

## IDENTIFICATION AND CHARACTERIZATION OF A LOCAL BACTERIAL STRAIN WITH HIGH KERATINOLYTIC ACTIVITY FROM CHICKEN FEATHERS

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### ABSTRACT

The present study was aimed at isolating a potential keratinase producing bacteria from chicken feathers collected from poultry waste sites in and around Coimbatore, Tamil Nadu, and optimising the parameters for the enzyme production. The strain was identified as *Bacillus licheniformis* KMBVP based on 16srRNA gene sequencing. The strain showed high keratinolytic activity when cultured in feather meal medium. Optimising parameters such as pH, temperature, incubation time, carbon and nitrogen sources were also determined. Keratinase production was maximum at temperature 40°C, pH 8.0 and on the 7<sup>th</sup> day of incubation. Feather meal and yeast extract were found to be the optimised carbon and nitrogen sources. The enzyme activity was strongly inhibited by PMSF, suggesting that it belongs to the family of serine proteases with a potential application in feather degradation.

### KEY WORDS

keratinase, chicken feather, *Bacillus licheniformis* KMBVP.

### INTRODUCTION

Enzymes produced by microorganisms play a significant role towards the prospects of industrial processes<sup>[1]</sup>. Especially, proteases have been reported to represent a resourceful class of enzymes with potential industrial applications, and have accounted for nearly 65% of the global enzyme sales<sup>[2]</sup>. Among these, high-alkaline proteases account for about 40% of the total sales<sup>[3]</sup>.

Large amounts of feather wastes are discharged every year from the poultry processing industry with an estimated global annual discharge of about millions of tons<sup>[4]</sup>. Insoluble fibrous keratin protein is the primary substance found in chicken feathers, and cannot be degraded by common proteases (trypsin, pepsin, papain)<sup>[5]</sup>. The mechanical stability of keratin and its resistance to biochemical degradation is based upon the firm linkage of the production chains. In  $\alpha$  – Helix ( $\alpha$ -keratin), or  $\beta$  – Sheet ( $\beta$  – keratin) structure are linked by disulfide bonds which fold into an initial

3-dimensional form<sup>[6]</sup>. Though keratin can be dissolved using reducing agents such as mercaptoacetate and copper sulphate, it cannot be used for large scale processes<sup>[7]</sup>.

The use of microbial enzymes overcomes the limitation and hence improves the nutritional value of feather wastes, of which, keratinases are a group of mostly extracellular serine proteases and are known for their potent activity to degrade keratin<sup>[8]</sup>. Keratin utilization has been reported in variety of organisms including non species of *Aspergillus*, *Onygena*, *Absidia* and *Rhizomucor*<sup>[9]</sup>, *Bacillus*<sup>[10]</sup> and the thermophilic *Fervidobacterium pennovorans*<sup>[11]</sup>. Keratinase has potential applications for removing hair and feather in the poultry industry<sup>[10]</sup> and for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry<sup>[12]</sup>.

The present study is focused on the isolation and identification of a local keratin producing *Bacillus* strain from poultry wastes in and around Coimbatore,

Tamil Nadu, India. Factors affecting the enzyme production such as pH, temperature, incubation period, carbon and nitrogen sources and inhibitors were also studied.

## MATERIALS AND METHODS

### Isolation and identification of keratinase producing bacteria

Poultry wastes such as chicken feathers and soil were collected from poultry waste dumping sites in and around Coimbatore, Tamil Nadu, India. Samples were serially diluted ( $10^{-1}$  to  $10^{-9}$ ) by adding 1 g of the soil sample or chicken feathers to 9 ml of sterile Tris-HCl buffer (pH 7.5). The sample suspensions were placed on milk casein agar plates for 1-2 days at 37°C for primary screening. The plates that showed casein hydrolysis activity were selected and further inoculated in a feather meal broth [(g/L): NH<sub>4</sub>Cl, 0.5; NaCl, 0.5; K<sub>2</sub>HPO<sub>4</sub>, 0.3; KH<sub>2</sub>PO<sub>4</sub>, 0.4; MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.1; Yeast extract, 0.1 and Feather, 10; pH 7.5] and incubated at 37°C for 7 days with shaking at 150 rpm. The culture which showed maximum feather degradation was selected for further identification by phylogenetic analysis using 16srRNA gene sequencing. Genomic DNA was extracted from the isolate and amplified by PCR<sup>[13]</sup>. 16s rRNA sequence analysis was performed with the aid of molecular evolutionary genetic analysis (MEGA) by using neighbour-joining method<sup>[14]</sup>.

