International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.3, pp 1294-1300, July-Sept 2013

Optimization of Keratinase Production by Keratinolytic Organisms under Submerged Fermentation

Srinivasan Balakumar*, Narayanan Mahesh, Mani Arunkumar, Ramalingam Sivakumar, V. Hemambujavalli

Department of Chemistry and Biosciences, Srinivasa Ramanujan Centre, SASTRA University, Kumbakonam, TamilNadu – 621001, India

***Corres. Author: balakumars_76@src.sastra.edu
9994708277.**

Abstract: Feathers contain over 90% protein and formed in huge amount as a poultry waste worldwide. Build up of feather leads to feather protein wastage and environmental pollution. In the present study we screen out for keratinase enzyme producing microorganism from contaminated poultry soil. Furthermore, the screened microorganism such as *Bacillus sp.* and *Aspergillus sp* were optimized for the hyper production of keratinase. Keratinolytic actions of these isolates were screened in feather basal medium with 1% feather, pH 7.5 and incubated at 37°C for 3 days and 100 rpm. Optimization of culture conditions with effect of pH, temperature, carbon, nitrogen and a few inhibitors on keratinase production was studied. Keratinase activity was determined by using essential amino acids like valine, methionine, threonine as well as ammonia are formed when feathers were used as substrates. Bacillus species showed maximum enzyme activity (156 U/ml) when compared to *Aspergillus* species. Keratinase have prospective applications in the bioconversion of feather wastes and eco-friendly dehairing in the leather industry.
Key words: Feather, keratin, keratinase, *Bacillus, Aspergillus.*

Introduction:

Keratin is a protein which belongs to the scleropeptides group that is strongly resistant to the chemical, biological and physical components. Keratin has high mechanical solidity and resistance to common proteolytic enzymes. Accumulation of feathers leads to feather protein wastage and environmental pollution[1]. Keratin is insoluble fibrous proteins found in feather, nail, hair, wool and horns. This is rich in beta helical coil linked through cysteine bridges. Cross-linking of protein chains by cysteine bridges confer extreme mechanical steadiness and confrontation to proteolytic degradation[2].

Keratins are distinguished into α keratin (α-helix of hair and wool) and β keratin (β-sheets of feathers). The α-helix constitutes an environmental problem. Their resistance to degradation by microbes is the result of the rigid stuffing of their polypeptide chains in α-helix structural and their linkages by disulphide bridges. Keratinases are serine metallo enzyme is able to degrade the structure of keratinous proteins in nature[3].

These enzyme had broad collection of substrate specificity as it can degrade the protein, elastin, fibrous, fibrin, collagen and other non-fibrous like casein, bovine serum albumin, gelatin etc. Consequently enzymatic biodegradation may be a superior substitute to recover their nutritive value. These enzymes have the capacity to degrade resident keratin into smaller peptides entities that can consequently be absorbed by cells. Keratin degrading enzymes are extensive in nature and are produced by several microbes. Most of them are isolated from poultry waste[4].

Microbial keratinases find a special place for de-hairing process in the leather industry due to its insolubility among keratin substrates. A keratinase enzyme has been shown to be helpful for biotechnological purpose, also for hydrolysis of poultry feathers and de-hairing of bovine pelts[5, 6, 7]. Microbial keratinases are promising biocatalysts for several purposes, including applications in fertilizer, feed, detergent, leather, textile industries, and also for biomedical and pharmaceutical applications. At present, the applications are aimed at environmental friendly activities.

Materials And Methods

Sample collection and Isolation:

The contaminated soil samples were collected from poultry farm house at Mayiladuthurai. Isolation of micro-organisms was carried out by broth dilution technique. For this present study, the two most predominant strains *Bacillus sp.* and *Aspergillus sp*. were used for the degradation process and keratin was used as the substrate.

Identification of bacteria:

The bacteria were identified using Bergey's manual of determinative bacteriology based on the morphological, physiological and biochemical tests.

Identification of fungi

The fungus was identified on the basis of the colony morphology on Potato Dextrose Agar (PDA) plates and mycelial pattern was identified by Lactophenol Cotton Blue staining and slide culture technique. The isolated fungus was maintained on PDA slants and stored at 4°C.

