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Isolation and characterization of feather degrading bacteria from poultry waste

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ABSTRACT:

The aim of this study was to characterize keratinolytic bacteria isolated from feather waste. Feather waste is generated in large amounts as a by-product of commercial poultry processing. This residue is almost pure keratin, which is not easily degradable by common proteolytic enzymes. The crude protein from feather has of high nutrient value and could be used as animal feed for livestock and fish feed in aquaculture. Feather constitutes over 90% protein, the main component being beta-keratin, a fibrous and insoluble structural protein extensively cross linked by disulfide bonds. This renders them resistant to digestion by animals, insects and proteases leading to serious disposal problems. It is degraded only by keratinase enzyme. These enzymes were produced by some species of *Bacillus*. In the present study, *B. licheniformis* was used for degrading keratin substrate such as feathers. Based on morphology and biochemical analysis, the isolates were identified as *Bacillus* spp. Fermentation using feather as a substrate was carried out on minimal salt media for seven days which resulted in almost complete degradation of feather. The optimum conditions for keratinase production were temperature 37°C, pH 7.0 and initial substrate concentration 1%. Maximum enzyme activity was found to be 100 U/L with the protein concentration of 4 µg/ml.

Keywords:
Feather, keratin, feather degrading bacterium, poultry waste, keratinase, keratinolytic activity.

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INTRODUCTION

Feathers, which are almost pure keratin proteins, are produced in large amounts as a waste by-product at poultry-processing plant. A total of 5-7% weight of mature chicken comprises of feathers (Avinash *et al*., 2011). Feather waste constitutes beta-keratin, a insolvable protein (Swetlana and Jain, 2011). In addition to this, feather waste is produced at the rate of 22 million kg per year (US alone) (Savitha *et al*., 2007). A group of proteolytic enzymes which are able to hydrolyze insoluble keratins more efficiently than other proteases are called keratinizes produced by some microorganisms. These bacterial strains produce enzymes which selectively degrade the beta-keratin found in feathers. These enzymes make it possible for the bacteria to obtain carbon, sulphur and energy for their growth and maintenance from the degradation of beta keratin (Muthusamy *et al*., 2011). Keratins are the most abundant proteins in epithelial cells of vertebrates and represent the major constituents of skin and its appendages such as nail, hair, feather, and wool. Keratins are grouped into hard keratins (feather, hair, hoof and nail) and soft keratins (skin and callus) according to sulphur content (Scott and Untereiner 2004). The protein chains are packed tightly either in α-helix (α-keratins) or in β-sheet (β-keratins) structures, which fold into final 3-dimensional form (Veslava *et al*., 2009)

Keratinases which are produced by these keratinolytic organisms could be used to degrade feather waste and further the digested products could be a excellent material for producing animal feed, fertilizers or natural gas (Tamilmani *et al*., 2008). Use of keratinolytic microorganisms for feather degradation is an economical, environmental friendly alternative. Keratinolytic proteases offer considerable opportunities for a low energy consuming technology for bioconversion of poultry feathers from a potent pollutant to a nutritionally upgraded protein feed for live stock (Jahan *et al*., 2010; Vigneshwaran *et al*., 2010). Most feather waste is land filled or burned which involves expense and can cause contamination of air, soil and water. Bacterial strains are known which are capable of degrading feathers (Savitha *et al*., 2007). Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking may not only destroy the amino acids (methionine, lysine and histidine) but also consume large amounts of energy. Utilizing poultry feathers as a fermentation substrate in conjunction with keratin-degrading microorganism or enzymatic biodegradation may be a better alternative to improve nutritional value of poultry feathers and reduce environmental waste (Veslava *et al*., 2009). These feathers constitute a sizable waste disposal problem. Several different approaches have been used for disposing of feather waste, including land filling, burning, natural gas production and treatment for animal feed. Most feather waste is land filled or burned which involves expense and can cause contamination of air, soil and water (Rai Sapna *et al*., 2011; Savitha *et al*., 2007). Feathers hydrolysed by mechanical or chemical treatment can be converted to feedstuffs, fertilizers, glues and foils or used for the production of amino acids and peptides (Jahan *et al*., 2010; Andrea *et al*., 1996). An alternative to decrease this pollution is the utilisation of feather constitutes that can be used as animal feed, preventing accumulation in the environment and the development of some types of pathogens (Veslava *et al*., 2009).

