

REGULAR ARTICLE

CHARACTERIZATION AND OPTIMIZATION OF KERATINOLYTIC BACTERIA *Pseudomonas stutzeri* C3 ISOLATED FROM POULTRY WASTE SOIL SAMPLE

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ABSTRACT

Microbial degradation of keratinous wastes is preferred over physicochemical methods, because it's a cost effective and eco-friendly. Novel habitats are promising for discovery of new microbial strains. Towards isolate a keratinolytic bacteria, screening of bacterial strains from a poultry farm soil in Namakkal district, Tamil Nadu, India was done and a promising the isolate C3 was found to degrade native chicken feather efficiently. It could grow over a pH range 7 at 40°C and in presence of potassium nitrate as a nitrogen source and citrate as a carbon source. Based on phenotypic characterization and 16S rRNA gene sequence analysis, the keratinolytic isolate was identified as *Pseudomonas stutzeri* C3. It produced 30.86±1.08 U/ml keratinase in raw feather medium and complete feather degradation at 5 days incubation periods.

Keywords: Microbial degradation, keratinase, poultry farm, *Pseudomonas stutzeri*

INTRODUCTION

Keratin is the most abundant protein in epithelial cells and forms major components of skin, hair, nail, feather and wool. Keratins are grouped into hard keratins, found in appendages such as feather, hair, hoof and nail and soft keratins found in callus and skin. The former have high disulfide bond content and are tough and inextensible whereas soft keratins such as skin and callus have low content of disulfide bridges and are more pliable (Gupta and Ramnani, 2006). Structurally, keratins are classified as α -keratins (hair, hooves, nails, etc.) and β -keratins (feather, silk fibrion, β -amyloid) (Voet and Voet, 1995; Akthar and Edwards, 1997).

Environmental wastes are found in large quantities in many countries. Although some of them contain a considerable amount of protein and various carbon compounds, little attention is given to utilizing or recycling these wastes in a technological way. Additionally, the accumulation of some of these wastes in nature is considered to be a serious source of pollution and health hazards. Therefore, their proper disposal may be considered as a means of avoiding environmental pollution. Recently, we have focused on the utilization of some polymeric wastes, mainly feather waste (Park and Son 2009). It is estimated that nearly 24 billion chickens are killed yearly (which is increasing per year) and leads to production of nearly 4 billion pounds of feathers as a waste from commercial (large and small scale) poultry industries around the world. Naturally a feather takes 3 to 4 years to get degraded due to solid structure of keratin protein. Disposal of feather waste is a major problem because simple dumping in the ground leads to the soil pollution and burning it adds to the SO₂ and CO₂ content in the environment and causes air pollution. This mammoth size of discarded feather, apart from polluting the soil or air, also causes various human ailments including chloresis, mycoplasma and fowl cholera (Revathi et al., 2013). Traditional ways to feather converted to feather meal through steam pressure and chemical treatment (Poole et al., 2009 and Freeman et al., 2009). Though making keratin waste more digestible, this chemical treatment process is both costly and destructive to certain amino acids (Xie et al., 2010, Syed et al., 2009 and Tatineni et al., 2008). The nutritional upgrading of feather meal with the treatment of microbial keratinase might lead to a significant increase in the availability of amino acids in feather keratin. Keratinase is an enzyme which was secreted by many microorganisms that hydrolysis keratin in to smaller molecule (Bin et al., 2009). Despite the recalcitrance keratin waste can be efficiently by degraded by a myriod of bacteria (Cheng et al., 2008; Bin et al., 2009); actinomycets (Amany et al., 2009) and fungi (Scott et al., 2004) due to the elaboration of keratinolytic proteases and keratinase (Onifade et al., 1998). In fact, keratinases have been successfully applied in several biotechnological processes, including the introduction of enzymatic dehairing in the leather industry (Marimuthu et al., 2016), production of slow-release nitrogen fertilizers in the agricultural industry (Siddharthan et al., 2017), and synthesis of biodegradable films and coatings in the biomedical industry (Anbu et al., 2007,

Freeman et al., 2009 and Brandelli et al., 2010). The present study aims at the degradation of feather by keratinase produced by *Pseudomonas stutzeri* C3, a keratinolytic bacteria isolated from poultry farm waste site and the optimization of keratinase production.

MATERIALS AND METHODS

Collection of Sample

The poultry waste soil sample was collected from Poultry Farm, Namakkal district, Tamil Nadu, India. The soil sample was collected in sterile polythene bag and transported to the laboratory maintaining temperature around 4°C as early as possible for analysis.

