International Journal of Pharmaceutical Research and Development (IJPRD)
Platform for Pharmaceutical Researches & Ideas
www.ijprd.com

STUDIES ON PRODUCTION OF KERATINASE FROM DIFFERENT KERATIN SUBSTRATES USING *BACILLUS LICHENIFORMIS* STRAINS

Tappa Mohammad Munawar[1]*, K.Aruna[1], A.V.N Swamy[1]

[1]JNTUA college of Engineering, Department of Biotechnology,Pulivendula-516390**,** Andhra Pradesh, India.

ABSTRACT

Keratin is an insoluble fibrous protein found in hair,wool,feathers,nails,horns and other epithelial coverings.Keratinase belong to the class hydrolase which are able to hydrolyze insoluble keratins more efficiently than other proteases.Keratinases are produced only in the presence of keratin containing substrate by some species of bacillus. In the present study, Bacillus Licheniformis was producing keratinase for the substrates feathers, feather meal and human hair. Basal medium was prepared and inoculated with the strain and incubated for 7 days at room temperature on a rotatary shaker at 100 rpm.Crude enzyme was obtained and it was assayed for keratinolytic activity and degree of feather degradation. Among the three keratin substrates employed maximum enzyme was produced from feather meal. The degree of degradation was also found to be maximum in feather meal. Thus from the present study inferred that feather meal produced the maximum amount of enzyme compared to other substrates.

Correspondence Author

Tappa Mohammad Munawar

JNTUA college of Engineering, Department of Biotechnology,Pulivendula-516390**,** Andhra Pradesh, India.

Email: munna686@gmail.com

KEYWORDS *: Keratin,Bacillus Licheniformis,Basal medium,Keratinase,feather meal.*

INTRODUCTION

Keratinases are a particular class of proteolytic enzymes that display the capability of degrading insoluble keratin substrates.These enzymes are gaining importance in the last years, as several potential applications have been associated with the hydrolysis of keratinous substrates among other applications. Keratinases are produced only in the presence of keratin containing substrate. It mainly attacks on the disulfide (-S-S-) bond of the keratin substrate. The keratinase productions by various microorganisms were reported by a number of workers. It was found that keratinase produce by fungi, *Streptomyces* and bacteria were

produced in nearly at alkaline pH and almost thermophilic temperatures. These enzymes have wide range of substrate specificity such as it can degrade other fibrous protein fibrin, elastin, collagen and other non fibrous protein like casein, bovine serum albumin gelatin etc(Williams *et al.,* 1991).

A significant amount of fibrous insoluble protein in the form of feathers, hair, nails, horn, and other are available as byproducts of agro industrial processing. Feathers are composed primarily of keratin. Because of a high degree of cross-linking by disulfide and other bonds, keratin is an insoluble protein and is not degraded by normal proteases such as trypsin, pepsin and papain.Thes keratin-rich wastes are difficult to degrade as the polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions, in addition to several disulfide bonds.

