CHAPTER III

MATERIALS AND METHODS

Materials

The research work was carried out at the Department of P.G Studies and Research in Microbiology, Gulbarga University, Gulbarga, Karnataka, India.

The materials used in the study comprises of chemicals, glass wares, substrates and samples. The chemicals used were of analytical grade, mainly from the brand names of Himedia, Merck, Sigma, Qualigens, SRL and SFDCL companies. The glass wares and plastic ware were of Borosil, Eppendorf, and Tarsons make. All the readymade media formulations were obtained from Himedia makes. All the microbiological methods for isolation, characterization, biochemical tests as per standard procedures described by Cappuccino, (1996); MacFaddin, (2000); Bergey’s Manual of Determinative Bacteriology (Brenner et al, (2004); Bergey’s Manual of Determinative Bacteriology Ludwig, (2005).

The instrumental facilities available at the Department of Microbiology, Biochemistry, Biotechnology and University Science Instrumentation Center (USIC) were utilized. The reference section of Central library and e-library was utilized for collection of literature and review articles and reference materials.
Methods

A. Isolation, screening and identification of proteolytic bacteria.

A.1 Collection of samples

Several soil samples were collected in sterile polythene bags from poultry waste dumping sites, slaughter house surroundings of Gulbarga (76°.04' and 77°.42 East longitude, and 17°.12' and 17°.46' North latitude), Karnataka and some animal faecal samples and field soil samples were also collected from Sirwar area (77°1’21” East longitude and 16°10’26” North latitude) of Raichur district, Karnataka and brought to the laboratory in sterile glass bottles.

A.2 Sample processing:

Respective sample (soil/faecal) suspension was prepared by mixing 10 g of sample with 100 ml of sterile normal saline and vortexted (Remi-India) the mixture for 10 min. Serial dilutions were made up to $10^{-7}$ dilution. 0.1 ml of the diluted sample suspension from $10^{-7}$ dilution was spreaded over the suitable solid agar plates using spread plate technique (Cappuccino, 1996). The inoculated plates were incubated at 35 (±2) °C temperature and observed for growth up to 4-5 days.

A.3 Isolation and screening of proteolytic bacteria by rapid plate assay method

Screening process for protease activity was conducted on skimmed milk agar (SMA) plates (containing g/L, peptone: 5.0; yeast extract: 3.0; skimmed milk: 100 ml; agar: 12, pH 7.5-8.5) under rapid plate assay and plates were kept for incubation at 35 (±2) °C for 96 hrs. Well isolated pure colonies with distinct haloes around the growth were re-streaked on freshly prepared SMA plates. The ratio of clearance zone diameter to colony size diameter (CZ/CS) was calculated (Pillai and Archana, 2008).

A.4 Selection of potent proteolytic strains

Based on large CZ/CS ratios (i.e. 3.5 and above) bacterial strains were selected for further microbial identification.

A.5 Identification of bacterial strains-phenotypic characterization

Bacterial identification till genus level was carried out by phenotypic (cultural morphological, physiological and biochemical) characterization of the selected strains as described by Cappuccino, (1996). Data was compared with Bergey’s Manual of Determinative Bacteriology (Brenner et al, 2004; Ludwig, 2005); Tindall et al, (2010), for confirmation of taxonomic position.
A.5.1 Cultural characteristics-Colony and cell morphology:

The bacterial strains were grown on skimmed milk agar plates and incubated at 35 (±2) °C. Observations were recorded for size, shape, configuration, elevation, margin and pigmentation after 24, and 48 h of incubation. Colony development on agar surfaces aids in identifying bacteria because individual species often form colonies of characteristic size, form, elevation, margin, opacity, pigment and appearance.

A.5.2 Morphological characteristics:

Gram staining:

Gram staining procedure divides bacteria into two separate groups based on staining properties. It is the most widely employed staining method in bacteriology. It is a differential staining procedure because it divides bacteria into two domains gram negative and positive. For cell morphology, Gram staining was performed. Bacterial cell morphology was viewed under bright field microscopy under oil emersion lens using a Nikon binocular microscope (Model: Nikon-E200).

A drop of normal saline (sterile) was placed on clean glass slide. Loop full fresh culture was taken and thin smear was made and fixed by heat. Slide was allowed to cool and stained with primary stain crystal violet for one min and then washed with distilled water. It was then mordated with Gram’s iodine for one min, washed with tap water. Then primary stain was then decolorized with ethanol (70%) which was washed off immediately. Counterstaining with safranin was done for 2 minutes. The slide was then washed with tap water, air dried and observed under microscope.

Endospore staining:

Endospores are not stained well by most dyes, but once stained, they strongly resist decolorization. Malachite green, being a strong stain can penetrate endospores. After malachite green treatment, the rest of the cell is washed free of dye with water and is counterstained with safranin. This technique yields a green endospore resting in a pink to red cell. A smear of the culture was prepared on a clean glass slide and heat fixed. The smear was flooded and kept saturated with 5% malachite green (w/v) stain where the slide was heated continuously for 5 min. The smear was then washed with distilled water followed by counter staining with safranin for 2 min. The slide was once again rinsed with water, air dried and observed under a microscope for spore presence or absence.

Motility test:

This test is used for assessing the ability of bacteria to move away from a line of inoculation due to presence of flagella. The motility media for the motility test was
prepared, poured into tubes to make slants accordingly after autoclaving. Then tubes were allowed to cool and to make butts and slants. The butts and slants were then inoculated with bacterial samples by stab inoculation technique using an inoculation needle and incubated at 35 (±2) °C for 24-48 h.

Hanging drop preparation followed by microscopy was also employed to determine motility of the isolates.

**A.5.3 Biochemical characteristics** (as per Mac Faddin, 2000)

**Catalase test:**

One drop of H$_2$O$_2$ (30%) was placed on a slide. One loopful of fresh bacterial culture from previously inoculated nutrient broth tubes was taken and placed on the drop of hydrogen peroxide; observed for bubble formation.

