratories, India)
The commercial reagent was diluted 3:1 with distilled water according to the manufacturer’s
instruction.

Lowry’s reagent
This was prepared by mixing 0.5 ml of each reagent B and reagent C to 99 ml of reagent A.

Bovine serum albumin standard (1mg/ml)
Bovine serum albumin (1 mg) was dissolved in 1ml of 50 mM phosphate buffer by mixing
gently.

Protease assay and total protein estimation
The enzyme activity was assayed using azocasein as the substrate, according to the
method of Sarath et al. (1989). The reaction mixture consisted of 0.25 ml of 50 mM sodium
phosphate buffer, containing 2.0% (w/v) azocasein and 0.15 ml of the enzyme solution. After
incubating at 37°C for 15 min, the reaction was stopped by the addition of 1.2 ml of 10.0% (w/v) TCA and incubated at room temperature, for an additional 15 min. Then the precipitate was removed by centrifugation at 8,000 × g for 5 min. To 1.2 ml of the supernatant, 1.4 ml of 1.0 M NaOH was added and its absorbance was measured at 440 nm. One unit of enzyme activity was expressed as giving an absorbance of 1.0, under the above condition. Protein content was estimated by the method of Lowry et al. (Lowry et al. 1951) using bovine serum albumin as standard. During chromatographic purification steps, the protein concentration was estimated as a function of its absorbance at 280 nm.

**SDS PAGE**

**12 % Resolving gel (15 ml)**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Water</td>
<td>4.9</td>
</tr>
<tr>
<td>2.</td>
<td>30% (29% Acrylamide + 1% BisAcrylamide)</td>
<td>6.0</td>
</tr>
<tr>
<td>3.</td>
<td>1.5 M Tris-Cl pH 8.8</td>
<td>3.8</td>
</tr>
<tr>
<td>4.</td>
<td>10 % SDS</td>
<td>0.15</td>
</tr>
<tr>
<td>5.</td>
<td>10 % APS</td>
<td>0.15</td>
</tr>
<tr>
<td>6.</td>
<td>TEMED</td>
<td>0.006</td>
</tr>
</tbody>
</table>

**5% Stacking gel (5 ml)**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Water</td>
<td>3.4</td>
</tr>
<tr>
<td>2.</td>
<td>30% (29% Acrylamide + 1% BisAcrylamide)</td>
<td>0.83</td>
</tr>
<tr>
<td>3.</td>
<td>1. M Tris-Cl pH 6.8</td>
<td>0.63</td>
</tr>
<tr>
<td>4.</td>
<td>10 % SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>5.</td>
<td>10 % APS</td>
<td>0.05</td>
</tr>
<tr>
<td>6.</td>
<td>TEMED</td>
<td>0.002</td>
</tr>
</tbody>
</table>
**5X Loading dye buffer (2 ml)**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 M Tris –Cl pH 6.8</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>2</td>
<td>SDS</td>
<td>0.2 g</td>
</tr>
<tr>
<td>3</td>
<td>DTT</td>
<td>0.154 g</td>
</tr>
<tr>
<td>4</td>
<td>Glycerol</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>5</td>
<td>Bromophenol Blue</td>
<td>0.01 g</td>
</tr>
<tr>
<td>6</td>
<td>Water</td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>

**10 X Tris glycine buffer (25 ml)**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tris base</td>
<td>3.0 g</td>
</tr>
<tr>
<td>2</td>
<td>Glycine</td>
<td>4.65 g</td>
</tr>
<tr>
<td>3</td>
<td>SDS</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Water was used to make up a final volume of 25 ml

**Sliver staining solution**

Fixing Solution: 50% Methanol and 12% Acetic acid (v/v)

Wash Solution: 30 % Methanol (v/v)

Sensitizer Solution: 0.02% Sodium thiosulphate (w/v)

Staining Solution: 0.2 % Sliver nitrite (w/v), 0.075 % Formalin (v/v)

Developing Solution: 6 % Sodium carbonate (w/v), 0.05 % Formalin (v/v) and 0.0004 % Sodium thiosulphate (w/v)

Stop Solution: 0.5% Glycine (w/v)
Protein purification

Ammonium sulphate precipitation

To 250 ml cell free culture supernatant, about 44 g of ammonium sulphate was added slowly at 20°C to attain 30% saturation, after the salt get completely dissolved, the supernatant was frozen at -20°C for 6 h and centrifuged at 13,000 x g for 20 min at 20°C. The pellet was then discarded and 30% saturated supernatant made up to 70% saturation by adding 69 g ammonium sulphate gently at 20°C and kept in -20°C for overnight and centrifuged at 13,000 x g for 20 minutes at 20°C. The pellet that was obtained at this stage was finally dissolved in 20 mM potassium phosphate buffer (pH 7.8).