### Production of keratinase enzyme

Keratinase production was carried out by seeding inoculums (2% w/v) in 500-mL Erlenmeyer flasks containing 100 mL fermentation medium (feather meal broth) and incubated done at 30°C on a shaker operated at 150 rpm for 7 days. After incubation, the culture medium was filtered through Whatmann No. 1 filter paper to remove the non-degraded residues. Further, the cells were removed by centrifugation at 10,000 rpm for 10 minutes and the culture supernatant was used as crude enzyme extract and examined for enzyme activity.

### Keratinase enzyme assay

The keratinase activity was assayed<sup>[15]</sup>. The mixture of 10 mg of feather powder suspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM CaCl<sub>2</sub> and 1 ml of culture filtrate was incubated at 45°C with

shaking at 300 rpm for 30 min in a water bath shaker. This elevated temperature was used for the enzyme incubation to accelerate substrate hydrolysis. The enzyme reaction was terminated by adding 2 ml of trichloroacetic acid (TCA) solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) into the reaction mixture. The mixture was then centrifuged at 3000 x g, 4°C for 30 min and the absorbance of the supernatant was spectrophotometrically measured at wavelength of 275 nm. The enzyme inactivated by TCA solution was used as a control. One unit (U) of keratinase activity was expressed as 1 μmol of tyrosine released per minute under the specific conditions.

### Biomass estimation

The culture broth (10 ml) was centrifuged at 8000rpm for 10 min. The cell pellet was washed with distilled water. The process was repeated again, following which, the pellet was dried at 60°C overnight and weighed.

### Factors affecting keratinase production

#### a) Effect of pH

Effect of pH on the enzyme activity was measured at various pH ranges (3.0 – 9). The pH was adjusted using the following buffers - acetate (50mM) (pH 3.0-4.0), phosphate (50mM) (pH 5.0-7.0), Tris-Cl (50mM) (pH 8.0) and glycine-NaOH (50mM) (pH 9.0) and the activity of the enzyme was measured as described previously.

#### b) Effect of Temperature

The activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 20, 30, 40, 50 and 60°C were studied. The activity of the enzyme was measured as described previously.

#### c) Effect of Incubation Period

The keratinase activity by the selected experimental microorganisms was determined individually was carried out individually at various incubation times ranging from 2 to 10 days.

#### d) Effect of Carbon and Nitrogen Sources

Glucose, maltose, lactose, starch and feather meal at 1% w/v were tested to determine the optimized carbon sources. The keratinase production was also optimized by supplementing different nitrogen sources individually at 1% concentration: peptone, soyabean meal, casein, and yeast extract.

#### e) Effect of inhibitors

Protease inhibitors (5mM concentration) phenyl methane sulphonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA),  $\beta$ -mercapto ethanol and 0.5% sodium dodecyl sulfate (SDS) were used. While PMSF was prepared by using isopropanol, all other inhibitors stocks were prepared in distilled water. Keratinase activity was determined as a percentage of residual activity relative to control.

## RESULTS AND DISCUSSIONS

### Isolation and Identification of Keratinase producing bacteria

In the present study, the strain that hydrolyzed casein (Figure 1) and showed feather degradation activity in feather meal broth (Figures 2A & 2B) was found to be a Bacillus strain and identified as *Bacillus licheniformis* (KMBVP) based on 16srRNA gene sequencing (Figure 3). The KMBVP strain revealed 99.93% identity to *Bacillus licheniformis*.

Keratinases have been reported to be produced by a variety of bacteria mainly by *Bacillus*<sup>[16][17][18]</sup>.

