

ISOLATION OF A NOVEL FEATHER-DEGRADING BACTERIUM AND OPTIMIZATION OF ITS CULTURAL CONDITIONS FOR ENZYME PRODUCTION

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ABSTRACT

Objective: A large amount of feather waste is generated from the poultry industry. This waste containing a considerable amount of keratin can be utilized by microorganisms thus leading to its environmentally safe disposal. The objective of the study was to isolate and identify a feather degrading bacterium from poultry farm soil, optimize the cultural conditions, followed by exploring the applications.

Method: Briefly, the study was carried out by collecting soil samples, enrichment using an appropriate enrichment medium, followed by screening, identification and then optimizing cultural conditions

Results: The isolated keratinolytic bacterium carried out complete degradation of the feather waste within 7 days and was found to be of *Bacillus sp.* by biochemical identification and showed 98% homology with *Bacillus sonorensis strain NRRLB-23154* by 16SrRNA sequencing. On optimization of growth medium, the enzyme production as determined by the enzyme activity increased.

Conclusion: The study shows the potential of the feather degrading isolate to carry out environmentally safe disposal of poultry waste and also to produce amino acids from a cheap raw material.

Keywords: Feather- degrading bacterium, Poultry waste, *Bacillus*.

INTRODUCTION

Tons of feather waste is left behind due to chicken consumption. Worldwide, around 18,500 lakh tons of poultry feather is generated annually, of which India's contribution alone is 3500 tons [1]. These feather wastes are biochemically rigid, and therefore tend to accumulate. The demand for poultry animals in the food sector would keep on increasing in the coming years, thus, feather waste generation would only increase, further adding to the environmental problems. Disposal of feather waste is quite challenging. The traditional methods for disposal of feather wastes include incineration and land filling [1]. However; these methods have extensive operating costs, consume energy, result in loss of natural resources and have extreme environmental implications [1].

Feathers represent approximately 5-7% of the total weight of poultry animals, form the exo-skeleton of birds and fulfil the functions of insulation, locomotion and protection. They contain a considerable amount of keratin (90%) [1, 3]. Keratin fibres from feathers are non-abrasive, eco-friendly, bio-degradable, insoluble in organic solvents and have good mechanical properties [3]. This property is due to the molecular configuration of the constituent amino acids, high degree of cross-linkages of disulphide bridges, hydrogen bonds and hydrophobic interactions [2, 3]. Therefore, native keratin is highly inert, water-insoluble and un-degradable by most proteolytic enzymes such as trypsin, pepsin, papain etc.

The high proportion of keratin in feathers makes feather waste useful for 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 using this feather waste. Up till now, feather waste is being converted to a readily digestible feather meal by hydrothermal treatment [5, 6]. The use of high temperature and pressure for this conversion makes the keratin easy to metabolize. Simultaneously, it also results in the loss of several essential amino acids such as lysine, methionine and tryptophan and generates non-nutritive amino-acids [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 rigid feather waste to a readily digestible feather meal.

Not only would it retain the existing valuable amino acid content of keratin, but it would also add to it, it involves microorganisms and microbial enzymes [6]. Thus, the feather meal obtained after such microbial treatment would have sufficient nutritional value. Also, the microbial technology would considerably bring down the cost since it would not require hydrothermal treatment. Moreover, feather waste would be a cheap raw material.

A diversity of microorganisms is capable of carrying out keratin degradation [10]. Many bacteria and fungi have been reported to carry out keratin degradation. Keratinolytic bacteria include *Actinomyces sp.*, *Bacillus sp.*, *Micrococcus sp.*, *Clostridium sp.*, etc [9, 10, and 11]. These keratinolytic micro-organisms exist in different ecological conditions all having their own preferences to solubilize keratin- containing substrates. Keratin degrading fungi include many dermatophytic fungi and non-dermatophytic keratinolytic fungi [12, 13]. The keratin degrading ability of the microorganisms can be attributed to extracellular protease, keratinases (EC 3.4.21/24/99.11) [14]. This category of protease is gaining added importance due to several associated applications such as hydrolysis of various 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

Nutrient Agar, Dextrose, Sucrose and Mannitol were purchased from Himedia, Mumbai, India. Agarose was purchased from Lonza, India. All other chemicals were purchased from S.D. Fine Chemicals, India.

