

RESEARCH ARTICLE

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Optimization of Keratinase Production and Utilization of *Bacillus pumilus* for Feather Degradation

S. Dhiva¹, C. Akshara¹, K. Afna¹, U. Dhanush¹, P. Arya¹, S. Saigeetha², S. Abirami³, Angel Abraham⁴, R. Emilin Renitta⁴  and Antony V. Samrot^{5*} 

¹Department of Microbiology, Sree Narayana College, Alathur, Palakkad - 678 682, Kerala, India.

²Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology, Shollinganallur, Rajiv Gandhi Salai, Chennai - 600 119, Tamil Nadu, India.

³Department of Microbiology, Kamaraj College, Thoothukudi, Thirunelveli - 628 003, Tamil Nadu, India.

⁴Department of Food Processing, Karunya Institute of Technology and Science, Coimbatore - 641 114, Tamil Nadu, India.

⁵School of Bioscience, Faculty of Medicine, Bioscience and Nursing, MAHSA University, Jalan SP2, Bandar Saujana Putra, 42610, Selangor, Malaysia.

Abstract

Soil samples were collected from the feather dumped area where *Bacillus pumilus* was isolated and used for keratinase production and keratinolytic activity. In the optimization study, optimal condition for enzyme production was observed at 144 h, pH 7, temperature 37°C. The organism was utilized for feather degradation study. The maximum degradation of 57% was obtained at 37°C, pH 7 and 6 days incubation. The size of keratinase was determined by SDS- PAGE and was observed as 52 KDa.

Keywords: Keratinase, feather, keratinolytic bacteria, SDS-PAGE

*Correspondence: antonysamrot@gmail.com

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INTRODUCTION

As a by-product of poultry waste, million tons of feathers are produced annually from the poultry Industries¹. Due to improper handling more than 5 million tons of Feather wastes are dumped into barren lands which leads a negative impact to environment thereby leading to various environmental pollutions². Since Feather is made of rich source of protein called keratin, such keratin cannot be used directly so physical and chemical treatment are required to destroy certain amino acids for better digestibility and get converted into a digestible dietary protein for animal feed^{3,4}.

Feather pollution can be controlled by alternate use of feather wastes⁵. But the drawback behind these methods is they are high energy consuming and also destructs certain other amino acids such as methionine, lysine and tryptophan^{5,6} which affects protein quality⁷. Many researchers isolated microorganisms like fungi, actinomycetes, and several bacterial species such as *Bacillus* sp., *Pseudomonas* sp., which are able to produce keratinase which indicates that they are capable of utilising the keratin found in the feather with a metabolic reaction⁸⁻¹¹.

Microbial degradation of feather improves the nutritional quality of the feather meal and it is a good alternative for Physio chemical treatment^{12,13}. Amongst all the proteolytic enzymes Keratinase has gained attention in last few years due to its potential application mainly in hydrolysis of keratin. Keratin and keratinases play various applications in biotechnology like feed for cattles, removal of hairs and feathers in leather and poultry industries, to generate natural gas, in textile industries for shrink proofing of wool and for removing blockages in sewage system¹⁴⁻¹⁶. So the present study was conducted to use *Bacillus pumilus* isolated from the feather contaminated soil, it was optimized for production of keratinase enzyme and the organism was used for effective degradation of feather.

MATERIALS AND METHODS

Collection of soil

Soil sample was collected from feather dumped area from the depth of 5-10 cm and transferred to the air tight containers. After serial dilution, the sample was transferred into *Bacillus*

Isolation agar. The isolated organism was subjected to Gram's staining and biochemical test based identification.

Processing of feather

Chicken feathers were collected from Broiler chicken from a slaughterhouse nearby Alathur, Kerala, India. Stains present in feathers were washed under tap water and kept for drying. After drying feathers are boiled in water for 30 min at 100°C and further dried at 60°C in hot air oven. The ground powder was obtained from the dried feather. The powder collected was used as a keratin source in this study.