**Oxidase test:**

Inoculated nutrient agar slants were incubated for 24-48h. 2-3 drops of freshly prepared p-aminodimethylaniline oxalate (1% aqueous) solution and 1% α-naphthol in ethanol added over the growth. Deep blue color formation is a positive reaction for oxidase test. Oxidase test was also carried out using disc/strip (Himedia) method as per manufacturer instructions.

**Indole test:**

Isolate culture was inoculated into tryptophan broth (containing g/L; tryptophan-7.0, CaCl$_2$-3.0, NaCl-3.0, dist. water-1 lit) and incubated at 35 (±2) °C for 48 hours. Then 0.5 ml of Kovac’s reagent was added and shaken the culture gently. The test tube was observed for colored ring formation which gets homogenized after shaking vigorously. Formation of red colour on the surface is positive test for indole production; (yellow or orange colour formation on the surface is negative for indole production).

**MR (Methyl Red) test:**

Glucose fermenting bacteria produce acid, which changes the pH of the medium. pH reaches typical acidic i.e. ≤ 4.5. MR test detects the production of acid. The isolated test sample was inoculated in glucose phosphate broth (containing g/L; peptone-, D-glucose-5, Na$_2$HPO$_4$-5, dist. water-1lit) and incubated at 37°C for 48 h. 4-5 drops of alcoholic methyl red solution (0.04% v/v) were added to the incubated culture, mixed well and the results were noted immediately (Appearance of red color is positive for acid production and MR test positive; appearance of yellow color is negative for acid production and MR test negative).
**Voges-Proskauer (VP) test:**

The isolated test sample was inoculated in glucose phosphate broth (containing g/L: peptone-5, D-glucose-5g, Na$_2$HPO$_4$-5, distilled water-1lit) and incubated at 37°C for 48 h. 3ml Barritts-A (solution of 5% of α-napthol in absolute alcohol containing 0.3% creatine) was added and left at for 2 min, then 1ml of Barritts-B (40% potassium hydroxide) and was added. (Appearance of red/pink colour is positive VP test; yellow colour appearance is VP negative test)

**Citrate Utilization test:**

The isolated test sample was inoculated in Simmon’s media slants (containing g/L MgSO$_4$-0.2g, NH$_4$H$_2$PO$_4$- 1, K$_2$HPO$_4$- 1, sodium citrate-2, NaCl-5, Bromothymol blue-0.08ml, agar- 20, dist. water-1lit). The slant was then inoculated with the respective isolates bacterial sample and incubated at 37°C for 48h. Colour change from green to Prussian blue is positive whereas; no colour change (green) indicates citrate utilization negative.

**Nitrate reduction test:**

The isolated test organism was inoculated in 5ml of nitrate broth (containing g/L; potassium nitrate-1, peptone-5, beef extract-3, Sodium chloride-5, dist. water-1 liter) and incubated at 37°C for 96 hours. Then we added 1ml of reagent-A (α-naphthyl, amine) and 1ml of reagent-B (sulphanilamide) and the results were recorded. (Appearance of red color is positive nitrate reduction test whereas, no red color formation is negative nitrate reduction test)

**Hydrolysis of casein:**

Milk agar (milk separately sterilized at 12 lbs pressure) medium was sterilized and poured into petri plates; solidified plates were streaked with isolated culture and kept it for 37°C for 24-48h. Observed for the halo formation around the colonies on skim milk agar plates.

**Gelatin liquefaction:**

Gelatin media was sterilized (separately) and poured in the tubes after sterilization, mixed without bubble formation. The isolated organisms were inoculated and tubes were kept for incubation at 35 (±2) °C for 24h. Tubes observed for gelatin liquefaction followed by incubation.
Hydrolysis of starch:

The isolated test organism was inoculated in starch agar (containing g/L, soluble starch-20.0, peptone-5.0, beef extract-3.0, agar-20.0, dist. water-1 lit). After incubation on starch agar, plates are flooded with iodine solution. A positive test is indicated by the colorless area around the organism growth; unutilized starch gives blue color with iodine solution.

A.5.4 Physiological characterization

Effect of pH:

The isolates pH range for growth was determined in nutrient broth or test medium at pH range of 6-11 with 0.5 pH increments. The isolates were inoculated in nutrient broth medium and incubated at constant temperature, 35 (±2) °C for 48-96 h. The optimum pH for the growth was then noted at the highest biomass production by optical density determination spectrophotometrically at 560nm.

Effect of temperature:

Temperature range for growth was determined in nutrient broth or test medium at the following temperatures (°C) 20, 25, 30, 40, 45 and 50 by keeping optimum pH constant from previous experiments. The isolates were then inoculated in the nutrient broth or test medium and incubated at different temperatures. The optimum temperature for the growth was noted at the highest biomass production by optical density determination spectrophotometrically at 560nm.

Effect of sodium chloride:

Nutrient broth medium or test medium was prepared with various concentrations of sodium chloride from (% w/v) 0, 2.5, 5.0, 7.5 and 10. The medium was poured in to 10 ml test tubes and autoclaved followed by inoculation with isolates after proper labeling. The tubes were incubated at respective optimum pH and temperature from previous experiments and growth pattern was examined after 4-5 days of incubation by optical density determination spectrophotometrically at 560 nm. The test was also conducted on solid nutrient agar medium with NaCl and growth was determined.

A.5.6 Identification of bacterial strains by 16S rRNA sequencing and phylogenetic analysis

Selected phenotypically identified proteolytic bacteria were reconfirmed till species level by 16S rRNA gene typing and phylogenetic analysis at Molecular Ecology Lab, NCCS-Pune. The PCR assay was performed using Applied Biosystems (Model ABI- 9800) with necessary PCR components as listed in table 1.
The PCR amplification of DNA was carried out using the reaction conditions for 30 cycles Genomic DNA extraction was performed as per the protocol of Ausubel et al, (2003).