Gel filtration chromatography

Dry powder of Sephadex G-75 (GE Healthcare, Sweden) was allowed to swell in MilliQ water for overnight. Then pre-swelled Sephadex G-75 beads were carefully packed into 50 cm by 1 cm column without air bubble. The packed column was operated through gravity. First the column was equilibrated with two column volumes of 20 mM potassium phosphate buffer (pH 7.8). About 2 ml of protein solution obtained from 30 % to 70 % ammonium sulphate precipitation was centrifuged to remove undissolved components and loaded onto the column. The sample was then eluted with two column volumes of 20 mM potassium phosphate buffer (pH 7.8), about 1 ml fractions were collected at a flow rate of 0.3 ml per min, absorbance measured for each fractions at 280 nm to detect presence of protein and protease assays were performed to all protein positive fractions. Fractions that showed activity were pooled in a single tube and concentrated to 2 ml volume by ultrafiltration with the use of 10 kDa membrane cut-off filter (Amicon, Beverly, MA, USA).

Ion exchange chromatography

Pre-swelled CM Sepharose beads (Sigma, USA) was packed into a 30 cm by 1 cm diameter column (GE Healthcare, Sweden), with a bed volume of 20 ml by using adaptor. The packed column was operated through AKTA purifier (GE Healthcare, Sweden). Column was washed with three to five column volumes of Milli Q water to remove ethanol and equilibrated
with three column volumes of 20 mM potassium phosphate buffer pH 7.8. Active fraction from gel filtration was injected and column was washed with two-column volume of 20 mM potassium phosphate buffer pH 7.8. Protease was eluted by 100 mM sodium chloride in phosphate buffer pH 7.8. Finally, all the strongly bound proteins were eluted by 1 M sodium chloride. Protease assay was performed for flow through, wash and elution fractions. The purified protein was concentrated to one ml volume by ultrafiltration using 10 kDa membrane cut-off filter (Amicon, Beverly, MA, USA).

**SDS PAGE**

The homogeneity and molecular weight of the purified protease was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using 5% stacking gel and 12% resolving gel. Electrophoresis was performed at 160 Volts for 3 hours. Protein bands were visualized by silver staining. Molecular weight of purified protein was estimated by comparing the relative mobility of proteins of different molecular weight using standard marker from Sigma-Aldrich (Cat # S8445).

**Effect of pH and temperature on protease activity**

The activity of the purified protease was measured at different pH values (7 - 12) using azocasein (2% w/v) as a substrate. The pH of the reaction mixture was adjusted to the desired value using 50 mM of the following buffers; potassium phosphate (pH 7.0 - 9.5) and glycine-sodium hydroxide (pH 10.0 - 12.0). The relative activities were quantified under standard assay conditions. The effect of temperature on the enzyme activity was examined at various temperatures at pH 9.5. Thermal stability was determined by incubating the purified enzyme at various temperatures, in the absence or presence of 5 mM Mn$^{2+}$. Aliquots were withdrawn at desired time intervals to test the remaining activity under the optimum conditions. The non-heated enzyme was considered to be a control and was assumed to have 100% activity.
Metal ions (Sisco Research Laboratories, India)

100 mM Calcium chloride
Calcium chloride (11 mg) in 1 ml distilled water.

100 mM Magnesium sulphate
Magnesium sulphate (25 mg) in 1 ml distilled water.

100 mM Manganese sulphate
Manganese sulphate (17 mg) in 1 ml distilled water.

100 mM Copper sulphate
Copper sulphate (25 mg) in 1 ml distilled water.

100 mM Zinc sulphate
Zinc sulphate (29 mg) in 1 ml distilled water.

100 mM Cobalt chloride
Cobalt chloride (24 mg) in 1 ml distilled water.

100 mM Barium chloride
Barium chloride (24 mg) in 1 ml distilled water.

100 mM Ferric chloride
Ferric chloride (27 mg) in 1 ml distilled water.

100 mM Mercuric chloride
Mercuric chloride (28 mg) in 1 ml distilled water.
Inhibitors (Sigma, USA)

100 mM AEBSF

AEBSF (25 mg) in 1 ml distilled water.

100 mM EDTA

EDTA (37 mg) in 1 ml distilled water.

10 mM E-64

E-64 (4 mg) in 1 ml distilled water.

5 mM Pepstatin

Pepstatin (3.5 mg) in 10 ml ethanol acetic acid (9:1) mixture.

100 mM PMSF

PMSF (2 mg) was dissolved in 1 ml distilled water.

