Materials and Methods
3.1 Materials:

Keratin azure, phenyl methyl sulphonyl fluoride (PMSF), leupeptin, pepstatin, para-chloro mercuric benzoate (pCMB) were purchased from Sigma Chemical Co. (St Louis, Mo, USA). Sephacryl S-100 gel filtration matrix was purchased from GE healthcare biosciences (NJ, US). Casein and other media components were purchased from Himedia, India. Other chemicals were procured from Qualigens, India.

3.2 Sample Collection:

Samples were collected from various places located in and around Hyderabad, AP, India. Soil samples were collected from dump yards of Food Corporation of India, slaughterhouse, Vijaya dairy farms, and banks of Hussein Sagar Lake. Poultry discarded waste materials were collected from poultry farms located near Hyderabad.

3.2.1 Isolation and Screening of Microorganisms:

A keratinolytic bacteria used in this study was isolated from dump yards of Food Corporation of India, slaughterhouse and poultry farms located in Andhra Pradesh, India. One gram of soil sample was suspended in 9ml of sterile distilled water, and 1 ml of suspension was used to inoculate 50 ml of screening medium (glucose, 10 g/L; peptone, 5 g/L; yeast extract, 5 g/L; KH₂PO₄, 10 g/L; NaCl 5.0 g/L) pH of the medium was adjusted to 7.2. The culture was incubated at 30°C for 48 hrs at 150 rpm. The culture was serially diluted and 0.1 ml of culture was plated on screening medium for isolation of bacterial pure cultures.
3.2.2 Screening for Proteolytic Activity:

Proteolytic activity from isolated pure cultures was screened by plating on Casein agar [Casein 0.3g, KNO₃ 0.2g, NaCl 0.2g, K₂HPO₄ 0.2g, MgSO₄ 0.005g, CaCl₂ 0.002g, FeSO₄ 0.001g, Yeast extract, 0.1g; agar 2g, distilled water 100ml]. Plates were incubated at 30°C for 24 hrs. Bacteria producing clear zones of caesinolysis on casein agar plates were identified as protease producers. The ratio of hydrolysis zone/growth zone was calculated which gives a measure of the caesinolytic activity. The strain producing maximum caesinolytic activity was selected and was maintained on slants/glycerol cultures at 4°C.

3.3 Nutritional Factors Affecting Growth and Protease Production:

The entire study on nutritional factors affecting the growth and protease production was performed as follows: 2.5 ml of 48 hr bacterial inoculum (OD₆₀₀=0.6) was inoculated in to 100 ml of culture medium and incubated at 30°C for 48 hrs. Basal medium containing Dextrose, 5.0 g/L; Peptone, 10.0 g/L; KNO₃, 0.6 g/L; NaCl, 5.0 g/L; K₂HPO₄, 0.5 g/L; MgSO₄. 7 H₂O, 0.5 g/L; CaCl₂, 1.0 g/L was used for inoculation. The culture was centrifuged at 10,000 rpm for 10 min at 4°C to separate the clear supernatant. Samples were withdrawn at 12h interval for protease production, bacterial count and biomass was determination. The supernatant was analyzed for protease activity by using casein as substrate (Kunitz et al., 1947). Bacterial count was determined by spread plate method, and biomass was calculated by wet weight (g/L).
3.3.1 Effect of Carbon Source on Protease Production:

To test the effect of different carbon sources on the protease production, dextrose in the basal medium was substituted with starch, glycerol, lactose, sucrose and maltose. All carbon sources were filter sterilized by 0.22 μm (Millipore, USA) membrane filter. After sterilization of media sugar was added and organism was inoculated and incubated for 48 hrs at 30°C, 150 rpm. This strain was checked for maximal caesinolytic activity and further checked for keratinolytic activity.

3.3.2 Effect of Organic Nitrogen Source on Protease Production:

To test the effect of different organic nitrogen sources on the protease production, peptone in the basal medium (Dextrose, 5.0 g/L; KNO₃, 0.6 g/L; NaCl, 5.0 g/L; K₂HPO₄, 0.5 g/L; MgSO₄·7H₂O, 0.5 g/L; CaCl₂, 1.0 g/L) was substituted with various organic nitrogen sources; beef extract, tryptone, yeast extract, casein, soy meal extract, skim milk powder. After sterilization organism was inoculated and incubated for 48 hrs at 30°C, 150 rpm. This strain was checked for maximal caesinolytic activity and further checked for keratinolytic activity.

