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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Production of an Extra Cellular Feather Degrading Enzyme by *Bacillus licheniformis* Isolated from Poultry Farm Soil in Namakkal District (Tamilnadu)

P. Tamilmani[1], A. Umamaheswari[2], A. Vinayagam[1] and B. Prakash[1]
[1]Department of Biochemistry, P.G.P. College of Arts and Science, Namakkal, Tamilnadu, India
[2]Department of Biotechnology, Prince Shri Venkateshwara Arts and Science College, Chennai, Tamilnadu, India

Abstract: Chicken feather is recognized as a solid waste generated from poultry farms and is abundant in Namakkal district, Tamilnadu, India. Feather is commonly treated by high temperature and pressure; it is used as animal food stuffs. However, feather degradation by biological methods has been increasingly interested because of environmental awareness. In this study, unidentified bacterial strains isolated from soil samples. They had show ability of feather degradation by making a clear zone around their colonies in FMA medium. The zoned isolates were identified by morphological and biochemical tests. The *Bacillus licheniformis* were also examined for keratinase production by shake flask fermentation in a basal medium containing 1% feather. The fermentation mediums were optimized. Fermentation process was carried out at 37°C for 7 days at 150 rpm. Crude keratinase were extracted and purified by salt precipitation, dialysis and column chromatography and measured the activity of keratinase.

Key words: Feather waste, keratin, *Bacillus licheniformis*, fermentation and keratinase production

Introduction

Feathers are produced in large amounts as a waste byproduct of poultry processing plant. A current value-added use for feathers is the conversion to feather meal, a digestible dietary protein for animal feed, using physical and chemical treatments. These methods can destroy certain amino acids and decrease protein quality and digestibility (Riffel and Brandelli, 2006).

Keratin is an insoluble macromolecule requiring the secretion of extra cellular enzymes for biodegradation to occur. Keratin comprises long polypeptide chains, which are resistant to the activity of non-substrate-specific proteases. Adjacent chains are linked by disulphide bonds though responsible for the stability and resistance to degradation of keratin (Safranek and Goos, 1982). The degradation of keratinous material is important medically and agriculturally (Shih and Williams *et al*., 1990; Shih, 1993; Matsumoto, 1996). Secretion of keratinolytic enzymes is associated with dermatophytid fungi, for which keratin is the major substrate (Matsumoto, 1996). However, the production of such enzymes is not exclusive to dermatophytes, since geophilic species have demonstrated keratinase production (Kushwaha, 1983; Kushwaha and Nigam, 1996).

World-wide poultry processing plants produce millions of tons of feathers as a waste product annually (Santos *et al*., 1996), which consists of approximately 90% keratin; the keratin is largely responsible for their high degree of recalcitrance. However, they also represent a potentially valuable source of protein as animal feedstock if keratinolysis can be achieved (Shih, 1993). Keratinolytic enzymes have been studied from a variety of fungi and, to a lesser extent, bacteria.

However, much current research is centered on the potential use of keratinase of bacterial origin for the industrial treatment of keratin-containing compounds, e.g. serine proteases produced by *Bacillus licheniformis* PWD - 1 (Lin *et al*., 1992) and *B. licheniformis* Carlsberg NCIMB 6816 (Evans *et al*., 2000). Such interest results from the broad substrate range of these bacterial enzymes, their rates of activity towards keratin-containing compounds and their thermostability. The present study describes the screening of bacterial isolates with known proteolytic activity for keratinolysis and the purification and characterization of an extra cellular keratinase produced by *Bacillus licheniformis*.

Materials and Methods

Isolation of microorganism: Samples (soil and feather) were taken from the natural composting wastes in the town of Namakkal (Tamilnadu). All the samples (500 g each) were transported in plastic bags to the laboratory (Ilham and Mohamed, 2003) Serial dilution from each sample was prepared by adding 1 g of the soil sample to 9 ml of saline water. This initial dilution was heat activated at 70°C during 15 min and dilution upto 10-9 in saline water was prepared in tubes. All the dilutions

were plated on Nutrient Agar medium and incubated at 35°C for 24 h. The appeared colonies were checked for spore presence and streaked on agar slants for further characterization.

Characterization: All the collected strains were grown on Nutrient Agar medium for fresh cultures. Spore production and localization were examined by microscopic observations. The identification was done according to the method described by Larpent and Larpent-Gourgaud (1985).

