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OPTIMIZATION OF CULTURAL CONDITIONS FOR EXTRACELLULAR KERATINASE PRODUCTION BY *BACILLUS* SPECIES ISOLATED FROM POULTRY FARM SOIL

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ABSTRACT

Poultry industry waste contains a major proportion of feathers, which comprise mainly of keratin.Keratin is an insoluble, fibrous protein, which makes the feathers resistant to proteolytic enzymes. Several microorganisms can carry out the degradation of such complex substrates. The present study exploits the ability of soil bacteria to degrade feather wastes. Bacteria can degrade feather keratin with their enzyme: keratinase. By screening poultry farm soil samples, two feather degrading isolates were obtained which showed degradation of 1% feathers within 7 days. On the biochemical identification and 16S rRNA sequencing, the isolates showed 99% homology with *Bacillus licheniformis* and *Bacillus subtilis.* Medium optimization for maximum enzyme production was performed. The optimized conditions showed significant increase in enzyme production, as determined by the activity i.e. 14.344U/ml/min for *B. licheniformis* and 12.934U/ml/min for *B. subtilis.* The study shows the potential of the isolates to carry out eco-friendly disposal of feathers.

KEYWORDS: **Keratinase, Poultry waste, Keratin, Proteolytic enzymes**

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INTRODUCTION

The consumption of chicken leaves behind tons of feathers as a waste. Worldwide, around 18,500 lakh tons of poultry feather is generated annually, of which India's contribution alone is 3500 tons[1]. Feathers are biochemically rigid, therefore tend to accumulate. With the increasing demand for poultry animals in the food sector in the coming years, feather waste generation would only increase, further adding to the environmental problems. Disposal of feather waste is quite challenging. The traditional methods are drastic such as incineration and landfilling[1]. However; these methods have extensive operating costs, consume energy, result in loss of natural resources and have environmental implications[1]. Feathers represent approximately 5-7% of the total weight of poultry animals, form the exo-skeleton of birds and fulfill the functions of insulation, locomotion and protection. They contain a considerable amount of keratin (90%) [2]. Keratin is a mechanically durable and chemically un-reactive protein. This property is due to the molecular configuration of the constituent amino acids, high degree of cross-linkages of disulphidebridges, hydrogen bonds and hydrophobic interactions[2, 3]. These properties make native keratin highly inert, water-insoluble and undegradable by most proteolytic enzymes: trypsin, pepsin, papain etc. Considering the high proportion of keratin in feathers and amino acid content, feather waste can have several value-added applications[2,4]. Thus, recycling of feather waste becomes a subject of interest. One such application is to obtain nutritionally upgraded animal feedstuff. Up till now, feather waste is being converted to a readily digestible feather meal by hydrothermal treatment[5, 6]. Basically, this treatment employs high temperature and pressure, thus making the keratin less complex and easy to digest. While the use of high temperature results in a readily digestible meal, it has also results in the loss of several essential amino acids such as lysine, methionine and tryptophan and generates non-nutritive amino-acids such as lysinoalanine and lanthionine[7, 8]. Thus, hydrothermal treatment does not sufficiently upgrade the nutritional content of the feather meal.

Microbial degradation of feathers appears to be a viable alternative to obtain a feather meal that would be nutritionally upgraded with essential amino-acids. This biotechnological approach would convert the indigestible feather waste to a readily digestible feather meal, and it would also retain the valuable amino acids previously present in the keratin. It would also add to the existing amino acids, since it involves microorganisms and microbial enzymes[6]. Thus, the feather meal obtained after such microbial treatment would be complete. Also, the technology would considerably bring down the cost since it would not require hydrothermal treatment. Moreover it uses feather waste which would be a cheap raw material. A great diversity of micro-organisms in nature is capable of carrying out keratin degradation [10]. Many bacteria and fungi have been reported and identified to carry out keratin degradation. Keratinolytic bacteria include *Actinomycetes* sp. and bacteria belonging to genus such as *Bacillus* sp., *Micrococcus* sp., *Clostridium* sp., etc[9, 10, and 11]. These keratinolytic micro-organisms exist in different ecological and environmental conditions all having different capacities to solubilize keratin- containing substrates. The keratin degrading fungi that are studied include many dermatophytic fungi and non-dermatophytic keratinolytic fungi[12, 13]. Microorganisms are capable of keratin degradation due to the keratinases (EC 3.4.21/24/99.11) which are generally extracellular proteases[14]. This category of protease is gaining added importance due to several associated applications such as hydrolysis of a significant amount of keratin containing by-products obtained from poultry industry, agro-industrial processing etc. The present study involves exploring the ability of