Isolation of Keratinolytic Bacteria

The soil sample was serially diluted with sterile distilled water upto 10⁻⁷ and the bacteria were isolated on the feather meal agar plates by the spread plate technique. Feather meal agar (FMA) plates containing: feather meal (0.5g), NH₃Cl₂ (0.05g), NaCl (0.05g), MgCl₂ (0.01g), K₂HPO₄ (0.03g), KH₂PO₄ (0.04g), yeast extract (0.1g), agar (1.7g) and distilled water (100 ml) with a pH of 7.5 (Saraniya and Baskaran, 2016). Plates were then incubated at 37°C for 24-36 h. Colonies forming transparent zones around the bacterial colony due to hydrolysis of keratin. The keratin hydrolyzed bacteria were screen to further process.

Screening of Keratinase Production

A raw feather basal medium containing: MgSO₄·7H₂O (0.2 g), K₂HPO₄ (0.3 g), KH₂PO₄ (0.4 g), CaCl₂ (0.22g), yeast extract (0.1g) and chopped raw feather (0.1g) in 100 ml distilled water. The medium was sterilized at 121°C for 15 minutes. The isolates which showed positivity in feather meal agar medium were inoculated and the flasks were incubated at 37°C for 5 days on a rotary shaker at 180 rpm. 5 ml of samples were, centrifuged at 5000 rpm for 30 minutes and used for keratinase determination. (Saraniya and Baskaran, 2016)

The keratinolytic activity was assayed as follows: 1.0 ml of crude enzyme properly diluted in phosphate buffer (0.05 mol/L, pH 8.0), was incubated with 1 ml keratin solution at 50°C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml of 0.4 mol/L trichloroacetic acid. After centrifugation at 4000 rpm for 30 min, the absorbance of the supernatant was determined at 280 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm (Gradisar et al., 2000) with the control for 0.01 per

minute under the conditions described above and calculated by the following equation:

$$U=4 \times n \times A280 / (0.01 \times 10)$$

Where n is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min) according to Cai et al., (2008).

Identification by 16S rRNA Sequencing

The maximum keratinase producing potential bacterial isolate was studied for its morphological, cultural and biochemical characteristics according to Bergey's Manual of Systemic Bacteriology. The strain was also identified by PCR using 16S rRNA as a molecular marker. The primers used were as follows: forward primer (5' CAGCCGCGGTAATAC 3'), and reverse primer (5' ACGGCGGTGTGTAC 3'). PCR was run for 30 cycles using the DNA thermal cycler. The PCR products were analyzed in a 1% (w/v) agarose gel with ethidium bromide before being sent for sequencing analysis. The DNA sequences were aligned and compared using the BLAST algorithm to find homologous sequences in the GenBank database of NCBI. The data were submitted to the GenBank database (Younes Ghasemi et al., 2012). A phylogenetic study of the selected strain was performed using the ClustalW program within MEGA6 software (Tamura et al., 2013). The branching pattern was designed based on the neighbor-joining method.

Optimization of Cultural conditions

Feather degradation was carried out in raw feather medium. The medium was inoculated with 5 % (v/v) inoculum. The inoculated flasks were kept incubated on a rotary shaker in 150 rpm at 30 °C. Time course of feather degradation was monitored by taking the fermentation broth at various time intervals and monitoring bacterial growth rate and keratinase production. Effect of feather concentration was monitored by adding feather at different concentrations. Effects of incubation temperature were also studied by growing C3 in raw feather medium at different temperatures. For evaluating effects of carbon and nitrogen supplements on feather degradation, various carbon sources viz. glucose, sucrose, citrate, corn starch and lactose were added in raw feather medium at 1 % (w/v) concentration and nitrogen sources viz. peptone, yeast extract, soybean meal, beef extract, KNO₃ and NH₄Cl were added at 0.5 % (w/v) concentration.

Microscopic Observation of Feathers

The potential bacterial strain was grown in FM and the fermentation broth was filtered with Whatman No.3 filter paper after incubation. The filtered feather was fixed with 2.5 % (v/v) glutaraldehyde and 2 % (v/v) formalaldehyde for 24 h. The specimens were dehydrated several times with 70–80 % acetone and dried at 50 °C for 10 min. The specimens were observed using FEI Quanta 250 scanning electron microscope and bright field microscope (Rahayu et al., 2012).

Statistical Analysis of Data

All the above experiments were repeated in triplicate and the final values have been presented as mean ± S.D.