MATERIALS AND METHODS

Enrichment

1 g of poultry waste was serially diluted in order to reduce the initial number of micro organisms. This dilution was then inoculated on minimal feather agar media. Feathers were washed, dried and hammer milled prior to being added to the medium. The medium was sterilized by autoclaving. All incubations were done at 37°C.

Screening

Skim milk agar (Himedia) was prepared and the above dilutions were streaked on milk agar plates for testing the caseinolytic activity of the organism. Bacteria were inoculated onto plates and incubated at 37°C for 24 h. Strains that produced clear zones in this medium were selected.

Subculturing

The organism screened with Keratin agar plates was subcultured by continuously growing the bacterium in minimal broth medium (3 days at 37°C, 120 rpm) and subsequently streaking on minimal agar medium (1.5% agar, 2 days 37°C).

Identification of Isolated feather degrading bacteria

Gram Stain, Spore staining, Motility test and Catalase Test.

Characterization of the isolate using Biochemical assays

IMViC Test, Hydrogen Sulfide Test, Urease Test, Lipid hydrolysis, Carbohydrate Fermentation, Starch hydrolysis and Gelatin liquefaction.

Production of keratinolytic enzyme

The bacterial isolate was cultivated in 100 ml minimal feather media. The samples were withdrawn and centrifuged at 6000 rpm for 10min. The supernatants were preserved at 4°C and assayed for protein.

Determination of keratinase activity

Azocasein hydrolysis was used as an alternative to the azokeratin hydrolysis. Keratinolytic protease activity was determined with azocasein (Sigma Co. St. Louis. Mo.) as a substrate by azocasein solution in 0.05 M Tris -HCI buffer at pH 8.5, which was incubated with 400 µl crude enzyme solution for 30 min at 37°C in a water bath with shaking. The reaction was terminated by addition of 1.4 ml of 10% trichloroacetic acid (TCA). After 15 min at 4°C, the reaction mixture was centrifuged at 10,000xg for 10 min. One ml supernatant was mixed with 1 ml of 0.5 N NaOH and the absorbance was read at 440 nm. The control was treated in the same way, except TCA was added before the addition of crude enzyme. One unit of caseinolytic activity was determined as the amount of enzyme that produces an increase in absorbance of 0.01 per min under the assay conditions. The soluble protein concentration in the culture supernatant was estimated according to the Bradford method (Bradford, 1976).

Table 1: Biochemical Characterization.

Sl. No	Bio-Chemical Test	*Bacillus* sp
1.	Indole production Test	Positive
2.	Methyl Red Test	Positive
3.	Voges proskaeur Test	Negative
4.	Citrate Utilization Positive	Positive
5.	Carbohydrate fermentation Test	Positive/ Presence of Air Bubbles
6.	Catalase	Positive
7.	Starch hydrolysis	Positive
8.	Urease	Positive

Enzyme characterization

Taking Temperature, pH, Substrate concentration, Activator and Inhibitor as parameters, characterization of enzyme was done.

Feather degradation

100 ml of Nutrient broth is prepared; 1 gm of feather is added to the media and sterilized. A loop full of inoculum is inoculated into the media and incubated for seven days at 37°C. Residual feathers were harvested from the fermentation media by filtering it over whatman filter paper No: 3. The harvested feathers were kept in hot air oven at 50°C until weight stabilized to constant value. The difference between the weight of residual feather obtained from the control and that of inoculated media has been used as measure of feather degradation. Degradation was expressed in percentage.

RESULTS

A screening program was employed to obtain bacterial isolates capable of producing feather degrading extracellular keratinase enzyme using feather (keratin) as sole carbon substrate. The potential isolate was then characterized and identified to its genus level.