these microorganisms to carry out biodegradation of feathers, therefore suggesting an alternative method for the treatment of the feather waste.

MATERIALS AND METHODS

Agar powder and Nutrient Agar were purchased from Himedia Laboratories, Mumbai, India. All the other chemicals were purchased from Qualigens Fine Chemicals, Mumbai, India.

1. Collection of Soil Samples, Enrichment and Screening

Soil samples were collected from poultry farms in Maharashtra: Nasik and Mumbai. Samples were collected in plastic bags and transported to the research center.Each soil sample was processed for three successive enrichments of 20 to 25 days each. For the first enrichment, 5 grams of the soil sample were inoculated in 100ml of Minimal Salts Medium in a 150 ml conical flask containing(g/1L): NaCl 0.05, K_2HPO_4 0.01, KH_2PO_4 0.01, $MgSO_4$ 0.002, $(NH_4)_2SO_4$ 0.001 and white chicken feathers10, as a sole source of carbon and nitrogen. White chicken feathers which were procured from a local poultry shop were first washed thoroughly under tap water, followed by subjecting them to a brief heat treatment in boiling water bath (approximately 100^oC) for removal of surface impurities and treatment with chloroform:methanol(1:1) for defatting. For the next enrichments, 10 ml of the supernatant from the previous enrichment was taken aseptically and transferred into 90 ml of fresh MSM with 1% feathers. The enrichments were carried out at room temperature under shaker conditions at 150 rpm/min. Regular viable counts were taken to assess the enriched microflora, and colonies, persistently appearing up till the third enrichment, were selected for the screening test. The screening test was performed on an agar based Minimal Salts Medium, containing finely chopped feathers (1-2mm)as a sole source of carbon and nitrogen. On this medium, selected organisms were spot inoculated and incubated at room temperature (30^oC ±2^oC) and they were observed for a zone of clearance around the spot inoculated culture. Cultures showing the zone of clearance were selected for identification.

2. Identification

Identification was done by microscopic, cultural and biochemical analysis as prescribed by the Bergey's Manuel of Systematic Bacteriology[15].

3. 16S rRNA Sequencing

The identification was confirmed by carrying out 16SrRNA sequencing. DNA was extracted according to the standard protocol for extraction of Bacterial genomic DNA (gDNA). The extracted gDNA was qualitatively analyzed by Agarose Gel Electrophoreses. Amplification of the 16SrRNA gene was performed as per the standard procedure for the PCR reaction of bacterial gDNA using universal forward primer: 8F (5'AGAGTTTGATCCTGGCTCAG3') and reverse primer: 1391R (5'GACGGGCGGTGTACA3') [16]. The amplified bacterial gDNA samples were sent for sequencing to GeneOmbio Research laboratories, Pune. The sequencing results were analyzed using NCBI BLAST tool.