Optimization of growth on feather containing medium

Incubation period optimization

The suitable media was designed by dissolving 0.1g of dried feather powder, 0.5g of K_2PO_4 , 0.04g of K_2HPO_4 , 0.2g of $MgSO_4$, at pH – 7.0 in 100 mL distilled water and sterilization was done for 15min at 121°C in autoclave. Media were added with 1 mL 24 h old bacterial culture and incubated at 37°C for 48- 168 h.

pH and temperature optimization

The optimum pH at 37°C was observed by incubating the cells in aforementioned media in optimal incubation time at different ranges of pH from 4- 9. The optimum temperature was estimated ranging from 30, 35, 37, 40 and 42°C respectively at optimal incubation time and pH. Effect of heavy metal in the production was carried in the presence of heavy metals (Lead, Mercury and Copper) were also studied in the optimal condition obtained from the above parameters.

Enzyme assay

Enzyme assay was performed by taking 1mL of 50 mM Tris HCl buffer at pH 8.0 as solvent in which 5mg keratin azure powder was dissolved. 1mL of (crude enzyme) culture supernatant obtained after centrifugation at 10000 rpm was added with the suspension and kept for 30 min at 50°C, then 0.4 M TCA of volume 2 ml was added for stopping the reaction. The mixture was centrifuged for 40 min at 5000 rpm and collected supernatant was measured spectrophotometrically and the optical density (OD) was read at 280nm. Enzyme activity was enzyme activity in Unit (U)' as described earlier³.

Percentage of degradation of chicken feather

Feather degradation percentage by the organism was found by sterilizing 10 g of feather to the above-mentioned media along with 10% of 24 h old *Bacillus pumilus* culture. Degradation percentage of dried feather was observed at regular interval.

Column chromatography

Culture grown in aforementioned medium in the optimal condition was centrifuged, supernatant was subjected for Column chromatography to purify the keratinase enzyme. Stationary phase was prepared with DEAE sephadox A - 50 and was allowed to set for 5 minutes and the sample was loaded on the top of the column and the 2 mL fraction was collected at 10 min interval and analyzed by taking the OD at 280 nm using spectrophotometer.

Characterization of enzyme

Molecular weight of the enzyme was identified by Polyacrylamide gel electrophoresis

(PAGE). Casting plate was assembled and plate was sealed using space aid. The separating gel was prepared with 1.5 M tris buffer, 30% of acrylamide, 10% ammonium per sulfate (APS), 10% SDS, TEMED and kept undisturbed for 2-5 minutes at room temperature for polymerization. 1M tris buffer was used instead of 1.5M tris buffer for the preparation of Stacking gel. The comb was fixed immediately and kept for 5- 10 min for polymerization. The casting tray was kept in the electrophoretic unit after removing the comb, and SDS buffer solution was added to electrophoretic unit. Prepared sample consists of 10 μ l of 2x SDS solution and 10 μ L of sample and incubated for 10 min at 90°C was loaded to the well along with marker. Electrophoresis was carried out for 4 hours at 50V, until the bromophenol blue dye reaches the bottom of the separating gel. After separating gel from the casting plate it was stained with 0.5% coomassie brilliant blue in water, acetic acid, methanol in the ratio 25:10:45 and kept in shaker at 60 - 70 rpm for overnight followed by the process of destaining (water : acetic acid : methanol - 25:10:4) and the result was estimated using gel documentation and the molecular weight was estimated.

