Available online at http://www.ijcrls.com

International Journal of Current Research in Life Sciences
Vol. 07, No. 02, pp.1018-1024, February, 2018

ISSN: 2319-9490

RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF NOVEL EXTRACELLULAR KERATINASE ENZYME FROM POULTRY FEATHER WASTE

[1]Mariyammal, P., *[,1]Ezhilarasu, A., [2]Karthy, E. S. and [3]Menaga, D.

[1]Department of Microbiology, Selvamm Arts & Science College, Namakkal, Tamilnadu, India
[2]AWE CARE, Analytical & Research Institute, Erode, Tamilnadu, India
[3]Vivekanandha College of Arts & Science for Women, Tiruchengode, Tamilnadu, India

Received 20th December, 2017; Accepted 27th January, 2018; Published Online 28th February, 2018

ABSTRACT

Keratinolytic microorganisms have great importance in the production of protein hydrolysates. Bacillus sp., a potential chicken feather degrading bacterium was isolated and identified by morphological and biochemical methods was used in the present study. The interactions of various factors such as temperature, pH, substrate concentration and composition of carbon and nitrogen sources as well as the level of inoculants were determined. The optimum conditions for keratinase production and feather degradation was at pH 6 and 31ºC. The maximum amount of enzyme production was observed in yeast extract 15.83 U/ml at 72 hours, fructose (5.64 U/ml) at 48 hours and 0.5 mg of chicken feather as substrate produced 11.44 U/ml at 96 hours in Bacillus sp. The maximum keratinase production was recorded at 96 hours in 100 µl of inoculum for specific activity 3.49 U/ml. Bacillus sp. can be used as an effective tool for the melanised feather degradation and gives a remedy for environmental concern over abundant feather waste conversion to value added product formation in biotech industry with its promising keratinolytic abilities.

Key words: Feather-degradation, Keratin, Keratinase, Poultry waste.

Copyright © 2018, Mariyammal et al. *This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.*

Citation: Mariyammal, P., Ezhilarasu, A., Karthy, E. S. and Menaga, D. 2018. "Purification and characterization of novel extracellular keratinase enzyme from poultry feather waste" *International Journal of Current Research in Life Sciences*, 7, (02), 1018-1024.

INTRODUCTION

Keratins are a family of important structural proteins found in feathers, wool, horn, hooves, nails, claws, beaks and hair. Feather keratin exhibits an elevated content of several amino acids such as glycine, alanine, serine, cysteine and valine. Because of a high degree of cross-linking by cysteine disulfide bonds, hydrogen bonding, and hydrophobic interactions, keratin is insoluble and not degradable by proteases such as trypsin, pepsin, and papain (Williams *et al*., 1990). Worldwide, millions of tons of feathers were released annually as a waste product mainly from poultry processing plants. The poultry feather are dumped, used for land filling and incinerated or buried, which involves problems in storage, handling, emission control and ash disposal (Mazotto *et al*., 2011). Discarded feathers also considered to be a serious source of pollution and cause various infections including chlorosis, mycoplasmosis and fowl cholera (Xu *et al*., 2009). There are several methods are available for degrading feathers but the major demerit of mechanical and chemical degradation methods over biological procedures that certain amino acids might be destroyed that leads to low protein quality and digestibility but also consume large amounts of energy (Xu *et al*., 2009). Keratinase is an extracellular enzyme used for the biodegradation of keratin. Keratinase is produced only in the presence of keratin substrate. Bacterial strains are known which are capable of degrading feathers. These bacterial strains produce enzymes which selectively degrade the beta-keratin present in feathers. These enzymes make it possible for the bacteria to obtain carbon, sulfur and energy for their growth and maintenance from the degradation of beta keratin. Biodegradation of poultry waste by keratinases is an environmentally friendly process that can play an important role in biotechnological such as enzymatic improvement of feather meal, production of rare amino acids (serine, cystein and proline) and peptides, used in the leather industry as well as medicine and cosmetic production. Alternatively, they can be used in the conversion of feathers into value-added products including fertilizers, glues, films and foils (Jin- Ha Jeong *et al*., 2010; Mohorcic *et al*., 2007; Zerdani *et al*., 2004). Additionally, the accumulation of some of these wastes in nature is considered to be a serious source of pollution and health hazards. The present work was designed to screen keratin disposal sites in Erode district for the isolation of keratinolytic bacteria and to determine their abilities to utilize keratin substrates from the feather wastes for the production of keratinases. One of the isolate, a *Bacillus* sp.,

Corresponding author: **Ezhilarasu, A.**
Department of Microbiology, Selvamm Arts & Science College, Namakkal, Tamilnadu, India.

demonstrated a high keratinolytic activity toward native keratin substrates, notably poultry feathers at room temperature, which makes it a promising tool for efficient biotechnological treatment of this waste.