Keratin is the insoluble structural protein of feathers and wool and is known for its high stability. The composition and molecular configurations of its constituent amino acids warrant structural rigidity. At least 30 different keratin polypeptides are known, falling into two Evolutionary families designated type I and type II. Within each polypeptide chain, the helical rod domain of about 310 amino acids is flanked by a shorter non helical head and tail domains, which are thought to have a flexible conformation. The keratin chain is tightly packed in the α-helix (α-keratin) or β-sheet (β-keratin) into a super coiled polypeptide chain, resulting in the mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin, and papain. Feather is pure keratin protein and is insoluble and hard to degrade due to highly rigid structure rendered by extensive disulphide bond and cross-linkages. (Riffel *et al.,* 2006).Physical and chemical treatments are used currently to increase the digestibility of feather keratin. However, these processes require consumption of large amounts of energy and they also destroy certain amino acids, thus yielding products of poor digestibility and variable nutrient quality (Papadopoulos, M.C., 1986). Dynamic hydrolysis by microorganisms that possess keratinolytic activity represents an attractive alternative to improve the nutritional value of feather wastes. Keratinolytic activity has been reported for various bacterial genera, such as Bacillus, Streptomyces Thermoanaerobacter,*Chryseobacterium*, *Flavobacterium* and *Vibrio*. (Savitha *et al.,* 2007).Keratinases are enzymes that hydrolyze keratin specifically. Keratinolytic enzymes may have potential roles in biotechnological processes involving keratin containing wastes from the poultry and leather industries through the development of nonpolluting processes. (Korkmaz *et al.,* 2004 and Kim *et al.,* 2001).Keratinolytic activity is well established among Bacillus spp. Several strains of B. *licheniformis* and *B. subtilis* are described as keratinolytic, and other species such as *B. pumilus* and *B. cereus* also produce keratinases. (Ramnani *et al.,* 2005).*B.licheniformis* is a gram positive, motile, spore forming, facultative anaerobe, and rod shaped organism. It is a nonpathogenic soil organism that is mainly associated with plant and plant materials in nature but can be isolated from nearly everywhere due to its highly resistant endospores that are disseminated with the dust.

Keratinolytic *B.licheniformis* strains are often capable of completely disintegrating feathers, and their proteolytic enzymes present a broad range of activity. (Manachini *et al.,1999*).Currently,feathers are converted to feather meal by steam pressure cooking, which require high-energy input. Feather meal has been used on a limited basis as an ingredient in animal feed, as it is deficient in methionine, histidine, and tryptophan.The utilization of a *B. licheniformis* feather-lysate with amino acid supplementation in test diets to fed growing broilers produced an identical weight gain to that of soybean meal. The use of crude keratinase significantly increased the amino acid digestibility of raw feathers and commercial feather meal.

This study was taken up with the objective for the production of keratinase enzyme from keratin substrate by a feather degrading *Bacillus licheniformis* strain, to determine the Degree of

feather degradation and to carry out a comparative analysis of keratinase production from three different keratin substrates namely, Feathers, Feather meal and Human hair.

MATERIALS AND METHODS

Microorganism and Maintenance of Culture: The organism used in the present study was *Bacillus Licheniformis* which kindly obtained from Microbial Type Culture Collection & Gene Bank (MTCC), Chandigarh, India.

Substrates and chemicals

The Feather substrates of different varieties i.e Feathers, Human hair were obtained from local market in Pulivendula, India. All other chemicals and reagents used were obtained from sigma chemicals Co.Ltd, India and were of analytical grade.

Preparation of the seed of *B. licheniformis*:

The seed medium was that of Mendel's. (Mandels. M& Weber.J., 1969).The organism were grown on synthetic media [composition (% w/v) (g/1000ml): Peptone,5g; Beef extract,1.4g; Nacl,5g], and incubated at 37^{o}C for 24 hrs.

Feather meal powder preparation

Poultry feathers were washed extensively and then boiled for 2-3 hours. These boiled feathers were then dried in a hot air oven for 4 h at 50^{o}C.The dried feathers were then pulverized and the powder was used as feather meal.

Cultivation and Keratinase production

Ten grams of feathers,feather meal and human hair was taken in a 500 ml Erlenmeyer flask seperately, moistened with 200 ml of the basal medium [composition (% w/v) (g/ 1000ml):NH_4Cl,0.5g,Yeast extract,0.1g; Nacl,0.5g, KH_2PO_4,0.4g, k_2HPO4,0.3g, $MgCl_2.6H_2O$,0.1g]. The pH was adjusted 4.8 with 0.1N NaOH. Erlenmeyer flask (500ml) containing 200 ml of the respective media were autoclaved at 121^{o}c,15 lb pressure for 15 minutes, cooled, inoculated with 1 ml of B.*licheniformis* strain and incubated at 35°C for 120 hras shown in Fig. 1.The cultures were incubated at 37°C in an orbital shaker (Remi, India) 220 rpm for 7 days. After incubation it was centrifuged (Remi cold centrifuge) at 5,000 rpm for 15 minutes at 4^{o}c.The supernatant were used as source of crude extracellular enzyme (Lin *et al.,* 1994).