Screening on skim milk agar plates[8]

Skim milk agar was prepared and above the dilutions of streaked on milk agar plates for testing the proteolytic activity of the organisms. Bacteria were inoculated on plates and incubated at 37°C for 24h.

Inoculum preparation

Two millimetres of the 24h bacterial suspension (12×10^6 cells /ml), grown in a nutrient broth at 35°C and 150rpm, was used as the inoculum. Fungal inoculums, 1% (v/v) of spore suspension (5×10^7 spores/ml) prepared by suspending the spores from the 7day old slant of *Aspergillus sp*. PDA slants in 10ml sterile saline contain 0.01%(v/v) tween80.

Keratinase production[9]

Experimental production was carried out in 500ml Erlenmeyer flasks containing 100ml of the culture medium (composition/1) (10g raw feather, NH_4Cl 0.5gm, NaCl 0.5gm K_2HPO_4 4gm, KH_2PO_4 0.3gm) for 3days at optimized temperature at 150rpm. After 3days of incubation, the culture medium was filtered through Whatman No.4 filter paper and the filtrate was collected. The filtrate was centrifuged at 10,000rpm for 10minutes at 37°C and culture supernatant were used as crude enzymes to examine keratinase activity and protein content.

Keratinase assay

The keratinase activity was assayed as follows: 1ml of the enzyme solution was incubated with keratin powder (1% w/v) in 2ml 0.1 M phosphate buffer pH 8.0 for 1h at 40°C in a shaking water bath. The reaction was blocked by adding 2ml of 20% TCA and followed by filtration to eliminate the substrate. The filtrate was spectrophotometrically measured for discharge of the protein at 595 nm. One unit (U) of keratinase activity was defined as the amount of enzyme causes 0.01 increases in absorbance among sample and control at 595 nm following one hour under the conditions given.

Protein Estimation:

Protein level in feather was estimated by the method using BSA as standard**.**[10]

Optimization of cultural condition

Effect of pH on Keratinase production:

The effect of initial pH of the medium on keratinase production was observed by using different pH like 4.0, 5.0, 6.0, 7.0, and 8.0.

Effect of temperature on keratinase production:

The effect of initial temperature of the medium on keratinase production was observed by using different temperature like 20°C, 30°C, 40°C, 50°C and 60°C.

Effect of carbon sources on keratinase production:

The feather basal medium was supplement with 1% (W/V) starch, lactose, glucose, maltose and sucrose to study the effects of different carbon source on keratinase production.

Effect of nitrogen sources on keratinase production:

Additional of equimolar amounts of different nitrogen sources such as peptone, yeast extract, sodium nitrate, ammonium sulphate and urea on keratinase production were also studied.

Results And Disscussion

The feather degrading microorganisms were isolated from the contaminated poultry soil from poultry farm house at Mayiladuthurai. The feather degrading organisms were isolated and maintained on the nutrient agar and potato dextrose agar medium. From the different isolates, the two most predominant strains *Bacillus spp*. and *Aspergillus spp*. were used for the feather degradation. Feather degrading organisms were isolated from poultry farm house soil using dilution techniques and identified the strains[10].

The isolated organisms *Bacillus* and *Aspergillus* were streaked on skim milk agar for screening and large clear zones were observed. This is preliminary screening for the isolation of proteolytic organisms. Skim milk agar plate was used for the screening process of Keratinolytic organism[11].

Keratinase production was carried out using the feather basal medium and its activity was measured by the standard assay procedure[12]. In this present study *Bacillus spp* have highest keratinase activity than *Aspergillus spp.,* as it produced 168 U/ml of keratinase activity. *Bacillus spp*. like *B. subtilis, B. licheniformis* were grown optimal conditions and showed maximum keratinase activity[12, 13, 14]. *Aspergillus spp* showed lesser keratinase activity than other fungal isolated strains, as it produced 10 µg/ml of keratinase activity (Fig.2&3).

Protein content measurement:

The protein content was measured by folin phenol reagent method. According to the keratinase activity on the feathers, the value of protein content was varied. Here, *Bacillus sp*. showed the highest protein content than *Aspergillus spp*. as it produced 1.1mg/ml and 0.98mg/ml of protein content respectively.

