Volume 65, Issue 7, 2021
Journal of Scientific Research
of
The Banaras Hindu University

Keratinase Enzyme Production from Bacillus Licheniformis KP9 Isolated from Chicken Feathers

Arun M. Patil[1], Jyoti P. Mahashabde[2], Leena P. Shirsath[3], Tushar A. Shinde[4], Sandip P. Patil[*3]

[1]Department of Physics, R. C. Patel Arts, Commerce and Science College, Shirpur-425405, India, ampatil67@gmail.com
[2]Department of Chemistry, R. C. Patel Arts, Commerce and Science College, Shirpur-425405, India, jyotimahashabde3@gmail.com
[*3]Department of Microbiology and Biotechnology, R. C. Patel Arts, Commerce and Science College, Shirpur-425405, [*] patilsandip3@gmail.com
[4]SVKM's NMIMS, MPSTME, Centre for Textile Functions, Shirpur-425405, India, tushar.shinde@nmims.edu

Abstract: **The aim of the present study was to isolate keratinolytic bacteria from chicken feathers, since it is rich in keratin. Microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin" recalcitrant to the commonly known proteolytic enzymes trypsin, pepsin and papain. The isolation of keratinolytic bacteria was performed by routinely used microbiological techniques and all the 11 isolates were screened for the keratinase production. The potent strain KP9 was characterized and identified by 16S r-RNA gene sequencing as *Bacillus licheniformis* KP9. The production of enzyme keratinase was studied by submerged fermentation process. The production of enzyme was optimized at various pH, temperature, incubation period and inoculum size. The maximum keratinase enzyme production by *Bacillus licheniformis* KP9 was recorded at pH 7.0, temperature 35ºC, 3% inoculum size and 48 h of incubation period.**

Index Terms: **Keratinase, Chicken feathers, Production, Optimization, *Bacillus licheniformis*.**

I. INTRODUCTION

Microbial keratinase is the type of protease enzyme capable of degrading the insoluble structural protein found in feathers, hair and wool known as keratin. Keratin is a fibrous and insoluble structural protein extensively cross linked with hydrogen, disulphide and hydrophobic bonds. It forms a major component of the epidermis and its appendages viz. hair, feathers, nails, horns, hoofs, scales and wool (Anbu et al., 2007; Kim, 2007).This protein is resistant to degradation by proteolytic enzymes such as trypsin, pepsin, papain due to the composition and molecular conformation of the amino acids found in keratin (Mukherjee et al., 2008; Rai et al., 2010). Feathers are produced in large amounts as a waste by-product at poultry processing plants, reaching millions of tons per year worldwide. Feathers contain over 90% crude protein in the form of keratin.

Keratinases (EC.3.4.99.11) belong to the group of serine proteases capable of degrading keratin. It is an extracellular enzyme produced in a medium containing keratinous substrates such as feathers and hair. Keratinases have applications in traditional industrial sectors including feed, detergent, medicine, cosmetics and leather manufacturers (Farag and Hassan, 2004), they also find application in more recent fields such as prion degradation for treatment of the dreaded mad cow disease (Langeveld et al., 2003), biodegradable plastic manufacture and feather meal production. Hence the present study focuses on the production of enzyme keratinase. Because of the numerous potential uses of keratinases, this study was undertaken to screen a bacterium that produces a highly active keratinase.

II. MATERIALS AND METHODS

A. Isolation of microorganisms

Samples of chicken feathers were collected from local poultry farms in Shirpur, India. The samples were inoculated for enrichment into the Luria-Bertani broth for 24 h at 37 ºC. After the enrichment process, samples were plated on the Luria-Bertani agar plates for the isolation of individual organisms. The plates were incubated at 37 ºC for 2 days until colonies appeared. Representative colonies were selected based on their morphology and colony colour. Selected colonies were isolated by transferring them on to the fresh LB agar plates (Suntomsuk and Suntomsuk, 2003).