RESULTS AND DISCUSSION

Isolation of Keratinolytic Bacteria

In the preliminary screening, 8 isolates showing keratinolytic activity by producing clear zones on feather meal agar plates (Fig.1) were selected for transfer to basal feather medium. However, 8 colonies growing well on this medium were chosen for further studies. Initial morphological identification showed that 5 isolates were Gram-positive, spore-forming bacilli and 3 were Gram-negative and rod-shaped.

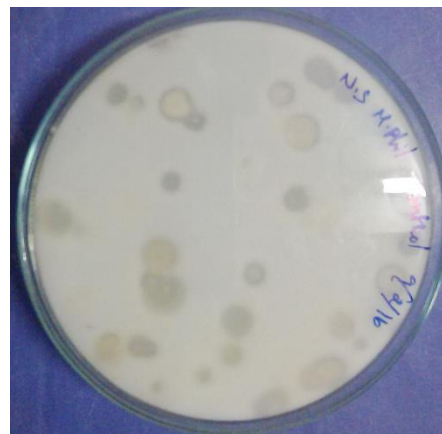


Figure 1 Isolation of Keratinolytic Bacteria from Feather meal agar Plate

Screening of Keratinase Production

The promising feather degrading isolates were selected for quantitative determination of enzyme activity using raw feather medium. The measurement of the keratinolytic activity revealed a range from 15.80±0.32 to 30.86±1.08 U/ml (Table.1). One isolate exhibited the highest keratinolytic activity (30.86±1.08 U/ml) in its culture supernatant. Moreover, it presented pronounced growth and complete hydrolysis of native chicken feathers. This isolate was designated C3 and was selected for identification, characterization and optimization studies. *Bacillus* sp FK 46 was maximum keratinase produced at 0.9 U/ml from soil sample (Suntornsuk and Suntornsuk, 2003). *Arthrobacter* sp 108 showed 5 U/ml keratinase activity on feather meal agar at 7 days of incubation periods (Kate and Archana, 2014).

Table 1 Keratinase Activity of Isolated Strains

S.No	Isolated Strains	Keratinase Activity (KU/ml)
1	C1	27.95±1.25
2	C2	15.81±1.31
3	C3	30.86±1.08
4	C4	15.80±0.32
5	C5	13.45±0.99
6	C6	25.13±0.34
7	C7	21.18±0.75
8	C8	18.93±0.50

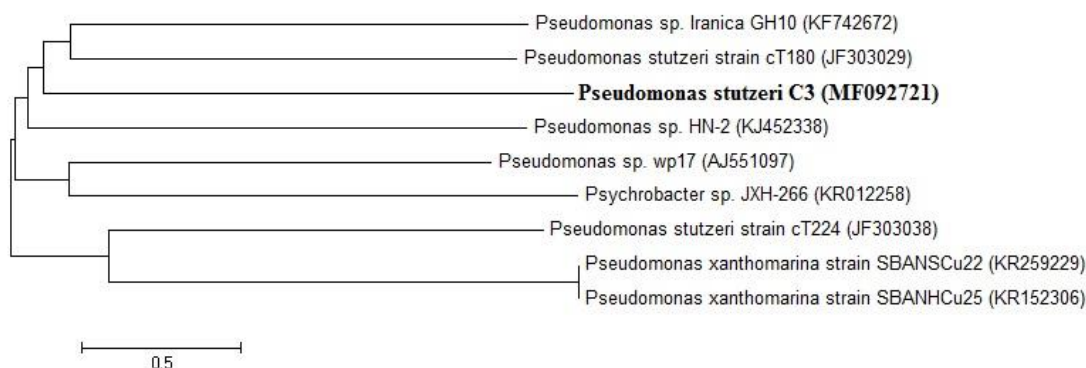
Identification by 16S rRNA sequencing

The cultural, morphological and physiological characteristics of strain C3 was summarized in Table 2. Briefly, the isolate *Pseudomonas stutzeri* C3, proved to be Gram negative straight rod-shaped cells with non-spore forming. It formed opaque creamy circular colonies on feather-agar plates whereas the colonies were irregular in shape and mucoid on nutrient agar plates. Based on morphological characterization and 16S rDNA sequence analysis, C3 was identified as a *Pseudomonas* sp. and designated as *Pseudomonas stutzeri* C3 (NCBI GenBank Accession No. MF092721). It showed closest 16S rRNA gene sequence similarities with *Pseudomonas stutzeri* (93 %) (Fig.2). However, earlier study rachises were completely degraded by strain *Pseudomonas stutzeri* at 5 days incubation, indicating complete degradation of chicken feathers (Venkatesh et al., 2013).