3.4 Identification of Feather Degrading Bacteria:

Colonies showing protease activity were grown in a medium containing trace salts [NaCl 0.5g, K₂HPO₄ 0.3g, KH₂PO₄ 0.4g, feather 10g, distilled water 1 liter] and whole feathers as carbon and nitrogen source [Riffel et. al., 2003]. The culture was incubated for 5 days at 30°C, 150rpm.
3.4.1 Preparation of Feather Substrate:

Chicken feathers collected from local poultry processing farm were washed with tap water and detergent. These were sterilized by autoclaving at 121°C for 30 min, and dried in a circulating air-drying oven at 50°C for about 12 hours. The dried feathers were then made powder by motor and pestle and stored at room temperature in a sealed bag until use.

3.4.3 Effect of Feather Concentration:

Effect of feather concentration was determined by using various concentration of feather in trace salt media. Different concentrations of feathers (1, 3, 5, 7, 10 g/L) were weighed and added to trace salt media. The media was incubated at 30°C 150 rpm for 5 days and checked for keratinolytic activity.

3.5 Scanning Electron Microscopy (SEM):

Feather samples from inoculated broth and control were removed on day 2, 3 and 4 of incubation and examined with SEM for their degradation. Feather samples were put on stubs and were fixed with osmium tetroxide. These fixed stubs were coated with platinum sputter target (Model: JEOL -JFC-1600 auto fine coater). Samples were scanned under SEM (Model: JOEL-JSM 5600) at an accelerating voltage of 6 kV and probe current of 150 P.A.

3.6 Characterization of Keratinolytic S7 Isolate:

The cultural, morphological and physiological characteristics of S7 isolate were investigated by various media, biochemical reactions. The S7 isolate was compared and identified according to Bergey's Manual of Determinative
Bacteriology (1974). This strain was further characterized on the basis of 16S rRNA gene sequencing.

3.6.1 Transmission Electron Microscopy (TEM):

Transmission Electron Microscopy (TEM) was carried out to determine the morphological structure of *Streptomyces* S7 sp. *Streptomyces* S7 sp cells grown in Malt yeast extract broth to mid log phase were centrifuged (5000g, 5 min) and the pellet was washed twice with sterile PBS. Specimen processing for electron microscopy includes the steps of fixation, embedding and ultra thin Sectioning. The bacterial cells were fixed by adding 1 % glutaraldehyde in 0.1 M sodium cacodylate buffer at 4 °C for of 4 hours with constant shaking at 100 rpm. The fixed cells were washed three times with 0.1 M phosphate buffer pH 7.4 at 4 °C for 30 min on a shaker and dehydrated in increasing grades of alcohol i.e. first in 30 % alcohol for 30 min at 4 °C followed by 50 % alcohol for 30 min at 4 °C, 70 % alcohol for 30 min at 4 °C and finally in 70 % alcohol + 50 % LR white (Acrylic Resin) for 30 min at 4 °C. Then the bacterial cells were kept in LR white overnight at 4 °C and then kept in fresh LR white for 2 h at room temperature. The bacterial cells were then embedded into closed, labelled, gelatine capsules with fresh LR white resin and polymerised at 56 °C for 24 h. After embedding ultra thins sections of 60 – 90 nm were cut using ultra microtome (Ultracut E, Reichert Jung). The ultra thin sections were transferred onto copper grids and immunogold staining was done. The grids were then observed in transmission electron microscope (Morgagri 268D, Fei, Netherlands) at 80 KV.
3.6.2 Tests for Morphological Characteristics of the Organism:

The morphology of the microbial cultures was studied in broth medium and solid agar medium. For the studies of growth on solid medium the microorganisms were grown on starch-ammonium sulphate agar and malt-yeast extract agar plates. The morphology of the bacterial colonies was studied after incubating the plates for 8-10 days.

3.6.2.1 Starch Ammonium Sulphate agar:

The medium is used to study the characteristic growth pattern of the microorganism by incubating it for 1 week. Starch solution [10g/500ml distilled water], ammonium sulphate and inorganic salts (K₂HPO₄ 1g, MgSO₄·7H₂O 1g, NaCl 1g, (NH₄)₂SO₄ 2g) were dissolved in 500ml of distilled water. The organism is inoculated by streaking and incubated at 30°C for one week. The color and morphology of the colonies in the surrounding medium was observed.

3.6.2.2 Malt-Yeast Extract Agar:

Malt yeast extract medium was prepared (Yeast extract 4g/L, Malt extract 10g/L, agar 18g/L; pH 7.2) and incubated at 30°C for 96 hours to study the colony morphology. The color of the surrounding medium was observed for pigmentation.
3.6.3 Biochemical Characterization:

Biochemical tests were performed to characterize the organism based on its biochemical properties.