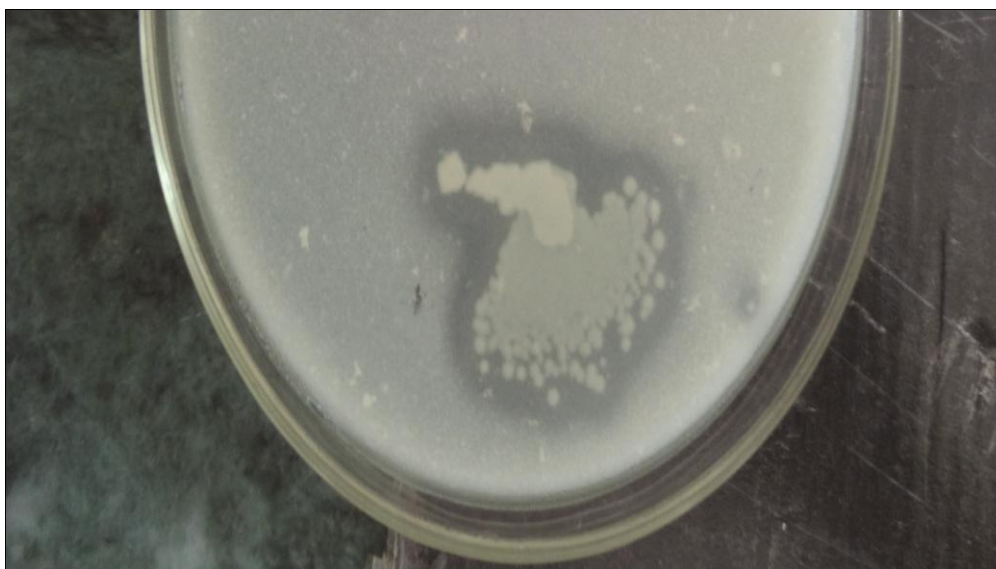


Figure 1: Casein hydrolysis by *B.licheniformis* KMBVP strain



Figure 2A: Feather in feather meal media (before incubation)

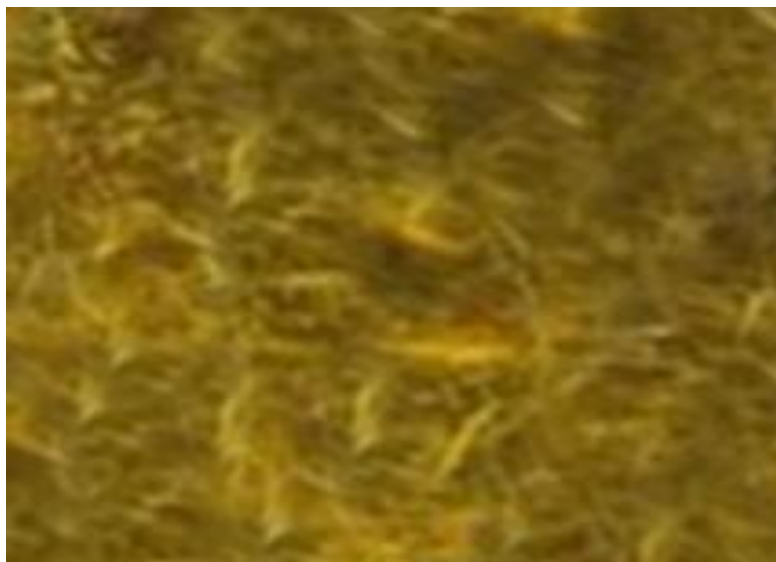


Figure 2B: Feather degradation in feather meal media by *B.licheniformis* KMBVP strain (After 6<sup>th</sup> day of incubation)

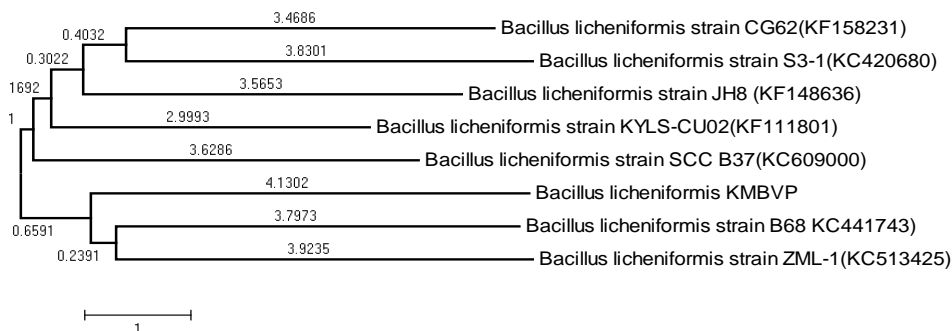


Figure 3: Identification of *B.licheniformis* KMBVP strain by neighbour joining phylogenetic tree based 16S rRNA gene sequences

#### Factors affecting keratinase production

The factors such as pH, temperature, incubation period, carbon and nitrogen sources and inhibitors were analysed to study the optimum conditions for the enzyme production by *B.subtilis* KMBVP strain.