Table 1: PCR reaction mixture components (as per Lane et al, 1991) and reaction conditions for PRC amplification.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water(Glass distilled)</td>
<td>15.76</td>
</tr>
<tr>
<td>Template</td>
<td>1.50</td>
</tr>
<tr>
<td>10X PCR buffer (with 1.5M MgCl\textsubscript{2})</td>
<td>2.50</td>
</tr>
<tr>
<td>dNTPs (2mM)</td>
<td>2.50</td>
</tr>
<tr>
<td>Forward primer(8F/27F 10 pm/µl)</td>
<td>1.25</td>
</tr>
<tr>
<td>Reverse Primer (1392R, 10 pm/µl)</td>
<td>1.25</td>
</tr>
<tr>
<td>Taq DNA polymerase (of 3U/µl)</td>
<td>0.20</td>
</tr>
<tr>
<td>Total volume</td>
<td>25.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Initial step hold</th>
<th>32 cycles</th>
<th>Final extension hold</th>
<th>Final step hold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting</td>
<td>Annealing</td>
<td>Extension</td>
<td></td>
</tr>
<tr>
<td>94 °C</td>
<td>94 °C</td>
<td>55 °C</td>
<td>72 °C 1.30 min</td>
</tr>
<tr>
<td>3 min</td>
<td>30 sec</td>
<td>30 sec</td>
<td>72 °C 10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 °C 30 min</td>
</tr>
</tbody>
</table>

Initially denaturation was accomplished at 94°C for 3 min followed by thirty-two cycles of amplification and the PCR product was purified by PEG-NaCl method. The sample was mixed with 0.6 times volume of PEG-NaCl, 20% [PEG (MW 6000) and 2.5 M NaCl] and incubated for 20 min at 37°C. The precipitate was collected by centrifugation at 3,800 rpm for 20 min. The pellet was washed with 70% ethanol, air dried and dissolved in 12 µl sterile distilled water.

The sample was sequenced using a 96-well Applied Biosystems sequencing plate as per the manufacturer’s instructions. The thermo-cycling for the sequencing reactions began with an initial denaturation at 94°C for 2 min, followed by 35 cycles of PCR consisting of denaturation at 94°C for 10s, annealing at 50°C for 10s, and extension at 60°C for 4 min using primers 704F and 907R (Lane et al, 1991). The samples were purified using standard protocols described by Applied Biosystems (Foster City, USA). To this, 10 µl of Hi-Di formamide was added and vortexed briefly.

The DNA was denatured by incubating at 95°C for 3 min, kept on ice for 5-10 min, and was sequenced in a 3730 DNA analyzer (Applied Biosystems-USA) following
the manufacturer’s instructions. Obtained nucleotide sequences of the selected bacteria were analyzed using Sequence Scanner (Applied Biosystems) software. The 16S rDNA sequences were generated using Chromas Pro and then analysed using online databases viz. NCBI-BLAST and Ribosomal Database Project (RDP) to find the closest match of the sequence. Duly annotated nucleotide sequences of the novel bacterial strains were deposited to NCBI’s Genbank (http://www.ncbi.nlm.nih.gov/) database for public access through Bankit sequence submission tool.

A.6 Screening for keratinolytic activity (by qualitative and quantitative methods) to select novel keratinolytic isolates:

Feathers (white) were collected from a local chicken retailer and thoroughly washed with mild detergent wash followed by tap water. Then they were soaked in chloroform and methanol mixture (1:1) to remove lipid content. These feathers were subjected for sun drying. Dried feathers were ball milled to fine powder to be used in feather meal (FMA/FMB) medium and all these preparations were preserved in dry containers till use.

A.6.1 Screening for keratinolytic bacteria by qualitative (rapid plate assay) method

Selected potent proteolytic bacteria were tested for their keratinolytic activity on chicken feather meal agar (CFMA) (containing g/L, NH₄Cl: 5, NaCl: 5, K₂HPO₄: 3, KH₂PO₄: 3, MgCl₂.6H₂O, feather meal: 10, agar: 15, pH 8.0 and temperature 35 °C) under rapid plate assay; CZ/CS ratios were measured, (Refai et al, 2005). Based on large CZ/CS ratios, potent keratinolytic bacterial strains were selected for further quantitative screening.

A.6.2 Screening for keratinolytic bacteria by quantitative method (In-situ feather degradation).

The microorganisms that were positive for halo formation on CFMA plates were inoculated in feather broth and incubated at 35 (±2) °C in an orbital shaker (160 rpm) up to 12 days, to verify visual hydrolysis of feather.

The ability of the isolates to degrade feather keratin was investigated. The assay consisted of chopped chicken feathers suspended in 100 ml of minimal medium (pH 7.0) sterilized at 121 °C for 15 min and then inoculated with 5% (v/v) inoculum. Different sets were incubated at 100 rpm for 8 days at temperature 35 °C. Control feather flasks without inoculums were also kept. The set ups were examined on a daily basis to
determine the percentage of degradation. To validate the visual result, the keratinolytic activity was quantified by measuring feather degradation as described by Jain et al, (2011) using the method of soluble protein measurement, as described by Lowry et al, (1951). After each day (experiment carried up to 12 days), contents of feather broth were centrifuged (5,000 rpm for 10 min) and the supernatant was measured for soluble protein, protease and keratinase activities.

A.7 Estimation of physico-chemical parameters

A.7.1 Estimation of feather degradation (percentage determination by weight loss method)

Strains which were screened in the qualitative rapid plate assay were subjected for feather degradation studies using feather broth with above mentioned composition. Flasks were kept for degradation (Lateef et al, 2010) under shaking (Remi) at 160 rpm for 12 days at 35 (±2) °C and feather degradation (calculated by weight loss method) was observed visually at regular intervals (Jain et al, 2011). Percentage of feather degradation was calculated by weight loss from the difference in residual (un-utilized) feather dry weight of control (without inoculation) and treated sample. Residual (un-hydrolyzed) feather was dried to a constant weight at 80°C for calculating percentage of feather degradation from the following formula,

\[ \text{Weight loss (WL)} = W_1 - W_2, \text{ where, } W_1 \text{ and } W_2 \text{ are initial and final feather weights (mg)}; \]

\[ \text{Percentage of feather degradation (\%)} = \frac{\text{WL}}{W_1} \times 100. \]

Based on the qualitative and quantitative screening, potent bacterial strain was selected for further feather degradation optimization studies under submerged fermentation.