4. Determination of Keratinase Activity

i. Preparation of DMSO-solubilized Keratin: - Preparation of soluble keratin was carried out by a simplified procedure by Wawrzkiewics *et al*., 1987[17]. Briefly, defatted chicken feathers were finely chopped with scissors into powder form. 10 grams of this fine feather powder was reacted with 500 ml of DMSO. This mixture was heated in an oven at 100^oC for 120 minutes, instead of refluxing in a reflux condenser. 1000 ml of cold acetone was then added to precipitate the soluble keratin. Precipitation was further carried out in a deep freezer for 2 hours. The precipitate was obtained by centrifugation at 10,000 rpm for 20 minutes. It was washed thoroughly, thrice, with distilled water and dried in a vacuum dryer at 40^oC. 1 gram of quantified precipitate was dissolved in 20 ml of 0.05N

NaOH solution. The pH of this solution was adjusted to 8 using 0.1 mol/L TrisHCl.

ii. Preparation of seed culture: - feather degrading organisms were grown in Minimal Salts Medium (MSM) containing 1% feathers as carbon and nitrogen source and the initial pH of the medium was adjusted to 7. The incubation was carried out at room temperature under shaker conditions (150 rpm) for 48 hours.1ml of this actively growing culture was used as the seed culture for further experiments. The cell free supernatant was used as a source of crude enzyme and also for quantification of proteins.

iii. Keratinase Assay: - The keratinase activity was assayed by reacting 1 ml of crude enzyme with 1 ml of the keratin solution in a water bath at 50^{o}C with shaking for 10 minutes. The reaction was then stopped by addition of an equal volume of 20% trichloroacetic acid. The precipitate was removed by centrifugation at 10,000 rpm for 15 minutes and the optical density of the supernatant was measured at 280 nm. One unit of keratinase activity was defined as an increase in the corrected absorbance (A_{280}) by 0.01units with respect to a control, per ml per minute under the conditions described above. It was calculated as: $U=4\times n\times A_{280}/(0.01\times 10)$, Where n is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min) [18]. During the present study, the enzyme production by the bacterial cells in broth has been quantified as Units of Enzyme Activity per Milliliter of cell free broth per minute.

5. Optimization of cultural conditions for maximum enzyme production

For optimization of medium, 1ml of fresh seed culture was inoculated into 25 ml of Minimal Salts Medium and incubation was carried out under shaker conditions at 150 rpm for 5 days after which the cell free supernatant was assessed for keratinase activity.

i. Investigation of the effect of additional Carbon source: - The addition of three different Carbon sources was investigated, one at a time: Glucose, Sucrose and Mannitol. Sugar solution was added in the growth medium to achieve its final concentration 1% in the MSM of which the feather concentration was maintained at 1% (w/v)

ii. Optimization of initial pH of the growth medium: - The bacteria were grown at different values of initial pH- 5, 6, 7, 8, 9.

iii. Optimization of growth temperature: - The cultivation of bacteria was carried out at different incubation temperatures: 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C.

iv. Optimization of Substrate (feather) concentration: - The bacteria were cultivated in the presence of different feather concentrations: 0.1%, 0.25%, 0.5%, 0.75%, 1%, 1.5% and 2% (w/v). All the above experiments were carried out in triplicates.

RESULTS AND DISCUSSION

1. *Isolation and identification*

Soil samples from poultry farms in Maharashtra were enriched and screened for feather degrading bacteria. On the basis of screening on Feather agar plates, two isolates were selected for identification. As per the cultural, microscopic and biochemical tests, these bacteria were found to belong to the genus *Bacillus* sp. (Tables 1, 2). Further identification was performed by 16S rRNA sequencing. The isolates 1 and 2 showed 99% homology with *Bacillus licheniformis* and *Bacillus subtilis* respectively. Previous studies by Cai *et al*., 2008 [19], Kim *et al*., 2001 [9]; Gessesse *et al*., 2003 [20]and El-Refai *et al*., 2005 [21] have reported keratinolytic bacteria belonging to the genus *Bacillus* sp. Both the isolates were able to degrade the feathers within 7 days of incubation. The observations of the present study with respect to time for complete degradation are comparable with previous studies by Agrahari *et al*., 2010 [22] and Naghi *et al*., 1998 [23].