Fig.1. Three different substrates used in comparative

Assay of keratinase activity

Preparation of keratin solution

Keratinolytic activity was measured with soluble keratin as substrate. Soluble keratin was prepared from chicken feathers. Native chicken feathers (5 g) in 250 ml of dimethyl sulfoxide were heated in a hot air oven at 100 °C for 2 h.Soluble keratin was then precipitated by addition of cold acetone (500L) at −20 °C for 2 h, followed by centrifugation at 5000 for 15 min. The resulting precipitate was washed twice with distilled water and dried at 40 °C.One gram of quantified precipitate was dissolved in 20 ml of 0.05M NaOH. The pH was adjusted to 7.0 with 0.1M Hydrochloric acid and the solution was diluted to 200 ml with 0.05 mol/L Phosphate buffer (pH 7.0)(Koramaz *et al.,* 2001).

Keratinase activity assay

Keratinolytic activity was assayed 1.0 ml of crude enzyme three times diluted in Phosphate buffer (0.05 M of pH 7.0) 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 0.4M Trichloroacetic acid (TCA).After centrifugation at 5000rpm for 15 min, the absorbance of the supernatant was determined at 570 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution as shown in Fig.2.One unit (U/ml) of keratinolytic activity was defined as

an increase of corrected absorbance of 570 nm (*A* 570) with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$U=4\times n\times A570/ (0.01\times T)$

Where *n* is the diluton rate; 4 is the final reaction volume (ml); T is the incubation time (min).

Fig.2 Feathers incubated in crude enzyme solution

Determination of degree of feather degradation (DFD)

The residual feather was washed, dried and scaled to calculate DFD using equation .

DFD (%) = (TF-RF)×100/TF

Where, TF is the total feather and RF is the residual feather.

RESULTS AND DISCUSSIONS

Degradation of feather by *Bacillus licheniformis*

Bacillus licheniformis was able to grow and produced keratinase in nutrient medium in which feather meal served as an additional carbon and nitrogen source and acted as enzyme inducer, resulted in nearly complete degradation of the feather after 7 days incubation at room temperature at 100 rpm. Keratinase activity was associated with growth at the maximum level of 43.2 U/mlas shown in Fig.3. Keratinolytic activity was measured in the absorbance at 570nm by the standard enzyme assay method (Cheng *et al.,* 1995).

Figure 3: After 7 days of incubation

Enzyme assay values

Keratinase was analyzed by using three feather substrates namely feathers,feather meal and human hair with final volume of the reaction mixture i.e.,4ml (1ml diluted enzyme+ 1ml keratin solution + 2ml TCA) ,Dilution rate (Crude enzyme diluted 3 times in phosphate buffer)and Incubation time 10 min **(**Manczinge *et al.,* 2003). From assay results that keratinase produced from feather meal was highest (43.2ml) among that produced by others (feathers, human hair) as shown in Table.1.

Table.1

SUBSTRATE	OD VALUES (at 570 nm)	UNITS OF ENZYME PER ml
FEATHERS	0.15	18
FEATHER MEAL	0.36	43.2
HUMAN HAIR	0.01	1.2

DFD values

From DFD calculations the feather meal was degraded to the maximum among others while human hair was found to be unaltered.Finally, from keratinase activity test observed the degradation of feathers by the crude enzyme solutions from both feathers and feather meal, but not from human hair as shown in Table.2.