A.7.2 Feather degradation under submerged fermentation

Preparation of inoculum

Well isolated distinct colony from a fresh nutrient agar plate was inoculated into nutrient broth and incubated for overnight (12-18 hrs) at 35 °C under 160 rpm shaking. Growth OD was measured at 660 nm.

Production medium and growth conditions:

Physiological studies related to feather degradation were carried out in feather broth medium (Refai et al, 2005). Overnight grown seed culture of *B. cereus* KER17 at 5% (v/v) (in nutrient broth, \( A_{660} \) was at 1.0 (±0.05) optical density was inoculated into 95 mL of the feather broth production medium (in 250 ml conical flasks) and flasks were kept in a shaking incubator (Remi) at 35 (±2) °C and 160 rpm. At regular intervals,
fermented FB was assayed for growth, enzyme activity and feather degradation percentage (from residual feather).

A.7.3 Enzyme recovery

At regular intervals, contents of the flasks were taken and filtered through Whatman No1 filter paper and centrifuged at 5000 rpm (Sigma) for 15 min at 4°C. Residual feathers were kept for sun drying and used for feather degradation percentage determination. Cell free supernatants (CFS) were preserved at 4°C for further analytical procedures.

A.7.4 Determination of cell growth by CFU counting method

Bacterial growth was monitored by measuring the CFU/ml x dilution factor, as described elsewhere (by Riffel et al, 2003). The bacterial suspension was diluted to $10^{-7}$ in normal saline. The samples were then homogenized and loaded (20 µl) onto nutrient agar plates, which were further incubated for 24h at 35 °C. Colonies formed were subsequently counted.

A.8 Analytical methods

A.8.1 Protease assay

Protease activity was determined using casein as a substrate according to the method as described by as per Refai et al, (2005).

- Unless otherwise stated, the reaction mixture in a total volume of 1 ml was composed of 0.5 ml of 0.625% casein (in 100 mM Tris–HCl, pH 8.0) and 0.3 ml of crude enzyme. Final volume made up to 1.0 ml using Tris-HCl buffer (pH 8.0).
- The reaction mixture was incubated in a water bath at 40 °C for 30 min. 1.0 ml of chilled TCA (10%) was then added and the mixture left for 20 min followed by centrifugation at 5000 rpm for 10 min.
- 0.5 ml of the supernatant was mixed with and 2.5 ml of 500mM sodium carbonate and incubated at 35 (±2) °C for 10 min.
- 0.5 ml of Folin-Ciocalteu reagent 1:3 (v/v) was added and incubated for 20 min followed by reading absorbance at 660 nm.
- A control was processed parallelly by adding the enzyme after incubation and TCA was immediately added.
- A standard graph was generated using standard tyrosine solutions of 10-100 µg/µl.
One unit of enzyme activity (U) can be defined as the amount of enzyme that liberates 1 µg of amino acid equivalent to tyrosine per min under the above described assay conditions.

**A.8.2 Keratinase assay**

Keratinase activity was determined by the method as described by V.P.Zambare et al, (2007) with minor modifications for caseinolytic enzyme where casein was replaced by keratin (Himedia, Mumbai).

- 2 ml of reaction mixture contained 1 ml of keratin (0.625% in 100 mM Tris–HCl buffer, pH 8.0) and 0.5 ml of CFS and the reaction mixture incubated for 10 min at 40°C in shaking water bath at 100 rpm agitation.

- 2.0 ml of chilled TCA (10%) was then added and the mixture was left for 20 min followed by centrifugation at 5000 rpm for 10 min.

- 0.1 ml of the supernatant was mixed with 0.9 ml distilled water and 0.5 ml of 500mM sodium carbonate and incubated at 35 °C for 10 min.

- 2 ml of Folin-Ciocalteu reagent (1:3 v/v) was added and incubated for 20 min followed by reading absorbance at 660 nm for the developed blue color, as per Lowry et al, (1956).

- A control was processed by adding the enzyme after incubation and TCA was immediately added.

- A standard graph was generated using standard tyrosine solutions of 10-100 µg/µl.

One unit of keratinolytic activity is defined as the amount of enzyme that liberates 1 µg of tyrosine equivalents per min under the described assay conditions.

**A.8.3 Fibrinolytic assay**

Fibrinolytic assay of the test sample carried out as described elsewhere by Hassanein et al, (2011). The steps involved are as follows;

- The reaction mixture contained 1 ml of 0.5 % bovine fibrin (Himedia, Mumbai, prepared in Tris-HCl buffer, pH 8.0) solution and 1 ml of CFS.

- The reaction mixture was incubated for 2 h at 37°C.
• Then the reaction was stopped by the addition of 2 ml of 10% (w/v) TCA. This was followed by centrifugation and assaying the solubilized amino acid tyrosine equivalents in the supernatant as described by Anson, (1939) method; briefly steps involved are as follows:
  • Add 2.5 ml 0.5M Sodium carbonate to 0.5 ml supernatant (from TCA precipitated fraction)
  • Leave it at room temperature for 5-10 min
  • Add 0.5 ml FC (Folin Ciocalteu) reagent (1:3 v/v)
  • Incubate it at 35 °C for30 min
  • Read the blue color developed at 660nm
  • Plot a standard graph using tyrosine as standard amino acid at concentrations from 10-100 µg/µl.

One unit of fibrinolytic activity (U) can be defined as the amount of enzyme required to liberate 1 µg of tyrosine/ml/min under described assay conditions. (For haemolytic assay the same methodology was adopted where fibrin was replaced by haemoglobin)

A.8.4 Protein estimation

Protein concentration from the CFS was determined by the method of Lowry et al, (1956). Steps involved are as follows:

  • Different aliquots of standard protein BSA (Bovine Serum Albumin) were taken at 10-100 µg/µl concentrations.