##### a) Effect of pH:

pH and temperature are the two vital factors that aid in providing a particular characteristic feature to the microorganism such as thermophilic, mesophilic, psychrophilic, alkaliphilic or acidophilic.

The maximum keratinase production by *B.licheniformis* KMBVP strain was observed at pH 8.0 (41.2 U/ml) with 50 mM Tris-HCl buffer, while pH 3.0 showed minimum enzyme production (6.46 U/ml) with 50mM acetate buffer (Table 1). The high optimum pH is a feature of alkaline proteases<sup>[19]</sup>.

*B.licheniformis* KMBVP strain is found to be alkalophilic keratinase producing bacterium. pH 8.5 was reported as optimum for keratinase production by *Bacillus* sp. SAAS<sup>[20]</sup>; by *B. licheniformis* strain<sup>[15]</sup>; by *B. licheniformis* K-508<sup>[21]</sup>; and by *B.licheniformis* FK 14<sup>[22]</sup>. Keratinase activity was maximum at pH 10.0 in *B.licheniformis* 1269 and *B.cereus* 1268<sup>[23]</sup>.

##### b) Effect of temperature:

The maximum keratinase production by *B.licheniformis* KMBVP strain was obtained at 40°C (46.63 U/ml), and the minimum keratinase activity was found at temperature 20°C (14.68 U/ml). Increase in temperature shows decrease in enzyme production. Maximum keratinase production at 37°C was found in *B. licheniformis* K-19<sup>[24]</sup>. Maximum keratinase activity was found at 50°C with *B.*

*licheniformis* K-508<sup>[22]</sup>; and at 40°C with *B.licheniformis* 1269 and *B.cereus* 1268<sup>[23]</sup>. The optimum temperature of keratinase from *B. licheniformis* FK 14 was 60°C<sup>[22]</sup>.

**c) Effect of incubation time:**

The commercial production of the enzymes always depends on the maximum enzyme yield. Keratinase activity of the *B.licheniformis* KMBVP strain was the maximum with 7 days of incubation time (8.64 U/ml). The culture extract was harvested on this day and used for further enzymatic studies. The minimum production of Keratinase was obtained with 24 hours incubation (1.82 U/ml). Maximum keratinase production was reported on day 5 and 7, respectively, with *Bacillus thurengensis* SN2<sup>[25]</sup> and *Bacillus* strain SAA5<sup>[20]</sup>.

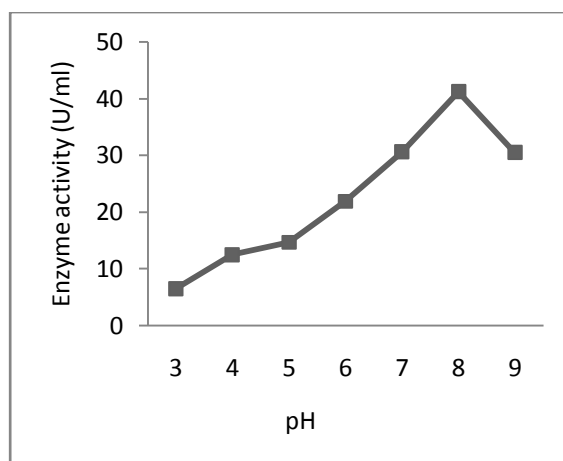
**d) Effect of carbon and nitrogen sources:**

*B.licheniformis* KMBVP strain showed high keratinase activity with feather meal (1.6 U/ml) and yeast extract