Table.2

SUBSTRATE	OD VALUES (at 570 nm)	UNITS OF ENZYME PER ml
FEATHERS	0.15	18
FEATHER MEAL	0.36	43.2
HUMAN HAIR	0.01	1.2

keratinase activity on feathers

After 7 days of incubation of feathers in keratinase crude enzyme solution small particles of degraded keratin were observed. The color of the clear light yellow solution changed into a thick yellow color as shown in Fig.4.

Figure 4: Keratinase activity on feathers

CONCLUSION

On the light of the obtained results, it could be concluded among the three keratin substrates employed maximum enzyme was produced from feather meal. The degree of degradation was also found to be maximum in feather meal. Thus from the present study inferred that feather meal produced the maximum amount of enzyme compared to other substrates. The degradation of feathers was found to be maximum in feather meal and observed that the degradation and production of keratinase from human hair was least.

ACKNOWLEDGMENT

The authors are sincerely thankful to the department of Biotechnology, JNTUA college of Engineering, Pulivendula, India, for providing facilities to carry out the research work.

REFERENCES

1. 1. Williams, C.M., C.S. Richter, J.M. Mackenzie, J.R. Jason and C.H. Shih(1991), "Isolation, Identification and Characterization of a Feather-Degrading Bacterium". *Appl. Environ.*
2. 2. Riffel, A. and A. Brandelli **(**2006) **,** "Keratinolytic bacteria isolated from feather waste". *Brazilian. J. Microbiol*, 37: 395-399.
3. 3. Papadopoulos, M.C. (1986), "The effect of enzymatic treatment on amino acid content and nitrogen characteristics of feather meal". *Anim. Feed Sci. Technol,*16:. 151-156.
4. 4. Savitha G. Joshi, M.M. Tejashwini, N. Revati, R. Sridevi and D. Roma (2007), "Isolation, Identification and Characterization of a feather degrading bacterium". *International journal of poultry science.,* 6(9): .689-693.
5. 5. Korkmaz, H., H. Hur and S. Diyncer(2004), "Characterization of alkaline keratinase of *Bacillus licheniformis* strain HK-1 from poultry waste". *Annals Microbiol*,(54) : 201-211.
6. 6. Kim, J.M., Lim, W.J. and Suh, H.J(2001*)* "Feather degrading Bacillus *sp.,* from poultry waste". *Process Biochemistry,*37: 287-291.
7. 7. Ramnani, P., R. Singh and R. Gupta (2005),"Keratinolytic potential of *Bacillus licheniformis* RG1: structural and Biochemical mechanism of feather degradation". *Can. J. Microbiol*., 51: 191-196.
8. 8. Manachini, P.L., Fortina, M.G. and Parini, C., "Thermostable alkaline protease produced by Bacillus thermoruber a new *sp*., of Bacillus", *Applied Microbiology and Biotechnology*, 1998 : 28: 409-413.
9. 9. Mandels.M (1981)**,**Cellulases;*Annual reports on Fermentation Processes*,5:35-78.
10. 10. Lin, X., C.G. Lee, E.S. Casale and H.J.C. Shih(1992) ."Purification and Characterization of a Keratinase from a Feather-Degrading *Bacillus licheniformis* Strain" *Appl. Environ. Microbiol*., 58: 3271-3275.
11. 11.Lin, X., Inglis, G.D., Yanke, L.J and Cheng, K.J(1999) , "Selection and characterization of keratinase from a feather degrading bacteria from canola meal compost" *Journal of Industrial Microbiology and Biotechnology*,23: 149-153.
12. 12. Cheng, S.W., Hu, H.M., Shen, S.W., Takagi, H., Asono, M. and Tsai, Y.C(1995), "Production and Characterization of keratinase of a feather degrading *Bacillus licheniformis* PWD-1", *Bioscience Biotechnology and Biochemistry*, (59):2239-2243.
13. 13. Manczinger, L., M. Rozs, V.G. Lgyi and F. Kevei(2003), "Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain" *World J. Microbiol. Biotechnol*., 19: 35-39.

*****