[*] Corresponding Author

B. Screening for the keratinase production

The isolates were screened for the production of extracellular keratinase. Eleven morphologically different bacterial colonies were streaked onto a sterile feather meal agar plate. The plates were then incubated at 37°C for 48 h. The strain that shows highest zone of clearance was selected (KP9) and it was sub cultured. The strain was further inoculated in nutrient broth containing feather meal and kept for incubation at 37°C for 4 days on shaking incubator at 180 rpm. Then the culture supernatant was assayed for keratinolytic activity (Yamamura et al., 2002; Williams et al., 1990).

C. Characterization and identification of potent isolate

The strain (KP9), which degrade keratin effectively, was characterized. Morphological, Biochemical characterization was carried out by performing Gram Staining, spore staining, motility test, IMViC test, urease test, catalase test, starch hydrolysis test and casein hydrolysis test (Manczinger et al., 2003; Yu et al., 1968). The strain was further identified by 16S r-RNA gene amplification and nucleotide sequencing performed at the National Center for Cell Science (NCCS-NCMR), Pune, India.

D. Production of enzyme keratinase

The isolated potent strain (KP9) was transferred to 25 ml of the seed medium containing (g/L) peptone, 5; yeast extract, 1.5; beef extract, 1.5 and sodium chloride, 5; (pH 7 ± 0.2) and incubated at 37°C on rotary shaker at 180 rpm for 24 h. This was used as the inoculum for the keratinase enzyme production process. Submerged fermentation process was carried out by inoculating pure culture of potent isolate into the production medium (Suntomsuk and Suntomsuk, 2003). The production medium contains (g/L) Feather meal, 10; Yeast extract, 0.1; MgSO4, 0.1; NH4Cl, 0.5; K2HPO4, 0.3; KH2PO4, 0.3; NaCl, 0.5. Initial pH of the medium was adjusted to 7.2 ± 0.2 with Tris–HCl buffer. The medium was sterilized in an autoclave for 15 min at 121°C. The production medium was inoculated with 5% (v/v) of 24 h old inoculum culture of potent isolate containing approximately 2×106 cells/ml. The flasks were incubated on a rotary shaker at 37°C and 180 rpm for 4 days. The 10 ml fermented broth was centrifuged at 10,000 rpm for 20 min and the supernatant was used as crude enzyme.

E. Keratinase enzyme assay

The keratinase enzyme activity was assayed by taking 1.0 ml of crude enzyme properly diluted in Phosphate buffer (0.05 M of pH 7.0) was incubated with 1 ml of keratin solution at 50 °C in a water bath for 10 min, and the reaction was ceased by the addition 2.0 ml 0.4 M Trichloroacetic acid (TCA). The resulted precipitate was removed by centrifugation at 10000 rpm for 20 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 keratinase enzyme activity was defined as an increase of corrected absorbance of 280 nm (Gradisar et al., 2005) with the control for 0.01 per minute under the conditions described above.

F. Optimization of keratinase enzyme production

1) Effect of pH

To investigate the effect of pH on enzyme production, the basal medium containing 1% feather meal was prepared. The medium was adjusted to the different pH values such as 3, 5, 7 and 9. The potent isolate KP9 was inoculated in to the medium. Production process was conducted and samples were assayed for enzyme activity (Priya et al., 2011).

2) Effect of temperature

To study the effect of temperature on enzyme production, the basal medium containing 1% feather meal was prepared. The potent isolate KP9 was inoculated in to the medium. The medium was incubated at different temperatures like 5^{o}C, 20^{o}C, 35^{o}C and 50^{o}C. Production process was conducted and samples were assayed for enzyme activity (Puri et al., 2002).

G. Effect of inoculum size

To study the effect of inoculum size on enzyme production, the basal medium containing 1% feather meal was prepared. The potent isolate KP9 was inoculated in different inoculum size such as 1%, 2%, 3% and 5% into the production medium. Production process was conducted and samples were assayed for enzyme activity (Ramnani and Gupta, 2004).