  • Final volume made up to 100 µl with distilled water.

  • 1 ml of alkaline copper sulfate in Sodium carbonate with sodium hydroxide was added

  • Folin Ciocalteau reagent, 0.5 ml was added to the above mixture, vortexed and incubated at 35 °C for 30 min.

  • Absorbance of the blue color developed in the test solutions were read at 750 nm.

  • A standard graph was generated using BSA as standard protein solutions at concentrations 10-100 µg/µl.

A.8.5 Thiol estimation

Free thiol groups from the spent medium were measured, as per Riffel et al, (2003).
• To 1 ml of CFS, 0.2 ml of ammonium hydroxide, 1 ml of sodium cyanide (0.5 g/L) and 1 ml of distilled water were added.

• The reaction mixture was incubated for 20 min at 25 °C. After incubation, 0.2 ml of sodium nitroprusside (0.5 g/L) and left for incubation at 25°C for 2 min.

• Absorbance of the purple color developed was read at 530 nm.

• A standard graph was generated by using cysteine as standard amino acid at concentrations from 10-100 µg/µl.

B. Fermentation optimization studies for enhanced feather degradation: Effect of physical and chemical parameters

Physical factors like fermentation time, inoculum’s age, size; aeration, fermentation-pH and temperature; different carbon, nitrogen, phosphate and metal ion sources are expected to influence the feather degradation by the selected strain (B. cereus KER17) were optimized by selecting one parameter at a time in feather mineral broth.

B.1 Effect of incubation period

In order to study the effect of incubation period on feather degradation, a selected strain, (B. cereus KER17) was grown on basal salt medium containing 1% feathers as described earlier. Inoculated basal feather medium flasks were incubated for period of 5 days at 35 (±2) °C on rotary shaker (160 rpm). Prior to sterilization, the pH of the medium was adjusted to 8. Degradation of feathers was estimated at every 12 h of incubation and the supernatant was examined for protease activity. Bacterial cell growth was measured in terms of CFU/ml count using nutrient agar (NA) plates as per (Riffel et al, 2003). The percentage of feather degradation was determined from the remaining/residual feather as described earlier. In all sets of experiments the culture conditions were kept optimum as determined in previous experiments. Un inoculated flasks with feather have been maintained as controls throughout the experiments.

B.2 Effect of inoculum age

To determine the effect of inoculum age on feather degradation, previously prepared B. cereus KER17 inoculum with different ages (12-48 h with 12 h inoculums age increments) was added to basal feather medium flasks and incubated up to 4d
fermentation period. Degradation of feathers was estimated at the end of fermentation time period from residual feather and the supernatant was examined for protease activity.

**B.3 Effect of inoculum size**

To determine the effect of inoculum size on feather degradation, previously prepared *B. cereus* KER17 inoculum was added at different volume (0.5 to 3.0 % v/v) at 0.5% increment to basal feather medium flasks and incubated till the end of fermentation period. Degradation of feathers was estimated as per (Jain et al, 2011) at the end of incubation time and the supernatant was examined for protease activity.

**B.4 Effect of aeration (agitation rate)**

The culture of *B. cereus* KER17 was grown in basal feather medium on a rotary shaker at different agitation rate from 100-200 rpm with 20 rpm increment and incubated for 4 days. Degradation of feathers was estimated at the end of fermentation time and the supernatant was examined for protease activity.

**B.5 Effect of fermentation pH**

Cultures of the test strain *B. cereus* KER17 were grown in basal feather medium individually at different pH range 6-10 with pH 1.0 increment for a period of 4 days on rotary shaker at 150 rpm. Degradation of feathers was estimated at the end of fermentation time along with bacterial cell growth measured in terms of CFU/ml count using freshly prepared nutrient agar (NA) plates. The cell free supernatant was examined for protease activity as previously mentioned.

**B.6 Effect of fermentation temperature**

To determine the optimum temperature for feather degradation and keratinolytic enzyme production, cultures of *B. cereus* KER17 was grown in basal feather medium under shaking at different temperatures, 20-50 °C with 5 °C increments. Degradation of feathers was estimated along with bacterial cell growth was measured in terms of CFU/ml count using freshly prepared nutrient agar (NA) plates at the end of fermentation period. Supernatant was examined for alkaline protease activity as previously mentioned.

**B.7 Effect of chemical parameters**

**B.8 Effect of different carbon sources**

In order to investigate the effect of culture conditions for the keratinolytic enzyme production by *B. cereus* KER17, various co-carbon sources such fructose, glucose, galactose, sorbitol, sucrose, mannitol, lactose, and starch were supplemented
individually to the basal feather medium containing 1% chicken feather; all these sources were added in the medium at a final concentration of 0.1 (% w/v).

**B.9 Effect of different nitrogen sources**

In order to investigate the effect of culture conditions for the keratinolytic enzyme production by *B. cereus* KER17, co-nitrogen supplements such as peptone, tryptone, gelatin, casein, skimmed milk, beef extract, yeast extract, urea, potassium nitrate, ammonium sulfate, ammonium chloride and sodium nitrate were supplemented individually to the basal feather medium containing 1% chicken feather; all these sources were added in the medium at a final concentration of 0.01 (% w/v).

**B.10 Effect of feather concentration**

In order to study the substrate concentration influence on feather degradation by *B. cereus* KER17, the strain was grown in the basal feather medium supplemented with different concentrations of feather (0.5-4% with 0.5% increment) individually and incubated for 4 days at 35 (±2) °C.

**B.11 Effect of different phosphates**

In order to study the influence of phosphates on feather degradation by *B. cereus* KER17, the strain was grown in the basal feather medium supplemented with different phosphates such as Potassium di-hydrogen phosphate, di-potassium hydrogen phosphate, Sodium di-hydrogen phosphate, and Ammonium phosphate at 0.1 % (w/v) final concentration individually and incubated for 4 days at 35 (±2) °C.