Effect of metal ions and inhibitors on protease activity

The effect of metal ions on purified enzyme was determined by treating purified enzyme with various metal ions like Ca$^{2+}$ (calcium chloride), Mg$^{2+}$ (magnesium sulphate), Mn$^{2+}$ (manganese sulphate), Cu$^{2+}$ (copper sulphate), Zn$^{2+}$ (zinc sulphate), Co$^{2+}$ (cobalt chloride), Ba$^{2+}$ (barium chloride), Fe$^{2+}$ (ferric chloride) and Hg$^{2+}$ (mercuric chloride) at concentration of 1 mM and 5 mM for 30 min at room temperature. The enzyme assay was subsequently performed as described earlier. The percentage relative enzyme activity was calculated with reference to the activity of the enzyme without these supplements. The effects of inhibitors give the most reliable information as to the catalytic type of a peptidase. To investigate the catalytic type of protease, purified protease was pre-incubated with different protease specific inhibitors from Sigma, namely AEBSF, EDTA, E-64, pepstatin and PMSF which are specific inhibitors of serine protease, metalloprotease, cysteine protease, acid protease and serine protease respectively at a concentration of 1 mM in various tubes for 30 min in room temperature. After pre-
incubation, protease assay was performed, purified protease without protease specific inhibitors was taken as control and its activity was considered as 100%.

**Detergents**

5 % Triton X 100 (v/v) (Sisco Research Laboratories, India)
5 % Tween 20 (v/v) (Sisco Research Laboratories, India)
5 % Tween 80 (v/v) (Sisco Research Laboratories, India)
10 % Sodium Dodecyl Sulphate (w/v) (USB, USA)

**Oxidizing agent (Sisco Research Laboratories, India)**

50 % Hydrogen peroxide (v/v)

**Reducing agent**

Beta Mercaptoethanol (Amersham Biosciences, USA)

**Effect of detergents, oxidizing and reducing agents on enzyme activity**

To study the effect of detergents on enzyme activity, purified protease was pre-incubated with various detergents such as Tween 20, Tween 80, Triton X 100 and SDS at 1% and 2% concentration for 30 min at room temperature in various tubes. After pre-incubation, protease assay was performed at 60°C for 15 min in pH 9.5. Detergents free pre-incubated purified protease was taken as a control and its activity was taken as 100%. To investigate the effect of oxidizing and reducing agents on enzyme activity, both β-mercaptoethanol and hydrogen peroxide were taken at 1 and 2 % (v/v) in various tubes. Enzyme and additive mixture were pre-incubated at room temperature for 30 min and then assayed for protease activity. The purified protease enzyme was pre-incubated under the similar conditions without additives and its activity was taken as 100%.

**Solvents (Sisco Research Laboratories, India)**

Ethanol
Acetone
Pyridine
n-Butanol
Benzene
Chloroform
Toluene
Xylene
Hexane

**Effect of organic solvents on stability of purified enzyme**

To determine the effect of solvents on protease stability, 3.0 ml of the enzyme solution in 50 mM phosphate buffer (pH 9.5) was mixed with 1 ml of different organic solvents such as ethanol, acetone, pyridine, n-butanol, benzene, chloroform, toluene, xylene, and hexane. The mixture was incubated at room temperature for 30 min with constant shaking. Residual activity was measured by assay method described earlier. All experiments were conducted three times and Standard errors are reported. The activity of purified protease enzyme that was pre-incubated under the similar conditions without solvents was taken as 100%.

**Evaluation of purified protease for dehairing**

Fresh piece of goat skin (about 3cm × 4 cm) was treated with the purified enzyme (2 U/g of skin), in the presence or absence of 5 mM PMSF and incubated at 37°C for certain time. Dehairing efficacy of the protease from TMS55 was compared with the commercially available dehairing proteases namely Biodart from Southern Petrochemical Industries Corporation (SPIC) Limited and Enzyme-A from Bioscience Ltd. All proteases were adjusted to azocasein unit of 2 U/g of skin. At the end of the process, the skin pieces were gently scraped with fingers to remove loose hairs. This procedure is necessary because rubbing in this laboratory scale process was not as vigorous in industrial drums. The quality of the dehaired skin was estimated according to the appearance observed by naked eye and microscope (Dayanandan et al. 2003).
**1 M Tris - Cl (pH 8.0)**

Tris (12.11 g) was dissolved in 80 ml of double distilled water, pH adjusted to 8.0 with HCl. The volume was made up to 100 ml using double distilled water.