MATERIALS AND METHODS

Feather Sample collection

Chicken feathers were collected from chicken shop. Collected feathers were extensively washed in tap water and finally with double distilled water. Washed feathers were dried under sunlight and then oven-dried at 75ºC for 8 hours. The dried feathers were kept at room temperature and used for further studies.

Isolation of Microorganisms

Soil sample was obtained from waste disposal yard at Anthiyur, Erode district. Then 1 g of soil sample was dispersed in 9 ml of sterile distilled water. About 0.1 ml of the aliquot was used to inoculate the feather meal medium for the selective growth of isolates using the spread plate method. The feather meal medium was prepared as follows NaCl - 0.25 g; NH_4Cl - 0.25 g; $MgCl_2$ - 0.05 g; KH_2PO_4 - 0.20 g; K_2HPO_4 - 0.15 g; Yeast extract - 0.05 g; Keratin substrate (feather) - 0.5 g and Agar agar. The pH of the medium was adjusted to 7.5 and the medium was sterilized at 121ºC for 15 min and then supplemented with 0.0 5 g of sterile nystatin to inhibit the growth of fungi. The plates were incubated at 37ºC for up to 3 days. Distinct colonies, observed using morphological features, were selected, isolated and purified on feather meal medium to obtain pure cultures. Pure cultures were stored on agar slants of feather meal medium. The pure cultures were identified by means of taxonomic schemes and descriptions (Venkata Nagaraju and Divakar, 2013).

Keratinase Enzyme Assay

After incubation the culture were centrifuged at 5500 rpm for 30 minutes and the supernatants were taken for keratinolytic activity which was assessed by a modified method of (Yu *et al*., 1968)Yu and co-author. About 20 mg of the clean chicken feather was suspended in 3.8 ml Tris HCL buffer. About 0.2ml of culture filtrate was added and the reaction in tubes was incubated at 30ºC for 1hour. Then the mixture was immersed in ice chilled water for 10 minutes. Feather was removed by filtration and the absorbance of the filtrate was read at 280nm in UV spectrophotometer.

Effects of pH and temperature on keratinolytic activities

The effect of the pH for keratinase production was determined by culturing the bacterium in the production media with various P^H 5, 6, 7, 8 and 9. The keratinase enzyme assay was carried out after 24, 48, 72 and 96 hours. The effect of temperature was measured by incubating the culture media at different temperature 28 and 45ºC at the optimum pH. The residual activity of the enzyme was determined after 24, 48, 72 and 96 hours (Venkata Nagaraju and Divakar, 2013).

Effects of carbon and nitrogen sources, inoculum and substrate concentration on keratinolytic activities

The feather meal medium was supplemented with various carbon and nitrogen sources separately. The additional carbon sources (1%) included glucose, sucrose, maltose, lactose and fructose. Then, additional nitrogen sources (1%) included yeast extract, ammonium nitrate, peptone, ammonium chloride and urea were selected individually. The bacterial inoculum was added at different concentration such as 100, 150, 200 and 250 µl and the keratin substrate (feather) was also added at different concentration such as 0.5, 1, 2, 4 and 6 mg to test its ability to induce keratinase production in the production medium (Venkata Nagaraju and Divakar, 2013).

Isolation and separation of plasmid DNA

The plasmid DNA was isolated from the multidrug resistant *Bacillus* sp. by following the method of Dharmadhikari, (Dharmadhikari and Beshwe, 2009). DNA was separated by agarose gel electrophoresis method. The power cord was connected to the electrophoretic supply at 50-75V and the samples were loaded in the wells in desired order for 3 hours. The visualization of plasmid DNA was observed under UV illumination.

Biochemical Analysis

Protein content was analysed using the Lowry method with bovine serum albumin as standard protein (Lowry *et al*., 1951). The total carbohydrate was estimated by Anthrone method with glucose as standard carbohydrate (Sadasivam and Manickam, 1992).