**B.12 Effect of different trace elements**

In order to study the influence of trace elements on feather degradation by *B. cereus* KER17, the strain was grown in the basal feather medium supplemented with different metal ion solution such as divalent chlorides of Mn, Fe, Hg, Ca, Zn; Aluminum chloride (trivalent) and Magnesium sulfate at a final concentration of 1mM individually and incubated at 35 (±2) °C for 4 days.

**C. Recording of results**

In all these experiments parameters for growth, keratin degradation and keratinolytic activity were measured at regular intervals specified for each experiments. The results were recorded and tabulated.

**D. Purification and biochemical characterization of keratinolytic protease**

All the steps were performed at 4 °C unless otherwise stated. Purification of an enzyme is carried out for the detailed study of an enzyme and in comparison to its crude form. The purified enzyme is used to know the parameters, such as the enzyme stability
and molecular characteristics—the molecular weight of the proteins, the protein subunit/s—monomer, dimer, trimer, etc metallo-enzymes, functional groups and protein sequence. These studies help to know its withstanding abilities in different conditions and the compatibility of the enzyme with its applications in futuristic approach.

There are several standard protocols, techniques and methods available, to purify and characterize biocatalysts. In developing an efficient downstream process for isolating and purifying an enzyme, the main objectives remain to bring down the cost, which requires strategic selection of the purification processes (Belter et al, 1988). Enzymes are purified by employing successive chemical and/or physical fractionation procedures. The object of each step is to retain as much of the desired enzyme as possible, while getting rid of other proteins. The efficiency of each step is given by the yield or recovery and the purification factor (the factor by which the specific activity of the preparation has increased). The objective is to optimize both factors. There are no general rules concerning the order of the purification steps, although optionally, heat treatment and ammonium sulphate precipitations are usually done in the purification sequence. Gel filtration can follow ammonium sulphate precipitation and thereby, serve to desalt the preparation as well as fractionate the proteins according to size. If the ion-exchange chromatography is to follow, ammonium sulphate step, then it is a good idea to dialyze the preparation first or pass the preparation through a rapid gel filtration column. The removal of the ammonium sulphate will facilitate the binding of the proteins to the ion-exchange column. Other steps that may be highly effective for certain enzymes include gel filtration and gel electrophoresis. The purity of final preparation should be checked by several methods before concluding that the preparation is homogenous. Unnecessary purification should be avoided, as each additional stage is costly in terms of equipment, manpower and loss of enzyme activity. As a result, some commercial enzyme preparations consist essentially of concentrated fermentation broth, plus additives to stabilize the enzyme activity (Segel, 2004; Sadasivam and Manikam, 2004).

D.1 Production medium and growth conditions

*B. cereus* KER17 was grown in an improved feather medium containing 1% (w/v) chicken feathers; D-Manitol (0.4%); Tryptone (0.06%); K₂HPO₄ and KH₂PO₄ (0.1% each); MgSO₄ ion (1mM) solution and distilled water 100 ml. The pH was adjusted to 9.0. 12 h old inoculum (prepared in nutrient broth, A660 ~1.0 OD) at 1.0% was added to feather medium and kept for fermentation at 160 rpm shaking for 3 days.
D.2 Enzyme recovery

At the end of 3d (72h) fermentation period, the cells and other solid feather residues were removed by centrifugation at 10,000 rpm for 15 min at 4 °C using a cold centrifuge. Cell free supernatant was used for further purification steps.

D.3 Ammonium sulphate precipitation and dialysis

The best method of the precipitation of proteins is achieved by solubilisation of the unwanted proteins in a stepwise process, at different concentration of the salt or a solvent. Precipitation of proteins using ammonium sulphate salt is useful and is a preferred method for the fractional precipitation of proteins, as it is less affected by the presence of interfering materials. Ammonium sulphate is available in highly purified form, has great solubility, allowing significant changes in the ionic strength and is inexpensive.

Changes in the ammonium sulphate concentration of a solution can be brought about either by adding solid substance or by adding a solution of known saturation, which increases the solubility of proteins. Increase in the ionic strength of the solution causes a reduction in the repulsive effect of like charges between identical molecules of a protein. It also reduces the forces holding the solvation shell around the protein molecules. As the ionic strength is increased, protein solubility also increases. This is referred to as ‘salting in’. Beyond a certain point, the solubility begins to decrease and this is known as ‘salting out’. These forces are sufficiently reduced, beyond which the protein will precipitate. Hydrophobic proteins are found precipitating at lower salt concentrations than hydrophilic proteins. This forms the basis for the fractional precipitation of proteins by means of salt (Chetan, 2007).

D.4 Gelfiltration and column chromatography

Partially purified enzyme preparations that have been clarified and concentrated by salt precipitation and dialysis are now suitable for further purification by chromatography. Chromatography is one of the techniques to separate biological molecules, using their molecular size, weight and affinity patterns. Majority of the chromatography is routinely carried out using the column mode. Chromatography is the most powerful technique to separate chemically, closely related substances into individual components on the basis of their physico-chemical properties. There are three principle types of chromatography utilizing the ion exchange, affinity and gel exclusion properties of the enzyme, usually in that order. Ion exchange and affinity
Chromatographic methods can both rapidly handle large quantities of crude enzyme. Ion exchange chromatography is based on the principle of separation of molecules based on exploiting the weak charges on the proteins for their separation. Ion exchange materials, generally cheaper, are preferred at an earlier stage of purification. Gel filtration chromatography invariably causes dilution of the enzyme, which must then, be concentrated later.

**Experimental**

750 ml of crude enzyme preparation was subjected to ammonium sulphate saturation (40–80%) by slow and continuous stirring at 4 °C cold conditions. The precipitated protein was dissolved in 100 mM Tris-HCl buffer at pH 7.8 and dialyzed against the same buffer using dialysis 150 membrane bag (Hi-Media) with two changes of 8 h interval. The dialyzed protein was concentrated by lyophilization and used for purification.