Collection of soil samples, Enrichment and Screening

Soil samples were collected from poultry farms in sterile plastic bags. 5 grams of soil sample was weighed and added aseptically into 100 ml of liquid Minimal Salts Medium (MSM) containing (grams/100ml): NaCl 0.5, K₂HPO₄ 0.1, KH₂PO₄ 0.1, (NH₄)₂SO₄ 0.1, MgSO₄ 0.02 and white chicken feathers 1, as a sole source of Carbon. Chicken feathers were obtained from a local poultry shop, washed thoroughly in tap water, subjected to a brief heat treatment using boiling water bath (approximately 100°C) followed by treatment with chloroform:water (1:1) for de-fatting. Three consecutive enrichments of 20-25 days were carried out for every soil sample at

room temperature (approximately 25°C) under shaker conditions (150 rpm). During the enrichment period, regular viable counts were performed using Nutrient Agar plates to assess the type of microflora getting enriched. Colonies that appeared consistently during the viability check were selected for screening on Feather Agar plates containing (grams/100ml) NaCl 0.5, K₂HPO₄ 0.1, KH₂PO₄ 0.1, (NH₄)₂SO₄ 0.1, MgSO₄ 0.02, Agar powder 1.5 and finely chopped white chicken feathers 1. Colonies showing a zone of clearance on feather agar plates were selected for identification.

Identification and 16S rRNA Sequencing and Phylogenetic Analysis

Identification of the feather degrading isolate was carried out by studying the microscopic, cultural and biochemical characteristics according to the Bergey's Manual of Systematic Bacteriology [15]. For further Identification by 16S rRNA sequencing, the genomic DNA (gDNA) was extracted and according to the standard bacterial gDNA extraction protocol [16]. After qualitative analysis by Agarose Gel Electrophoresis, gDNA was amplified by Polymerase Chain Reaction (PCR) using universal bacterial primers: 8F forward primer (5'AGAGTTTGTATCCTGGCTCAG3') and 1391R reverse primer (5'GACGGGCGGTGTACA3')[16]. The gDNA was amplified using thermal cycler - Applied Bio systems. The amplified gDNA sample was outsourced for sequencing to GeneOmBio Research Laboratory, Pune. The 16S Ribosomal gene sequence obtained was then analysed using NCBI BLAST tool. Alignment of the 16S rRNA gene sequence with 20 closely related gene sequences was performed using CLUSTALW2 and distance based phylogenetic tree was constructed using neighbour joining (NJ) algorithm. The sequence was submitted in the database GenBank (BANKIT) under the sequence ID 1634439 and accession number that was procured is KF306097.

Determination of Keratinolytic Activity

a) Preparation of DMSO- Solubilized Keratin Solution: Preparation of soluble keratin was carried out as described previously[17] by a simplified procedure by Wawrzkievicz *et al.* 1987.

b) Preparation of Seed Culture: Preparation of seed culture was performed as described previously and the cell free supernatant was used as a source of crude enzyme and also for quantification of proteins and amino acids.[17]

c) Keratinase Assay: The keratinase activity was assayed as described previously[17, 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.

Studying the feather degradation by the isolate using Scanning Electron Microscopy

In order to study the degradation pattern of the feather degrading isolate at microscopic level, Scanning electron microscopy was used. For examination of a degraded feather under a scanning electron microscope, the feather sample from a culture broth (after incubation of 7 days) was first washed using distilled water, then treated with acetone for dehydrating followed by air-drying. Similar treatment was given to untreated feather sample (for comparison). This sample was then placed on a stub, subjected to sputter coating with platinum using JEOL JFC-1600 sputter coater. The feather sample was examined using JEOL JSM - 7600F Scanning Electron Microscope.