H. Effect of incubation period

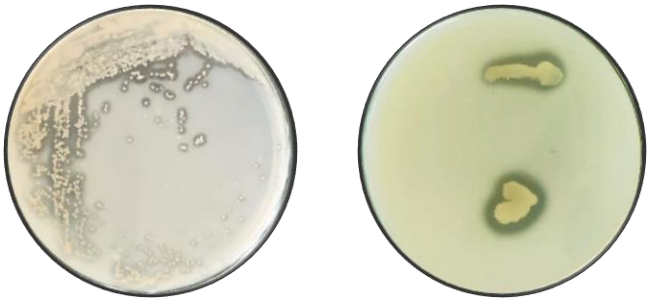
To study the effect of incubation period on enzyme production, the basal medium containing 1% feather meal was prepared. The potent isolate KP9 was inoculated in to the medium. The medium was incubated at 37^{o}C for different incubation periods like 24h, 48h, 60h and 72h. Production process was conducted and samples were assayed for enzyme activity (Sen and Satyanarayana, 1993).

III. Results and Discussion

A. Isolation, screening and identification of potent isolate

A total of eleven different bacterial strains were isolated from chicken feather sample collected from local poultry farm at Shirpur, India. These all the 11 isolates were screened for the keratinase production on basal agar medium plates containing 1% keratin. It was found that the strain KP9 shows highest zone of clearance, indicates its ability to produce enzyme keratinase (Fig.1). The strain KP9 was selected and sub cultured for further study. The strain KP9 appeared singly and was a straight rod, Gram positive and endospore forming organism. It was aerobic, motile, and catalase positive. Additional morphological, physiological and biochemical characteristics were checked as shown in Table-1. On the basis of the results of morphological, physiological and biochemical tests the strain was belongs to *Bacillus* sp. After performing 16S r-RNA gene sequencing and phylogenetic studies by using MEGA6, it was confirmed that, the

strain KP9 was identified as *Bacillus licheniformis* KP9.



A. Isolation on LB agar plate B. Potent isolate KP9

Fig. 1. Isolation of potent isolate KP9 on Luria-Bertani agar medium

Table-1. Morphological and biochemical characteristics of *Bacillus licheniformis* KP9

Characteristics/Test	Results
Shape	Rod
Gram character	Gram positive
Acid fastness	Non-acid fast
Endospore staining	Spore forming
Motility test	Motile
Indole production	Negative
Methyl red test	Negative
Vogus-Proskauer test	Positive
Citrate utilization	Positive
Catalase activity	Positive
Oxidase activity	Negative
Starch hydrolysis	Positive
Casein hydrolysis	Positive
Urease activity	Negative
Nitrate reduction	Positive
Hydrogen sulphide	Negative

B. Production of enzyme keratinase

Production of enzyme keratinase by using *Bacillus licheniformis* KP9 isolated from chicken feathers was studied by submerged fermentation process (Fig.2). The production studies were carried out at 37ºC, for 24 h on rotary shaker at 180 rpm. The 10 ml fermented broth was centrifuged at 10,000 rpm for 20 min and the supernatant was used as crude enzyme. This crude enzyme was assayed spectrophotometrically at 280 nm. Production of enzyme keratinase was optimized at various pH, temperature, inoculum size and incubation period.

Fig. 2 Submerged production of enzyme keratinase

1) Effect of pH

pH value	Enzyme activity (U/ml)
3	43 ± 1.2
5	77 ± 2.1
7	263 ± 0.9
9	141 ± 1.3

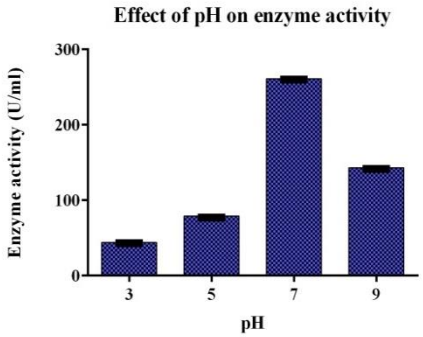


Fig. 3A. Effect of pH on enzyme activity

2) Effect of Temperature

Temperature (ºC)	Enzyme activity (U/ml)
5	13 ± 1.4
20	55 ± 1.1
35	210 ± 0.9
50	130 ± 0.8

