CHAPTER 5

CONCLUSION AND FUTURE PROSPECTIVE

We report the isolation, purification, characterization and optimization of extracellular caseinolytic enzyme and keratinolytic enzyme produced by soil microbes. The caseinolytic, keratinolytic thermostable enzyme is capable of utilizing feather a major waste byproduct of poultry processing plant.

Soil sample was collected from Ghazipur poultry waste site, Ghaziabad, India, a feather dumping site. Soil sample was inoculated in three enrichment media, our results depict that optimal medium for caseinolytic enzyme and keratinolytic enzyme production is feather meal media 2 and colonies producing clear zone in feather meal agar were selected and identified as *B. megaterium* SN1, *B. thuringenesis* SN2, *B. pumilis* SN3 were able to degrade chicken and pigeon feathers. They produced extracellularly keratinolytic enzymes in enrichment media with 10% feather meal powder.

All bacterial isolates of *Bacillus sp.* SN1, SN2, SN3 crude showed presence of caseinolytic activity and Milk clotting activity in crude and ammonium sulphate fraction. Highest ratio (520.84) of milk clotting activity to caseinolytic activity was seen in presence of MnSO₄. 30-60% ammonium sulphate of *Bacillus sp.* SN1, 0-30% ammonium sulphate fraction of *Bacillus sp.* SN1, 0-80% ammonium sulphate fraction of *Bacillus sp.* SN3 and *Bacillus sp.* SN2 too showed milk clotting activity. Antibacterial activity against *Bacillus subtilis* (MTCC 1789), *Bacillus amyloliquifaceance* (MTCC 1270) and *Escherichia coli* (MTCC 1695) and *Bacillus sp.* SN2 could inhibit *M. luteus* and *Bacillus subtilis, Bacillus amyloliquifaceance, Escherichia coli* whereas *Bacillus sp.* SN3 showed against *Bacillus subtilis* (MTCC 1789), *Pseudomonas fluroscence* (MTCC 2421).
The strain *B. megaterium* SN1, *B. thuringenesis* SN2 produces extracellular caseinolytic enzyme and keratinolytic enzyme in feather meal media 2 that was maintained at 30°C, 160 rpm for 72 hrs and 96hrs respectively. Enzyme of *B. megaterium* SN1 was purified by ammonium sulphate precipitation and 25 Q sephrose chromatography and casein zymography studies showed that enzyme is having molecular weight 30 kDa. The optimum pH for the proteolytic and keratinolytic activity was pH 3 and at 60°C and 70°C temp respectively. Interestingly Mn$^{2+}$ (10mM) strongly activated caseinolytic enzyme and keratinolytic enzyme activity by 2.1, 1.17 fold respectively. While Hg$^{2+}$ strongly inhibited caseinolytic enzyme and keratinolytic enzyme activity.

Enzyme for *B. thuringenesis* SN2 was purified by ammonium sulphate precipitation and casein zymography studies showed that enzyme is having molecular weight 80 kDa, 60 kDa and 40 kDa. We report optimum pH for caseinolytic enzyme activity was at pH 5 and 40°C where as keratinolytic enzyme activity at pH 3 and 50°C. Interestingly Mn$^{2+}$ strongly activated caseinolytic enzyme activity by 3.74 fold. Ba$^{2+}$ strongly activated keratinolytic enzyme activity by 1.9 fold and was strongly inhibited caseinolytic enzyme activity, Whereas Fe$^{2+}$ could inhibit the keratinolytic enzyme activity.

To develop a process for the optimum production of caseinolytic enzyme from poultry feather, standardization of media components is crucial. Selected strain of *Bacillus megaterium* SN1 that is competent of rapidly degrading native feather for optimization. We have varied the various components in the media and the specific activity of the enzyme was determined. RSM and the Resilient back propagation- *RPROP*, neural network were used to predict the best combination which was then validated. To optimize these three significant medium constituents viz., NaCl, Yeast extract and Feather were chosen in our experimental design. The Optimization studies suggest NaCl, Yeast extract are insignificant variables and however, Feather had a profound effect on keratinolytic enzyme for yield. The optimum values of the tested variables by response surface methodology were; NaCl, 0.2%; Yeast extract, 1%; Feather, 15% found to be optimum for keratinolytic enzyme production. The keratinolytic enzyme production of 21.4 U/ml could be further increased by approximately 2.5 fold. On comparison with the prediction method used, it is found that the trained network is a better option to predict new data points thus
providing a mathematical alternative to quadratic polynomial required for data derived from statistically designed experiment [33, 34].

In future, optimization of enzymes of *B. thuringenesis* SN2 with other factors can be studied and predicted. Purification of enzymes of *B. thuringenesis* SN2. Amino acid sequence determination of purified enzyme from *B. megaterium* SN1, *B. thuringenesis* SN2 would be performed and checked for innovative application in other biotechnology industries.

Future studies regarding upgrading the caseinolytic enzyme production technology from laboratory to a large-scale process is to be performed.

The caesinolytic activity and the milk clotting activity can be further optimized. The thermostability and wide pH range shown for the caesinolytic activity suggests its application in dairy industry. Since the caesinolytic activity is inhibited by barium chloride we suggest that the addition of barium selenate can be used to inhibit the enzyme activity in the manufacturing process of cheese and also increasing its nutraceutical value.

The quality of both the enzyme and the milk curd formed is needed to further confirm the usefulness in dairy processing. Future studies regarding upgrading the caesinolytic enzyme production technology from laboratory to large-scale process after taking into consideration the various operational and nutritional factors is required for Milk clotting enzyme production.

Following the confirmation of application, this enzyme and its hydrolysate produced from waste can be fed to chickens as a high-protein supplement, resulting in the recycling of this product. Need less to say if practical methods of enzyme production at large scale and application are developed and implemented, the poultry industry could utilize this technology to reduce the overall production of feather-keratin waste too.