Optimization of Cultural Conditions for maximum Enzyme Production

The optimization study was performed using 25ml of MSM, under shaker conditions (150 rpm). As inoculum, 1ml of fresh seed culture was added to sterile MSM, and assessment of enzyme activity and release of amino acids was carried out on the fifth day post inoculation [19]. All experiments were carried out in triplicate.

a) Optimization of Carbon Source: The enzyme production was assessed in the presence of three simple carbon sources in addition to 1% feathers at the final concentration of 1% (w/v): Glucose,

Sucrose, and Mannitol and compared to that in the absence of any additional carbon source.

b) Optimization of MgSO₄ Concentration: The isolate was cultivated in the presence of different concentrations of MgSO₄ i.e. 0.01%, 0.02%, 0.04% and 0.06%, and the enzyme activity was assessed.

c) Optimization of (NH₄)₂SO₄ Concentration: The isolate was cultivated in the presence of different concentrations of (NH₄)₂SO₄ i.e. 0.01%, 0.05% and 0.10% and the enzyme activity was assessed.

d) Optimization of Initial pH of Medium: The initial pH of the growth medium was adjusted to different values: 5, 6, 7, 8 and 9 and the enzyme activity was assessed.

e) Optimization of Temperature: The incubation was carried out at 25°C, 30°C, 35°C, 40°C and 45°C.

f) Optimization of Feather (Substrate) Concentration: The enzyme activity was assessed by incubating the isolate in the presence of different concentration of feather (substrate) i.e. 0.1%, 0.25%, 0.5%, 0.75%, 1%, 1.5% and 2%.

Statistical Analysis

All the optimization parameters were conducted in triplicate and the data was analysed using single factor analysis of variance (ANOVA). All the data are graphically presented as mean ±S.D. of triplicates. The statistical analysis software GraphPad Prism 5 was used to perform ANOVA. P values < 0.05 were considered significant.

RESULTS

Isolation and Identification

The feather-degrading bacterium isolated from poultry farm soil was identified by studying its microscopic, biochemical and cultural characteristics (Table 1&2). It was a rod-shaped, Gram-positive, and sporulating *Bacillus* spp., capable of carrying out 1% feather degradation completely within 7 days. Images of the electron micrographs clearly (figure 2) show the breakdown and weakening of the feather structure by the isolate thus indicating keratinolysis. The 16SrDNA sequencing and phylogenetic analysis (figure 1) revealed that the feather degrading isolate was found to have maximum similarity (98%) with the strain *Bacillus sonorensis* strain NRRL.

Optimization of Growth Conditions for Maximum Enzyme Production

I. Effect of Additional Carbon source on Enzyme Production and Amino acids Production

The addition of simpler carbon sources in the growth medium suppressed enzyme activity. Similarly there was a reduced release of amino-acids in the cell free supernatant as compared to that in case of feathers as a sole Carbon source (Figure 3).

II. Effect of Varying Concentrations of MgSO₄ on Enzyme Production

Maximum Enzyme production was observed at 0.2% MgSO₄ concentration. However, an excess concentration of MgSO₄ resulted in repression of the protease. (Figure 4)

III. Effect of Varying Concentrations of (NH₄)₂SO₄ on Enzyme Production

An increase in enzyme production and feather degradation was observed up to 0.1% concentration of ammonium sulphate which was found to be optimum; a further increase however leads to decreased enzyme production (Figure 5)

IV. Effect of Initial pH of Medium on Enzyme Production and Amino acids Production

Maximum enzyme production was observed at pH 7, while maximum amino-acids production was observed at pH 8 (figure 6&7). At pH values higher and lower than the optimum, there was a

decrease in enzyme production. Thus the bacterium has a moderate pH range for keratinase production.

V. Effect of Temperature on Enzyme production and Amino acids Production

Maximum enzyme production and amino acids production was observed at 35°C (figure 8&9). As the temperature increases, the enzyme production and release of amino-acids decreased. This shows the organism's preference for mesophilic temperatures. Also, due to the mesophilic nature, an increase in temperature would not

be required to assist enzyme production, thus lowering production costs, if used at a higher scale.

Table 1: General Characteristics of the Isolate

Shape of the cell	Rod shaped
Gram Nature	Gram positive
Motility	Motile
Oxygen Requirement	Facultatively anaerobic
Endospore	Endospore observed
Position of Spore	Sub-terminal