Table 1. Biochemical testing of isolated organism

Sl. No.	Biochemical test	Optical Density(nm)
1	MR	Positive
2	VP	Negative
3	TSI agar	Acid butt, acid slant and gas production, No H ₂ S production
4.	Mannitol utilization	Positive

RESULTS AND DISCUSSION

Keratin degrading microbiota load are normally observed in the feather dumped soil, as the feather is rich in keratin^{17,18} and poultry waste samples were reported to have keratinolytic bacteria^{19,20}. The organism isolated from the

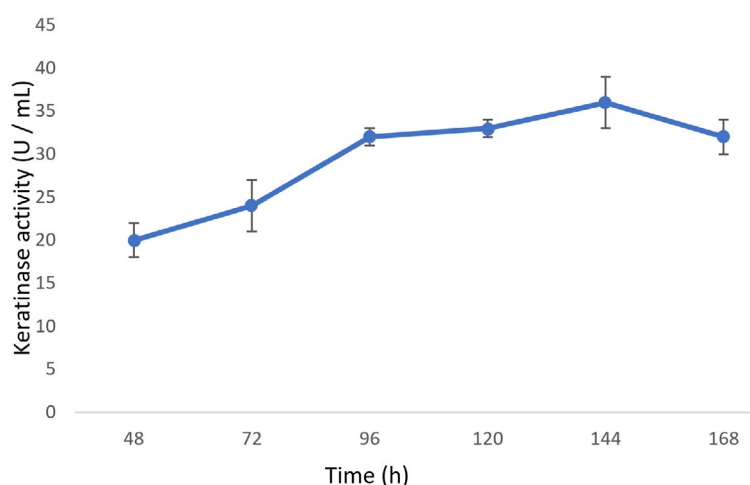


Fig. 1. Optimization of keratinase enzyme production at different incubation time.

soil after it grew on *Bacillus* isolation agar, the organisms was identified as *Bacillus pumilus* based on Gram's staining and biochemical test as follows – the organism was Gram positive rod and the biochemical test is listed in Table 1. Abdel-Fattah et al.²¹, also isolated *Bacillus licheniformis* from soil which was found to be keratinolytic.

Optimization of keratinase production

Growth of the organism on the designed media, showed the maximum production of keratinase on 6th day (Fig. 1). Optimal pH and temperature was found to be pH 7 and 37°C respectively (Fig. 2 and 3). Effective keratinase production was observed at 37°C (Fig. 3). Dhiva et al.⁵ found *Pseudomonas aeruginosa* SU-1 to

produce keratinase at 30°C. The presence of heavy metal in medium was having negative influence on the enzyme production, it might be the impact of heavy metal on the growth pattern of the organism (Fig. 4).

Degradation of feather

Feather degradation at different incubation time

Degradation of keratin was observed at 37°C, where 57% of degradation was observed in 6 days (result not shown here). Degradation of keratin was due to keratinase enzyme produced by Keratinolytic organisms. Similar work was reported by Intagun et al.¹⁹ and Lakshmi et al.²².

Feather degradation at different pH

At pH 5, 16% of feather was degraded

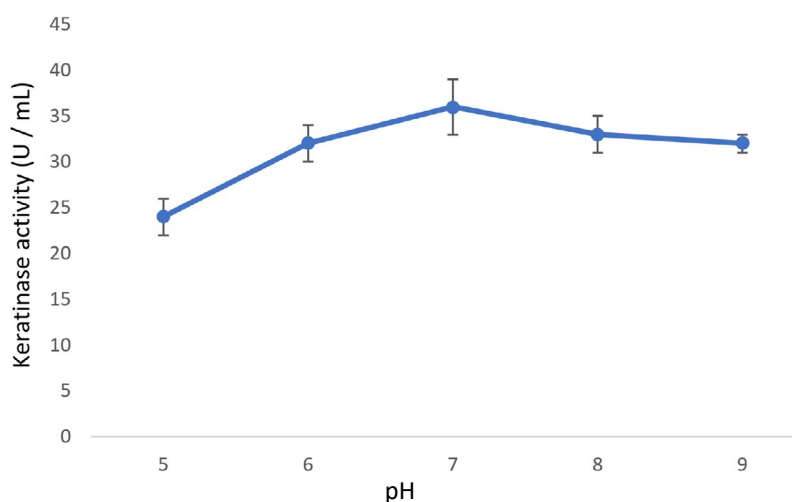


Fig. 2. Optimization of keratinase enzyme production at different pH.