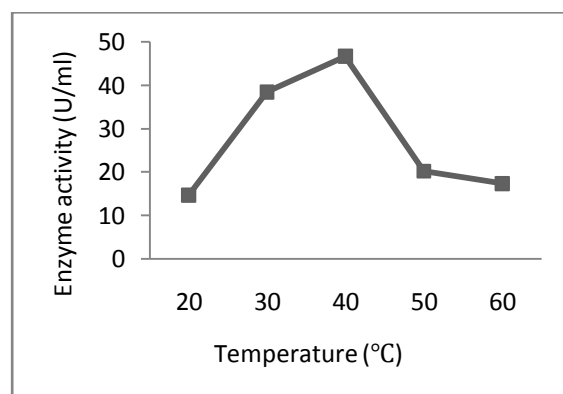
0.1% (25.44 U/ml) as carbon and nitrogen sources respectively. The maximum keratinase production by *B. megaterium* took place with 1.5% feather meal concentrations (carbon source) and in 0.1% yeast extract (nitrogen source)<sup>[26]</sup>. 1% feather meal and 0.1 % yeast extract achieved maximum keratinase production by *Bacillus* sp. JB 99<sup>[27]</sup>. 1% feather powder gave the highest keratinase activity for *B. licheniformis* PWD-1<sup>[15]</sup>. Maximum keratinase production in *B. licheniformis* YJ4 was with 0.5% feather meal after 72 h incubation<sup>[28]</sup>.

**e) Effect of inhibitors**

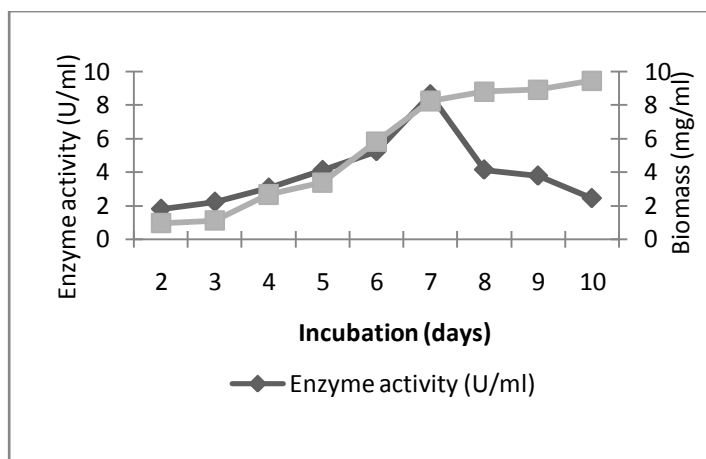
The enzyme activity was strongly inhibited by PMSF, followed by EDTA, indicating the presence of the serine group in the enzyme active site. The keratinolytic proteases produced by *Bacillus* sp. are often serine proteases, such as those produced by *B. licheniformis*<sup>[29]</sup> and *B. pseudofirmus*<sup>[30]</sup>.



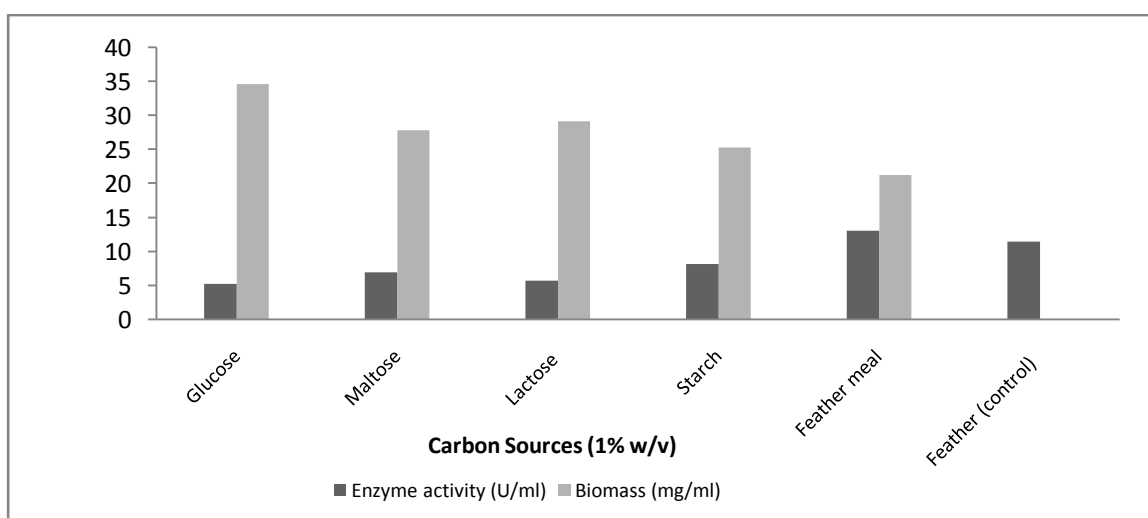
a. Effect of pH on keratinase activity by *B.licheniformis* KMBVP strain