Screening: The bacterium used in this study was a patented strain of *B. licheniformis* isolated in our laboratory (Williams *et al.*, 1990; Shih and Williams, 1990). All culture conditions and the feather culture medium were as previously described. The medium contained, per liter, the following: 0.5 g of NH_4Cl, 0.5 g of NaCl, 0.3 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.1 g of $MgCl_2.6H_2O$, 0.1 g of yeast extract, and 10 g of Poultry chicken feathers. The pH was adjusted to 8.0. Feathers were washed, dried, and cut into smaller pieces (2-3 cm) prior to being added to the medium. The medium was sterilized by autoclaving. The bacterium was cultured in a test tube containing 10 ml of culture medium. After 7 days of incubation, 10 ml of the culture medium was transferred to a 3-liter Fern Bach flask containing 1.0 liter of medium. After 7 days of incubation, the medium was collected for keratinase purification. All incubations were done at 35°C with shaking at 150 rpm in a controlled-environment shaker (SAI Scientific and Co, India Pvt. Ltd.)

Enzyme purification: The culture medium was prefiltered through Whatmann No.1 filter paper to remove residual undegraded (Xiang *et al.*, 1992). The medium was then filtered through a 0.45-, µm-pore-size membrane with a Pellicon cassette system (Millipore Corp., Bangalore.) to remove bacterial cells and other particles. The filtrate was concentrated by membrane ultra filtration (molecular weight cutoff, >10,000) with the same (Pellicon) system. The crude concentrated keratinase solution was applied to a column of Diethyl amino ethyl cellulose (DEAE-cellulose) at 4°C. The column was equilibrated with buffer A (25 mM potassium phosphate buffer (pH 5.8). Approximately 50 mg of protein was loaded on the column. Elution was beginning with 5 ml of buffer A. The eluted proteins were monitored by measuring the A280 of the fractions. When the 280nm reading was a continuous baseline, elution with buffer B (25 mM potassium phosphate buffer (pH 6.2), 20 mM NaCl) was begin. Approximately 300 ml was used for this elution. Finally, buffer C (25 mM potassium phosphate buffer (pH 6.8), 20 mM NaCl) was used. The elution flow rate was 0.4 ml/min, and 5-ml fractions were collected. Fractions exhibiting proteolytic activity were assayed for keratinase activity. Fractions were screened with the milk-agarose plate assay, and then the active fractions were assayed for keratinase activity with the azokeratin hydrolysis test.

Keratinase activity assay: Keratinolytic activity of culture filtrates was measured spectro-photometrically according to the method of Hamaguchi *et al.* (2000). with some modifications (using keratin powder instead of guinea pig hair as a keratin source). Keratin powder (20 mg), 3.0 ml phosphate buffer (28 mM, pH 7.8) and 2.0 ml culture filtrate were incubated in a shaker water bath at 150 rpm at 37°C for 1 hour. After the addition of 10% trichloroacetic acid (TCA) and centrifugation at 10,000 g for 15 min, the optical absorption of the supernatant was measured at 280 nm wavelengths using a double-beam spectrophotometer toward the blank. The blank was treated in the same way except for the addition of TCA which done before the initiation of enzyme reaction. The increase of 0.1 unit absorption is equal to one unit of enzyme activity. Protein content was measured according to the Lowry's method (Lowry *et al.*, 1951).

Enzyme characterization: Enzyme characterization was done by Allpress *et al.* (2002) method.

Temperature: The effect of temperature on keratinase activity was determined by the addition of 20 µl keratinase (3 mg ml 1 protein) to 1.5 ml phosphate buffer (100 mmol 1, pH 7.5) containing 15 mg powdered keratin and incubating at a range of temperatures (25, 30, 35, 40, 45 and 50°C) for 10 h. Peptide release was determined spectro-photometrically (280 nm; 1 unit of activity (U) the amount of enzyme causing an increase of 1.0 A280 unit within 1 minute).

pH: The effect of temperature on keratinase activity was determined by the addition of 20 µl keratinase (3 mg ml 1 protein) to 1.5 ml phosphate buffer (100 mmol) with different pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) containing 15 mg powdered keratin, and incubating at 35°C for 10 h. Peptide release was determined spectro-photometrically (280 nm; 1 unit of activity (U) the amount of enzyme causing an increase of 1.0 A280 unit within 1 minute).

The molecular weight of the enzyme was determined by using Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) method.

Results

Isolation of microorganism: Feather degrading microorganisms were isolated from soil samples in near poultry farms by serial dilution, spread plate method and streak plate method.

The master plate contained microorganisms exhibited distinct colony morphologies when streaked into nutrient

agar plate. One type contained rod shaped bacteria and another was a coccus, which appear singly and in chain. When streaked into the feather meal agar plate the rod shaped bacteria's produced clear zones. This rod shaped bacteria was identified and characterized by staining and biochemical methods. The identified bacterium was *Bacillus licheniformis*.