Protein Mass Determination- SDS PAGE Analysis

In order to study the protein profile of lipolytic enzymes both lipase and amylase producing bacteria in cell free broth, performed electrophoresis by the method of Laemmli (1970). Gel was casted by using discontinuous buffer system having 10% resolving gel and 5% stacking gel. The resolving gel was poured and overlaid with few drops of deionizer water and kept in the incubator at 37ºC for some time till polymerized and then the water was poured off. The stacking gel was poured onto the resolving gel, the comb was fixed and the gel was allowed to polymerization. One micro liter of 25% bromophenol blue (tracking dye) and few crystals of sucrose were added in each sample and mixed by vortex. 75 µl of crude protein samples were loaded. The electrophoresis was carried out at 100 volts (constant) and 25 mA for 2 hours in electrode buffer (pH 8.3) containing 0.05 M Tris-HCl and 0.24 M glycine. After electrophoresis, the gel was carefully placed in staining tray and soaked in about 100 ml of Coomassie Brilliant Blue staining solution.

RESULTS AND DISCUSSION

Several bacteria belonging to the genus *Bacillus* were obtained during the course of this research, where obtained from a chicken-feather disposal site, was found to produce high titres of enzyme during the period of cultivation. Feather degrading microorganisms were isolated from soil samples collected near the poultry farm by spread plate method. The colonies that showed a clear zone on casein plates (Fig. 1) were regarded as enzyme producers. Based on microscopic and biochemical tests, the isolate was identified as *Bacillus* sp. After one week incubation time, feather powder appeared in the medium and feather was fractured to shorter pieces, but not separated from rachis.The feathers were degraded thoroughly after 2 weeks of incubation in feather meal medium (Fig. 2).

Fig. 1. Casein agar plate showing the proteolytic activity

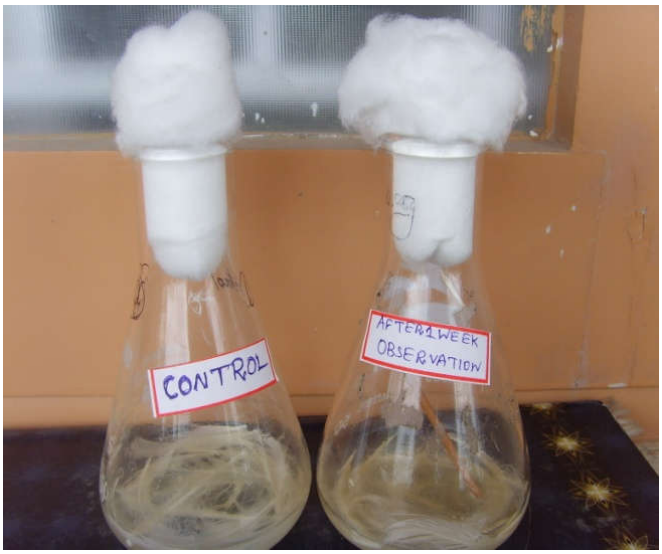
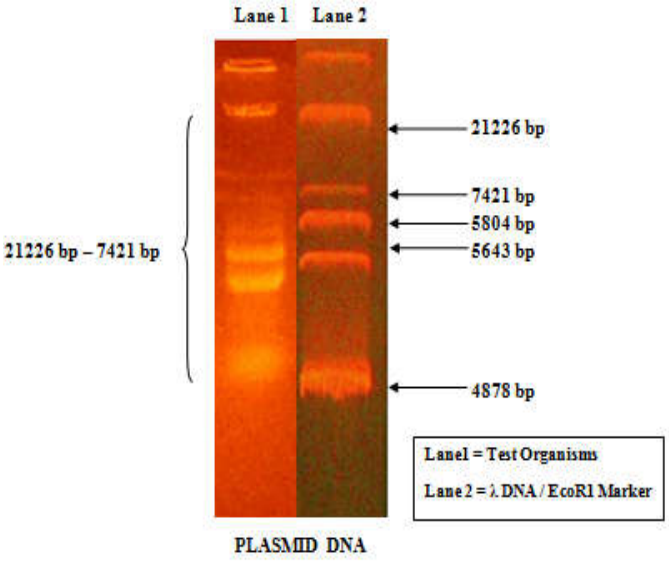
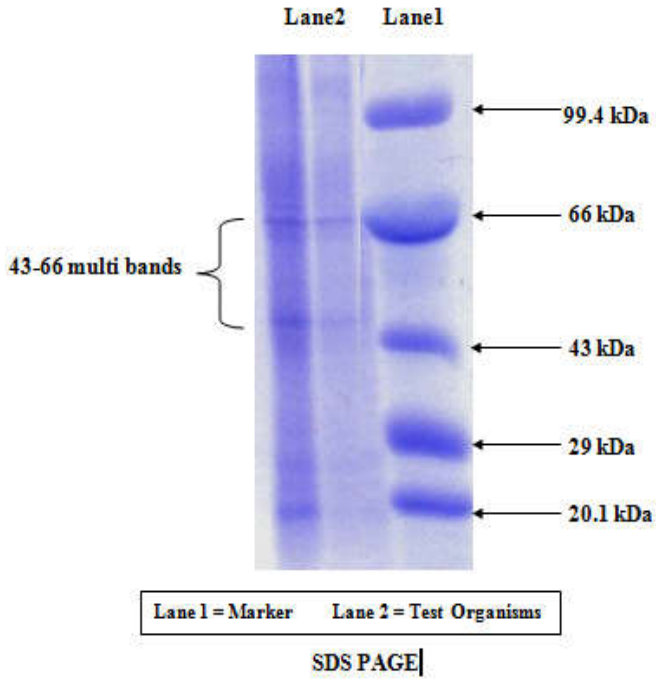