The protein pellet obtained after saturation with ammonium sulphate between 60% and 80% was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-200 (1.5 × 24 cm, Sigma-USA) equilibrated with Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 60 mL/h with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. From the elution profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak at a NaCl concentration of 0.6M Fractions (21-29) with high protease activities were pooled, dialyzed, and concentrated by lyophilization and used for further studies. All the enzyme purification steps were performed at 4°C unless and otherwise stated as per (Adinarayana et al, 2003).

**D.5 Molecular weight determination by SDS-PAGE analysis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to determine the purity and molecular weight of the enzymes as described by Laemmli (1970) and Zhang et al, (2009b) using a 5% (w/v) stacking and a 12% (w/v) separating gels. Samples were prepared by mixing purified enzymes in the ratio of 1:5 (v/v) with glass distilled water containing 10 mM Tris-HCl (pH 8.0), 2.5% SDS, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were heated at 100 °C for 5 min before electrophoresis. Purified protein samples were run on a SDS-PAGE along with a concurrent run of the standard protein markers Rabbit Phosphorylase b (97.4 kDa), Bovine Serum Albumin (66.2kDa), Bovine Carbonic Anhydrase (31.0 kDa),
Soyabean Trypsin inhibitor (20.1 kDa) and Lysozyme (14.3 kDa) were procured from Genei, Bangalore, India.

**D.6 Effect of pH on the enzyme activity**

The enzyme activity was determined by the assay method as described previously in methods section. To study the pH optimum, the purified enzyme was dissolved in the below mentioned buffers and kept at 40 °C for 1 h. The optimum pH was determined at 35 °C using the following buffers (50 mM): sodium phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH 7.5-9.0), and Glycine/NaOH buffer (pH 9.0-11.0); Gessesse et al, (2003).

**D.7 Effect of temperature on the enzyme activity**

The reaction mixture was incubated at different temperatures ranging from 20 to 80 °C for 1 h after which, the activity was determined as previously described in methods section. To study the enzyme activity at different temperatures, the enzyme was dissolved in 50 mM Glycine/NaOH buffer (pH 9.5), pre-incubated at different above mentioned temperatures ranging from 20 to 80 °C for 30 min, rapidly cooled and residual activities were measured as per the assay procedure described earlier.

**D.8 Effect of metal ions on the enzyme activity**

To determine the effect of divalent cations on keratinolytic activity, the purified fraction was incubated in the presence of metal chlorides like Fe$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Ba$^{2+}$ and Hg$^{2+}$, Cu$^{2+}$, Mn$^{2+}$ and Al$^{3+}$ at, (5 mM each) for 1 h at 40 °C as described by Bernal et al, (2006). Protease assay was conducted as described earlier (without metal ion solution served as control activity which was taken as 100 % activity).

**D.9 Effect of inhibitors and reducing agents on the enzyme activity**

The purified protease were pre-incubated in 50mM Glycine/NaOH buffer (pH 9.5) at 45 °C (optimum temperature for enzyme activity) for 1 h with β-mercaptoethanol, dithiothreitol (DTT), ethylene diamine tetra acetic acid (EDTA) and phenyl methylsulfonyl fluoride (PMSF) at 5 mM each. The activity of the control without addition of any additive was taken as 100%; (Bernal et al, (2006).

**D.10 Substrate specificity of keratinolytic protease**

Substrate specificity of *B. cereus* KER17 keratinolytic protease enzyme was determined using natural substrates (2mg/ml) such as casein, BSA, haemoglobin, fibrin, and keratin. Protease assay was assayed as described earlier. After 10 min, they were centrifuged for 15 min at 10,000 rpm. The increase in absorbance due to the hydrolysis and release of peptide was measured under the corresponding wavelength of the initial
substrate. The relative protease activity toward casein was taken as a control (Adinarayana et al, 2003; Zouari et al, 2010).

D.11 Effect of different commercial brands of detergents on the enzyme activity

The potential of the protease from B. cereus KER17 as a detergent additive, its compatibility towards some commercial laundry detergents available in the local market such as, Rin (HU Lever Ltd., India), Surf excel, Wheel, Ariel (Procter & Gamble, India), Henko and Nirma (Nirma Ltd., India) were examined. Protease of B. cereus KER17 was mixed with the protease denatured detergent in the ratio of 1:1 (v/v), incubated at 45°C for 30 min, followed by measuring the residual protease activity by described assay conditions and compared with the control (enzyme diluted to 1:1 in tap water without detergent). Aqueous solutions of detergents (0.7% stimulate washing condition) were heated at 100 °C for 90 min to denature the indigenous protease activity, if any, and confirmed by protease assay. The residual activity was expressed in percentage activity considering the activity of control as 100% as described by Adinarayana et al, (2003).

E. Scanning Electron Microscopy (SEM) studies of feather degradation

Culture broth was filtered with Whatman No. 3 filter paper. The filtered feathers were dried and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C and post-fixed in aqueous osmium tetroxide (2%) for 4 h in the same buffer. Post-fixation, samples were dehydrated in series of alcohols (extra pure grade) and then dried to critical point with electron microscopy Critical Point Drier (CPD) unit. The dried samples were then mounted over the stubs with double sided carbon tape. A thin layer of gold was coated over the samples by using an automated sputter coater (JEC-550 (JEOL TECHNIC LTD., Tokyo, Japan). The specimens were observed using a scanning electron microscope (JEOL JSM - 6390LV) at an accelerating voltage of 15kV, Jeong et al, (2010b).

F. Biochemical analysis of spent medium by TLC and HPLC

F.1 Amino acid analysis by TLC

Amino acids from the CFS were detected by thin layer chromatography (TLC) using pre-coated Silica-gel TLC plates (Merck, Germany). The following mixture was employed as a mobile phase: n-butanol/glacial acetic acid/water (4:1:1 ratio). The revealing/spraying agent is ninhydrine 0.2% (p/v) in acetone with the addition of 5 drops of pyridine. Samples were collected after 4 days of fermentation time from the
culture broth. The following amino acids were used as standards: proline, phenyl alanine, lysine, glutamine; cysteine, leucine, methionine; arginine and valine at 2mg/ml concentration, Nagal and Jain, (2010).

