PRODUCTION OF ENZYMES AND DEGRADATION OF FEATHERS BY SOIL MICROBES

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By

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Enzymes are a very well established product in biotechnology [1], sales from US have been from $1.3 billion in 2002 to US $5.1 billion in 2009 and is anticipated to reach $7 billion by 2013 [2, 3, 4, 5, 6]. A recent survey on world sales of enzymes ascribes 31% for food enzymes, 6% for feed enzymes and the remaining for technical enzymes [7]. This pattern corresponds to a rise in global demand of about 6% yearly [4, 6]. This includes enzymes required in large scale for application in food and feed [8, 9], where the amino acids segment will have the share of the market at $7.8 billion in 2013 [10]. Other technical enzymes are used in the detergent, personal care, leather, textile and pulp, and paper industries [11]. Major enzyme producers are located in Europe, USA and Japan. Denmark is dominating, with major players like Novozymes (45%), Danisco (17%), Genencor (USA), DSM (The Netherlands) and BASF (Germany) [7, 8, 10]. The pace of development in emerging markets suggested that companies from India and China can join this restricted party in a very near future [12, 13, 14, 15].

Feathers are produced in large amount as a waste by poultry product processing plants; it reaches millions of tons per year worldwide [16]. They can be degraded by keratinolytic bacteria. A number of keratinolytic microorganisms have been reported, including some species of fungi such as *Microsporum* [17], *Trichophyton* [18], *Bacillus* [19, 20, 21], *Streptomyces* [22, 23, 24] and Actinomycetes [25, 26].Till date most of the purified keratinase cannot completely degrade keratin, their exact nature and uniqueness for keratinolysis is still not clear, so there is a requirement to isolate new sources of microbial keratinases to meet the industrial demand.

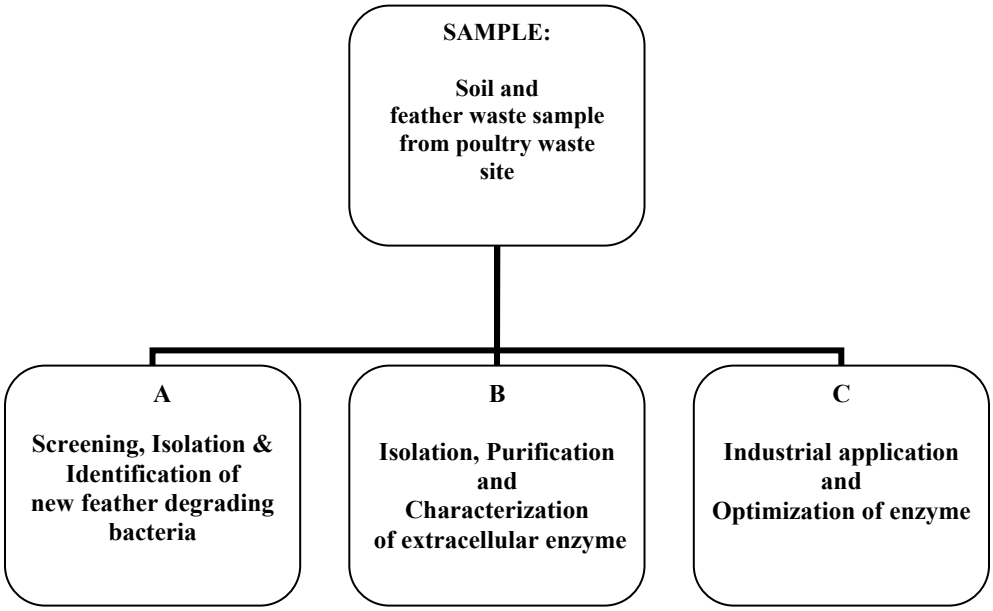
The innovative aspect of the present work is to identify new sources of keratinases producing microbes from soil of feather dumping sites and this can have positive effect in solid waste management.