**0.5 M EDTA (pH 8.0)**

Na$_2$EDTA.2H$_2$O (18.61 g) was added to 80 ml of double distilled water, stirred vigorously and adjusted to pH 8.0 with NaOH pellets. The volume was made up to 100 ml using double distilled water.

**Sucrose TE**

Sucrose (10.3 g), 2.5 ml of 1M Tris-Cl (pH 8.0) and 5 ml of 0.5 M EDTA were added to 92.5 ml of double distilled water and autoclaved at 121°C for 20 min.

**10 % SDS**

SDS (10 g) was added to 80 ml of sterile double distilled water and heated for 65°C for dissolution. The volume was then made up to 100 ml with sterile double distilled water.

**50X Tris Acetate EDTA (pH 7.7)**

Tris (242.2 g) (2 M) and 37.2 g (0.1 M) of Na$_2$EDTA. 2H$_2$O were dissolved in 600 ml of double distilled water; pH was adjusted to 7.7 with glacial acetic acid (about 57 ml) and volume was made up to one litre with distilled water.

**Ethidium bromide (10 mg/ml)**

Ethidium bromide (100 mg) was dissolved in 10 ml of sterile double distilled water by stirring overnight. The container with the solution was wrapped in aluminum foil and stored at 4°C.
**6X Gel loading dye**
Sucrose (4 g) (40% w/v), 25 mg (0.25%) of bromophenol blue and 25 mg (0.25%) of xylene cyanol were added to 8 ml of distilled water and dissolved by mixing well. The volume was made up to 10 ml with distilled water and the solution was autoclaved and stored at 4°C.

**Equilibration of phenol**
The saturated phenol was equilibrated using 0.5 M Tris-Cl (pH 8.0). Then 0.1 M Tris-Cl pH (8.0) was added and equilibrated till it reached a pH greater than 7.8. To the equilibrated phenol, 0.1 M Tris-Cl (pH 8.0) containing 0.2% β-mercaptoethanol was added and stored at 4°C.

**Phenol: Chloroform (1:1)**
Equal volume of equilibrated phenol and chloroform was mixed before use.

**Chloroform: Isoamyl alcohol (24:1)**
To 96 ml of chloroform, 4 ml of isoamyl alcohol was added and stored in amber bottle.

**Enzyme stocks**

**Lysozyme (10 mg/ml)**
Lysozyme (10 mg) was dissolved in 1 ml of 10 mM Tris-Cl (pH 8.0).

**Proteinase K (20 mg/ml)**
Proteinase K (20 mg) was dissolved in 1 ml of solution containing 50 mM Tris-Cl (pH 8.0) and 1.5 mM calcium acetate. The stock solution was stored at -20°C.

**RNase A (10 mg/ml)**
RNase A (10 mg) was dissolved in 1 ml of 0.01 M sodium acetate (pH 5.2), kept in boiling water bath for 15 min and allowed to cool slowly to room temperature. 0.1 volume of 1 M Tris-Cl (pH 7.4) was added to the RNase A solution. The stock was stored at -20°C.
Genomic DNA isolation

The strain TMS55 was used to inoculate LB broth and incubated overnight at 37°C. The culture (1.5 ml) was spun at 6000 x g for 3 min. The pellet was resuspended in 400 μl of Sucrose TE. Lysozyme of a final concentration of 8 mg/ml was added to the suspension and the mixture was incubated for 1 h at 37°C. To the mixture, 100 μl of 0.5 M EDTA (pH 8.0), 60 μl of 10% SDS and 3 μl of proteinase K from 20 mg/ml stock were added and incubated at 55°C overnight. After incubation, the mixture was extracted with an equal volume of phenol: chloroform mix (1:1), centrifuged at 8,000 x g for 10 min and the supernatant transferred to a sterile tube. The supernatant was extracted twice with phenol: chloroform, once with chloroform : isoamylalcohol (24:1) and finally ethanol precipitated. The DNA pellet was resuspended in sterile water and stored at 4°C (for immediate use) or at -20°C (for long-term storage). Genomic DNA was checked on 0.8% agarose gel.

Plasmid DNA isolation

Solution I

2.5 ml of Tris-Cl (1 M, pH 8.0), 2 ml of EDTA (0.5 M, pH 8.0) and 0.9 g of glucose were added to 75 ml of distilled water and made up to 100ml and autoclaved.

Solution II

0.4 N NaOH

NaOH (pellets) (1.6 g) was dissolved in 75 ml of sterile distilled water and made up to 100 ml with sterile distilled water.

2% SDS

SDS (2 g) was dissolved in 100 ml of sterile distilled water and stored at room temperature. Equal volume of 0.4 N NaOH and 2% SDS were mixed just before use.
Solution III

3 M Potassium acetate (pH 4.8)

Potassium acetate (29.442 g) was dissolved in 20 ml of distilled water and the pH was adjusted with glacial acetic acid. The volume was then made up to 100 ml with distilled water.