Screening and feather degradation: The isolated and identified *Bacillus licheniformis* was inoculated into the feather meal broth which contained feathers and incubated at 35° C for seven days at 150 rpm in a shaker (Table 1).

Table 1: Chemical composition of the feather

Component	Percentage (%)
Protein	81.0
Fat	1.2
Dry matter	86.0
Ash	1.3

Downstream processing: The crude enzyme sample was precipitated by ammonium salt precipitation method. The precipitated proteins were dialyzed and eluted by ion exchange chromatography. The activity of the enzyme was determined. Protein was determined by Lowry's method. The molecular weight of the enzyme was characterized by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE). Enzyme immobilization was also done (Fig. 1, 2 and 3).

Characterization of keratinase: The strain *Bacillus licheniformis* grew well and completely degraded poultry feathers in the medium. This intense feather degrading activity was achieved in room temperature, 35°C and with initial pH adjusted from 7.0 to 8.0. Similar growth curves were observed with in this range of temperature and pH. Maximum enzyme activity and enzyme yield were observed at 35°C in alkaline pH 8.0. In all cases, the pH values and temperatures increased from 6.0 to 9.0 and 25 to 50°C respectively. The effect of various substrates on Keratinase production was investigated. Production of Keratinase activity was similar when *Bacillus licheniformis* was grown in raw feathers or feather meal, but decreased with other pertinacious substrates. The effect of concentration of three growth substrates on Keratinase production was tested. Keratinase was produced at a similar level at different concentration of raw feathers and feather meal. Increased amounts of feather and feather meal resulted in higher bacterial growth (Fig. 4 and 5).

Discussion

Bacteria were isolated from a poultry processing plant that owned keratinolytic activity and ability to degrade keratin wastes. These bacteria present different

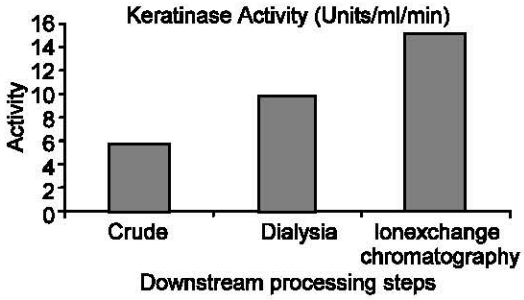


Fig. 1: Enzyme activity.

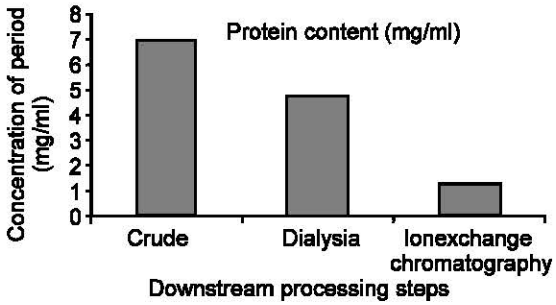
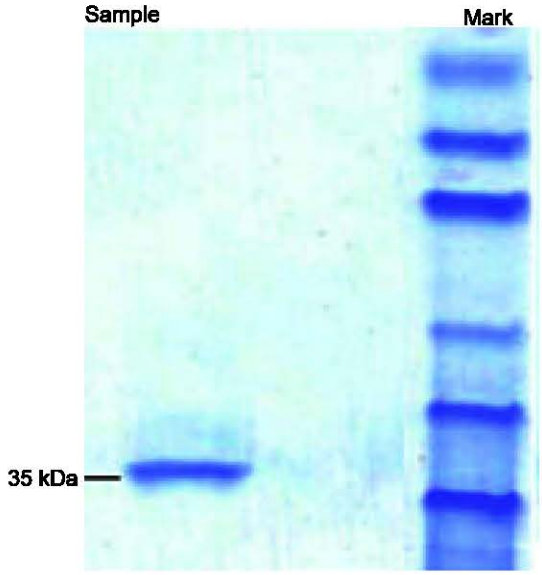


Fig. 2: Estimation of protein



Lane M: Molecular weight marker proteins
a_2 Macroglobulin (170KD)
ß-Galactosidase (116.4KD)
Fructose-6-phosphate kinase (85.2KD)
Glutamate dehydrogenase (55.6KD)
Aldolase (39.2KD)
Triosephosphate isomerase (26.6KD)
Trypsin inhibitor (20.1KD)
Lysozyme (14.3KD)

Fig. 3: Characterization of enzyme

characteristics, such as a broad temperature range of growth. The optimal proteolytic activities were detected between 30 and 37°C, whereas previously described keratinolytic bacteria mostly have feather-degrading activity at elevated temperatures. However, these strains behave similar to a Vibrio strain, previously isolated from decomposing feathers. An optimum keratin-degrading activity at mesophilic temperatures should be a desirable characteristic because these microorganisms may achieve hydrolysis with reduced energy input.