The objective of the present work was

i. To identify the new sources of keratinolytic bacteria from soil sample of feather dumping site at Ghazipur poultry waste site near our institute.
ii. Isolation, characterization, purification and optimization of enzymes produced by isolated bacteria and check for its application

We describe the protocol for production of caesinolytic enzymes and keratinolytic enzymes from microbes of soil found at dumping site of Ghazipur poultry processing plant, Ghaziabad. The purified enzyme has application in food and feed industry. Optimization studies for production of enzyme were also performed.

The strategy followed is as below



We describe in our work that

- *On Screening for keratinolytic enzyme and caesinolytic enzyme producing microbes*. Three microbial sources after initial screening the enzyme were selected and identified as *B. megaterium* SN1, *B. thuringenesis* SN2, *B. Pumilis* SN3, these produced acidic enzyme extracellulary as reported by Agrahari et. al. 2010 [28].

- *Our Studies involving application of isolated enzymes*: We report that feather was degraded in cultivation media with the isolated microbes separately and enzymes

isolated from this media was found to have milk clotting activity Agrahari et. al. 2010 [29]. These enzymes have been purified and characterized. Agrahari et. al. 2010 [32].

- *Our studies involving optimization of enzyme production from B. megaterium* SN1. We have varied the various components in the media and the specific activity of both the enzymes was determined. Resilient back propagation- *RPROP,* neural network was used to predict the best combination. Asawa et. al. 2010 and Wadhwa et. al. 2010 [31, 35].

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 caesinolytic 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 [28]. Earlier studies from our lab involving screening of micro-organism from same soil sample of dumping site of Ghazipur poultry processing plant, we have reported isolation of *Pseudomonas thermaerum* GW1, GenBank accession GU95151, this bacteria showed proteolytic activity but not keratinolytic activity [30].

All bacterial isolates of *Bacillus sp.* SN1, SN2, SN3 crude should presence of caesinolytic activity and milk clotting activity in crude and ammonium sulphate fraction. Highest ratio (520.84) of milk clotting activity to caesinolytic activity was seen in presence of $CaCl_2$ and $MnSO_4$. 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) as reported by Agrahari et. al. 2010 [29].

The strain *B. megaterium* SN1, *B. thuringenesis* SN2 produces extracellular caesinolytic 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 25Q 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 caesinolytic enzyme and keratinolytic enzyme activity by 2.1, 1.17 fold respectively. While Hg^{2+} strongly inhibited caesinolytic enzyme and keratinolytic enzyme activity [32, 33].

Caseinolytic activity from B. *thuringenesis* SN2 is reported in 0-80% ammonium sulphate precipitation. Casein zymography studies showed that enzyme has molecular weight of 80 kDa, 60 kDa and 40 kDa. We report optimum pH for caesinolytic enzyme activity was at pH 5 and 40ºC where as keratinolytic enzyme activity at pH 3 and 50ºC. Interestingly Mn^{2+} strongly activated caesinolytic enzyme activity by 3.74 fold. Ba^{2+} strongly activated keratinolytic enzyme activity by 1.9 fold. Whereas Ba^{2+} and Fe^{2+} strongly inhibited caesinolytic enzyme activity and keratinolytic enzyme activity respectively [34].

To develop a process for the optimum production of caseinolytic enzyme from poultry feather, standardization of media components is crucial. We selected *Bacillus megaterium* SN1 that is competent of rapidly degrading native feather for our optimization studies. The various components in the media was varied and the specific activity of the enzyme was determined. Resilient back propagation- *RPROP,* neural network was used to predict the best combination and 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, however Feather had a profound effect on yield of keratinolytic enzymes NaCl 0.5gm, Yeast extract 0.13 gm and Feather 15g (-1, 1, 1) yielded the maximum amount of caesinolytic enzyme (24.292 U/mg) and for keratinolytic enzyme production, it is evident that presence of feather is significant. The optimum combination being NaCl 0.5gm, Yeast extract 0.1 gm and Feather 10g (-1,-1, 0) yielded the maximum amount of keratinolytic enzyme (17.2314 U/mg protein).

The prediction method used 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 [31, 35].

In future optimization with other factors can be studied and predicted. 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 caesinolytic and keratinolytic enzyme production technology from laboratory to a large-scale process is to be performed.

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