Optimization of cultural conditions:

Effect of pH on keratinase production:

Keratinase production was optimized with different pH concentration such as 4, 5, 6,7 and 8. The highest keratinase production was observed at pH 7 and 8 by *Bacillus spp. (*156 U/ml) and fungi showed highest production at pH 5 (96 U/ml). On comparison of both the organisms, *Bacillus spp.* more promisisng than *Aspergillus spp.,* The lowest production was observed at pH 4 by *Bacillus spp* and *Aspergillus spp.* at pH8, as they produced 30U/ml and 18 U/ml respectively. *Bacillus spp.* grown on feather basal medium and optimized with different pH parameters[15,16]. At pH 7 and 8, *Bacillus subtilis* showed higher keratinase production (Fig.1). Some of the researchers indicating that keratinase were produced by *Bacillus* sp at pH between 7 and 8[17].

Effect of temperature on keratinase production:

To check the keratinase production and optimized with various temperature such as 20°C, 30°C, 40°C, 50°C and 60°C. The highest keratinase production was observed at 30°C by *Bacillus spp* and *Aspergillus spp.* at 50°C, 54 U/ml (Fig.2). Both strains yield maximum production, but *Bacillus spp.* showed best production than *Aspergillus spp. Bacillus subtilis* inoculated in medium and optimized the production at different temperature. The increased production was identified at 30°C.

Effect of carbon sources on keratinase production:

Glucose, fructose, maltose, lactose and sucrose were supplemented with feather basal medium at 1gm concentration for the optimization of keratinase. Maltose was found to be sole carbon source for the production of keratinase by both *Bacillus spp* and *Aspergillus spp.* The lowest keratinase production was observed at sucrose and glucose by both strains (Fig.3). Sucrose was optimized with medium and selected as sole carbon source for the maximum keratinase production[6].

Effect of nitrogen sources on keratinase production:

To optimize the keratinase production with different nitrogen sources such as yeast extract, peptone, ammonium nitrate, sodium nitrate and urea. 1gm of nitrogen sources were supplemented with feather basal medium. Ammonium nitrate was optimized with medium and selected as sole carbon source for the keratinase production by both *Bacillus spp* showed maximum production than *Aspergillus spp.* The lowest keratinase production was observed at sodium nitrate by both organisms, 48 U/ml &18 U/ml respectively (Fig.4). Ammonium nitrate was the best nitrogen source for optimum keratinase production[14].