Feather degradation in liquid medium

Figure 1
Flask on left showing intact feathers in liquid medium before inoculation and flask on right showing complete degradation within seven days.

Table 1
General Characteristics of Isolates

Characteristics	Isolate 1	Isolate 2
Gram Nature	Gram Positive	Gram Positive
Microscopic Morphology	Endospore forming *Bacilli*	Endospore forming *Bacilli*
Sporulation	Central Endospore	Sub-terminal Endospore
Motility	Motile	Motile
O_2 Requirement	Facultative Anaerobic	Facultative Anaerobic

Table 2
Biochemical Characteristics

Isolates	Isolate 1	Isolate 2
Tests to distinguish between aerobic and anaerobic breakdown of carbohydrates		
O/F aerobic	Positive (utilization of sugar)*	Positive (utilization of sugar)*
O/F anaerobic	Positive (utilization of sugar)*	Positive (utilization of sugar)*
Tests to show degradation of range of carbohydrates and related compounds		
Glucose	Acid Production	Acid Production
Sucrose	Acid Production	Acid Production
Lactose	No Acid/Gas Production	No Acid/Gas Production
Maltose	Acid Production	No Acid/Gas Production
Mannitol	Acid Production	Acid Production
Xylose	No Acid/Gas Production	No Acid/Gas Production
Tests for Specific Breakdown Products		
Methyl Red	Negative	Positive
Vogues-Proskauer	Positive	Negative
Tests to show Ability to utilize particular Substrate		
Starch	Positive	Positive
Citrate	Negative	Negative
Tests for Metabolism of Proteins and Amino-acids		
Indole Production	Negative	Negative
Arginine dihydrolyase	Positive	Negative
Gelatin hydrolysis	Positive	Positive
Tests for Enzymes		
Catalase	Positive	Positive
Oxidase	Negative	Negative
Urease	Positive	Positive
Nitrate Reduction	Positive	Positive
Combined Tests		
Triple Sugar Iron (TSI) reaction	K/A**	K/A**

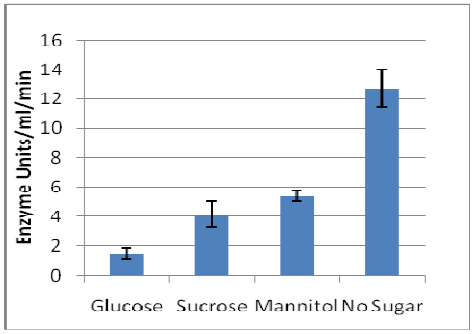
Key: *Utilization of carbohydrate resulted in yellow coloration in the medium, indicating aerobic as well as anaerobic fermentation. ** Alkaline Slant, Acidic butt.

2. *Optimization of Media for maximum enzyme production*

Investigation of presence of additional Carbon source in the growth medium: On supplementation with simpler carbon sources i.e. 1% Glucose, 1% Sucrose and 1% Mannitol in the growth medium, the enzyme production as determined by the activity decreased. Addition of these simpler, readily utilizable carbon sources, led to decreased degradation of feathers, which is a comparatively difficult substrate to degrade. However, in the absence of these additional carbon sources, feathers remained the sole source of carbon, leading to a significantly higher enzyme production and also better degradation. Efficient feather degradation in the absence of a simpler carbon source (Graph 1, 2) suggests the inducible characteristic of the keratinase. The results are in agreement with the studies carried out by Santos *et al*., 1996[25], Ignatova *et al*., 1999[26], and Laba *et al.* 2010[24] in which addition of simpler carbon sources have led to decreased feather degradation and reduced enzyme production. Thus, the isolates produced a lesser active keratinase in the presence of other simpler carbon sources. In order to achieve a better degradation of feathers, absence of a simpler carbon source was preferred.