Table 2: Biochemical tests performed for the isolate

Type of test	Result obtained
Tests to distinguish between Aerobic and Anaerobic Breakdown of Sugars	
1. Oxidative/Fermentative test (glucose)	Positive
Utilization of Carbohydrates:	
1. Glucose	Acid
2. Production	Acid Production
2. Sucrose	Acid Production
3. Mannitol	Acid Production
4. Maltose	No Acid/Gas Production
5. Lactose	No Acid/Gas Production
6. Xylose	No Acid/Gas Production
Tests for specific Breakdown Products	
1. Methyl Red for production of acid	Positive
2. Voges-Proskauer for production of acetoin	Negative
Tests for Metabolism of Proteins and Amino-acids	
1. Indole Production/Tryptophan metabolism	Negative
2. Arginine Dihydrolyase	Positive
3. Gelatin Hydrolysis	Positive
Tests for Utilization of particular Substrate	
1. Starch	Positive
2. Citrate	Negative
Tests for Enzymes	
1. Catalase	Positive
2. Oxidase	Negative
3. Urease	Positive
4. Nitrate Reduction	Positive
Combined Tests	
Triple Sugar Iron (TSI) Agar Test	(K/A)**

*Oxidative and fermentative breakdown of carbohydrate

**Alkaline Slant/Acidic Butt

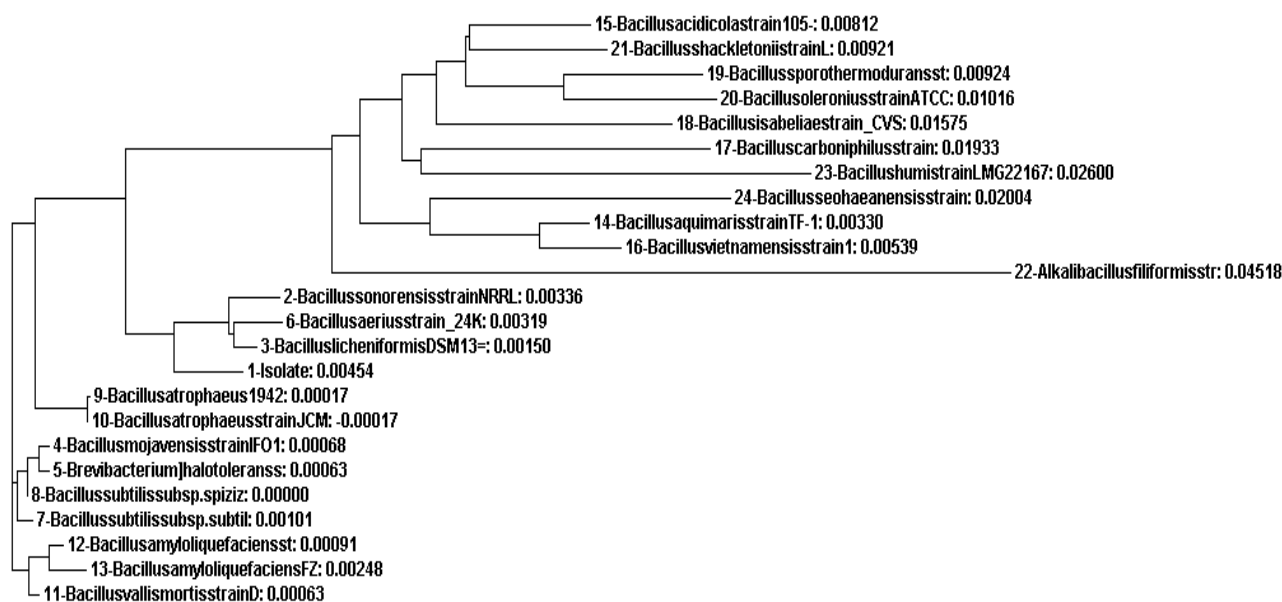
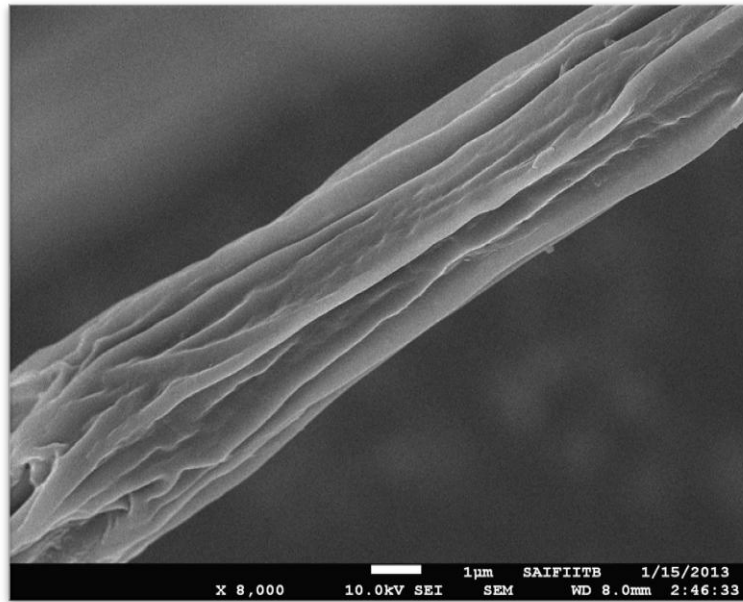
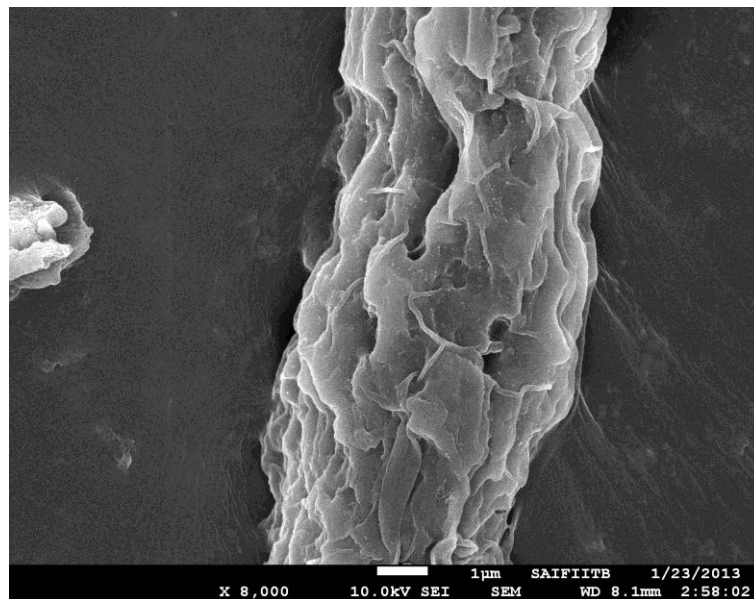