Fig. 2. Feather degradation in feather meal medium after 1 and 2 weeks of incubation time

Casein agar assay is a useful tool for the screening of proteolytic microorganisms and determination of proteolytic and keratinolytic activities. Casein is a highly stable and hydrophobic non-fibrous protein which does not coagulate and denatured by heat but can be hydrolysed by certain proteases (Cheng *et al*., 1995). Based on these characteristics we selected casein hydrolysis assay for the identification of proteolytic microorganisms that can hydrolyse casein and other structurally similar substrates such as keratins.





Coomassie blue-stained SDS-polyacrylamide gel after electrophoresis

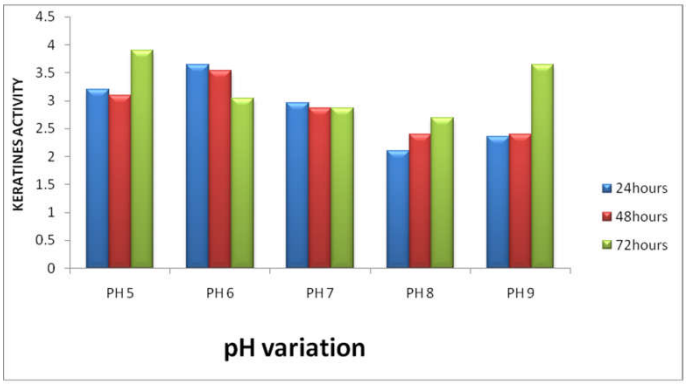


Fig. 3. Effect of various P^H on keratinase production

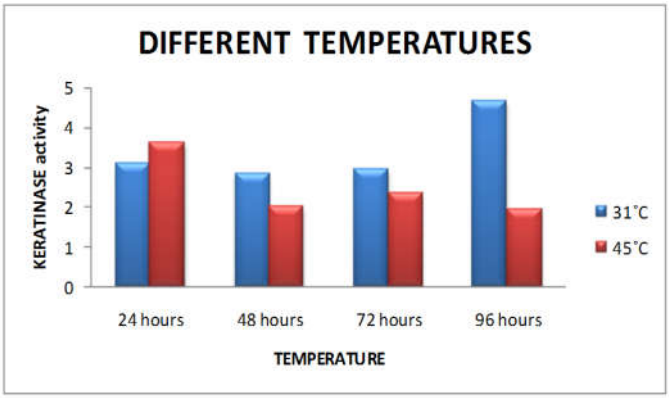


Fig. 4. Effect of various temperatures on keratinase production

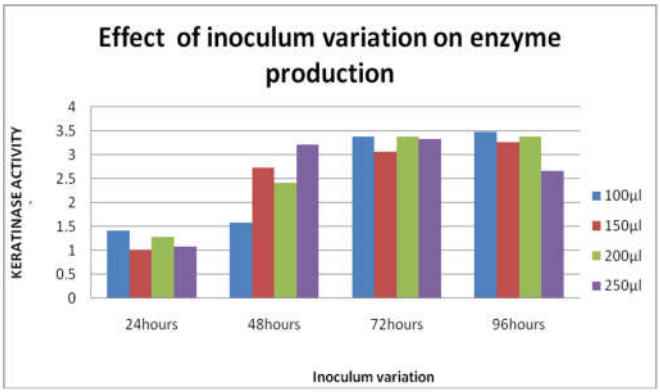


Fig. 5. Effect of various inoculum levels on keratinase production

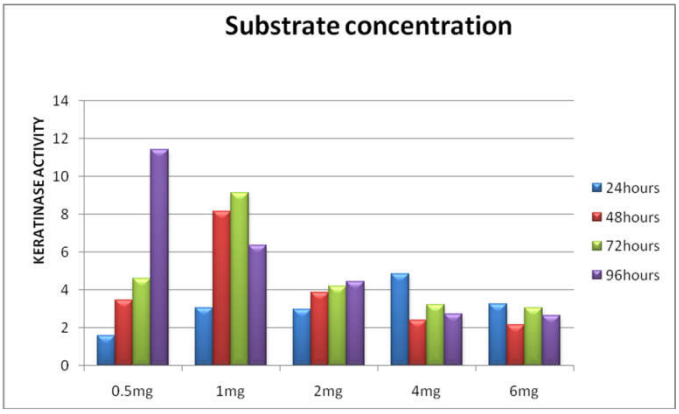


Fig. 6. Effect of substrate concentration on keratinase production

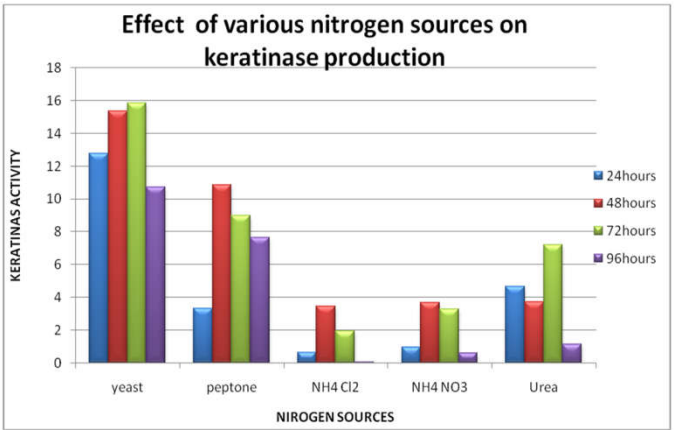


Fig. 7. Effect of various nitrogen sources on keratinase production

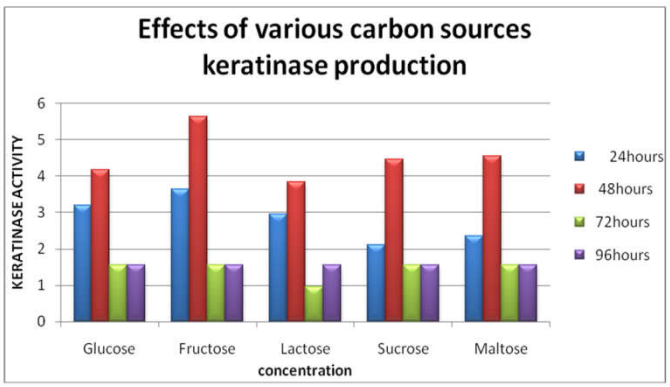


Fig. 7. Effect various carbon sources on keratinase production

It is important to note that since not all proteases can degrade keratin, an evaluation of keratinolytic activity of potential keratinase producers should be confirmed by the keratinolytic assay which is highly specific for determination of keratinase activity. *Bacillus* sp. is known to produce a number of hydrolytic enzymes including keratinase, which is able to degrade feather, wool, and hair (Daroit *et al*., 2009). *Bacillus* sp. was found to express significant keratinase degradation activity. Isolated strain was identified as a Gram-positive, rod shaped, endospore forming bacterium displaying a number of characteristics similar to the *Bacillus* species listed in the Bergey's Manual of Determinative Bacteriology. The aerobic, mild and easily manageable growth conditions could make this bacterium an attractive candidate for biotechnological applications. The interaction of various factors such as temperature, pH, substrate concentration, composition of carbon and nitrogen sources as well as the level of inoculants, influences cell growth and survival, and the level of protease synthesis in a microbial culture (Singh *et al*., 1975). Thus, optimisation of these factors is important for an effective synthesis of biological entities such as keratinases.