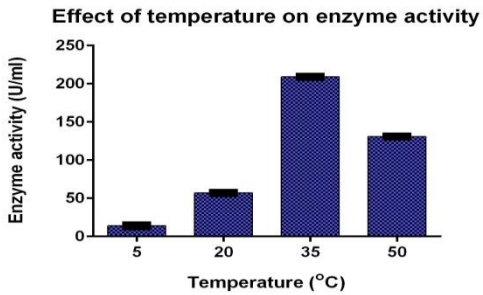


Fig. 3B. Effect of temperature on enzyme activity

4) Effect of Inoculum size

Inoculum size (%)	Enzyme activity (U/ml)
1	74 ± 1.3
2	101 ± 1.8
3	195 ± 1.6
5	150 ± 1.4

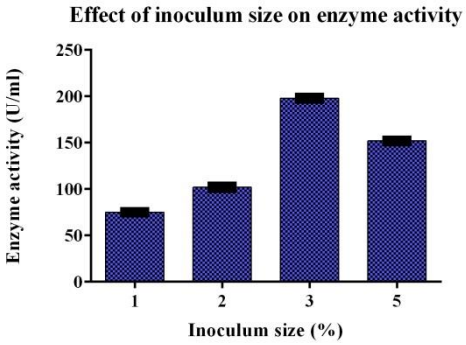


Fig. 3C. Effect of inoculum size on enzyme activity

5) Incubation period

Incubation period (h)	Enzyme activity (U/ml)
24	187 ± 1.3
48	239 ± 1.7
60	86 ± 1.2
72	47 ± 1.4

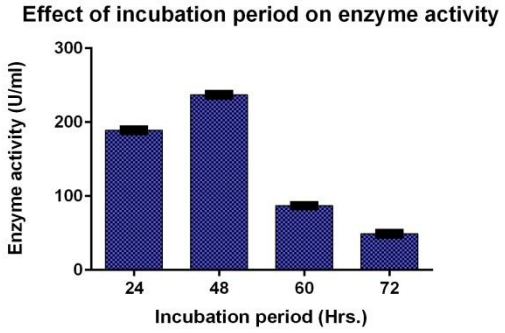


Fig. 3D. Effect of incubation period on enzyme activity

Maximum enzyme production (263 ± 0.9 U/ml) was observed at pH 7.0 i.e. at neutral pH, whereas the minimum enzyme activity (43 ± 1.2 U/ml) was seen at pH 3.0 (Fig. 3A) (Hossain et al., 2007; Najafa, 2006). In the optimization of temperature for the keratinase enzyme production, maximum activity (210 ± 0.9 U/ml) was recorded at 35ºC (Fig. 3B) (Cheng et al., 1995). Incubation period for the maximum enzyme production was recorded as 48 h. After 48 h of incubation period the keratinase enzyme production was decreased (Fig. 3D) (Lin et al., 1992). The size of inoculum at which *Bacillus licheniformis* KP9 showed maximum enzyme production is 3% (Fig. 3C) (Nilegaonkar et al., 2007).

CONCLUSION

The objective of the present study was to isolate the keratinase producing bacteria from chicken feathers, since it is rich in keratin. The production of enzyme keratinase and optimization of various parameters for the enzyme production were studied. This novel keratinolytic *Bacillus licheniformis* KP9 could be a potential candidate for the degradation of feather keratin and also in dehairing process in leather industry. This isolate could be used to produce keratinase for biotechnological applications and effectively used in the large scale production of enzyme for commercial purposes.

REFERENCES

Anbu, P., S. C. B. Gopinath, A. Hilda, T. Lakshmipriya, G. Annadurai, (2007). Optimization of extracellular keratinase production by poultry farm isolates *Scopulariopsis brevicaulis*. Bioresour. Technol. 98:1298-1303.

Cheng, S.W., H.M. Hu, S.W. Shen, H. Takagi, M. Asano and Y.C. Tsai, (1995). Production and characterization of a feather degrading *Bacillus licheniformis* PWD-1, *Biosci. Biotechnol. Biochem*. 59: 2239-2243.