Identification of bacteria was based on morphological, cultural and biochemical tests comparing the data with standard species (Hoq *et al*., 2010). Morphological and physiological characteristics of the bacteria were compared with the Bergey's Manual of Systemic Bacteriology. The isolate was Gram positive, rod shaped and spore-former and were able to utilize both glucose and sucrose but not lactose. They were also catalase positive, oxidase negative. MR test is positive and VP test negative for these organisms. The organisms were unable to utilize citrate and all were able to reduce nitrate to nitrite. The isolate showed typical characteristics of *Bacillus licheniformis*.

Characterization of Enzyme

Characterization was done by determining the effect of pH, Temperature, Activator, Inhibitor and Substrate.

Effect of Temperature and pH on Keratinase enzyme

The activity of the enzyme at various temperatures and pH was studied and graphs are plotted. The optimum temperature and pH is 37°C and 7 respectively (Fig 1 and 2).

Effect of Activator and Inhibitor on Keratinase enzyme

The enzyme samples were checked for the effect of activator and Inhibitor. Zinc Chloride is used as activator and EDTA is used as an inhibitor. It was observed that as the concentration of the activator increased, activity of the enzyme increased where as the concentration of the inhibitor increases the activity of the enzyme decreases (Fig 3 and 4).

Table 2 : Rate of feather degradation

Time in weeks	Concentration of feather in gms	Rate of degradation in %
1	1	25
2	1	79
3	1	85

Effect of Substrate on Keratinase enzyme

The activity of the enzyme at different concentration was carried out and the graph is plotted (Fig 5).

Rate of degradation

Bacillus sp was able to grow and produce keratinase in nutrient medium in which feather meal served as an additional carbon and nitrogen source and acted as enzyme inducer, resulted in 85% of feather degradation in seven days at 37°C. Kerotinolytic activity was measured in the absorbance at 440 nm by the standard enzyme assay method.

DISCUSSION

A bacterium isolated from poultry waste has been shown to degrade feather keratin. The identification of the keratinolytic bacteria was based on cell morphology, colony morphology, and several other methods. These results suggested that the strain belong to genus *Bacillus* (Saritha Agrahari and Neeraj Wadhwa 2010). The most studied keratinolytic bacteria are *Bacillus* spp., which have been described to possess feather-degrading activity (Kim *et al*., 2001; Lin *et al*., 1999). Through the strategy of isolation of keratinolytic