Keratinolytic activities were checked at feather meal medium at pH (5-9). The keratinolytic activity was observed at the pH 5. Maximum enzyme production was observed between pH 6 and 9, indicating its predominant alkaline character. Additionally, high enzyme production was achieved at the pH 5 and 6 indicating the potential versatility of such enzyme preparations for diverse applications (Table 1 and Fig. 3). Maximum activity in the alkaline range suggests a positive biotechnological potential in the detergent and leather industries (Gupta *et al*., 2002). To explore the effect of temperature on keratinolytic activity, *Bacillus* sp. was cultivated under different temperatures. Table 2 and Fig. 4 shows the maximum keratinase production was recorded at 31ºC (specific activity 4.67 U/ml at 96 hours). Minimum keratinase production was recorded at 45ºC (specific activity 3.61 U/ml at 48 hours). The present study was in agreement with Gupta and Ramnani, (2006) who reported that the optimum temperature of most keratinases is in the range of 30-80ºC. able 3 and Fig. 5 show the initial inoculum level has played an important role in keratinase production by *Bacillus* sp. The maximum keratinase production was recorded at 96 hours in 100 µl of inoculum for specific activity 3.49 U/ml. Minimum amount of keratinase production was observed at 24 hours in 150 µl of inoculum for specific activity (1.01 U/ml). Keratinolytic activity was examined using 0.5 to 6 mg of chicken feather as substrate for 96 hours in *Bacillus* sp. The maximum enzyme production was observed in 0.5 mg substrate (11.44 U/ml) at 96 hours (Table 4 and Fig. 6).

Table 1. Effect of various P^H on keratinase production

S.No.	Different P^H	OD Value/ Percentage of Transmission (%) (hours)							
		24		48		72		96	
1.	P^H 5	3.20	48.1	3.10	49.90	3.90	40.7	3.90	51.3
2.	P^H 6	3.64	43.1	3.54	51.00	3.04	49.5	3.04	54.2
3.	P^H 7	2.96	50.5	2.86	51.2	2.87	51.5	2.87	51.2
4.	P^H 8	2.10	59.20	2.40	52.31	2.69	53.5	2.69	50.7
5.	P^H 9	2.36	58.0	2.40	52.31	3.64	43.2	3.64	48.2

Table 2. Effect of various temperatures on keratinase production

S. No.	Different Temparature	OD Value/Percentage Of Transmission (%) (hours)							
		24		48		72		96	
1.	31ºC	3.10	49.9	2.86	51.7	2.96	50.5	4.67	34
2.	45ºC	3.61	43.5	2.03	62.5	2.36	58.0	1.94	63.9

Table 3. Effect of inoculum variation on keratinase production

S.No.	Inoculum variation (µl)	OD Value /Percentage of Tranmission (%) (hours)							
		24		48		72		96	
1.	100	1.41	72.1	1.59	69.2	3.38	45.8	3.49	44.7
2.	150	1.01	79.2	2.73	53.3	3.07	49.2	3.26	47.2
3.	200	1.28	74.3	2.41	57.2	3.39	45.7	3.38	45.8
4.	250	1.09	77.7	3.21	47.7	3.34	46.3	2.66	54.1

Table 4. Effect of substrate concentration on keratinase production

S.No.	Substrate concentration (mg)	OD Value/Percentage of Tranmission (%) (hours)							
		24		48		72		96	
1.	0.5	1.59	69.2	3.47	44.9	4.59	34.7	11.44	7.1
2.	1	3.07	49.2	8.17	50.2	9.12	12.2	6.35	23.1
3.	2	2.99	50.1	3.85	41.2	4.20	36.6	4.44	35.9
4.	4	4.83	32.8	2.41	57.2	3.21	47.7	2.73	53.3
5.	6	3.24	42.7	2.14	61	3.07	49.2	2.65	54.2

Table 5. Effect of various nitrogen sources on keratinase production

S.No.	Nitrogen soruces	OD Value/Percentage of Transmission (%) (hours)							
		24		48		72		96	
1.	Yeast	12.76	5.2	15.36	2.9	15.83	2.6	10.72	8.4
2.	Peptone	03.33	46.4	10.84	8.2	8.97	12.6	7.64	17.1
3.	Ammonium chloride	00.64	86.2	3.48	44.8	1.96	63.5	0.08	98.2
4.	Ammonium nitrate	00.97	80	3.67	42.9	3.28	46.9	00.63	86.4
5.	Urea	04.68	34	03.73	42.3	7.19	19	1.13	76.9

Table 6. Effect various carbon sources on keratinase production

S.No.	Carbon sources	OD Value/Percentage of Transmission (%) (hours)							
		24		48		72		96	
1.	Glucose	3.20	48.1	4.16	38.3	1.55	70.0	1.55	70.0
2.	Fructose	3.69	43.1	5.64	35.8	1.55	70.0	1.55	70.0
3.	Lactose	2.96	50.5	3.84	41.2	0.97	80.0	1.55	70.0
4.	Sucrose	2.10	59.20	4.46	35.8	1.55	70.0	1.55	70.0
5.	Maltose	2.36	58.6	4.55	35.0	1.55	70.0	1.55	70.0