3.6.3.1 Citrate Utilization Test:

The citrate utilization test is used to determine the ability of an organism to use citrate as sole carbon source. Simmon's citrate agar medium is used in the present study. The medium contains sodium citrate as carbon source and ammonium ions as nitrogen source for the microorganisms, bromothymol blue is indicator in medium. Citrate utilization results in the change of the medium from green to blue. Organism was inoculated and, an uninoculated tube was kept as a control.

3.6.3.2 Urease Test:

Microorganisms can be differentiated on the basis of their ability to hydrolyze urea by the enzyme urease to produce ammonia. The resulting raise in the pH of the medium above 8.4 is detected by the change in color of the indicator phenol red to dark pink. Organism was inoculated and, an uninoculated tube was kept as a control.

3.6.3.3 Tyrosine Degradation:

Isolate S7 was inoculated in tyrosine agar and incubated for 2-3 weeks. It was then examined periodically under a low power microscope for the disappearance of tyrosine crystals around the bacterial growth. The plates were
also checked for pigmentation of the medium due to the growth of microorganism.

3.6.3.4 \( \text{H}_2\text{S} \) Production:

Hydrogen sulfide, \( \text{H}_2\text{S} \), production is a natural by-product of metabolism in some microorganisms. Microorganisms form \( \text{H}_2\text{S} \) by reduction of sulfates, sulfites and elemental sulfur. Various formulations of iron agar, iron with carbohydrate and iron with lysine medium are used for this test. The production of \( \text{H}_2\text{S} \) was detected by growing isolate in SIM medium. The medium contains cysteine, sulphur containing amino acid, and sodium thiosulfate with peptonized iron or ferrous sulfate. \( \text{H}_2\text{S} \) production results in reaction of iron or ferrous sulfate in the medium, leaving a black precipitate. Organism was inoculated and, an uninoculated tube was kept as a control.

3.6.3.5 Nitrate Reduction:

This test is used to identify microorganisms that convert nitrates (\( \text{NO}_3^- \)) to nitrite (\( \text{NO}_2^- \)) or some other nitrogenous compound, such as molecular nitrogen (\( \text{N}_2 \)), using the enzyme nitrate reductase. This process is the result of either anaerobic respiration or denitrification. Potassium nitrate is the source of nitrate in the media. The nitrite production is detected by the addition of sulfanilic acid (Solution A) and a-naphthylamine (Solution B), producing a red. Organism was inoculated and, an uninoculated tube was kept as a control. After incubation, a few drops of reagents, Solution A and Solution B were added to the test tubes. An uninoculated tube was kept as a control.
3.6.3.6 Phenylalanine Degradation:

This test differentiates microorganisms that possess an enzyme phenylalanine deaminase. This enzyme phenylalanine deaminase converts phenylalanine to phenylpyruvic acid by the removal of the terminal amide group of the amino acid. This phenylpyruvic acid is detected by the addition of 10% ferric chloride solution. Change in color from yellow to green indicates positive result. Organism was inoculated and, an uninoculated tube was kept as a control. After incubation few drops of 10% ferric chloride solution was poured over the growth and observed for color change.

3.6.3.7 Starch Hydrolysis:

Starch is hydrolyzed by the extracellular enzyme amylase synthesized by the microorganisms. After incubation of the inoculated medium, a dilute solution of iodine is poured over the culture in the plate. Blue colouration results when iodine reacts with starch. Starch hydrolysis is thus detected by the presence of clear zones against a blue background. Organism was inoculated and, an uninoculated tube was kept as a control. After incubation the plates were flooded with dilute iodine solution.

3.7 Isolation of Genomic DNA:

Chromosomal DNAs were isolated by a versatile quick-prep method for genomic DNA of Gram-positive bacteria (Pospiech & Neumann, 1995), with some modifications. Mycelia (1–2 ml) grown in a nutrient broth shake culture were centrifuged, rinsed with TE (Tris EDTA buffer, pH 7.5) and resuspended
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in 0.4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysozyme was added to a concentration of 1 mg ml\(^{-1}\) and incubated at 37 °C for one hour. Then 0.1 vols 10% SDS and 0.5 mg Proteinase K ml\(^{-1}\) were added and incubated at 55 °C with occasional inversion for two hours. One-third volume 5 M NaCl and 1 vol. chloroform were added and incubated at room temperature for 30 min with frequent inversion. The mixture was centrifuged at 4500 g for 15 min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of 1 vol. 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70% ethanol, dried under vacuum and dissolved in a suitable volume (about 100 µl) of distilled water. The dissolved DNA was treated with 20 µg RNase A ml\(^{-1}\) at 37 °C for one hour. Samples were extracted in the same volume of phenol/chloroform/isoamyl alcohol (25: 24:1) and precipitated with 2.5 vols cold ethanol and 0.1 vols 3 M sodium acetate. The pellets were washed with 70% ethanol, dried and dissolved in TE or distilled water. Purity of DNA was checked by electrophoresis and the DNA was subsequently used for 16S rRNA gene amplification, and ribotyping.

3.8 PCR Amplification of the 16S rDNA and Sequence Determination:

A PCR was performed in order to amplify the 16S ribosomal DNA of *Streptomyces S7* isolate. The primers used were 63f 5′CAGGCCTAAACATGCAAGTC3′ and 1387r 5′GGCGGTGTGTAACAGGC-3′ (Julian *et al.*, 1998). The DNA sequence of the PCR products was determined by using DNA thermal cycler. Sequencing reaction products were
analyzed with automated DNA sequence (Applied Biosystems, USA). Databases (GenBank, ClustalW, etc.) were searched for sequences similar to the 16S rRNA.

3.9 Inoculum Preparation:

The organism showing keratinase activity was then transferred into liquid medium for preparation of inoculum. 100 ml of nutrient broth was prepared and dispensed into 250 ml Erlenmeyer flasks. The media were autoclaved at 15 lbs and 121°C for 20 minutes. Organism was inoculated and incubated for 3 days at 30°C, 150 rpm.

3.9.1 Enzyme Preparation:

Initial inoculum was prepared by inoculating a part of the colony in 10 ml nutrient broth and incubating for 24 hours at 20°C, 150 rpm. 0.5 ml of 24 hr culture was used to inoculate 500 ml Erlenmeyer flasks containing 100 mL of trace salt broth containing feather (NaCl 0.5 g/L, K2HPO4 0.3 g/L, KH2PO4 0.4 g/L, feather 10 g/L) and incubated for 48 hours at 20°C, 150 rpm. The culture was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant obtained was lyophilized and used as crude enzyme preparation for further steps.

3.10 Analytical Methods:

3.10.1 Determination of Proteolytic Activity:

Protease activity was determined by modified method Kunitz et al., (1947) using casein [Himedia] as substrate. 50 µl of enzyme was added to 450 µl of substrate solution (1% (v/v) casein with 50 mM carbonate buffer pH 10.0 and incubated at 30°C for 30 min. The reaction was stopped by adding 750 µl of 5%
TCA mixture (5% TCA, 9% sodium acetate, 9% acetic acid) followed by 30 min incubation at room temperature and centrifugation (10,000 rpm, 15 min). The absorbance of supernatant was measured at 280nm (Shimadzu UV-2450). One unit of enzyme activity was defined as the amount of enzyme, which releases 1 µmol of tyrosine per min under the assay conditions. The amount of tyrosine was determined from the Tyrosine standard curve.

3.10.2 Construction of Standard Graph for Tyrosine:

3.10.2.1 Preparation of Stock Solution:

50 mg of tyrosine was dissolved in 100ml of distilled water, making the final concentration to 500 µg/ml. Into a series of tubes 1,2,3,4 and 5 ml of standard stock solution was taken and made up to 10 ml by distilled water. To this one ml of two-fold dilution of Folin Ciocalteau reagent was added. After 30 min, the color developed was measured at 660 nm against reagent blank prepared in same manner. A standard curve was constructed with concentration of tyrosine (µg/ml) on X-axis and corresponding optical density on Y-axis.

3.10.3 Protein Estimation by Lowry method:

The protein content of the enzyme preparation was estimated by Lowry method using Bovine serum albumin as standard (Lowry et al, 1951).

3.10.4 Keratinase Assay:

Keratinase activity was determined by modified method of Letourneau et al (1998) using keratin azure [Sigma chemicals, USA] as substrate. The keratin azure was suspended in carbonate buffer at a concentration of 4mg/ml. The reaction mixture contained 1ml of enzyme and 1ml of keratin azure suspension.
The reaction was carried out at 37°C, 300 rpm for 1 hour. After incubation, the mixture was kept in ice for 15 min followed by centrifugation at 5000 rpm for 15 min to remove unutilized substrate. The supernatant was spectrophotometrically measured for the release of the azo dye at 595 nm. A control was kept with enzyme and buffer without substrate. One unit (1U) of keratinase was defined as the amount of enzyme causing an increase of 0.1 absorbance between sample and control at 595 nm in an hour under the given conditions [Letourneau et. al., 1998; Suntornsuk and Suntornsuk, 2003].

3.11 Screening of Different Combinations of Salts for Feather Degradation and Keratinase Production:

Various combinations of experiments were conducted to screen the combination of mineral salts that give maximal concentration of keratinase by *Streptomyces* sp7 from poultry feather. In these experiments various combination of salts viz. K$_2$HPO$_4$, KH$_2$PO$_4$, NaCl, MgSO$_4$, (NH$_4$)$_2$SO$_4$, CaCO$_3$ were tested with a constant feather meal concentration. Poultry feather serves as carbon and nitrogen source (Gousterova et. al., 2005). All the experiments were conducted in 250 ml Erlenmeyer flasks according to the combinations given in Table 3.1 with feather concentration of 5 g/l and pH 6.5. Flasks were inoculated with 3 days old inoculum (1%) and incubated in a rotary shaker incubator at 120 rpm and temperature 30°C. Samples were drawn from the flasks at the end of 4 days and relative activity of the keratinase was estimated. Based on the results obtained from the above experiments SMC-5 was found good for maximal production of keratinase from feather by *Streptomyces* sp7. Hence this
The medium composition selected for optimization studies by RSM was had the following variables $K_2HPO_4$ (0.3 g/l), $KH_2PO_4$ (0.4 g/l), NaCl (0.5 g/l), feather meal 5 g/l and pH 6.5.

**Table 3.1:** Different salt media combination tested for keratinase production by *Streptomyces S7* sp.

<table>
<thead>
<tr>
<th>Salts combinations</th>
<th>K$_2$HPO$_4$ (0.3 g/l)</th>
<th>KH$_2$PO$_4$ (0.4 g/l)</th>
<th>NaCl (0.5 g/l)</th>
<th>MgSO$_4$ (0.3 g/l)</th>
<th>(NH$_4$)$_2$SO$_4$ (0.4 g/l)</th>
<th>CaCO$_3$ (0.2 g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMC-1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SMC-2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SMC-3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SMC-4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SMC-5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SMC-6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SMC-7</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SMC-8</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>SMC-9</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>SMC-10</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Present, - Absent, SMC- Salt medium combination
3.1.1 Response Surface Methodology Studies:

A two-step response surface methodology (RSM) study was conducted. In first step (first experimental design) keratinase production from poultry feather by *Streptomyces* sp7 was optimized and in the second step (second experimental design) keratinase enzyme activity conditions were optimized. The statistical software package STATISTICA 6.0 (Stat Soft, Inc USA) was used for regression analysis of experimental data, ANOVA and to plot contour plots was used to estimate the statistical parameters.

3.1.2 Experimental Design and Optimization for Maximum Production of Keratinase:

Production media optimization with favorable levels of five variables from previously defined medium composition, viz. feather meal, K₂HPO₄, KH₂PO₄, NaCl, and pH on production of keratinase by *Streptomyces* sp7 by means of RSM. The RSM consists of a group of empirical techniques devoted to the evaluation of relationships existing between a cluster of controlled experimental factors and measured responses according to one or more selected criteria. The levels of five medium variables viz feather meal, K₂HPO₄, KH₂PO₄, NaCl, and pH were selected and each of the variables were coded x₁, x₂, x₃, x₄ and x₅ at five levels, -2, -1, 0, 1, and 2 by using equation 1.

\[ x_i = \frac{X_i - X_0}{\Delta X_i} \]  

(1)

Where \( x_i \) is the coded value of an independent variable, \( X_i \) is the real value of an independent variable, \( X_0 \) is the real value of an independent variable at the center point, and \( \Delta X_i \) is the step change value. The range and levels of
experimental variables investigated in this study are presented in Table 3.2. In these studies a full factorial central composite design was used. According to this design, the total number of treatment combinations was \(2^k \cdot 2k + n0\). Where, \(k\) is the number of independent variables and \(n0\) is the number of repetitions of the experiments at the center point. For this study, a \(2^4\) factorial design (32 points) with 10 six star points and 6 replicates at the central points were employed to fit the second order polynomial model given by equation 1, which indicated that 48 experiments were conducted (Table 3.3). The keratinase relative activity (%) was considered as the dependent variable or response \((Y_i)\).

The data on keratinases production was subjected to analysis of variance (ANOVA) appropriate to the design of the experiments by using STATISTICA 6.0 (Stat Soft, Inc USA). The keratinase production \((U/ml)\) was considered as the dependent variable or response \((Y_i)\). The quadratic model for predicting the optimal point is expressed according equation 2.

\[
Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ijk} x_i x_j x_k - - - (2)
\]

The mathematical relationship of the independent variable and the response (Keratinase production) was calculated by the second order polynomial equation 3;

\[
Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \beta_{11} x_1^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{15} x_1 x_5 + \beta_{22} x_2^2 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{25} x_2 x_5 + \beta_{33} x_3^2 + \beta_{34} x_3 x_4 + \beta_{35} x_3 x_5 + \beta_{44} x_4^2 + \beta_{45} x_4 x_5 + \beta_{55} x_5^2 - - - (3)
\]
Where, Y is the response, keratinase relative activity, %; β₀, intercept; β₁, β₂, and β₃, are linear coefficients; β₁₁, β₂₂ and β₃₃ are squared coefficients; β₁₂, β₁₃, and β₂₃ interaction coefficients.

Table 3.2: Experimental range and levels of the five independent variables used in RSM in terms of actual and coded factors for keratinase production optimization.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Variable name</th>
<th>Range and levels</th>
<th>ΔX</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁, g/l</td>
<td>Feather meal</td>
<td>-2   -1   0   1  2</td>
<td>2</td>
</tr>
<tr>
<td>X₂, g/l</td>
<td>K₂HPO₄</td>
<td>0   0.1  0.3  0.5 0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>X₃, g/l</td>
<td>KH₂PO₄</td>
<td>0.2  0.3  0.4  0.5 0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>X₄, g/l</td>
<td>NaCl</td>
<td>0.1  0.3  0.5  0.7 0.9</td>
<td>0.2</td>
</tr>
<tr>
<td>X₅</td>
<td>pH</td>
<td>5.5  6    6.5  7  7.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Where ΔX is step increment in each variable values.
3.12 Degradation of Black Feathers:

Black feathers were collected from local poultry forms. These feathers were washed and autoclaved at 15 lbs, dried in oven at 60°C overnight. The dried feathers were powdered by motor and pestle and stored at room temperature in a sealed bag until use. 0.5 ml of inoculum, was added to the medium containing NaCl 0.05g, K₂HPO₄ 0.03g, KH₂PO₄ 0.04g, feather 1g, distilled water 100 ml. This was later incubated at 30°C 150 rpm for about 5 days.

3.13 Enzyme Purification:

All subsequent purification steps were carried out at 0–4°C.

3.13.1 Ammonium Sulphate Fractionation:

Protein in the retentate was precipitated by adding solid ammonium sulphate in the following saturation ranges: 0–20, 20–40, 40–60, 60–80 and 80–100%. The precipitate was collected by centrifugation at 8,500 rpm for 30 min. The precipitate was dissolved in a minimal volume of 50 mM Tris buffer, pH 7.5. The enzyme solution was desalted by dialysis in 50 mM Tris buffer at pH 7.5 by three changes of buffer for 24 hours.

3.13.2 Gel Filtration:

The dialyzed fraction of the ammonium sulfate precipitation was checked for azokeratin hydrolysis and the fractions containing enzyme activity was loaded onto Sephacryl S-100 column (3 x 100 cm) previously equilibrated with 50mM phosphate buffer (pH 7.2) and then eluted with the same buffer. Fractions of 1.0 ml each were collected at a flow rate of 0.2ml/min. Protein concentration of each fraction were determined at O.D 280 nm. Purity was checked by SDS--
PAGE analysis and zymography. Fractions were screened for proteolytic activity on casein-agar plate assay. Fractions exhibiting proteolytic activity were assayed for keratinase activity with the keratin azure hydrolysis test. Keratinase active fractions were pooled and concentrated.

3.14 Experimental Design for Optimization of Keratinase Activity:

In second step experiments were conducted to optimize the keratinase enzyme activity by using RSM. The experimental design chosen for this study was a full factorial central composite design (CCD) that helps in investigating linear, quadratic, and cross-product effects of three factors. The CCD used for the present investigation includes a $2^3$ full factorial of 8 experiments, 6 star point experiments and 6 central point experiments for replication. The experiments were conducted at five levels for three process variables pH, Temperature and rpm. Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the co-efficients in a mathematical model and predicting the response and checking the adequacy of the model.

A second order polynomial function was fitted for the keratinase activity and is given by equation 4.

$$Y = \beta_0 + \beta_1 * x_1 + \beta_2 * x_2 + \beta_3 * x_3 + \beta_{11} * x_1 * x_1 + \beta_{12} * x_1 * x_2 + \beta_{13} * x_1 * x_3 + \beta_{22} * x_2 * x_2 + \beta_{23} * x_2 * x_3 + \beta_{33} * x_3 * x_3$$

(4)

Where $Y$, is the predicted response (keratinase enzyme activity); $\beta_0$, constant; $X_1$, pH; $X_2$, temperature ($^\circ$C); $X_3$, rpm; $\beta_1$, $\beta_2$ and $\beta_3$, linear co-efficients; $\beta_{11}$,
\( \beta_{22} \) and \( \beta_{33} \) quadratic co-efficients; \( \beta_{12}, \beta_{13}, \) and \( \beta_{23} \), interaction co-efficients; pH, Temperature and rpm were chosen as the variables and designated as \( X_1, X_2 \) and \( X_3 \). The levels of three variables were coded \( x_1, x_2 \), and \( x_3 \) at five levels, \(-2, -1, 0, 1, \) and \( 2 \) by using equation 1 and listed in Table 3.4. A total of 20 experiments were necessary to find the second order polynomial model (Table 3.5). The maximum predictable response for keratinase activity was thus obtained. The data on keratinases enzyme activity was subjected to analysis of variance (ANOVA) appropriate to the design of the experiments by using STATISTICA software (Stat Soft, Inc USA).

Table 3.4: Experimental range and levels of the five independent variables used in RSM in terms of actual and coded factors for keratinase enzyme activity conditions optimization.

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Variable name</th>
<th>Range and levels</th>
<th>( \Delta X )</th>
</tr>
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<tr>
<td>( X_1 ) pH</td>
<td></td>
<td>-2 -1 0 1 2</td>
<td></td>
</tr>
<tr>
<td>( X_2 ), °C</td>
<td>Temperature</td>
<td>30 35 40 45 50</td>
<td>5</td>
</tr>
<tr>
<td>( X_3 ) Rpm</td>
<td></td>
<td>150 200 250 300 350</td>
<td>50</td>
</tr>
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Where \( \Delta X \) is step increment in each variable values
Table 3.5: Design matrix of full factorial Central composite design for keratinase enzyme activity conditions optimization.

<table>
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<tr>
<th>Run number</th>
<th>x1</th>
<th>x2</th>
<th>x3</th>
<th>Factorial no experiments</th>
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<td>-1</td>
<td>-1</td>
<td>2⁰ full factorial experiments</td>
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3.15 Enzyme Characterization:

3.15.1 Molecular Weight Determination: SDS-PAGE analysis:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) using 12% crosslinked polyacrylamide gel on an amersham gel electrophoresis unit (Amersham Biosciences, Minnesota USA). Silver staining was used to visualize protein bands on the gels (Morrissey 1981). Molecular weight of the proteases was estimated by comparing the relative mobility of proteins of different molecular size using a standard molecular marker 220-14 kDa (Bio-rad-USA).

3.15.2 Silver Staining:

Gel was soaked for 10 min in fixing solution containing (40ml methanol, 13.5 ml formalin, 46.5 ml water). The gel was washed twice in deionised water for 5 min. After washing the gel was soaked for 1 min in 0.02% Na₂S₂O₃ (Sodium thiosulfate). The gel was washed twice in water for 20 sec. The gel was finally soaked in 0.1% AgNO₃ (Silver nitrate) for 10 min. This was rinsed with water and again with a small volume of developing solution (3% sodium carbonate, 0.05% formalin, 0.000016% Na₂S₂O₃). Gel was then soaked in fresh developing solution until band intensities were adequate (~1-3 min). 2.3 M citric acid (or 6 g solid citric acid) solution was added to stop the reaction. The gel was finally washed in water, and then soaked in water for 30 minutes or longer before drying.
3.15.3 Zymography with Gelatin:

Activity staining of keratinase was performed by Gelatin-SDS-PAGE zymography (Heussen and Dowdle, 1980) using 10mg/ml gelatin in 10% polyacrylamide mixture. After electrophoresis, the gels were soaked thrice for 20 min in 2.5% (v/v) Triton X-100 at room temperature. Enzyme activity was visualized by incubating the gel for 12 h in substrate buffer (0.1M glycine - NaOH buffer pH 8.3) at room temperature. The gel were stained with 0.5% of Coomassie brilliant blue R-250 and bleached with methanol- glacial acetic acid - water (30:10:60).

3.15.4 Zymography with Feather Meal:

SDS-PAGE was performed in 9% gels containing 0.2% feather meal. In order to prevent a quick sedimentation of feather meal, the components without ammonium persulfate (APS) were preincubated in a 60°C water bath for 5 min. APS was added while stirring, and the solution was immediately transferred to the gel chamber system. Because of preheating of the solution, polymerization starts immediately, and the distribution of feather meal is nearly homogeneous. Protein bands possessing keratinolytic activity were visualized by staining the gel with Coomassie brilliant blue. Clear bands were visible at the positions where protein bands showed keratinolytic activity (Riessen and Antranikian, 2001).

3.16 Determination of pH optima and Stability of Keratinase:

The pH optimum for keratinase activity with azure keratin substrate was assayed at a pH range of 5-12 using azokeratin. The pH optima for keratinolytic
activity was checked using the buffers Acetate buffer (pH 5.0, 6.0), Tris-HCl buffer (pH 7), Glycine-NaOH buffer (pH 8.0, 9.0), carbonate buffer (pH 10.0-12.0). pH stability for keratinase was investigated by pre-incubating the enzyme in buffers of different pH values (5-12) at 30°C for 4 hr. The residual activities were quantified under standard assay conditions. One unit (U) of keratinase was defined as the amount of enzyme causing an increase in 0.1 absorbance between sample and control at 595nm in an hour under the given conditions (Letourneau et al 1998).

3.17 Isoelectric Focusing:

The isoelectric point (pI) of purified keratinase was determined using a vertical mini-gel system (Robertson et al., 1987) and 1.5 mm polyacrylamide gels consisting of 5%(w/v) 30:1 acrylamide/bisacrylamide, 10%(v/v) glycerol and 2%(w/v) pH 3.0-8.0 ampholytes. Electrode solutions were cooled to 4°C prior to electrophoresis. The cathode solutions were 1M β-alanine and anode solution was 0.1m glutamic acid and 0.3M H3PO4. Samples and protein standards were mixed with an equal volume of 50%(v/v) glycerol and 4%(v/v) ampholytes in the 3.0-8.0 range. Pre run was carried out at 4°C for 15 min at 200v. Electrophoresis was at 4°C and 300v for 8 hours. The gels were fixed with 11.5% trichloro acetic acid for 30 min and subsequently transferred to acetic acid, ethanol and H2O (0.8:2.5:6.7) after rinsing with Milli-Q water, the gels were stained with silver staining method.
3.18 Determination of Temperature Optima and Stability on Keratinase:

To study the temperature optima of enzyme, the enzyme reaction mixture was incubated at different temperatures ranging from in 20°C to 70°C in glycine-NaOH (9.0) carbonate bicarbonate buffer (pH 10-12) buffer using azokeratin as substrate (Letourneau et al 1998). For determining thermal stability, the enzyme was pre-incubated for 1h at 20-70°C, and residual activity was measured after incubating with the azokeratin under the same conditions described above.

3.19 Hydrolysis of Protein Substrates:

Protease activity of the keratinase with protein substrates like BSA, casein, and gelatin was assayed by mixing 100 μl of enzyme and 400μl of assay buffer containing protein substrates (1 mg/ml). After incubation at 45°C for 30 min, the reaction was stopped by adding 400μl of 10% trichloro acetic acid (TCA) and allowed to stand at room temperature for 10 min. The undigested protein was removed by centrifugation and peptides released were assayed. The specific protease activity towards casein was taken as control.

3.20 Effect of Protease Inhibitors on Keratinase Activity:

Protease inhibitors were added to the purified enzyme and pre-incubated for 30 min at 30°C before being tested for keratinase activity. Protease inhibitors (5mM) used in the reaction were phenyl methyl sulphonyl fluoride (PMSF), pepstatin, para-chloro mercuric benzoate (pCMB). After 30 min of incubation, azokeratin was added and residual activity of the enzyme was measured as described above (Letourneau et al 1998).
3.21 Effect of Chemicals on Keratinase Activity:

Chemicals were added to the purified enzyme and pre-incubated for 1-4 hrs at 30°C before being tested for keratinase activity. Chelators (5mM) like ethylene diamine tetra acetic acid (EDTA) dithiothreitol (DTT), solvent like Dimethyl sulfoxide (DMSO) (0.1%, 0.5% v/v), and detergents like SDS and Triton X-100 (0.1%, 0.5% w/v, v/v) were used in reaction mixture. After incubation, azokeratin was added and residual activity of the enzyme was measured as described above (Letourneau et al 1998). A parallel control was kept with enzyme and substrate, with out any added chemicals was taken as control. One unit (1U) of keratinase was defined as the amount of enzyme causing an increase of 0.1 absorbance between sample and control at 595nm in an hour under the given conditions.

3.22 Effect of Metal Ions on Keratinase Activity:

Metal ions like Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Mg$^{2+}$, Mn$^{2+}$ Na$^{+}$ and Zn$^{2+}$ were tested by pre-incubating enzyme in a working concentration of 5mM. After 1 hr of incubation, azokeratin was added and residual activity of the enzyme was measured as described above (Letourneau et al 1998).