Table 2 Morphological and Biochemical Characteristics of Potential Strain

S.No	Characteristics	Result
1	Gram staining	Negative
2	Shape	Rod
3	Motility test	Motile
4	Capsule staining	Non capsulated
5	Spore forming	Non spore forming
6	Pigment	Positive
7	Catalase	Positive
8	Oxidase	Positive
9	Indole	Negative
10	Methyl red	Negative
11	Voges proskauer	Negative
12	Citrate test	Positive
13	Urease test	Negative
14	H ₂ S production	Negative
15	Gas production	Positive
16	Nitrate reduction test	Positive
17	Starch hydrolysis	Positive
18	Coagulase test	Negative
19	Cetrimide agar	Positive

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 12.52928651 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 911 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

**Figure 2** Phylogenetic Analysis of Potential Strain

Optimization of Keratinase Production

Feather concentration was affected to bacterial growth and feather degradation. Bacterial growth also increased as the feather concentration increased, while the percentage of feather degradation was inversely proportional to the feather concentration. Keratinase was produced at a similar level at different feather concentrations. It was demonstrated that high feather concentrations may cause substrate inhibition /repression of keratinase production, resulting in a low percentage of feather degradation. The results were similar to a previous report of Cheng et al. (1995) which showed that the minimum amount of feather powder (1%) yielded the highest keratinase activity from *B. licheniformis* PWD-1. The initial pH of the medium also greatly affected bacterial growth, percentage of feather degradation and keratinase production. The optimum pH for bacterial growth, feather degradation and keratinase production was 7. The growth, feather degradation and keratinase production of *Pseudomonas stutzeri* C3 were optimal at an incubation temperature of 40°C (Fig. 3). Similarly maximum keratinase production was observed at pH 7 and 40°C, inoculum size of 5% (v/v) and inoculum age of 7 d (Ningthoujam et al., 2016). Citrate as a carbon source and a potassium nitrate was a nitrogen sources to obtained maximum enzyme production. Among the carbon sources tested, sodium citrate was found to be the best carbon supplement for keratinase production followed by glucose, corn starch and sucrose (Kshetri and Ningthoujam, 2016).

Microscopic Observation of Feathers

Scanning electron microscopy was applied to visualize and confirm the keratinolytic degradation of feather. As illustrated on Fig.4, the degree of feather degradation increased when feather was incubated with *Pseudomonas stutzeri* C3. The pictures show the degradation of feather barbules along with disintegration of the feather rachis structure after incubation. Similarly Pedersen et al. (2015) reported the protease from *B. subtilis* highly feather degrading compared with 3 commercial available proteases.