Farag, A. M. and M. A. Hassan, (2004). Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae.* Enzyme Microb. Technol. 34:85-93.

Gradisar H, Friedrich J, Krizaj I, Jerala R., (2005). "Similarities and specificities of fungal Keratinolytic proteases: comparison of keratinase of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases". *Appl Environ Microbiol*; 71 (7): 3420-3426.

Hossain, M.S., A.K. Azad, S.M. Abu, G. Mostafa and M.M. Hoq, (2007). Production and partial characterization of feather degrading keratinolytic serine protease from *Bacillus licheniformis* MZK, *J. Biol. Sci.* 7(4): 599-606.

Kim J.D., (2007). Purification and characterization of a keratinase from a feather-degrading fungus, *Aspergillus flavus* strain K-03, *Microbiol*., 35(4), 219-225.

Langeveld, J. P. M., J. J. Wang, D. F. M. Van de Wiel, G. C. Shih, G. J. Garssen, A. Bossers, J. C. H. Shih, (2003). Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. *J. Infect. Dis.* 188:1782-1789.

Lin, X., C.G. Lee, E.S. Casale and J.C.H. Shih, (1992). Purification and characterization of a keratinase from feather degrading *Bacillus licheniformis* strain, *Appl. Environ Microbiol.* 58: 3271-3275.

Manczinger, L., M. Rozs, C. Vagvolgyi, F. Kevei, (2003). Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. World *J. Microbiol. Biotechnol.* 19:35-39.

Mukherjee, A.K., H. Adhikari and S.K. Rai, (2008). Production of alkaline protease by a thermophilic *Bacillus subtilis* under solid-state fermentation (SSF) condition using Imperata

cylindrical grass and potato peel as low-cost medium: characterization and application of enzyme in detergent formulation. *Biochem. Eng. J.*, 39: 353-361.

Najafa, M.F., (2006). Enzymatic properties of a novel highly active and chelator resistant protease from a Pseudomonas aeruginosa PD100, Enz. Microbial Technol. 39: 1433-1440.

Nilegaonkar, S. S., V. P. Zambare, P. P. Kanekar, P. K. Dhakephalkar, and S. S. Sarnaik, (2007). Production and partial characterization of dehairing protease from *Bacillus cereus* MCMB-326, *Bioresource. Technol.* 98: 1238-1245.

Priya, P., G. Sweta Mandge and G. Archana, (2011). Statistical optimization of production and tannery applications of a keratinolytic serine protease from *Bacillus subtilis* P13. *Process Biochem*., 46: 1110-1117.

Puri, S., Q. K. Beg, R. Gupta, (2002). Optimization of alkaline protease production from *Bacillus* sp. By response surface methodology. *Curr. Microbiol*. 44:286-290.

Rai, S.K. and A.K. Mukherjee, (2010). Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. *Biochem. Eng. J.*, 48: 172-180.

Ramnani, P. and R. Gupta, (2004). Optimization of medium composition for keratinase production on feather by *Bacilluslicheniformis* RG1 using statistical methods involving response surface methodology, Biotechnol. *Appl. Biochem.*, 40: 191-196.

Sen, S. and T. Satyanarayana, (1993). Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S40. *Indian J. Microbiol*. 33:43-47.

Suntornsuk, W. and L. Suntornsuk, (2003). Feather degradation by *Bacillus* species FK 46 in submerged cultivation. Bioresour. *Technol.* 86:239-243.

Williams, C. M., C. S. Richter, J. M. Mackenzie, J. C. H. Shih, (1990). Isolation, identification and characterization of a feather degrading bacterium. *Appl. Environ. Microbiol*. 56:1509- 515.

Yamamura, S., Y. Morita, Q. Hasan, S. R. Rao, Y. Murakami, K. Yokoyama, E. Tamiya, (2002). Characterization of a new keratin-degrading bacterium isolated from deer fur. *J. Biosci. Bioeng*. 93:595-600.

Yu, R. J., S. R. Harmon, F. Blank, (1968). Isolation and purification of an extracellular keratinase of *Trychophyton mentagrophytes*. *J. Bacteriol.* 96:1435-1436.

***