**F.2 Amino acid analysis by HPLC**

Amino acids from the CFS of post fermented broth of 4 days were quantified by High Performance Liquid Chromatography (HPLC) by using Phenyl Iso ThioCyanate (PITC) acts as coupling reagent. PITC as a means for quantitative precolumn derivatization of amino acids and several reverse phase HPLC systems for separation of the resulting phenylthiocarbamyl (PTC) derivatives. The properties of PITC are well known, and this reagent fulfills the desired criteria for the derivatization step. Results thus obtained from analysis of HCl hydrolyzates of several peptides and proteins compare favorably both in sensitivity and precision with those from state-of-the-art ion-exchange analyzers. During the process, 3.5µl Agilent columns (4.6 × 150 mm) were employed.

**Procedure:**

- 1ml of supernatant was taken and centrifuged at 13000 rpm for 10 minutes. Supernatant was collected to a fresh tube.
- Equal amount of 10%TCA (Tricarboxylic acid) was added to the above supernatant and left it for 20 minutes at -20 °C.
- Again centrifuged at 13000 rpm for 10 minutes.
- Resulting supernatant was collected.
- Supernatant dried under vacuum until completely it dries.
- Then 10µl of PITC reagent was added.
- Then it was kept at 45°C in thermomixer for 1 hour.
- Again it was dried until it completely dries.
- 200µl of 10 mM Sodium acetate (pH adjusted to 6.4) buffer was added to the above dried sample and then 20µl was injected to HPLC sample injector.
- Sample flow rate was maintained as 1 ml/min. Stop time was 82 min, (Robert and Stephen, 1984; Jeong et al, 2010).
G. Evaluation of the utilization of different keratin wastes for keratinolytic activity.

All locally available keratin materials (human hair and nails collected from a barber shop; buffalo horns collected from Gulbarga slaughter house, sheep wool from mutton processing outlet) were thoroughly washed with tap water followed by mild detergent wash, sun dried for 1 day and later soaked in chloroform, methanol (1:1) to remove lipid content later washed with distilled water and sun dried for 12 hrs. Sheep wool and human hair were cut into 2-3 cm fragments. Horns and nails were grinded to fine powder and preserved in dry containers.

1% v/v inoculum (overnight grown culture; $A_{660}$ with optical density 1.0 ±0.05,) was added to 50 ml basal mineral medium (250 ml conical flasks) supplemented with keratin waste (1% each) in different flasks. Un inoculated flasks were kept as control at each case. Fermentation was carried at 35 (±2) °C and 160rpm agitation up to 14 days. On visual basis, contents of the flasks were filtered through Whatman filter paper followed by centrifugation and supernatant was used for analytical procedures as described earlier in experimental methods section; (Refai et al, 2005; Anbu et al, 2008).

H. Applications of crude keratinolytic protease enzyme from selected isolate.

H.1 Goat skin de-hairing studies

The crude keratinase enzyme produced by $B$.cereus KER17 strain was applied for deharing of the goat skin. Freshly salted goat hide was chopped into pieces of approximately 4×4 cm and washed with distilled water repeatedly to remove salt and extraneous matter. After brief air drying, the hide transferred to 40-ml capacity glass tubes containing 10 ml Tris-HCl buffer (50 mM, pH 8.6) supplemented with 2.0% (v/v) of crude keratinase enzyme as positive control and negative control (without enzyme) and kept at 37 °C. The enzyme treated skin samples were washed thoroughly under tap water and observed for the deharing. Incubations were carried out for 12 to 30 h after which hair was scraped off gently from the hides. Traditional tannery deharing was also performed using lime (1.5%) and sodium sulfide (1.5%) as described by Pillai and Archana, (2008); Gurav and Jadhav, (2012).

H.2 Feather hydrolysis (in-vitro) studies

10 mg of autoclaved whole chicken feather was taken in a test tube and incubated with 25 ml crude enzyme of $B$.cereus KER17 up to 30 h at 40 °C. The feather tubes were observed for degradation visually against distilled water and un inoculated feather broth solutions were kept as controls as described by Bockle et al, (1997).
H.3 Blood stain cleansing / wash performance studies

Application of *B. cereus* KER17 protease as a detergent additive was studied on white cotton cloth pieces (5 × 5 cm) which was previously stained with blood sample. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied:

1. Flask with distilled water (100 ml) + stained cloth (cloth stained with blood)
2. Flask with distilled water (100 ml) + stained cloth (cloth stained with blood) + 1 ml wheel detergent (7 mg/ml).
3. Flask with distilled water (100 ml) + stained cloth (cloth stained with blood) + 2 ml enzyme solution.
4. Flask with distilled water (100 ml) + stained cloth (cloth stained with blood) + 1 ml wheel detergent (7 mg/ml) + 2 ml enzyme solution

The above flasks were incubated at 45°C for 15 minutes. After incubation, cloth pieces were taken out, rinsed with water, and dried. Visual examination of various pieces exhibited the effect of enzyme in removal of stains. Untreated cloth pieces stained with blood were taken as control; Adinarayana et al, (2003).

H.4 Fibrinolytic activity

Cell free supernatant of *B. cereus* KER17 from fermented feather medium was collected after centrifugation and about 20 µl CFS was pipetted into pre made wells (6 mm diameter) on fibrin agar plates 0.5% (v/v) in 100 mM Tris-HCl buffer, (pH 8.0). The plates were then incubated at 40°C for 6-12 h and zones of clearance were observed, (Allpress et al, 2002; Hassanein et al, 2011).

I. Statistical analysis

The data presented in the tables and graphical figures corresponded to the mean values of triplicates based on ANOVA analysis (Analysis of variables), unless and otherwise specified.

Thus, the experiments, estimations and assays were conducted and carried out as per standard procedures described above. Care has been taken to repeat the experiments to get consistency trend in the observations. The tabulated results are given in the form of tables and graphs. Wherever necessary, copies of photographic figures have been included to chance visual observation.