Fig. 1: Phylogenetic tree of the feather degrading isolate. From this phylogenetic tree, it is seen that the isolate is closely related to the strain *Bacillus sonorensis*.



A:



B:

Fig. 2: Scanning Electron Micrographs at 8000X magnification of the feather degraded by the isolate. A: untreated feather, B: feather degraded after seven days.

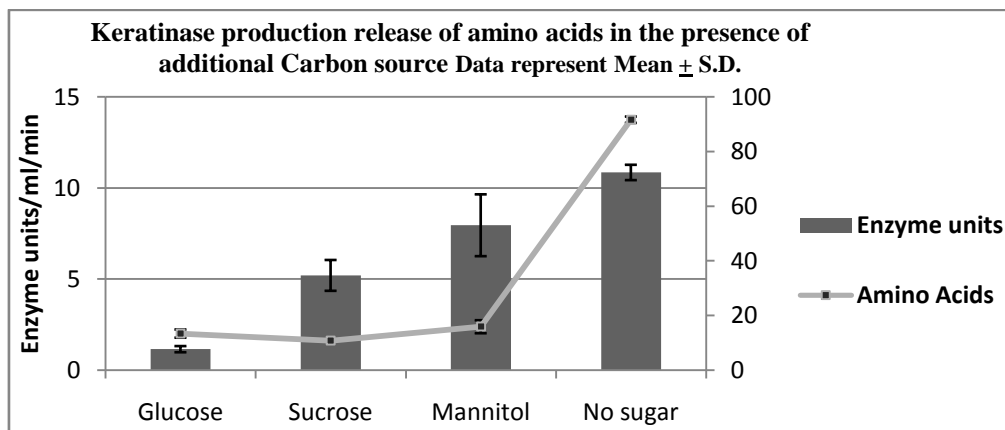


Fig. 3: Effect of additional carbon source on Enzyme production and amino acids production