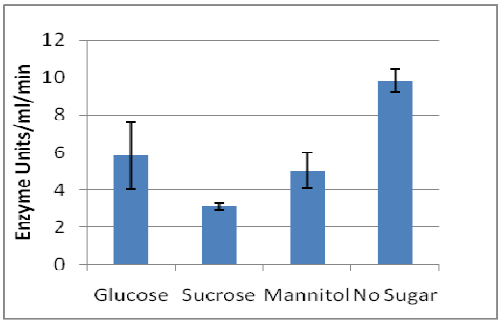
Graph 1

Effect of additional carbon source on enzyme production in the growth medium of isolate 1



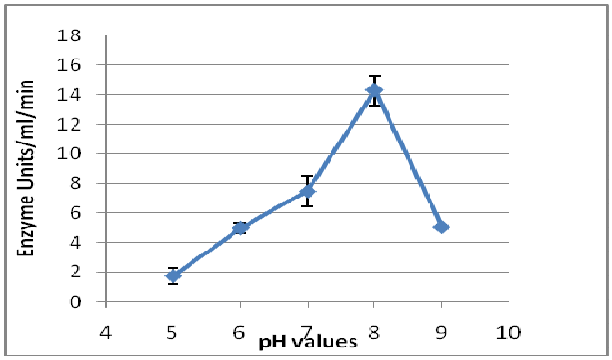
Graph 2

Effect of additional carbon source on enzyme production in the growth medium of isolate 2

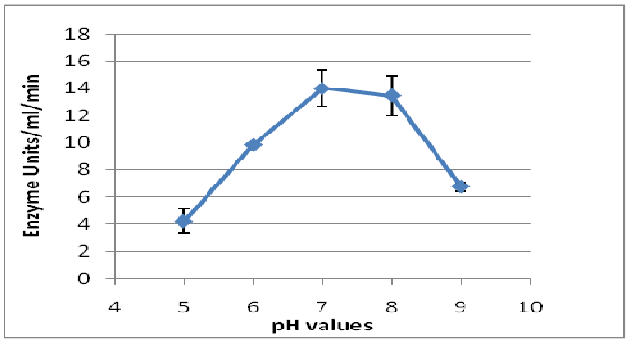


i. Investigation of effect of initial pH of the growth medium: The effect of five different initial pH values was studied: pH 5, pH 6, pH 7, pH 8 and pH 9. For isolate 1, the maximum enzyme production and best degradation was achieved at pH 8 (Graph 3) which is in accordance to previous reports of alkaline keratinases[20, 27], whereas for isolate 2, this was achieved at pH 7-8 (Graph 4). Few reports showed optimum keratinase production at neutral pH[9]. This explains that the initial pH of the growth medium does affect the production and activity of the enzyme.