A keratinase was produced by *Chryseobacterium* sp. kr under different growth conditions. This enzyme has been shown to be useful for biotechnological purposes such as hydrolysis of poultry feathers and de-haring of bovine pelts (Riffel and Brandelli, 2006). For production of keratinase by *B. licheniformis* and a recombinant *B. subtilis*, uncontrolled pH operation was more favorable than the controlled pH operations (Wang and Shih, 1999). The same was observed for alkaline protease production by *B. licheniformis* (Calik *et al*., 2002).

Preliminary identification tests indicate that belong to the *Bacillus* or *Cytophaga-Flavobacterium* group. In agreement with this data (Lucas *et al*., 2003), noted that feather degrading Gram - negative bacteria isolated from soils belonged to the *Bacillus* or *Cytophaga flavobacterium* group. The most studied keratinolytic bacteria are *Bacillus* spp., which have been described to possess feather-degrading activity. *Bacillus licheniformis* is a well known keratinolytic organism, possessing the gene ker A, which has been cloned and sequenced. However, data on Gram-negative bacteria are relatively scarce and feather-degrading activity has been described only recently.

An increase in pH values was observed during feather degradation, a trend similar to other microorganisms with large keratinolytic activities. This trend may be associated with proteolytic activity, consequent deamination reactions and the release of excess nitrogen as ammonium ions. The increase in pH during cultivation is pointed as an important indication of the keratinolytic potential of microorganisms. Micro-organisms growing on medium containing feather meal as a unique carbon and nitrogen source presented variable activity on keratin, suggesting that this enzyme may be inductive. Substrate levels in the medium may regulate enzyme secretion. *Bacillus licheniformis* showed to be more adapted to keratinase production using keratin as substrate, since the maximum keratinase activity of the isolate was observed during early growth, and the strain displayed a higher total activity during incubation.

High substrate concentrations may cause substrate inhibition or repression of keratinase production. This was observed when soy meal was used as substrate, similar to that described by Joo *et al*. (2002) during protease production by *Bacillus horikoshii*. This

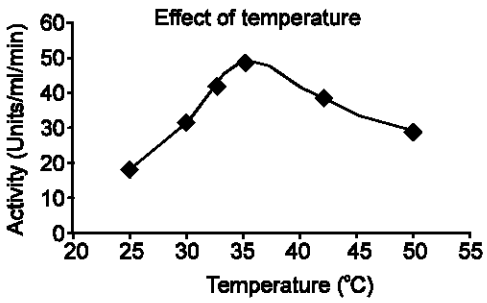


Fig. 4: Effect of temperature

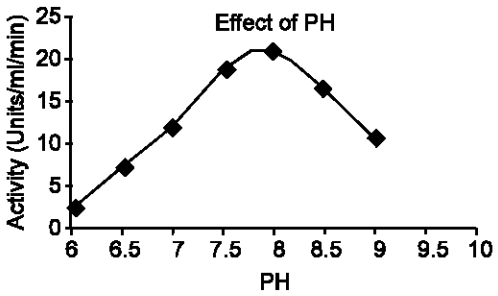


Fig. 5: Effect of pH

indicates that smaller amounts of substrate are preferred since they result in higher yields of product per substrate (Ug-1 substrate). It was previously observed for the strain kr6 that substrate and metabolite levels in the extracellular milieu can regulate enzyme secretion1 (Riffel *et al*., 2003). As the strain grows in soy meal faster than in feather substrates, different kinetics for keratinase production would be expected, depending on the type and amount of substrate.

The keratin hydrolysis ratio was higher for *Bacillus licheniformis*, suggesting preferred utilization of keratin as substrate. Through the strategy of isolation of keratinolytic microorganisms utilized in this work, bacteria presenting high keratinolytic activities were selected.

Considering that feather protein has been showed to be an excellent source of metabolizable protein and that microbial keratinase enhance the digestibility of feather keratin, these keratinolytic strains could be used to produce animal feed protein. In addition, the selected isolates were able to grow and display keratinolytic activity in diverse keratin wastes. This would be beneficial for the utilization of these residues. This novel isolates present potential biotechnological use in processes involving keratin hydrolysis.

Conclusion: It can be concluded that the feather waste as a renewable source for the production of keratinase. In this study, the optimum conditions for keratinase, synthesized by *Bacillus licheniformis*, were determined which was essential step for the production of adequate

amount of enzyme. It applied for animal feed in poultry industries.

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