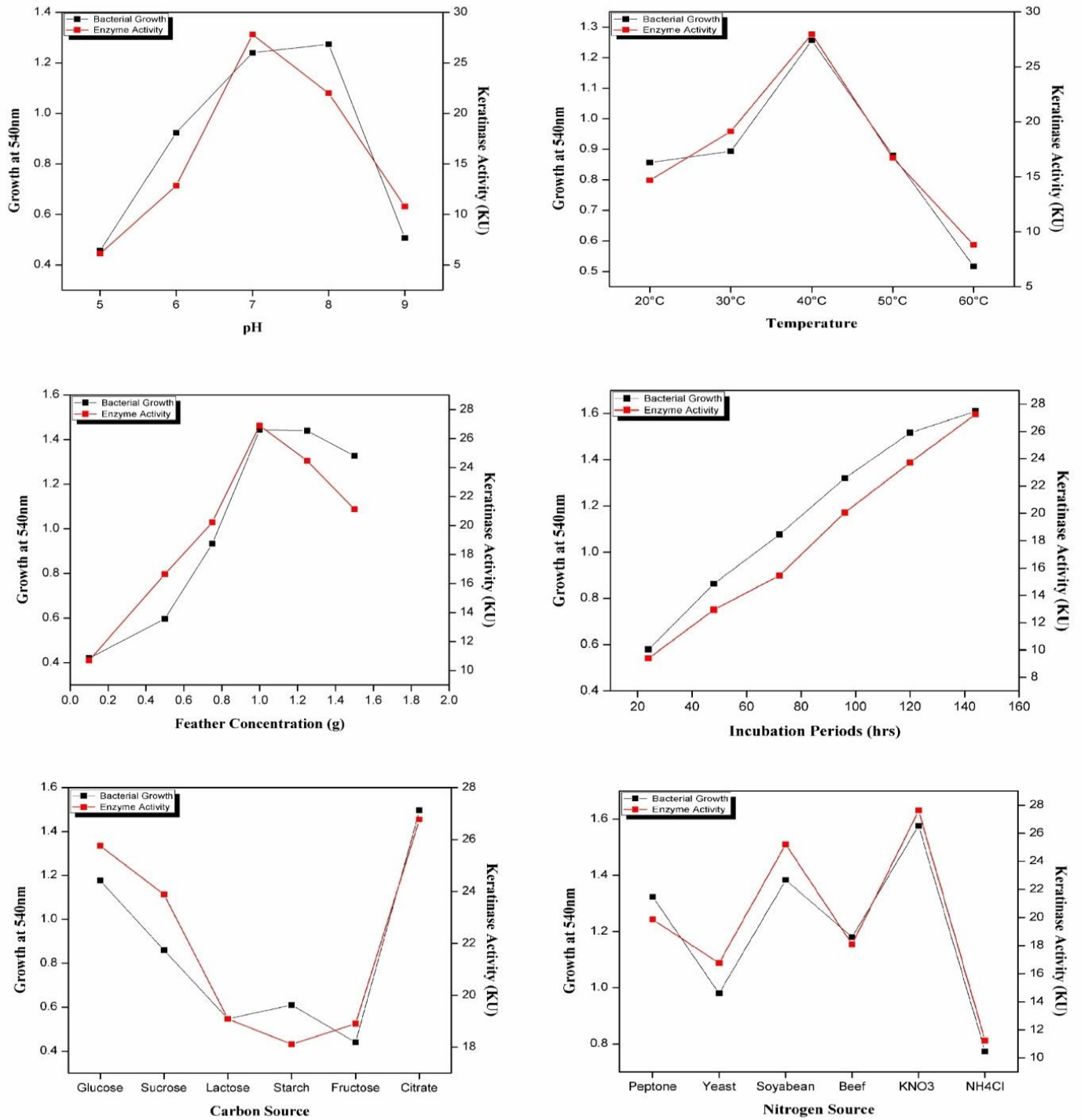


Figure 3 Optimization of Potential Strain and Enzyme Production

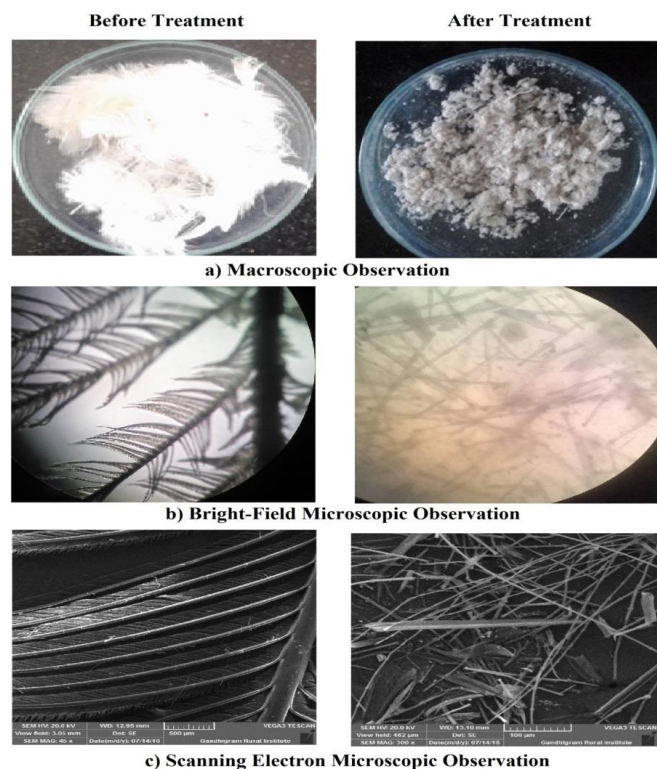


Figure 4 Macroscopic and Microscopic Observations of Feather Degradation

CONCLUSION

In conclusion, this present study was isolated *Pseudomonas stutzeri* C3 possesses high keratinolytic activity and is effective in feather degradation in a short period of 5 days at 40°C and pH 7, potential uses for biotechnological processes. This study also demonstrate the potential useful of feather as a cheap source fermentation substrate for the production of industrial enzyme and as a source of valuable amino acids and soluble proteins that can be used as a digestible feed for animal.

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