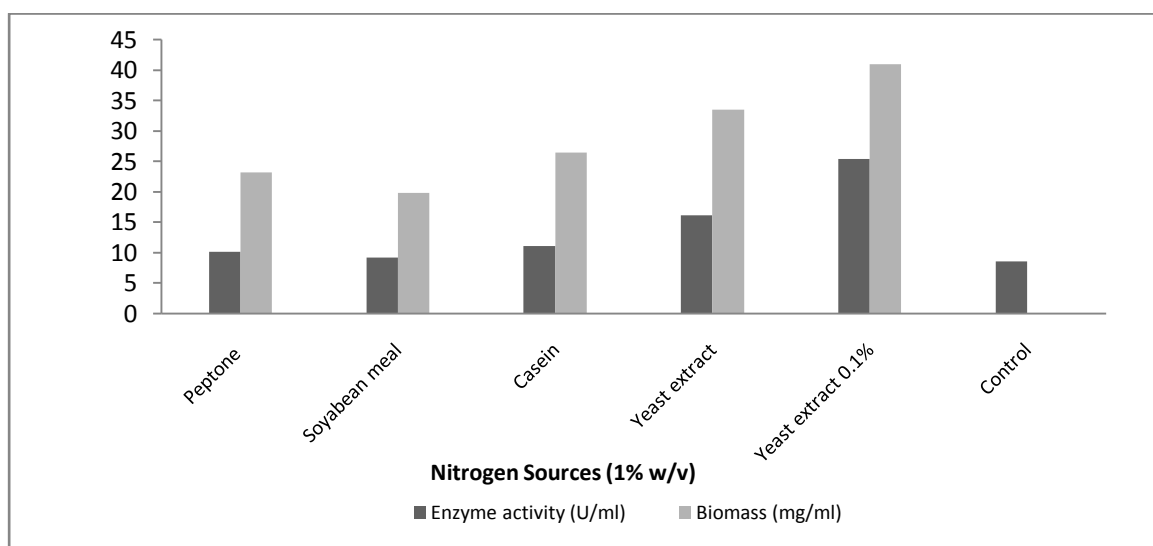
b. Effect of temperature on keratinase activity by *B.licheniformis* KMBVP strain



c. Effect of incubation period on keratinase activity by *B.licheniformis* KMBVP strain