Graph 3
Effect of Initial pH on Enzyme Production of Isolate 1

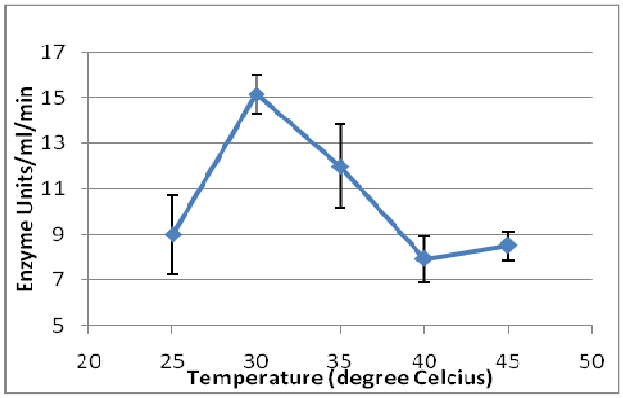


Graph 4
Effect of Initial pH on Enzyme Production of Isolate 2

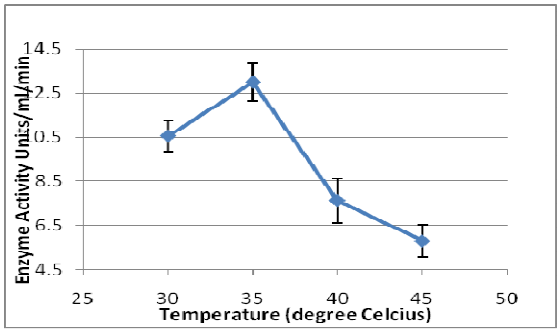


ii. Investigation of effect of incubation temperature: For isolate 2 the optimum temperature for enzyme production was found to be 35°C, while for isolate 1, the optimum temperature was found to be 30°C (Graph 5, 6). The present study involves isolates belonging to *Bacillus* species, which are known to have growth temperature ranging between 30°C up to 55°C. The results are comparable with the studies carried out by Kumar *et al.* 2011[27], however, there are reports indicating keratinolytic bacteria having thermophilic optimum temperatures[9, 28].

Graph 5
Effect of Incubation temperature on Enzyme Production of Isolate 1

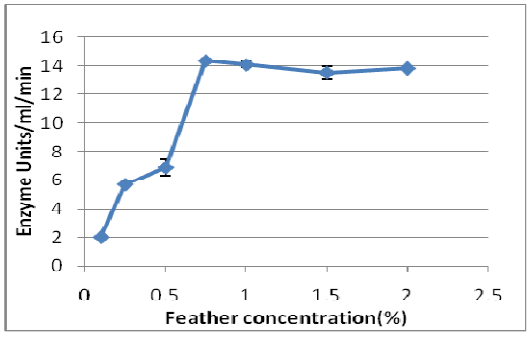


Graph 6
Effect of Incubation temperature on Enzyme Production of Isolate 2

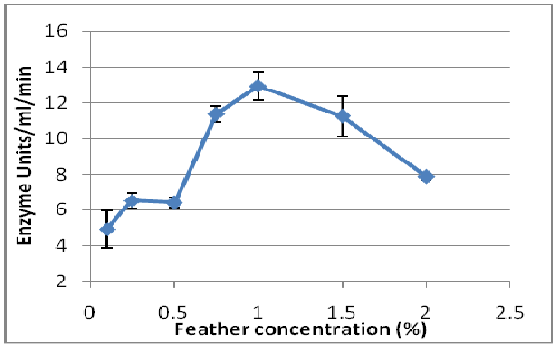


iii. Investigation of effect of Substrate (feather) concentration of the growth medium: The effect of seven different feather concentrations was studied: 0.1%, 0.25%, 0.5%, 0.75%, 1%, 1.5% and 2% (w/v) of chopped white chicken feathers was added into the growth medium and the enzyme production was determined. In case of the isolates under study, there was steady increase in the enzyme production as determined by the activity up to 0.75% and 1% respectively after which there was saturation for isolate 1 (Graph 7) and decrease in activity for isolate 2 (Graph 8). Substrate concentration is a prime factor that affects enzyme production. To achieve the best degradation and maximum enzyme production, therefore, the concentration of the substrate was maintained at 0.75% and 1% respectively.

Graph 7
Effect of Feather concentration on Enzyme Production of Isolate 1



Graph 8
Effect of Feather concentration on Enzyme Production of Isolate 2



CONCLUSION

The feather degrading isolates were capable of utilizing feathers as a primary source of carbon and nitrogen, and therefore capable of degrading them. Complete degradation of feathers was achieved within 7 days. Thus, these isolates show a potential for biodegradation of feather waste. The media conditions were optimized, which resulted in a significant increase in the enzyme production (Isolate 1: 14.344 U/ml/min and Isolate 2: 12.934 U/ml/min) and improved degradation. These data are crucial for the mass production of keratinase for further studies i.e. purification and characterization of keratinase and enzyme kinetics. Also, microbial degradation of feathers by the isolated *Bacillus* species can be scaled up for synthesis of amino-acids as a product of keratin degradation in a commercial way using feather waste as a cheap raw material.

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