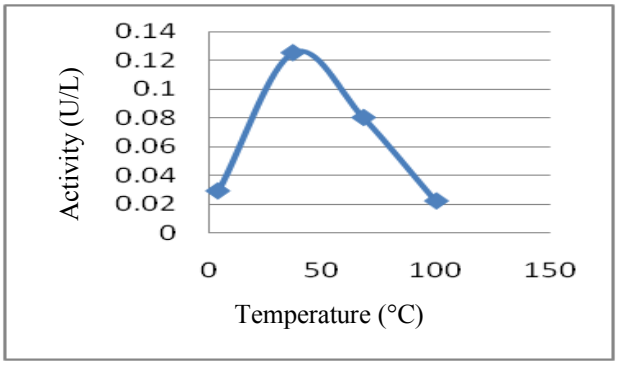

Fig 1: Effect of Temperature

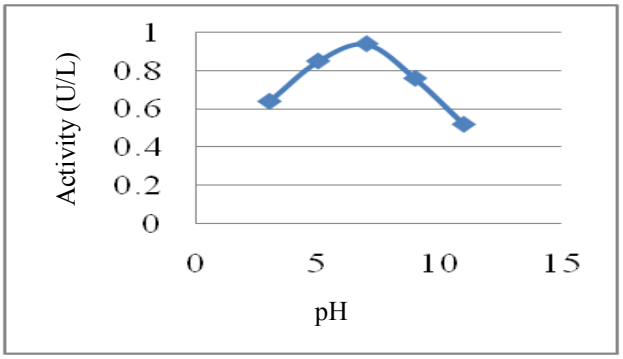

Fig 2: Effect of pH

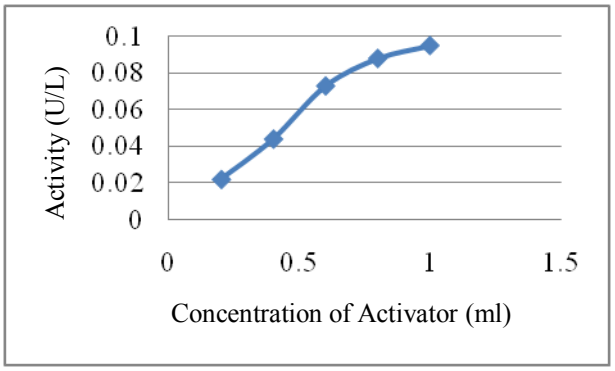

Fig 3: Effect of Activator

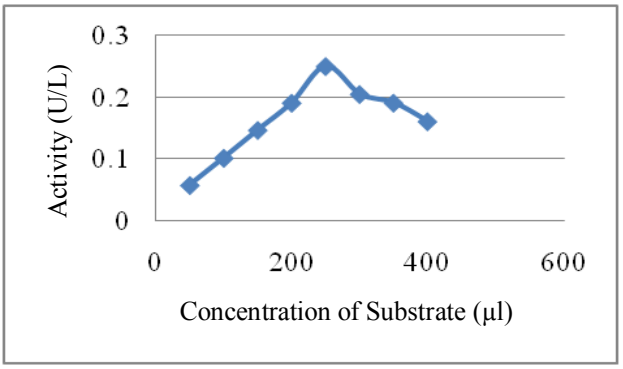

Fig 5: Effect of Substrate

microorganisms utilized in this work, bacteria presenting high keratinolytic activity were selected. Considering that feather protein has been showed to be an excellent source of metabolizable protein (Klemersrud *et al*., 1998), and that microbial keratinases enhance the digestibility of feather keratin (Lee *et al*., 1991; Odetallah *et al*., 2003), these keratinolytic strains could be used to produce animal feed protein. The enzyme was stable at the pH range of 6-8 (Cheng *et al*., 2007). The activity decreased at pH 3.0 and 8.0. The isolated bacterium showed Optimum keratinolytic activity at 37°C and pH 7.0. The enzyme also showed to be stable at 60°C and pH 9.0 (Kurt Cotanch and Grant 2007). In the present study, it has been recorded that the *Bacillus licheniformis* degraded the feather at a rate of 85% at 37°C in seven days where as Williams *et al*., (1990) reported that *Bacillus licheniformis* PWD-1 degraded chicken feather completely at 50°C in 10 days. (Bockle *et al*.,1995) demonstrated that Streptomyces pactum DSM40530 partially degraded native chicken feather at 50°C, the maximum feather degrading activity was at 50°C.

On increasing the feather concentration the extent of feather degradation decreases because of a decrease in keratinase activity. It indicates that at higher substrate concentration repression of keratinase production can take place. This observation is similar to previous studies which concluded that a low concentration of substrate is optimum for yielding maximum enzyme activity (Avinash *et al*., 2011).

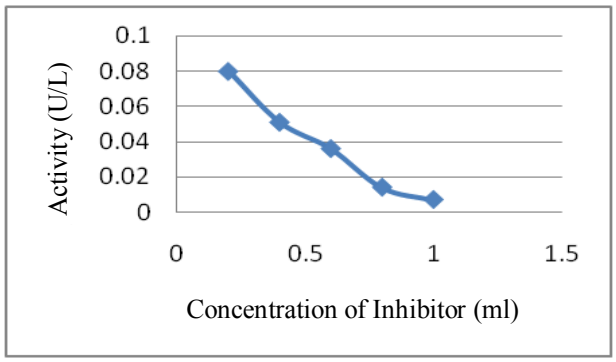

Fig 4: Effect of Inhibitor

CONCLUSION

In the present study, we isolated the *Bacillus* sp. capable of producing keratinase from habitats where keratin containing substrate is decomposed under natural conditions. The isolate exhibited highest keratinase activity is the most potential isolate which degraded feather at a rate of 85% after seven days at 37°C. The keratinolytic microorganisms isolated in this study therefore could play an important role in the production of animal feed protein in addition to the biodegradation of poultry wastes. The degradation of feathers with keratinolyticmicro organisms is the best eco friendly approach in the poultry feather waste management. This novel keratinolytic isolate could be a potential candidate for the degradation of feather keratin and also in de-haring process at leather industry.

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