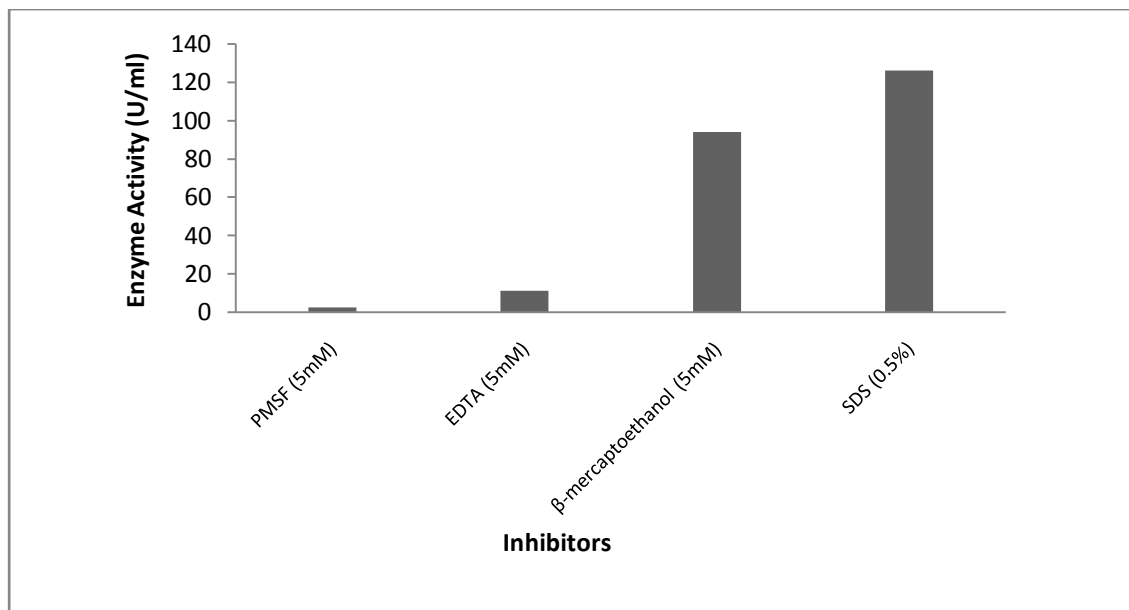


d. Effect of carbon sources on keratinase activity by *B.licheniformis* KMBVP strain



d-a. Effect of nitrogen sources on keratinase activity by *B.licheniformis* KMBVP strain





e. Effect of inhibitors on keratinase activity by *B.licheniformis* KMBVP strain

### CONCLUSION

The results suggest that the keratinolytic enzyme from the *Bacillus licheniformis* KMBVP strain belongs to alkaline serine protease family. *B. licheniformis* KMBVP strain could be a potential candidate for the degradation of feather keratin and also in de-hairing process; and can be effectively used in the large scale production of enzyme for commercial purposes.

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### REFERENCES

- Cherry, J. R., Fidantsef A. L. Directed evolution of industrial enzymes: an update. *Curr. Opin. Biotechnol*, 14: 438-443, (2003)
- Anonyme. World enzymes to 2011 (2229). *Focus on Catalysts*, 2(2), (2007)
- Kirk, O., Borchert, T. V., and Fuglsang, C. C. Industrial enzyme applications. *Curr Opin Biotechnol*, 13: 345-351 (2002)
- Manczinger, L., Rozs, M., Vagvolgyi, C. S., and Kevei, F. Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. *World J. Microbiol. Biotechnol*, 19: 35-39, (2003)
- Gushterova, A., Vasileva-Tonkova, E., Dimova, E., Nedkov, P., and Haertle, T. Keratinase production by newly isolated Antarctic actinomycete strains. *World Journal of Microbiology and Biotechnology*, 1: 831-834, (2005)
- Brandelli, A., Daroit, D. J., and Riffel, A. Biochemical features of microbial keratinases and their production and applications. *Applied Microbiology and Biotechnology*, 85: 1735-1750, (2010)
- Krilova, V. and Popov, V. A method for production of protein hydrolysate from a keratin source, SU Patent: 161-064, (1983)
- Jan P.M., Langeveld Dick, F.M., and Vande wiel, G. Jan Garsen and Alex Bosseres. Enzymatic degradation of prion protein in Brain stem from infected cattle and sheep. *The Journal of Infectious Diseases*, 188: 1782-1789, (2003)
- Friedrich, J., H. Gradisar, D. Mandin, and J.P. Chaumont. Screening fungi for synthesis of keratinolytic enzymes. *Lett. Appl. Microbiol*, 28: 127-130 (1999)
- Takami, H., N. Satoshi, R. Aono, and K. Horikoshi. Degradation of human hair by a thermostable alkaline protease from alkaliphilic *Bacillus sp.* no. AH-101. *Biosci. Biotechnol. Biochem*, 56: 1667-1669, (1992)
- Friedrich, A.B. and G. Antranikian. Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilic anaerobic species of the order *Thermotogales*. *Appl. Environ. Microbiol*, 62: 2875-2882, (1996)
- Williams, C.M., C. G. Lee, J. D. Garlich, and J.C.H. Shih. Evaluation of a bacterial feather fermentation product, feather-lysate, as a feed protein. *Poultry Sci*, 70: 85-94, 1991