4 M Sodium chloride

Sodium chloride (11.6 g) was dissolved in 30 ml of distilled water and the volume made up to 50 ml with distilled water and autoclaved.

Miniprep plasmid DNA isolation

LB medium (2 ml) was inoculated with a single colony of the strain TMS55 and incubated at 37°C overnight. Plasmid DNA was extracted by the alkaline lysis method. The culture was spun at 6,000 x g for 2 min. To the pellet, 200 μl of Solution I was added and vortexed. To the reaction mixture 300 μl of Solution II was added and the mixture was kept in ice for 5 min. To the lysate 300 μl of ice-cold Solution III was added and the mixture was incubated in ice for 10 min. Chloroform (400 μl) was further added, mixed gently and spun at 13,000 x g for 10 min. The plasmid DNA was precipitated with 2.5 volumes of absolute ethanol and centrifuged at 13,000 x g for 10 min. The pellet was washed with 70% ethanol. The pellet was air dried and dissolved in 30 μl of sterile water. The quality of the plasmid was checked on 1% agarose gel.

Cloning and sequencing of MASPT of B. pumilus

Genomic DNA from B. pumilus TMS55 was used for the amplification of the protease gene from B. pumilus TMS55 using the primers A1:5’-TTT CCA AGC GAC TTA ATT CC-3’; A2:5’-CGC GTC GAC GGC ATC AAG AAC CGT GCA GC-3’ (SalI) that were used to amplify intact alkaline serine protease of B. pumilus UN-31-C-42 (Huang et al. 2003). The PCR products were purified using GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences) according to the manufacturer’s instructions and cloned in pTZ57R/T cloning vector (InstAclone™ PCR Product Cloning Kit, #K1214, MBI Fermentas). Competent E. coli DH5α cells (Novagen, USA) were then transformed with the ligation mix and the transformants were analyzed by PCR and were
further confirmed by restriction enzyme digestion for the release of the insert. The intact alkaline serine protease gene was sequenced and processed and analysed.

**Analysis of MASPT protein sequence**

The translation of nucleotide sequence was performed with ExPASy Molecular Biology server (http://www.expasy.com). Amino acid composition was computed using ProtParam tool (Gasteiger et al. 2003). The hydrophobic profile was performed using ProtScale tools with Kyte and Doolittle method (Kyte and Doolittle 1982). SignalP was used to predict signal peptide (Bendtsen et al. 2004). Sequence homologs were retrieved from sequence databases using BLAST search against non-redundant protein sequence database with an E-value of 0.001 (Altschul et al. 1997). The conserved motifs were identified by ScanProsite (Gattiker et al. 2002).

**Homology modeling**

Subtilisin E from *Bacillus subtilis* (PDB code: 1scj) was selected as a template for the homology modeling of MASPT (Jain et al. 1998). The template selection was performed using BLAST search against PDB database (Berman et al. 2000). The target and the template sequences were aligned using T-COFFEE (Notredame et al. 2000). Homology modeling was performed by MODELLER (Sali and Blundell 1993). Energy minimization for the homology model was performed using PHENIX program (CCP4 package) (Adams et al. 2010). HBOND program from JOY suite was used to identify hydrogen bonds within the protein structure and between protein and ligand (Mn) (Mizuguchi et al. 1998). Catalytic site prediction was done based on the method of Pugalenthi et al. (2008). The generated 3D-model was assessed using RAMPAGE (Lovell et al. 2003), VERIFY3D (Eisenberg et al. 1997) and HARMONY (Pugalenthi et al. 2006).

**Docking analysis with Mn²⁺**

Docking calculations were carried out using DockingServer (Bikadi and Hazai 2009). Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were merged, and rotatable bonds were defined. Docking calculations were carried out on CAS
(Chemical Abstract Service) 28029-54-1 manganese (2+) salt model. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the aid of AutoDock tools (Morris et al. 1998). Affinity (grid) maps were generated using the Autogrid program (Morris et al. 1998). AutoDock parameter set- and distance-dependent dielectric functions were used in the calculation of the van der Waals and the electrostatic terms, respectively. Docking simulations were performed using the Lamarckian genetic algorithm (LGA) and the Solis & Wets local search method (Solis and Wets 1981). Initial position, orientation, and torsions of the ligand molecules were set randomly. All rotatable torsions were released during docking. Each docking experiment was derived from 2 different runs that were set to terminate after a maximum of 250000 energy evaluations. The population size was set to 150. During the search, a translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied.