CHAPTER 3

MATERIALS AND METHODS

3.1 Soil Sample collection for isolation of Protease and Keratinase enzyme from Microbial sources

Soil was collected from a regular feather dumping site of Ghazipur poultry processing plant, Ghaziabad, India in sterilized sampling bags. The samples were brought in winter to the laboratory and processed for analysis on the same day.

3.2 Isolation and Screening of Protease and Keratinase producing bacteria

3.2.1 Growth condition and isolation

Soil samples were suspended in Peptone broth (Peptone Broth - Beef extract 3g/l, Peptone 5g/l, NaCl 7.5g/l) and kept for growth at 30°C for 3 days. This suspension was reinoculated in three media, Horikoshi media, Feather meal media 1 and Feather meal media 2 at 30°C at 160rpm for 7 days. They were used for protease and keratinase production. They contained the following constituent:

I. Horikoshi media (g/L): Soluble starch, 5; Peptone, 5; Glucose, 5; K₂HPO₄, 1; MgSO₄ 7H₂O, 0.2; Na₂CO₃, 1; Yeast extract, 5 and Feathers, 10; pH 7.5.

II. The Feather meal media 1 (g/L): NaCl, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.4; MgCl₂·6H₂O, 0.1; Yeast extract, 0.1 and Feather, 10; pH 7.5.

III. The Feather meal media 2 (g/L): NH₄Cl, 0.5; NaCl, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.4; MgCl₂·6H₂O, 0.1; Yeast extract, 0.1 and Feather, 10; pH 7.5.

The flask was incubated at temperature of 30°C on a rotary shaker at 160rpm for 7 days. Feather degradation in culture broth was confirmed visually. After 24 hrs of incubation at regular
intervals of 6 hrs the activity of protease and keratinase was measured and sample showing maximum activity was screened for protease and keratinase producing strains.

The culture broth in which feather degradation was confirmed was screened for keratinolytic activity. Feather meal agar that composed of (g/L): NH₄Cl, 0.5; NaCl, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.4; MgCl₂.6H₂O, 0.1; Yeast extract, 0.1 and Feather meal powder (see 3.2.2), 10, agar powder, 20 and pH was maintained at 7.5 at 30°C for 72hrs, Strain which exhibited the largest clearing zones were selected, identified and grown in cultivation media for enzyme production.

3.2.2 Feather meal powder preparation
Poultry feathers was washed extensively, boiled at 30-40 psi for 2-3hrs. Dried in hot air oven for 4 hours at 50°C. The dried feathers were pulverized and the powder was used as feather meal.

3.3 Studies of Bacterial isolates
In this study we report the isolation of three mesophilic bacteria that produce keratinolytic enzymes, which can efficiently degrade chicken and pigeon feather. The isolate SN1, SN2, SN3 was identified as a strain of Bacillus megaterium, Bacillus thuringiensis, Bacillus pumilis respectively based on Morphological, cultural and Biochemical characteristics. Earlier studies from our lab involving screening of micro-organism from same soil sample of dumping site of Gazipur poultry processing plant, we have reported isolation of Pseudomonas thermaerum GW1, GenBank accession GU95151, this bacteria showed proteolytic activity but not keratinolytic activity [48].

3.3.1 Identification of Bacterial isolated strain
Bacterial identification was conducted on morphological, physiological and biochemical tests. Results were compared with Bergey’s Manual of Determinative Bacteriology, 8th edition [348]. Genus Bacillus: Agriculture Handbook No. 427 [349]. The strains were also identified by chromogenic method on the bacillus differential agar from Himedia, India, M1651 recommended for rapid identification of Bacillus species from a mixed culture. The medium contains peptic digest of animal tissues and meat extract, which provide nitrogenous compounds. Mannitol
serves as the fermentable carbohydrate, fermentation of which can be detected by the pH indicator phenol red. Mannitol fermenting organisms like *B. megateruim* yield yellow colored colonies, *B. thuringiensis* will grow as blue colonies and *B. pumilis* will also grow as green colonies on this medium. Growth determination of bacteria was done taking absorbance at 600nm of bacterial growth media at regular intervals

### 3.3.2 Morphological characteristics

#### 3.3.2.1 Gram Staining
A drop of sterile saline water was placed on the microscope slide. A light suspension of the test culture was made with normal saline and heat fixed. The slide was allowed to cool and flooded with crystal violet for 60 seconds and then washed with distilled water. It was then flooded with Gram’s iodine for 60 seconds, washed with water and excess water was drained off. The stain was then decolorized with acetone which was washed off immediately. Counterstaining was done with safranin for 2 minutes. The slide was then washed, air dried and observed under a microscope.

#### 3.3.2.2 Endospore Staining
A smear of the culture was prepared on a clean slide and heat fixed. The smear was flooded and kept saturated with 5% malachite green stain while the slide was heated continuously for 5 minutes. The smear was then rinsed with distilled water and counter stained with safranin for 2 minutes. The slide was once again washed with water, air dried and investigated under a microscope for endospore formation.

### 3.3.3 Biochemical Test

#### 3.3.3.1 Oxygen Requirement
Depending on the growth of the bacteria are distributed as aerobic, micro-aerobic and anaerobic. The isolates were inoculated into nutrient broth to study the oxygen requirement.
3.3.3.2 IMViC Test (Indole Methyl red Voges-proskauer Citrate Test)

3.3.3.2.1 Indole Production
The bacterial isolate was inoculated into tryptone broth (tryptone-10g/l, NaCl-5g/l, distilled water-1 liter) and incubated at 37°C for 48 hours. Then 0.5 ml of Kovac’s reagent was added and shook the culture gently. The test tube was observed for color reaction.

3.3.3.2.2 Methyl Red Test
Bacteria produce acid by fermentation of glucose, which changes the pH of the medium. It falls and maintained below 4.5. This test detects the production of acid. The isolated test sample was inoculated in glucose phosphate broth (Protease peptone-5g/l, D-glucose-5g/l, Na₂HPO₄-5g/l, distilled water-1liter) and incubated at 37°C for 2 days. Five drops of 0.04% solution of alcoholic methyl red solution were added to the culture, mixed well and the results were read immediately.

3.3.3.2.3 Voges-Proskauer Test
The isolated test sample was inoculated into 5ml glucose phosphate broth (Protease peptone-5g/l, D-glucose-5g/l, Na₂HPO₄-5g/l, distilled water-1liter) and incubated at 37°C for 48 hours. Then 1ml of 40% potassium hydroxide (KOH) containing 0.3% creatine and 3ml of 5% solution of α-napthol in absolute alcohol were added. The results were read immediately.

3.3.3.2.4 Citrate Utilization Test
This test is used to study the ability of an organism to utilize citrate present in Simmon’s media (MgSO₄-0.2g/l, NH₄H₂PO₄-1g/l, K₂HPO₄-1g/l, sodium citrate-2g/l, NaCl-5g/l, Bromothymol blue-0.08ml, agar-15g/l, distilled water-1liter) as a sole source of carbon for growth. The Simmon’s media was prepared, sterilized and poured into the tubes. The media was allowed to cool and solidify in the form of slants. The slant was then stabbed with the isolated bacterial sample using a needle and incubated at 37°C for 36 hours.
3.3.3.3 Motility Test
This test used to check for the ability of bacteria to migrate away from a line of inoculation due to physical features like flagella. The motility media for the motility test was prepared, sterilized and poured into tubes. The media was allowed to cool and solidify in the form of slants. The slants were then stabbed with the isolated bacterial sample using an inoculation needle and incubated at 37°C for 24 hours.

3.3.3.4 Nitrate Reduction Test
The enzyme nitrate reductase reduces nitrate to nitrite, ammonia, nitrous oxide, nitrogen, etc. This test is used to detect the production of nitrate reductase. The isolated test organism was inoculated in 5ml of nitrate broth (beef extract-3g/l, peptone-5g/l, NaCl-5g/l, potassium nitrate-1g/l, distilled water-1 liter) and incubated at 37°C for 96 hours. Then we added 1ml of α-naphthylamine reagent and 1ml of sulphanilamide reagent and the results were immediately read.

3.3.3.5 Catalase test
One drop of 30% hydrogen peroxide was placed on a slide. One loopful of the fresh bacterial culture was taken by a sterile needle and placed on the drop of hydrogen peroxide. Observed for bubble formation.

3.3.3.6 Gelatinase test
Gelatin media was sterilized and poured in the plates. The isolated organism was inoculated by spot inoculation method. Kept it for incubation at 37°C for 24 hours. After incubation observed for liquefication by gelatin gives positive test for gelatinase.

3.3.3.7 Urease Test
This test detects the ability of an organism to produce urease enzyme. The test organism was inoculated on the slant of Urea agar and incubated at 37°C for 24 hours. After incubation period observed the color change from yellow to pink.
3.3.3.8 Caesinase Test
The skim milk agar was sterilized and poured in petriplates, allowed to solidify. After solidification streaked on the plate with isolated culture and kept it for 37°C for 24 hours. Observed for the zone of hydrolysis on skim milk agar.

3.3.3.9 Cellulase Test
Potato dextrose agar was prepared and sterilized in autoclave. Poured in petriplate and allowed to solidify. After incubation if the clear zone was observed surrounding the growth of bacteria then the test was positive.

3.3.3.10 Deaminase Test
Peptone broth (Beef extract 3g/l, Peptone 5g/l, NaCl 7.5g/l) was prepared and sterilized. Sterile 4% peptone broth tube were inoculated with the test culture and incubated at 37°C for 24 hours. After incubation add Nessler’s reagent. The orange color developed indicated that the test was positive.

3.3.3.11 Sugar Fermentation
Prepare Glucose, Lactose and Mannitol broth (beef extract-3g/l, peptone-5g/l, glucose/lactose/mannitol-5g/l) and take about 10ml in test tube fill the durham’s tube with broth and put it into the test tube taking care that no air bubble enter into it. Sterilized the broth and inoculated with isolated culture and then incubated at 37°C for 24 hours. Tube was observed for acid and gas production. Production of acid was indicated by change in color from blue to yellow and production of gas was indicated by formation of bubble in durham’s tube.

3.3.3.12 Triple Sugar Iron Agar (TSI)
To determine the ability of an organism to attack a specific carbohydrate incorporated into a basal growth medium, with or without the production of gas, along with the determination of possible hydrogen sulphide (H₂S) production. TSI medium (Beef Extract – 3g/l, Yeast extract – 3g/l, Peptone - 15.0 g/l, Proteose peptone -5.0 g/l, D-glucose monohydrate - 1.0 g/l, Lactose - 10.0 g/l, Sucrose - 10.0 g/l Ferrous Sulphate
(FeSO₄·7H₂O) - 0.2 g/l, Sodium Chloride - 5.0 g/l, Sodium thiosulphate pentahydrate - 0.5 g/l, Phenol Red, 0.5 g/l aqueous solution- 48.0 ml, Agar - 12.0 g/l) was inoculated with an inoculating needle by stabbing the butt and streaking the slant. The tubes were then incubated at 37°C for 24 hours.

3.4 Enzyme production

3.4.1 Growth condition for Protease and Keratinase production
Seed culture of the three isolated strains were prepared in 500 ml Erlenmeyer conical flask containing 100 ml of the culture media that was maintained at 30°C at 160 rpm and washed feather 10% in cultivation media. After five days of incubation, the crude culture broth was centrifuged (10,000g, 4°C, 30 min) and cell free supernatant was subjected to 0-80% ammonium sulphate precipitation. After chilling at 4°C for 1 hr, the resulting precipitate was collected by centrifugation (10,000g, 4°C, 30 min) and dissolved in a minimal volume of Tris–Cl buffer 10mM (pH 8.0) and dialyzed overnight against 4 liters Tris–Cl buffer 10mM (pH 8.0). The dialysed protein fraction was checked for protease and keratinase activity (as in section 3.8.1, 3.8.2). Standard strain of Bacillus licheniformis (MTCC 1483) was also studied for comparative purposes.

3.5 Enzyme activity Determination for Bacillus sp. SN1, SN2, SN3

3.5.1 Protein concentration determination

3.5.1.1 Preparation of Bradford Reagent
100mg of Coomassie brilliant Blue G250 dissolved in 50 ml of 95% ethanol then 100ml of 85% of Ortho-Phosphoric acid was added. Final volume to 1 liter was made up with distilled water.
3.5.1.2 Protein determination

Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard [354]. (Bovine Serum Albumin) was used as a standard protein for the estimation. A stock of BSA was prepared with 1mg / ml concentration. Take 10µg/ml – 100µg/ml dilutions and makeup the volume 1 ml with distilled water). 5 ml of Bradford reagent was added in each tube. After 10 min, OD was measured at 595 nm. The absorbance against protein concentration was plotted to get a standard curve. The absorbance of unknown sample was checked and the concentration of the unknown sample was determined by using the standard curve.

3.5.2 Determination of keratinase activity

The keratinase activity was assayed by the modified method of Cheng et. al. 1995 [352] by using keratin as a substrate. The reaction mixture contained 200µl of enzyme preparation and 800µl of 20µg/ml keratin in 10mM Tris buffer, pH 8. The reaction mixture was incubated at 45ºC for 20min and the reaction was terminated by adding 1ml of 10% chilled trichloroacetic acid. The mixture was centrifuged at 10,000g for 5min and the absorbance of the supernatant fluid was determined at 440 nm. All assays were done in triplicate. One unit (U) of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm in 20 min at 45ºC.

3.5.3 Determination of protease activity

Protease activity was assayed by a modified method of Tsuchida et al., 1986 [20] by using casein as substrate. 100µl of enzyme solution was added to 900µl of substrate solution (2 mg/ml casein in 10 mM Tris–Cl buffer, pH 8.0).The mixture was incubated at 50ºC for 20 min. Reaction was terminated by the addition of an equal volume of 10% chilled trichloroacetic acid then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The supernatant was separated by centrifugation at 10,000 rpm for 10 min at 4ºC, the acid soluble product in the supernatant was neutralized with 5 ml of 0.5 M Na₂CO₃ solution. The color developed after adding 0.5 ml of 3 fold diluted Folin–Ciocalteau reagent was measured at 660 nm. All assays were done in triplicate. One protease unit is defined as the amount of enzyme that releases 1 µmol of tyrosine per ml per minute under the above assay conditions. The specific activity is expressed in the units of enzyme activity per milligram of protein.
3.5.4 Hydrolysis of protein substrates
Protease activity with various protein substrates including keratin, casein, gelatin and bovine serum albumin (2 mg/ml) was assayed by mixing 100 µl of the enzyme and 900 µl of assay buffer containing the protein substrates. After incubation at 50°C for 20 min, Reaction was terminated by the addition of an equal volume of 10% chilled trichloroacetic acid then the reaction mixture was allowed to stand in ice for 15 min to precipitate the insoluble proteins. The undigested proteins were removed by filtration or centrifugation at 10,000 rpm for 5 min and amino acid released was assayed. The supernatant was separated by centrifugation at 10,000 rpm for 10 min at 4°C; the acid soluble product in the supernatant was neutralized with 5 ml of 0.5 M Na₂CO₃ solution. The color developed after adding 0.5 ml of 3 fold diluted Folin–Ciocalteau reagent was measured at 660 nm. All assays were done in triplicate.

3.6 Microbial strain *Bacillus megaterium* SN1

3.6.1 Production of enzyme in cultivation media for *Bacillus megaterium* SN1
Seed culture of the isolated strains were prepared in 500 ml Erlenmeyer conical flask containing 100 ml of Feather meal media that composed of (g/L): NH₄Cl, 0.5; NaCl, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.4; MgCl₂.6H₂O, 0.1; Yeast extract, 0.1 and washed feather 10% and pH was maintained at 7.5 at 30°C at 160 rpm for 72hrs and the fresh overnight culture was inoculated in cultivation media.

i. Chicken feathers (10%) + basal medium for enzyme production
ii. Pigeon feathers (10%) + basal medium for enzyme production
iii. Hair (10%) + basal medium for enzyme production
iv. Nail (10%) + basal medium for enzyme production

The above cultivation media were checked for keratinolytic and caesinolytic enzyme activity (as in section 3.8.1, 3.8.2)
3.7 Microbial strain *Bacillus thuringiensis* SN2

3.7.1 Production of enzyme in cultivation media for *Bacillus thuringiensis* SN2

Seed culture of the isolated strains were prepared in 500 ml Erlenmeyer conical flask containing 100 ml of Feather meal media that composed of (g/L): NH₄Cl, 0.5; NaCl, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.4; MgCl₂.6H₂O, 0.1; Yeast extract, 0.1 and washed pigeon feather 10% and pH was maintained at 7.5 at 30°C at 160 rpm for 96hrs and the fresh overnight culture was inoculated in cultivation media.

The above cultivation media were checked for keratinolytic and caesinolytic enzyme activity (as in section 3.8.1, 3.8.2)

3.8 Protease and Keratinase Purification

3.8.1 Crude Extract Preparation

Microbes were allowed to grow in 500 ml conical flask containing 100 ml of the culture media that was maintained at 30°C at 160 rpm for 72 hrs of *B. megaterium* SN1 and 96hrs of *B. thuringenesis* SN2, *B. Pumilis* SN3 both and fresh culture was inoculated in cultivation media [46]. Cells were harvested by centrifugation at 10,000g, 4°C, 10 min. the cell-free supernatant was collected.

3.8.2 Ammonium Sulphate Precipitation

3.8.2.1 Microbial strain *B. megaterium* SN1

The supernatant was subjected for Ammonium sulphate precipitation. Supernatant was precipitated with 0-30%, 30-60%, and 60-90% ammonium sulphate. Ammonium sulphate was poured slowly into the supernatant over a period of ten minutes, allowing the salt to slowly dissolve. The supernatant was continually stirred at 4°C for an additional 1 hour. The precipitate was collected by centrifugation (10,000 rpm for 10 minutes at 4°C), and dissolved in a small volume (1/50) of 10 mM Tris-HCl buffer (pH 8.0), and dialyzed against 4 liters of same buffer
for 12 hrs at 4°C. This step was repeated twice. The protease activity and protein concentration was measured and specific activity calculated.

3.8.2.2 Microbial strain B. thuringenesis SN2
The supernatant was subjected for Ammonium sulphate precipitation. Supernatant was precipitated with 0-80% ammonium sulphate. Ammonium sulphate was poured slowly into the supernatant over a period of ten minutes, allowing the salt to slowly dissolve. The supernatant was continually stirred at 4°C for an additional 1 hour. The precipitate was collected by centrifugation (10,000 rpm for 10 minutes at 4°C), and dissolved in a small volume (1/50) of 10 mM Tris-HCl buffer (pH 8.0), and dialyzed against 4 liters of same buffer for 12 hrs at 4°C. This step was repeated twice. The protease activity and protein concentration was measured and specific activity calculated.

3.8.3 Chromatographic Techniques

3.8.3.1 Ion-Exchange Chromatography for B. megaterium SN1
The protein pellet obtained after saturation with 30-60% ammonium sulphate was dissolved in 10 mM Tris-HCl buffer and loaded onto a column of 25 Q sepharose stong ion exchange chromatography column equilibrated with Tris–HCl buffer 10 mM (pH 8.0) at 4°C. After washing with the same buffer, adsorbed proteins were eluted with a linear gradient of NaCl 2 – 4 mM in the same buffer at a flow rate of 1ml/min. One ml fractions were collected and checked for protein and protease and kerainase activity. The maximum enzyme activity was found in the flow-through. All the fractions with high enzyme activity were pooled, dialyzed, and concentrated by lyophilization and used for further studies. All steps were conducted at 4°C.

3.9 Molecular Weight Determination

3.9.1 Polyacrylamide Gel Electrophoresis for microbial strains
SDS-PAGE was performed on a slab gel containing 10% (w/v) polyacrylamide by the method of Switzer et al 1979 [355].
### Resolving Gel (10%)
- Distilled Water- 4.1 ml
- 1.5M Tris (pH: 8.8)-2.5ml
- 10%SDS-50μl
- Acrylamide-3.3 ml
- 10%ammonium persulphate-50μl
- Temed-5μl

### Stacking Gel (4%)
- Distilled water- 3.075ml
- 1.5M Tris (pH:6.8)-1.25ml
- 10%SDS-25μl
- Acrylamide-670μl
- 10%ammonium persulphate-25μl
- Temed-5μl

All the above reagent of resolving gel was mixed and after polymerization of the resolving gel all the component of the stacking gel was mixed and poured inside the glass plate, wells were made with the help of comb. After that protease sample was loaded in each well and electrophoresed at 100V for 2 hour after electrophoresis completed, gel was removed from the glass plates placed in the staining container and then

1. Washed with methanol: acetic acid: distilled water (5:1:4) for 20 minutes
2. Washed with 20% ethanol for 10min.
3. Washed with water for 1 hr. by frequently changing water.
4. Rinse with 0.03% sodium thiosulphate 1min.
5. Rinse with water twice.
6. Immerse in 0.1% AgNO3 at 4 C for 20min.
7. Rinse with water twice.
8. Add chill developer (0.04% formaldehyde and 2% sodium carbonate) shake until developed yellow continue with fresh cold developer.
9. To stop development, add 5% acetic acid to solution
10. Store in 1% acetic acid

### 3.9.2 Zymography
Casein zymography was performed in 10% polyacrylamide slab gels containing SDS and casein (0.12% w/v) as co-polymerized substrate, as described by Choi et al, [356]. After electrophoresis, the gel was incubated for 30 minutes at room temperature on a gel rocker in 50 mM Tris-Cl (pH 7.4), which contained 2.5% Triton X-100 to remove SDS. The gel was then
incubated in a zymogram reaction buffer (30 mM Tris-HCl, pH 7.4, 200 mM NaCl and 10 mM CaCl$_2$) left at 37°C for 12 hrs on rocker shaker. The gel was stained with Coomassie brilliant blue (0.5% w/v) for 30 min. The activity band was observed as a clear colourless area depleted of casein in the gel against the blue background when destained in 10% methanol and 5% acetic acid for a limited period of time.

3.10 Characterization of Proteases and Keratinase of isolates

3.10.1 Hydrolysis of Protein Substrates
Protease activity with various protein substrates including keratin, casein, gelatin and bovine serum albumin (2 mg/ml) was assayed by mixing 100 µl of the enzyme and 900 µl of assay buffer containing the protein substrates., (as in section 3.8.1) All assays were done in triplicate.

3.10.2 Effect of pH on Enzyme Activity
Effect of pH on the purified enzyme activity was measured at various pH ranges (2.0 – 12). The pH was adjusted using the following buffers- acetate (50mM) (pH 2.0- 4.0), phosphate (50mM) (pH 5.0-7.0), Tris-Cl (50mM) (pH 8.0) and glycine-NaOH (50mM) (pH 9.0-12.0) and the activity of the enzyme was measured as described previously.

3.10.3 Effect of Temperature on Enzyme Activity
The activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 20, 30, 40, 50, 60, 70 and 80°C were studied. The activity of the enzyme was measured as described previously.

3.10.4 Effect of Various Metal Ions on Enzyme Activity
The effects of metal ions on enzyme activity (e.g., Ca$^{2+}$, Mg$^{2+}$, Fe$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Hg$^{2+}$, and Cu$^{2+}$ [10mM] were investigated by adding them to the reaction mixture. The activity of the enzyme was measured as described previously.
3.11 Antimicrobial activity

An antimicrobial test for the enzyme fractions was carried out by disk diffusion method [376]. The sensitivity of various microorganisms like *Micrococcus luteus*, *Bacillus subtilis*, *Bacillus amyloyligenes*, *Pseudomonas fluorescens*, *E. coli*. 100 µl of suspension containing $10^8$ colony forming units (CFU)/ml of bacteria spread on nutrient agar (NA) medium. The sterilized filter paper discs (whatman filter paper no.1, 6mm in diameter) impregnated with 10 µl of dialyze ammonium sulphate fraction purified from *Bacillus megaterium* SN1 (50 µg/disk) were placed on the inoculated agar. Tetracyclin (30 mcg) was used as standard antibiotics (HIMEDIA Laboratories, Mumbai, India). The positive controls were used to determine the sensitivity of one strain/isolated in each microbial species tested. The inoculated plates were incubated aerobically at 30°C (Gram negative) and 37°C (Gram positive) according to strain for 24 h. Antimicrobial activity was evaluated by measuring the zone of inhibition that had been caused by the enzyme preparation against the test organisms as dicussed by Edris et al and Kordali et al [377, 378]. Each activity was repeated twice.

3.12 Milk Clotting Activity (MCA)

3.12.1 Preparation of milk clotting activity substrate

10 g of skimmed milk powder was dissolved by stirring on magnetic stirrer in 100ml of 10mM CaCl$_2$ and MnSO$_4$. The pH of milk substrate was adjusted to 6.5 with 0.1 N NaOH or HCl.

3.12.2 Assay of milk clotting activity

Milk-clotting activity was determined according to the method of Arima [167], which is based on the visual evaluation of the appearance of the first clotting flakes, and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1ml of a solution containing 0.1 g skim milk powder in 40 min at 35°C. In brief, 0.5 ml of tested materials was added to a test-tube containing 5ml of reconstituted skim milk solution (10 g dry skim milk/100 ml, 10mM CaCl2 and 10mM MnSO$_4$) preincubated at 35°C for 5min. The mixture was mixed well and the clotting time $T$ (s), the time period starting from the addition of test material to the first appearance of clots of milk solution,
was recorded and the clotting activity was calculated using the following formula:
SU = 2400×5×D/T×0.5; T = clotting time (s); D = dilution of test material. [379]

3.13 Optimization of media

3.13.1 Resilient Back Propagation Based Yield Prediction of enzyme from *Bacillus megaterium* SN1

3.13.2.1 Bacterial strain *Bacillus megaterium* SN1

Bacterial strain of *Bacillus megaterium* were identified and maintained in our lab and glycerol stock was preserved. Bacterial identification was conducted by morphological, cultural and biochemical tests. Results were compared with *Bergey’s Manual of Determinative Bacteriology*, 8th edition [348]. Genus Bacillus: Agriculture Handbook No. 427 [349]. The strain was also identified by chromogenic method on the bacillus differential agar from Himedia, India, M1651 recommended for rapid identification of *Bacillus* species from a mixed culture. [46]

3.13.2.2 Media and Culture Conditions

Seed culture of the isolated strain *B. megaterium* SN1 was prepared in 500 ml Erlenmeyer conical flask containing 100 ml of the culture media containing: NH₄Cl, 0.5; NaCl, 0.5; K₂HPO₄, 0.3; KH₂PO₄, 0.4; MgCl₂.6H₂O, 0.1; Yeast extract, 0.1 and pH was maintained at 7.5 at 30°C for 72hrs and washed feather (10%) in cultivation media. After 72 hrs of incubation, the crude culture broth was centrifuged (10,000g, 4 C, 30 min). The above cultivation media were checked for proteolytic activity by the modified method of and Tsuchida et al [20].

3.13.2.3 Biological design for protease and keratinase production

Twelve experimental setups comprising of different initial values of NaCl, Yeast extract and Feather were set up in 250 volume flask with the working volume of 50 ml. The range of the three variables is mentioned in Table 3.1. Identified variables along with; KH₂PO₄, 0.3; K₂HPO₄, 0.4 and pH 7.5 of the basal media remained constant. These production media (50 ml in a 250-ml Erlenmeyer flask) were inoculated with the 5% (v/v) microbial culture (20 hrs old
cultures) and were kept for further 120 hours in cultivation media at 30°C, 160 rpm. The cell-free supernatant was obtained by centrifugation at 10,000 rpm for 10 min at 4°C and the Protease and keratinase production was determined in the cell-free supernatant.

<table>
<thead>
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<th>Variable</th>
<th>Actual</th>
<th>Code</th>
<th>Actual</th>
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<th>Actual</th>
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<td>+1</td>
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<tr>
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<td>0</td>
<td>0.3g</td>
<td>+1</td>
</tr>
<tr>
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<td>10g</td>
<td>0</td>
<td>15g</td>
<td>+1</td>
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### 3.13.2.4 Protein concentration
Protein concentration was determined by the method of Bradford MM 1976 [354] with bovine serum albumin as standard.

### 3.13.2.5 Determination of protease and keratinase activity
Protease activity was assayed by a modified method of Tsuchida et al and Cheng et. al. 1995 as described previously. (As in section 3.8.1, 3.8.2) All assays were done in triplicate. Values of specific activity measured during the experiments as obtained from the production media setups were used for prediction of specific activity for other combinations of media.

### 3.13.2.6 Experimental Design and Statistical Analysis ANN
Artificial neural network is a system loosely modeled on the human brain. The network usually consists of an input layer, some hidden layers, and an output layer [380, 381]. Back-propagation training algorithms gradient descent and gradient descent with momentum are often too slow for practical problems because they require small learning rates for stable learning. In addition, success in the algorithms depends on the user-dependent parameters learning rate and momentum constant. Faster algorithms such as conjugate gradient, quasi-Newton and
Levenberg–Marquardt (LM) use standard numerical optimization techniques, where as resilient back-propagation and variable learning rate algorithms based on heuristics. Multilayer neural networks typically use sigmoid transfer functions in the hidden layers. These functions are often called "squashing" functions, because they compress an infinite input range into a finite output range. Sigmoid functions are characterized by the fact that their slopes must approach zero as the input gets large. This causes a problem when we use steepest descent to train a multilayer network with sigmoid functions, because the gradient can have a very small magnitude and, therefore, cause small changes in the weights and biases, even though the weights and biases are far from their optimal values.

The purpose of the Resilient Back propagation training algorithm (RPROP) introduced by Riedmiller M. 1993; Sameh A. 2005 [382, 383], is to eliminate these harmful effects of the magnitudes of the partial derivatives. Only the sign of the derivative can determine the direction of the weight update; the magnitude of the derivative has no effect on the weight update. The size of the weight change is determined by a separate update value. The update value for each weight and bias is increased by a factor delt_inc whenever the derivative of the performance function with respect to that weight has the same sign for two successive iterations. The update value is decreased by a factor delt_dec whenever the derivative with respect to that weight changes sign from the previous iteration. If the derivative is zero, the update value remains the same. Whenever the weights are oscillating, the weight change is reduced. If the weight continues to change in the same direction for several iterations, the magnitude of the weight change increases.

The design of neural architecture and the training parameters are shown in Table 3.2 and Table 3.3 for prediction of the specific activity of protease production and in table 3.4 and 3.5 for prediction of specific activity of keratinase production. The network trained from the values obtained from the 12 experimental setup and predicted values revalidated experimentally.
For Protease-

Table 3.2: Network training parameters for protease

<table>
<thead>
<tr>
<th>Training parameters</th>
<th>Range of values</th>
<th>Best value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nos of neurons in input layer</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Nos of neurons in hidden layer</td>
<td>6-12</td>
<td>9</td>
</tr>
<tr>
<td>Nos of neurons in output layer</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Activation function</td>
<td>Tangent Sigmoid, Log Sigmoid, Pure Linear</td>
<td>Hidden layer - Tangent Sigmoid Output layer - Pure linear</td>
</tr>
<tr>
<td>Performance, Time</td>
<td>0-45.1, 8, input</td>
<td>0.321, 0.8 Sec</td>
</tr>
<tr>
<td>Epoch size and Epoch</td>
<td>0-100</td>
<td>1, 1000</td>
</tr>
<tr>
<td>Nos of training datasets</td>
<td>10-20</td>
<td>12</td>
</tr>
</tbody>
</table>

Gradient and related parameters

Gradient 0.0252
Minimum gradient $10^{-10}$
increment 1.2
decrement 0.5
maximum 50.
**Table 3.3a:** Weights and Bias terms obtained from the trained network for protease

### 3a-Input to hidden layer weights

<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>Yeast extract</th>
<th>Feather</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.3503</td>
<td>23.9614</td>
<td>4.0677</td>
</tr>
<tr>
<td>2</td>
<td>-61.0897</td>
<td>-60.7841</td>
<td>-20.9075</td>
</tr>
<tr>
<td>3</td>
<td>40.7541</td>
<td>42.2172</td>
<td>-20.2406</td>
</tr>
<tr>
<td>4</td>
<td>1.2833</td>
<td>-1.3411</td>
<td>-0.96223</td>
</tr>
<tr>
<td>5</td>
<td>-20.8603</td>
<td>-21.3852</td>
<td>-20.1762</td>
</tr>
<tr>
<td>6</td>
<td>-3.0203</td>
<td>-0.35336</td>
<td>-0.97504</td>
</tr>
</tbody>
</table>

**3.3b:** Hidden to Output to layer Weight (specific activity of enzyme in units/mg protein)

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17704</td>
<td>-0.22918</td>
<td>-0.88262</td>
<td>-2.3182</td>
<td>-0.54995</td>
<td>0.32938</td>
<td></td>
</tr>
</tbody>
</table>

**3.3c:** Bias terms

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>b1</td>
<td>-4.8553</td>
</tr>
<tr>
<td>b2</td>
<td>40.0895</td>
</tr>
<tr>
<td>b3</td>
<td>-0.54715</td>
</tr>
<tr>
<td>b4</td>
<td>-2.0671</td>
</tr>
<tr>
<td>b5</td>
<td>-0.7979</td>
</tr>
<tr>
<td>b6</td>
<td>-5.02</td>
</tr>
<tr>
<td>c1</td>
<td>1.1346</td>
</tr>
</tbody>
</table>
For Keratinase -

Table 3.4: Network training parameters for keratinase

<table>
<thead>
<tr>
<th>Training parameters</th>
<th>Range of values</th>
<th>Best value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nos of neurons in input layer</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Nos of neurons in hidden layer</td>
<td>3-6</td>
<td>6</td>
</tr>
<tr>
<td>Nos of neurons in output layer</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Learning rate</td>
<td>0.2-0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>Activation function</td>
<td>Tangent Sigmoid, Log Sigmoid, Pure Linear</td>
<td>Hidden layer - Tangent Sigmoid Output layer - Pure linear</td>
</tr>
<tr>
<td>Performance and Time</td>
<td>0-215</td>
<td>1.88e-18, 08Sec</td>
</tr>
<tr>
<td>Epoch size and Epoch</td>
<td>1 and 0- infinite</td>
<td>1 and 1000</td>
</tr>
<tr>
<td>Nos of training datasets</td>
<td>10-20</td>
<td>2</td>
</tr>
<tr>
<td>Gradient and related parameters</td>
<td>Gradient 9.75e-08 Minimum gradient 1 e-010 Delta_inc 1.2 Delta_dec 0.5 maximum 50</td>
<td></td>
</tr>
<tr>
<td>Validation check</td>
<td>0-6</td>
<td></td>
</tr>
<tr>
<td>Data division</td>
<td>Random</td>
<td></td>
</tr>
<tr>
<td>Training</td>
<td>RPROP</td>
<td></td>
</tr>
<tr>
<td>Performance</td>
<td>Mean Squared Error</td>
<td></td>
</tr>
</tbody>
</table>
**Table 3.5a:** Weights and Bias terms obtained from the trained network for keratinase

<table>
<thead>
<tr>
<th>3a-Input to hidden layer weights</th>
<th>NaCl</th>
<th>Yeast extract</th>
<th>Feather</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3823</td>
<td>1.8389</td>
<td>1.4941</td>
</tr>
<tr>
<td>2</td>
<td>0.386</td>
<td>-21.0107</td>
<td>-21.0516</td>
</tr>
<tr>
<td>3</td>
<td>24.1862</td>
<td>21.7499</td>
<td>3.1175</td>
</tr>
<tr>
<td>4</td>
<td>1.0433</td>
<td>-0.88071</td>
<td>-1.8536</td>
</tr>
<tr>
<td>5</td>
<td>-0.37883</td>
<td>-21.3852</td>
<td>-1.2421</td>
</tr>
<tr>
<td>6</td>
<td>-2.9626</td>
<td>-0.84299</td>
<td>-1.532</td>
</tr>
</tbody>
</table>

**3.5b:** Hidden to Output to layer Weight (specific activity of enzyme in units/mg protein)

| 1.1164 | 0.74175 | -0.58663 | -1.086 | -0.22563 | 0.58276 |

**3.5c:** Bias terms

<table>
<thead>
<tr>
<th>b1</th>
<th>-3.2797</th>
</tr>
</thead>
<tbody>
<tr>
<td>b2</td>
<td>-0.36846</td>
</tr>
<tr>
<td>b3</td>
<td>-2.6294</td>
</tr>
<tr>
<td>b4</td>
<td>-2.0163</td>
</tr>
<tr>
<td>b5</td>
<td>1.1119</td>
</tr>
<tr>
<td>b6</td>
<td>-5.5954</td>
</tr>
<tr>
<td>c1</td>
<td>0.60455</td>
</tr>
</tbody>
</table>
The optimization of medium components NaCl, Yeast extract and Feather, which have been predicted to play an important role in the production of acidic protease and keratinase were selected as main variables to optimize the process. Therefore, a Box-Behnken design with three factors were used in the design of experiments and Minitab Statistical Software Release 13 in the analysis of data and generation of response surface graphics. Total of 27 experiments in three blocks were performed in the media optimization part of the study. Block variables were three different incubators to be able to run three different runs at the same time. Additional 12 media optimization experiments were designed in Box-Behnken design. The response variable in two sets of experiments was enzyme activity. The actual factor levels corresponding to the coded factor levels are presented in Table 3.1. After running the experiments and measuring the activity levels, a second order full model (Eq. 3.1) including interactions was fitted to the
3.15 General methodology

3.15.1 Preparation of Dialysis Tubing
Dialysis tubing was cut into convenient length and boiled for 10 min in 500 ml of 2% sodium bicarbonate and 1mM EDTA (pH 8.0) solution after that the tubing was rinsed thoroughly in distilled water and boiled for 10 min in 1 mM EDTA then the tubing was allowed to cool and stored at 4°C in 1mM EDTA buffer.

3.15.2 Preparation of Electrophoresis Reagents

<table>
<thead>
<tr>
<th>Resolving Gel (10%)</th>
<th>Stacking Gel (4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water- 4.1 ml</td>
<td>Distilled water- 3.075ml ml</td>
</tr>
<tr>
<td>1.5M Tris (pH: 8.8)-2.5ml</td>
<td>1.5M Tris (pH:6.8)-1.25ml</td>
</tr>
<tr>
<td>10%SDS-50μl</td>
<td>10%SDS-25μl</td>
</tr>
<tr>
<td>Acrylamide-3.3 ml</td>
<td>Acrylamide-670μl</td>
</tr>
<tr>
<td>10%ammonium persulphate-50μl</td>
<td>10%ammonium persulphate-25μl</td>
</tr>
<tr>
<td>Temed-5μl</td>
<td>Temed-5μl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acrylamide- Bisacrylamide solution</th>
<th>Staining Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide – 30.0 gm</td>
<td>Coomassie brilliant blue – 1.25 gm</td>
</tr>
<tr>
<td>Bisacrylamide – 0.8gm</td>
<td>Glacial acetic acid – 35 ml</td>
</tr>
<tr>
<td>Distilled water – 100 ml</td>
<td>Methanol - 200 ml</td>
</tr>
<tr>
<td></td>
<td>Final vol. - 500 ml (Distilled Water)</td>
</tr>
</tbody>
</table>
Destaining Solution
Methanol - 45 ml
Acetic Acid – 10 ml
Distill Water – 45 ml

Reservoir Buffer
Tris - 3.0 gm
SDS – 1.0 gm
Glycine – 14.4 gm
Distilled water – 1 liter
pH – 8.3

Sample Buffer (2X)
1M Tris-HCl (pH-6.8) – 12.5 ml
SDS – 4.0 gm
β – mercaptoethanol – 10.0 ml
Glycerol – 20.0 ml
1% Bromophenol blue – 4.0 ml
Final volume made up up to 500 ml with distilled water

Sample Buffer (2X)
for Zymography
0.5 M Tris-HCl, pH 6.8
SDS - 10
Glycerol - 20%
Bromophenol blue - 0.5%

Staining Solution for Zymography
Coomassie brilliant blue - 0.5%

Destaining Solution for Zymography
Methanol - 10%
Acetic acid - 5%

Zymogram Reaction Buffer
30 mM Tris-HCl, pH 7.4,
200 mM NaCl,
10 mM CaCl$_2$
3.15.3 Source of Chemicals, Media and Reagents

<table>
<thead>
<tr>
<th>S.nos</th>
<th>Name of chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acrylamide</td>
<td>Sigma</td>
</tr>
<tr>
<td>2</td>
<td>acetic acid</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>3</td>
<td>Acetone</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>4</td>
<td>Agar</td>
<td>HiMedia</td>
</tr>
<tr>
<td>5</td>
<td>Ammonium persulphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>6</td>
<td>Ammonium sulphate</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>7</td>
<td>4-(2-aminoethyl)benzene sulfonyl fluoride</td>
<td>Sigma</td>
</tr>
<tr>
<td>8</td>
<td>Beef extract</td>
<td>Sigma</td>
</tr>
<tr>
<td>9</td>
<td>Benzene</td>
<td>Sigma</td>
</tr>
<tr>
<td>10</td>
<td>Bisacrylamide</td>
<td>Sigma</td>
</tr>
<tr>
<td>11</td>
<td>Bromophenol blue</td>
<td>Sigma</td>
</tr>
<tr>
<td>12</td>
<td>BSA</td>
<td>Sigma</td>
</tr>
<tr>
<td>13</td>
<td>Casein</td>
<td>Sigma</td>
</tr>
<tr>
<td>14</td>
<td>Cetrimide agar</td>
<td>HiMedia</td>
</tr>
<tr>
<td>15</td>
<td>CoCl$_2$</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>16</td>
<td>Coomassie brilliant blue R-250</td>
<td>Sigma</td>
</tr>
<tr>
<td>17</td>
<td>Coomassie brilliant blueG-250</td>
<td>Sigma</td>
</tr>
<tr>
<td>18</td>
<td>DEAE-cellulose</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>19</td>
<td>3,4-dichloroisocoumarin</td>
<td>Sigma</td>
</tr>
<tr>
<td>20</td>
<td>D-glucose monohydrate</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>21</td>
<td>Ethanol</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>22</td>
<td>Ethyl acetate</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>23</td>
<td>Ferrous Sulphate pentahydrate</td>
<td>Merck</td>
</tr>
<tr>
<td>24</td>
<td>Folin–Ciocalteau reagent</td>
<td>Merck</td>
</tr>
<tr>
<td>25</td>
<td>Gelatin</td>
<td>Merck</td>
</tr>
<tr>
<td>26</td>
<td>Glacial acetic acid</td>
<td>CDH,Qualigens</td>
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<tr>
<td>27</td>
<td>Glycerol</td>
<td>CDH,Qualigens</td>
</tr>
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<td>28</td>
<td>Glycine</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>29</td>
<td>Hexane</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
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<td>Hydrogen peroxide</td>
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<td>Sigma</td>
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<td>32</td>
<td>Isopropanol</td>
<td>Merck</td>
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<tr>
<td>33</td>
<td>K$_2$HPO$_4$</td>
<td>CDH,Qualigens</td>
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<tr>
<td>34</td>
<td>KH$_2$PO$_4$</td>
<td>CDH,Qualigens</td>
</tr>
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<td>35</td>
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<td>CDH,Qualigens</td>
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<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>---</td>
<td>---------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>36</td>
<td>LB broth</td>
<td>HiMedia</td>
</tr>
<tr>
<td>37</td>
<td>Methanol</td>
<td>Merck</td>
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<tr>
<td>38</td>
<td>MgCl₂</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>39</td>
<td>MgSO₄</td>
<td>CDH,Qualigens</td>
</tr>
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<td>40</td>
<td>MgSO₄·7H₂O</td>
<td>CDH,Qualigens</td>
</tr>
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<td>41</td>
<td>Motility Agar</td>
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</tr>
<tr>
<td>42</td>
<td>Na₂CO₃</td>
<td>Merck</td>
</tr>
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<td>NaOH</td>
<td>CDH,Qualigens</td>
</tr>
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<td>44</td>
<td>Pepstatin</td>
<td>Sigma</td>
</tr>
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<td>45</td>
<td>Peptone</td>
<td>HiMedia</td>
</tr>
<tr>
<td>46</td>
<td>1,10-Phenanthroline</td>
<td>Sigma</td>
</tr>
<tr>
<td>47</td>
<td>Phenol red</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>48</td>
<td>Phenylmethylsulphonyl fluoride</td>
<td>Sigma</td>
</tr>
<tr>
<td>49</td>
<td>Phosphoric acid</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>50</td>
<td>Potassium sulphate</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>51</td>
<td>Protein molecular weight marker</td>
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<tr>
<td>52</td>
<td>Proteose peptone</td>
<td>HiMedia</td>
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<tr>
<td>53</td>
<td>SDS</td>
<td>Sigma</td>
</tr>
<tr>
<td>54</td>
<td>Sephadex G-250</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>55</td>
<td>Simmon’s media</td>
<td>HiMedia</td>
</tr>
<tr>
<td>56</td>
<td>Skim milk powder</td>
<td>HiMedia</td>
</tr>
<tr>
<td>57</td>
<td>Sodium Chloride</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
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<td>Sodium thiosulphate</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>59</td>
<td>Starch</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>60</td>
<td>Sucrose</td>
<td>CDH,Qualigens</td>
</tr>
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<td>61</td>
<td>TEMED</td>
<td>Sigma</td>
</tr>
<tr>
<td>62</td>
<td>Toluene</td>
<td>Merck</td>
</tr>
<tr>
<td>63</td>
<td>Trichloroacetic acid</td>
<td>CDH,Qualigens</td>
</tr>
<tr>
<td>64</td>
<td>Tris</td>
<td>Sigma</td>
</tr>
<tr>
<td>65</td>
<td>Triton X-100</td>
<td>Sigma</td>
</tr>
<tr>
<td>66</td>
<td>Tryptic soy broth</td>
<td>HiMedia</td>
</tr>
<tr>
<td>67</td>
<td>Urea agar</td>
<td>HiMedia</td>
</tr>
<tr>
<td>68</td>
<td>Yeast extract</td>
<td>HiMedia</td>
</tr>
<tr>
<td>69</td>
<td>β-mercaptoethanol</td>
<td>Sigma</td>
</tr>
</tbody>
</table>