The keratinase production was depended on substrate concentration and cultivation conditions. In the present study highest keratinase production was observed on substitution with 0.5 and 1 mg of chicken feather meal. This confirms that high substrate concentration reduced keratinase production. The use of feather waste as growth substrates for keratinase-producing microorganisms will invariably serve as one of a most capable means of managing the major amounts of feather wastes produced by the poultry processing industry (American Chemical Society, 2011). Table 5 and Fig. 7 shows the effect of different kinds of organic and inorganic nitrogen sources on keratinase production after 96 hours of incubation period at 28ºC. The maximum amount of enzyme production was observed in yeast extract 15.83 U/ml at 72 hours for feathers. The minimum enzyme activity was observed in peptone 10.84 U/ml at 48 hours. Table 6 and Fig. 8 shows the effect of carbon source on keratinase production after 24 – 96 hours of incubation period at 28˚C. The maximum keratinase production was recorded in fructose (5.64 U/ml) at 48 hours supplemented medium and minimum keratinase production was recorded in lactose (0.97 U/ml) at 72 hours.

Park and Son, (2009) reported that the addition of mono or disaccharides to the culture medium might result in higher enzyme production. When specific inorganic nitrogen sources were added to culture media containing organic nitrogen sources, it results in increased keratinase production (Anbu e*et al*., 2007). On the other hand, the presence of inorganic nitrogen sources was reported to inhibit keratinase production in *Myrothecium verrucaria* (Gioppo *et al*., 2009). Therefore,

the effects of extra carbon and nitrogen sources on keratinase production vary according to the species, substrate and carbon and nitrogen concentration. Hence, the optimization of medium composition for keratinase production should be performed on a case-by-case basis (Cai and Zheng, 2009). Different molecular weight of plasmid DNA was observed, its range from 7421 to 21226 bp (Fig.9). The molecular weight of the enzyme was characterized by sodium dodecyl sulfate – polyacrylamide gel electrophoresis. In SDS-PAGE partially purified enzyme showed one clear band having keratinolytic activity and number of diffuse bands of different proteins. On comparison with the standard molecular weight markers, the apparent molecular weight of keratinase was found to be 43 to 46 kDa (Fig.10). The molecular weight of keratinase varies between bacteria. Kumar *et al*. (2007) reported that the molecular weight of the bacterium *Bacillus* sp. as 65 kDa whereas, Brandelli, (2007) proposed 38kDa from *Chryseobacterium sp*. kr6.

Conclusion

Bacillus sp. showed a remarkable potential for the biodegradation of feather keratin, with the concomitant production of keratinases. However, further study is needed for the evolution of biotechnological application of the keratinolytic enzyme from selected bacteria which requires more detailed perceptive of the factors that enable this enzyme for complete degradation of chicken feathers. Therefore, additional researches have to be done for purification and characterization of keratins and kinetic enzyme studies. The present study clearly indicates that the feather degradation by *Bacillus* sp. is not only economical but also a viable process for better management of feather landfills.

REFERENCES

American Chemical Society, "Advance towards Making Biodegradable Plastics from Waste Chicken Feathers". Science Daily. Online at: http://www.sciencedaily.com/ releases/2011/03/110331142204.htm. Last accessed 19.04. 12, 2011.

Anbu, P., Gopinath, S.C.B., Hilda, A., Lakshmipriya, T., Annadurai, G., "Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis"*. *Bioresource Technology*, vol. 98, pp. 1298-1303, 2007.

Brandelli, A., "Bacterial keratinases: Useful enzymes for bioprocessing agro industrial wastes and beyond". *Food Bioprocess Technol*., vol. 1, pp. 301-305, 2007.

Cai, C.G., Zheng, X.D., "Medium optimization for keratinase production in hair substrate by a new *Bacillus subtilis* KD-N_2 using response surface methodology". *Journal of Industrial Microbiology and Biotechnology*, vol. 36, pp. 875-883, 2009.

Cheng, S.W., Hu, H.M., Shen, S.W., Takagi, H., Asano, M., Tsai, Y.C., "Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1". *Bioscience Biotechnology and Biochemistry*, vol. 59, pp. 2239-2243, 1995.

Daroit, D.J., Correa, A.P.F., Brandelli, A., "Keratinolytic potential of a novel *Bacillus sp*. P45 isolated from the Amazon basin fish *Piaractus mesopotamicus"*. *International Biodeterioration and Biodegradation*, vol. 63, pp. 358-363, 2009.