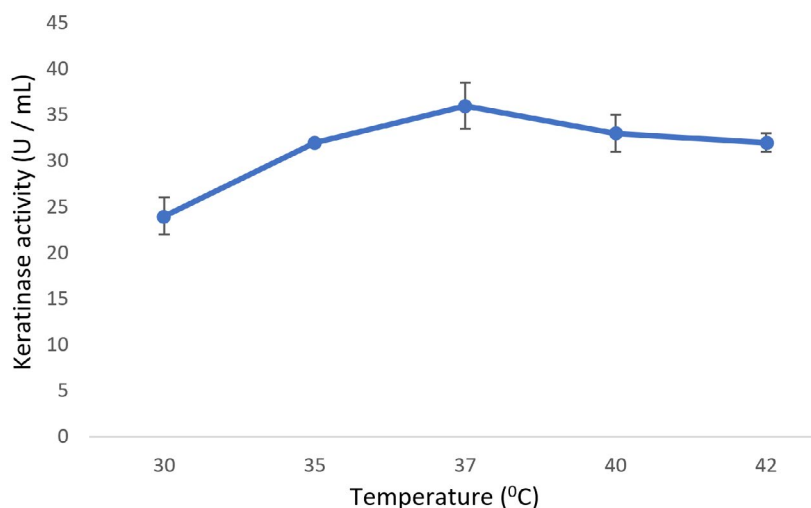


Fig. 3. Optimization of keratinase enzyme production at different Temperature.

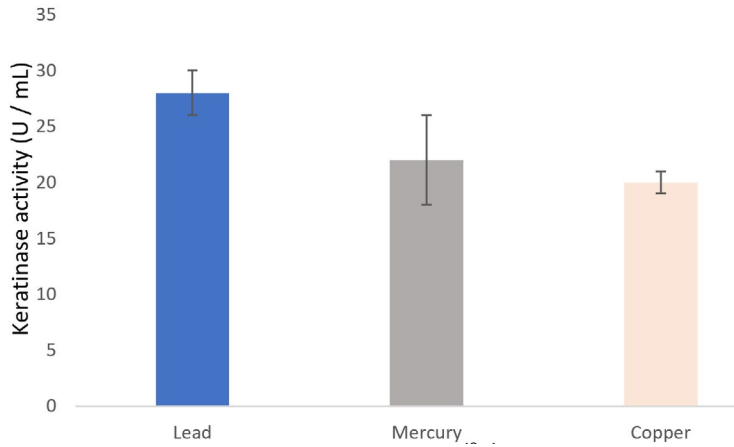


Fig. 4. Optimization of keratinase enzyme production presence of heavy metals.

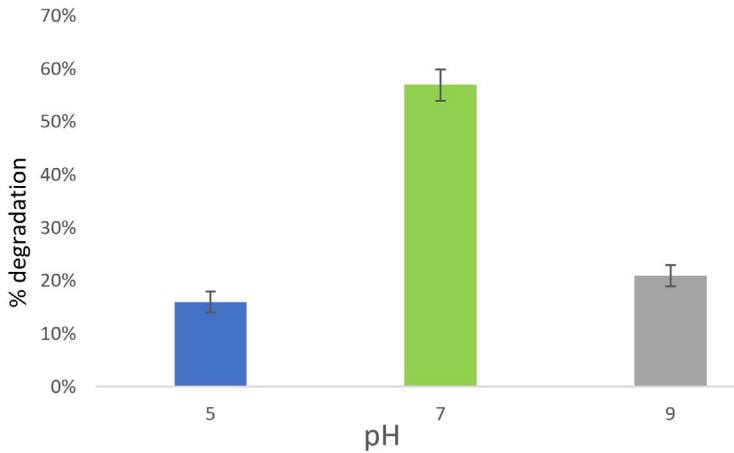


Fig. 5. Feather degradation by *Bacillus pumilus* at different pH.