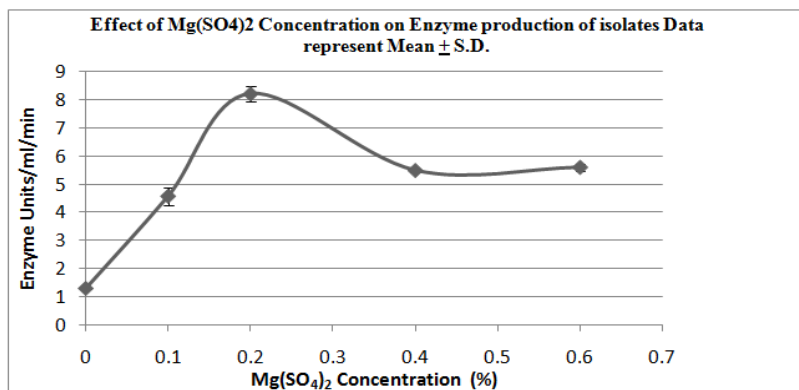


Fig. 4: Effect of Varying Concentrations of MgSO₄ on Enzyme Production

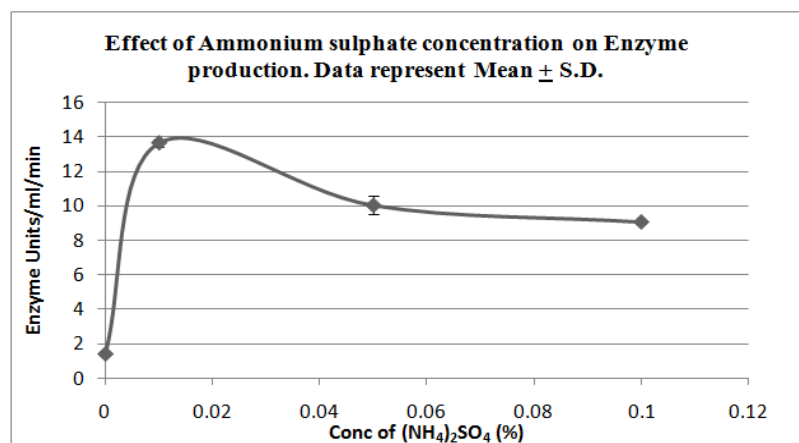


Fig. 5: Effect of varying concentrations of (NH₄)₂SO₄ on Enzyme Production

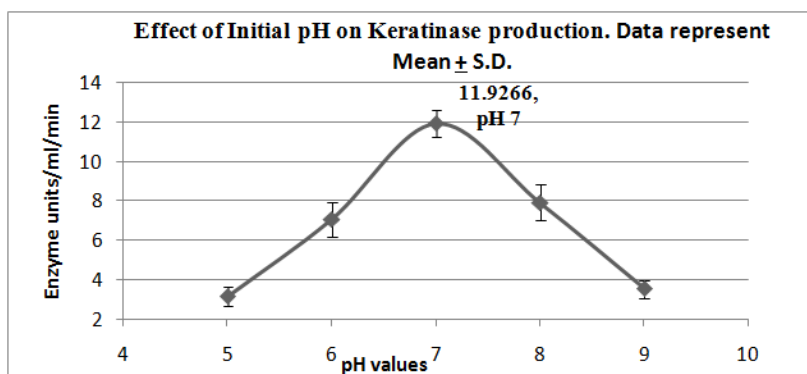


Fig. 6: Effect of Initial pH of Medium on Enzyme

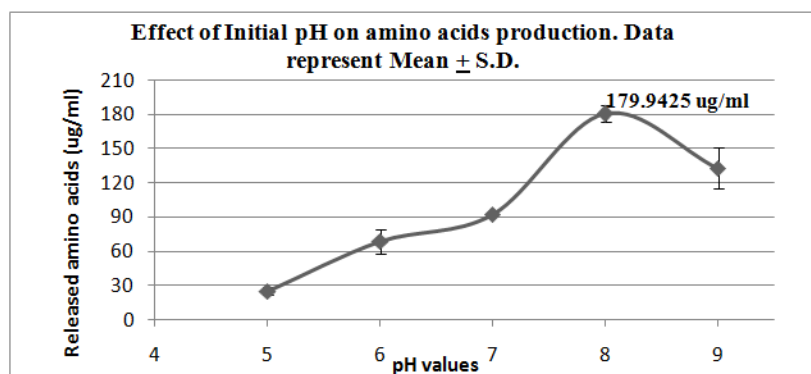


Fig. 7: Effect of initial pH of medium on amino acids production

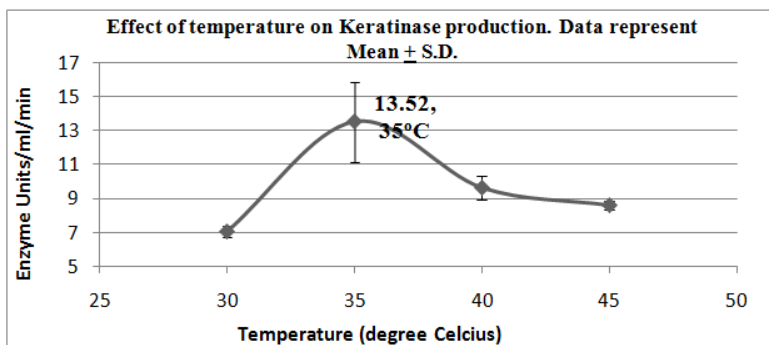


Fig. 8: Effect of temperature on Enzyme production

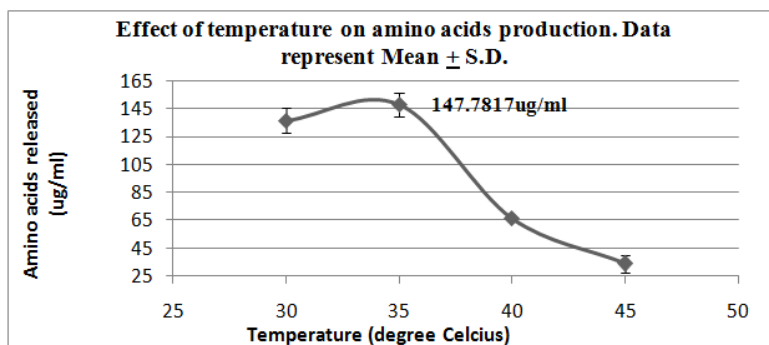