Figure 1: Effect of pH on keratinase production

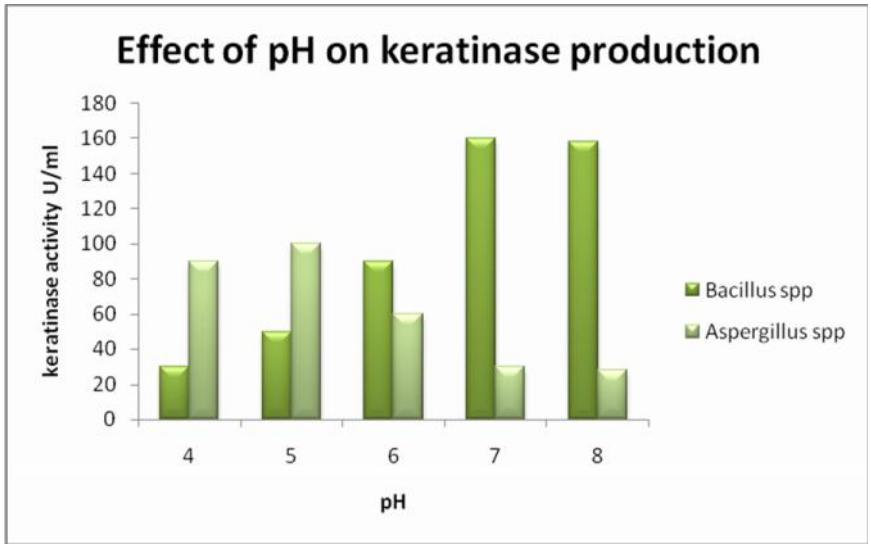


Figure 2: Effect of temperature on keratinase production

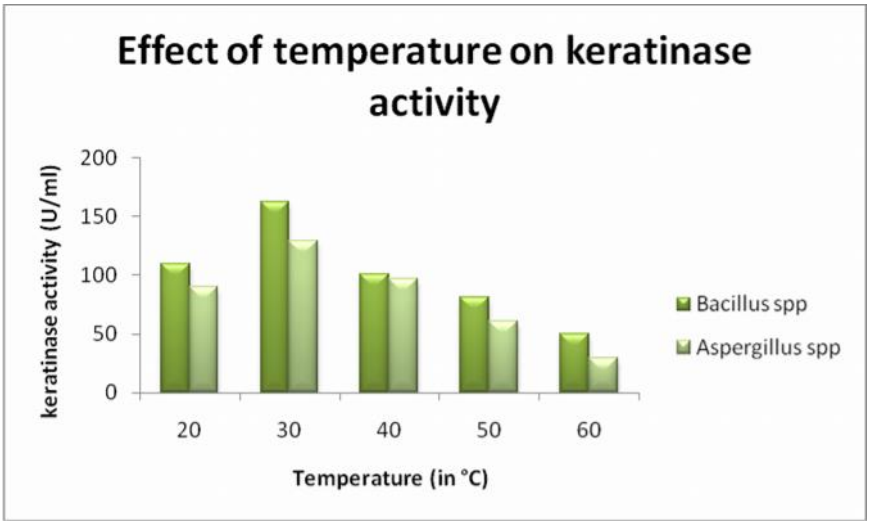


Figure 3: Effect of carbon sources on keratinase production

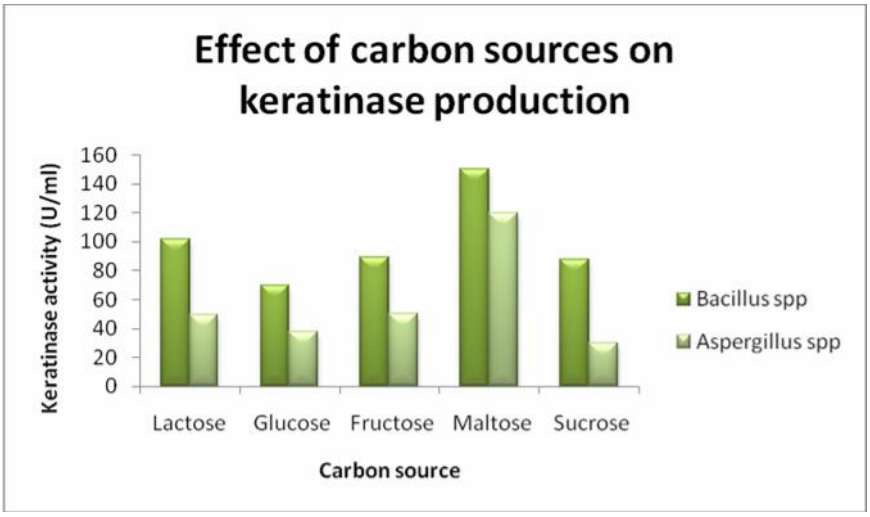
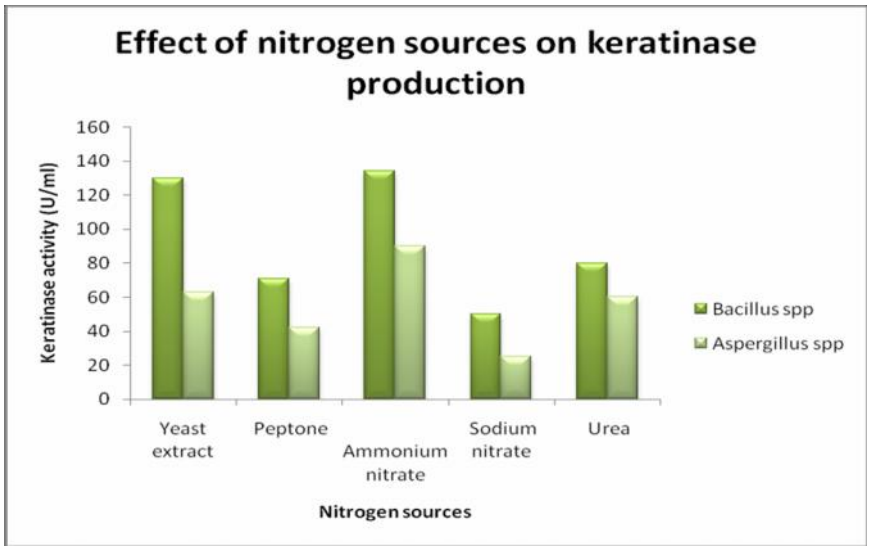