13. Innis, D.H. Gelfand, J.J. Sninsky, & T.J. White (Eds.), PCR Protocols: A Guide to Methods and Application (pp. 3–12). San Diego, Academic Press: ISBN 0-123-72180-6, (1990)
14. Saitou N, Nei M. The neighbour joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 4:406-425, (1987)
15. Cheng, S.W., H.M. Hu, S.W. Shen, H.Takagi, M. Asano, and Y.C. Tsai. Production and characterization of keratinase of a featherdegrading *Bacillus licheniformis* PWD-1. *Biosci. Biotechnol. Biochem.*, 59: 2239-2243, (1995).
16. Lin, X., C.G. Lee, E.S. Casale and H.J.C. Shih. Purification and Characterization of a Keratinase from a Feather-Degrading *Bacillus licheniformis* Strain. *Appl. Environ. Microbiol.*, 58: 3271-3275, (1992).
17. Kim, J.M., Lim, W.J. and Suh, H.J. Feather degrading *Bacillus sp.*, from poultry waste. *Process Biochemistry*, 37: 287-291, (2001)
18. Savitha G. Joshi, M.M. Tejashwini, N. Revati, R. Sridevi and D. Roma. Isolation, Identification and Characterization of a feather degrading bacterium. *International journal of poultry science.*, 6(9): 689-693, (2007)
19. Adinarayana, K., P. Ellaiah and D.S. Prasad. Purification and Partial Characterization of Thermostable Serine Alkaline Protease from a Newly Isolated *Bacillus subtilis* PE-11. *AAPS Pharm. Sci. Tech.*, 4(4): Article 56, (2003)
20. Srivastava A., Sharma A. and Suneetha V. Feather waste biodegradation as a source of amino acid, *Eur. J. Exp. Biol.*, 1(2), 56-63, (2011)
21. Rozs M., Manczinger L., Vagvlgyi C. and Kevei F. Secretion of a trypsin-like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*, *FEMS Microbiol. Lett.*, 205, 221-224, (2001).
22. Suntornsuk, W., Tongjun, J., Onnim, P., Oyama, H., Ratanakanokchai, K., Kusamran, T., and Oda, K. Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium. *World Journal of Microbiology & Biotechnology*, 21: 1111–1117, (2004)
23. Mazotto, A. M., Ana Cristina N. de Melo., Macrae, A., Rosado, A. S., Peixoto, R., Sabrina M. L. Cedrola., Couri, S., Russolina B. Zingali., Ana Lu´cia V. Villa., Rabinovitch, L., Jeane Q. Chaves., Alane B. Vermelho. Biodegradation of feather waste by extracellular keratinases and gelatinases from *Bacillus* spp, *World J Microbiol Biotechnol.*, 27:1355–1365, (2011)
24. Xu B., Zhong Q., Tang X., Yang Y. and Huang Z. Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability, *Afr. J. Biotechnol.*, 8(18), 4590-4596, (2009)
25. Agrahari S. and Wadhwa N. Degradation of chicken feather a poultry waste product by keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant, *Int. J. Poult. Sci.*, 9(5), 482-489, (2010)
26. Saibabu, Francois Niyongabo Niyonzima and Sunil S. More. Isolation, Partial purification and Characterization of Keratinase from *Bacillus megaterium*, *Int. Res. J. Biological Sci.*, 2(2): 13-20, (2013)
27. Kainoor P.S. and Naik G.R. Production and charecterization of feather degrading keratinase from *Bacillus* sp. JB 99, *Indian J. Biotechnol.*, 9, 384-390, (2010)
28. Lin H.H. and Yin L.J. Feather meal and rice husk enhanced keratinase production by *Bacillus licheniformis* YJ4 and characterization of produced keratinases, *J. Mar. Sci. Technol.*, 18(3), 458-465, (2010)
29. Lin X, Kelemen DW, Miller ES, Shih JCH. Nucleotide sequence and expression of kerA, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1. *Applied and Environmental Microbiology*, 61(4): 1469–1474, (1995)
30. Kojima M, Kanai M, Tominaga M, Kitazume S, Inoue A, Horikoshi K. Isolation and characterization of a feather-degrading enzyme from *Bacillus pseudofirmus* FA30-01. *Extremophiles*, 10(3): 229–235, (2006)





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