Fig. 9: Effect of temperature on amino acids production

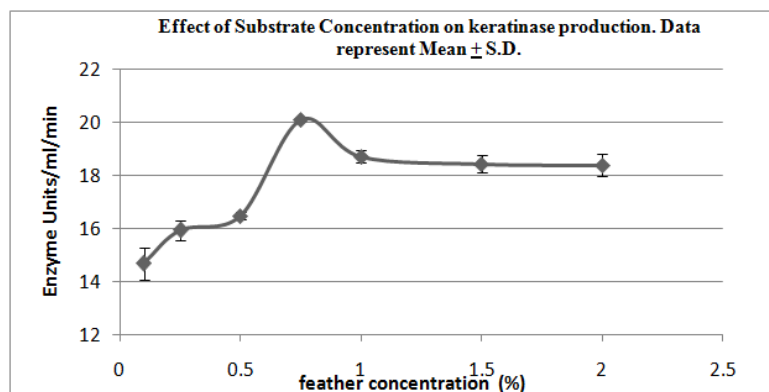


Fig. 10: Effect of Feather concentration on Enzyme production

VI. Effect of Substrate (feather) Concentration on Enzyme Production and Amino- acids Production

Enzyme production increased with respect to Substrate concentration upto 0.75%, which was found to be the optimum for

keratinase production (figure 10). Further increase in substrate concentration, caused a minute decrease in enzyme activity. Maximum release of amino acids was observed at 2% substrate concentration.