Figure 4: Effect of different nitrogen sources on keratinase production



Conclusion:

Recently *in vitro* extraction of keratinase enzymes from keratinolytic microorganisms have been employed for animal feed processing, sewage treatment and even environmental bioremediation of animal waste polluted areas. A feather degrading organism was isolated from poultry waste. Optimum culture conditions for keratinase production keratin as substrate. The maximum keratinase activity was obtained at pH 7&8 and temperature at 30°C. When optimized with different carbon sources, maltose stimulates keratinase production and showed highest activity. Similarly various nitrogen sources supplemented with medium for optimization of production. Ammonium nitrate was found to be the best source for the keratinase production by both strains. When compared to the two strains, *Bacillus* spp. showed best growth and higher activity on feathers than *Aspergillus* spp. This current research work elucidated that the evaluation of biotechnological use of the keratinase from selected keratinolytic organisms requires more comprehensive perceptive of the factors that make possible this enzyme for complete degradation of resident keratinous substrates.

References:

1. Cai C, Ji-shuang Chen, Jiong-jiong Qi, Yun Yin, and Xiao-dong Zheng Purification and characterization of keratinase from a new *Bacillus subtilis* strain J Zhejiang Univ Sci B, 9(9): 713–720, (2008).
2. Riffel, A., Lucas, F., Heeb, F. and Brandelli A. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Arch Microbiol, 179:258-265 (2003).
3. Ramnani, P. and Gupta, R. Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG1 using statistical methods involving response surface methodology. Biotechnol and Appl Biochem, 40:491-496 (2004).
4. Sharma R and Gupta R. Thermostable, thiol activated keratinase from *pseudomonas aeruginosa* KS-1 for prospective application in Prion decontamination. Research J Microbiol, 5:954-965, (2010).
5. Kumar, D., Savitri, Thakur, N., Verma, R. and Bhalla, T.C.. Microbial Proteases and application as laundary detergent additive. Research Journal of Microbiology, 3:661-672, (2008).
6. Riffel, A., Brandelli, A., Bellato, C. M., Souza, G. H. M. F., Eberlin, M. N. and Tavares, F.C. A. Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium sp.* kr6. J. Biotechnol, 128: 693-703 (2007).
7. Rouse, J.G. and Van Dyke, M.E. A review of keratin-based biomaterials for biomedical applications. Materials, 3:999-1014, (2010).
8. 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).
9. Kansoh, A.L., Hossiny E.N. and Abd EL-Hameed E.K. Keratinase production from feathers wastes using some local *Streptomyces* isolates. Australian Journal of Basic and Applied Sciences, 3:561-571, (2009).
10. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951) Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265-275.
11. 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: 229–235, (2006).
12. Riffel, A., & Brandelli, A. Keratinolytic bacteria isolated from feather waste. Brazilian Journal of Microbiology, 37: 395–399, (2006).
13. Matikeviciene V, Masiliuniene D, Grigiskis S. Degradation of keratin containing wastes by bacteria with keratinolytic activity. Environ Technol Resour, 1:284-289, (2009).
14. Cai, C and Zheng X. Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N2 using response surface methodology**.** J Ind microbial Biotechnol, 36:875-883 (2009).
15. Macedo, A.J., Silva, W.O.B and Termignoni, C. Properties of a non collagen degrading *Bacillus subtilis* keratinase. Can J Microbiol, 54:180-188, (2008).

16. Cai C, Ji-shuang Chen, Jiong-jiong Qi, Yun Yin, and Xiao-dong Zheng Purification and characterization of keratinase from a new *Bacillus subtilis* strain J Zhejiang Univ Sci B, 9(9): 713–720, (2008).
17. Selvam K., Vishnupriya B. and Yamuna M.. Isolation and description of keratinase producing marine actinobacteria from South Indian Coastal Region. African Journal of Biotechnology, 12(1): 19-26, (2013)
18. Deivasigamani B. and Alagappan K.M. Industrial application of keratinase andsoluble proteins from feather keratins. J Environ Biol, 29: 933-936, (2008).

*****