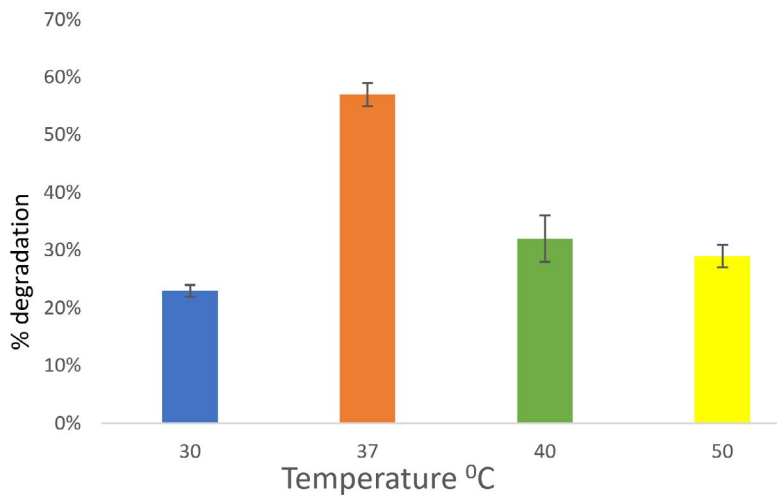


Fig. 6. Feather degradation by *Bacillus pumilus* at different temperature.

Table 2. OD value of fractions obtained using column chromatography

Sl. No.	Tube Name	Optical Density(nm)
1	B1	0.024
2	B2	0.022
3	B3	0.042
4	B4	0.049
5	B5	0.060
6	B6	0.029
7	B7	0.015
8	B8	0.013

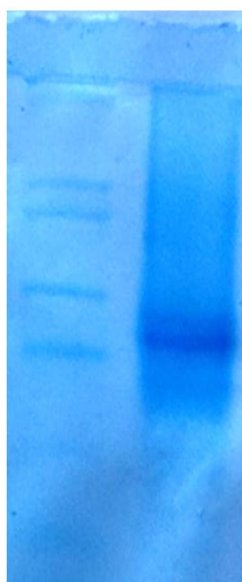


Fig. 7. SDS- PAGE Lane 1. Marker, Lane 2. Sample

after incubated for 6 days. At pH 7, 57% feather was degraded after incubated for 6 days. At pH 9, 21% feather was degraded after incubated for 6 days. Maximum feather degradation was observed at pH 7. So, pH 7 is the optimum pH for the activity of keratinase enzyme (Fig. 5).

Feather degradation at different temperature

At temperature 30°C, 23% of feather was degraded after incubated for 6 days. At temperature 37, 40 and 50°C, 57%, 32% and 29% respectively was degraded after incubated for 6 days (Fig. 6). Vigneshwaran et al.²³, reported *Bacillus licheniformis* to degrade feathers in 7 days at 40°C. Abirami et al.⁴ also found *Lichtheimia corymbifera* AS1 to degrade cattle hooves efficiently.

Column chromatography

When the fraction obtained through column chromatography was subjected for absorbance at 280 nm, the highest absorbance was recorded in the B6 fraction where the absorbance was found to be 0.06 (Table.2). It states that the highest protein was obtained at that time.

SDS- PAGE

The enzyme was characterized by SDS-PAGE. The organism used in this study produced enzyme of 52 KDa (Fig. 7). The produced keratin enzyme was 58 KDa in size from the organism *Bacillus* sp. CBNRBT⁵.

CONCLUSIONS

In this study, *Bacillus pumilus* was isolated from soil and used for keratinase production. The optimized conditions were determined as Temperature 37°C, pH 7 and incubation time 6 days. The estimated size of keratin was 52KDa. The organism was capable of degrading 57% of feather on 6 days.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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