Dharmadhikari, S.M. and Beshwe,S.A., "Molecular level studies on multiple antibiotic and serum resistance in UTI pathogens". *J.Indian J. of Biotech*., vol. 8, pp.40-45,2009.

Gioppo, N.M.R., Moreira-Gasparin, F.G., Costa, A.M., Alexandrino, A.M., Souza, C.G.M., Peralta, R.M., "Influence of the carbon and nitrogen sources on keratinase production by *Myrothecium verrucaria* in submerged and solid state cultures". *Journal of Industrial Microbiology and Biotechnology*, vol. 36, pp. 705-711, 2009.

Gupta, R. and Ramnani, P. 2006. "Microbial keratinases and their prospective applications: an overview". *Applied Microbiology and Biotechnology*, vol. 70, pp. 21-33.

Gupta, R., Beg, Q.K. and Lorenz, P. 2002. "Bacterial alkaline proteases: molecular approaches and industrial applications". *Applied Microbiology and Biotechnology*, vol. 59, pp. 15–32.

Jin- Ha Jeong., O- Mi Lee., Young-Dong Jeon., Jeong-Do kim., Na-Ri Lee., Chung- Yeol Lee and Hong Joo Son., 2010. "Production of keratinolytic enzyme by a newly isolated feather-degrading *Streptophonomas maltophilia* that produces plant growth promoting activity". *Process Biochemistry,* vol. 45., pp. 1738-1745.

Kumar, A.G., Swarnalatha, S., Gayathri, S., Nagesh, N. and Sekaran, G. 2007. "Characterization of an alkaline active-thiol forming extracellular serine keratinase by the newly isolated *Bacillus pumilus"*, J. Appl. Microbiol., vol. 104, pp. 131.

Laemmli U. K. 1970. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4". Nature, vol. 227, pp. 680 – 685.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall., 1951. "Protein measurement with the Folin phenol reagent". *J. Biol. Chem*., Vol. 193, pp. 265-275.

Mazotto, AM., Coelho Rosalie, RR., Cedrola, SM., de Lima, ML., Couri, S., Paraguai de, SE. *et al*. 2011. "Keratinase production by three *Bacilllus spp.* using Feather Meal and Whole Feather as Substrate in a submerged Fermentation". *Enzyme Res*., Vol. 2011, pp. 7.

Mohorcic, M., Torkar, A., Friedrich, J., Kristl, J. and Murdan, S. 2007. "An investigation into keratinolytic enzymes to enhance ungula drug delivery". *Int. J. Pharm*., vol. 332, pp. 196-201.

Park, G.T. and Son, H.J 2009. "Keratinolytic activity of *Bacillus megaterium* F7-1, a feather-degrading mesophilic bacterium". *Microbiological Research*, vol. 164, pp.478-485.

Sadasivam, S. and Manickam, A. 1992. "In: Biochemical methods for Agricultural Sciences", Willey Eastern Limited, New Delhi, pp. 10 – 11.

Singh, D.P., Sing, R. and Vyas, S.R 1975. "Effect of pH, temperature, nitrogen source glucose concentration on acid protease production by *Aspergillus niger* mutant". Indian Journal of Microbiology, vol. 15, pp. 109-113.

Venkata Nagaraju and Divakar, G. 2013. "Production of keratinase by using Pseudomonas aeruginosa isolated from poultry waste". *IIPCBS*, vol. 3, no. 1, pp. 79-86.

Williams, CM., Richter, CS., Kenziejr, M. and Shih, JCH., 1990. "Isolation, identification, and characterization of a feather degrading bacterium". App. Environ. Microbial. Vol. 56, pp.1509-15.

Xu, B., Zhong, Q., Tang, X., Yang, Y. and Huang, Z. 2009. "Isolation and characterization of a new keratinolytic bacterium that exhibits significant feather-degrading capability". *Afr J Biotechol*., Vol. 8 no. 18, pp. 5490-6.

Yu, R. J., Hormon, S. R. and Blank, F. 1968. "Isolation, purification of an extracellular keratinase of Trichophyton mentagrophytes". *J. Bacteriol*., 96: 1435-1436.

Zerdani, I., Faid, M. and Malki, A. 2004. "Feather wastes digestion by new isolated strains *Bacillus sp* in Morocco". *Afr J Biotechnol*., Vol. 3, no.1, pp. 67-70.

****