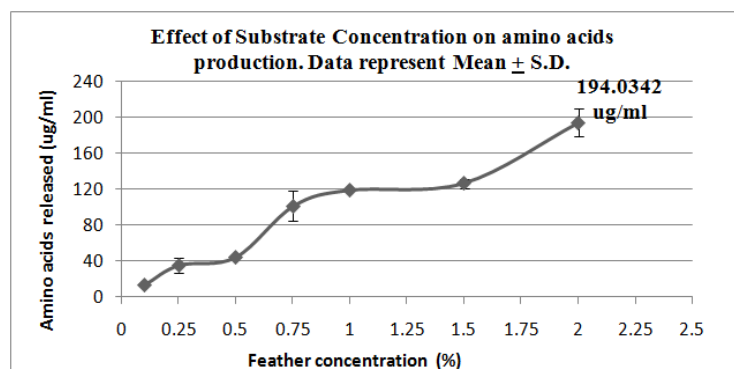


Fig. 11: Effect of feather concentration on Amino acids production

DISCUSSION

Soil samples of 5poultry farms of Maharashtra were screened to obtain six feather degrading isolates, of which one isolate showing the most rapid and intense feather degradation was selected for further studies. The isolate belonged to the genus *Bacillus sp.* While a variety of bacteria are capable of keratinolysis, several studies have reported isolates of the genus *Bacillus* as feather degraders [20, 21]. On further identification by 16SrRNA sequencing, it was determined that the isolate showed 98% homology with *Bacillus sonorensis* NRRL. This species is not previously reported as a feather degrading bacterium; however it shows close resemblance with *B.licheniformis* which is a reported keratinolytic bacterium.

The addition of simpler carbon source in the growth medium resulted in decreased enzyme production which is in agreement with previous scientific findings [6, 22, 23, and 24] but not in complete inhibition of the enzyme which is in contrast with the previous reports [25]. Presence of simpler carbon source in addition to a rigid protein such as feather keratin, results into catabolite repression i.e., the bacterium would first breakdown an easier nutrient source, and after its exhaustion, would start utilizing the difficult nutrient source. In this case, glucose, sucrose or mannitol in the growth medium are readily utilizable sources of energy as compared to feathers, which are highly rigid. Till the exhaustion of the simpler carbohydrate, utilization of feathers may not be required, thus resulting in late and less enzyme production expressed as enzyme activity. The suppressed enzyme activity resulted in decreased feather degradation and therefore a decrease in the release of amino acids which are the by-products of feather degradation. The ability of the isolate to show the maximum enzyme production and amino acids production in a growth medium containing feathers as a sole source of Carbon, explains its ability to utilize and degrade feathers.

Trace elements play an important role in protease production. Mg, a divalent ion, acts as a co-factor for several enzymes, and is present in cell walls and membranes, thus playing an important role for cell mass build-up and enzyme activity and stability. Maximum enzyme activity was observed at 0.02% $MgSO_4$ concentration. The role of ammonium sulphate in the growth medium is that of inorganic Nitrogen Source. Maximum enzyme production was observed at 0.1% concentration of $(NH_4)_2SO_4$. The results are comparable with previous reports which have shown enhanced keratinase production in the presence of $(NH_4)_2SO_4$. [23]

Culture pH strongly affects many enzymatic processes and transport of several nutrients across the cell membrane. Enzymes are normally active only within a certain pH interval and the total enzyme activity of the cell is therefore a complex function of the environmental pH. The optimum pH for keratinase production was determined to be 7. While keratinase production has been reported to be optimum at alkaline pH [24] the current findings corroborate with reports by, Matikevičienė *et al* 2011 [23], Brandelli *et al* 2005 [25] and Kim *et al* 2001 [26] which have shown optimum keratinase production at neutral pH range.

Temperature is another physical parameter which affects enzyme production. Temperature controls enzyme synthesis and energy metabolism [22]. The optimum temperature for enzyme production and amino acids production was determined to be 35°C. *Bacillus* species are known to have growth temperature ranging between 30°C up to 55°C. Although keratinolytic bacteria often display optimal growth and activity at higher temperatures [26, 27], the results are consistent with the studies carried out by Brandelli *et al.*, 2005 [25] where mesophilic temperature has favoured maximum keratinase production.

On studying the role of various substrate concentrations for enzyme production, it was observed that, increase in feather content showed a corresponding rise in enzyme activity as well as release of amino acids. The optimum feather concentration was determined to be 0.75%, which is in line with previous findings by Matikevičienė *et al* 2011 [23] who have reported highest keratinase production at 0.7% Chicken feather meal. Previous reports have determined 1% [28] and 2% [29] feather concentration as optimum. As a result, feathers

which contain keratin, act as substrate and inducer for keratinase production. Keratinase production thus depends upon the presence of keratin and also its concentration in the medium, thus confirming the inducible characteristic of the enzyme. However, after the optimum level of the substrate, further increase in substrate concentration would result in inhibition or saturation of the enzyme.

The isolated bacterium can potentially be used for treatment of feather waste thus bringing about effective waste management of feather waste using an eco-friendly approach. The optimized parameters can be implemented, and the process can be scaled up to use this microbial technology. Thus, the study provides an alternative approach for poultry waste management.

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