ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Study of efficiency of Keratinase production by *Arthrobacter creatinolyticus KP015744* isolated from leather sample

Savita Kate[1] and Archana Pethe[2]

1 Department of Biotechnology, ShivChhatrapatiCollege, Aurangabad-431001,M.S., India

2.Department of Microbiology, ShivajiCollege of Arts, Commerce and Science,Akola-444001,M.S.,India

Manuscript Info

Manuscript History:

Received: 19 September 2014
Final Accepted: 26 October 2014
Published Online: November 2014

Key words

Collagenase, keratinase, caseinase, gelatinase, leather waste, Keratin

Corresponding Author

Savita Kate

Abstract

Keratin is an insoluble structural protein having high mechanical stability and resistance to proteolytic degradation due to disulphide, hydrogen bonds and other cross linkages. Keratinase is used in removal of hair and feathers in leather and poultry industry, in agro industrial waste degradation and to enhance drug delivery.

In the present study, the keratinase producing bacteria isolated from deteriorated leather sample using feather meal as a sole source of carbon and nitrogen. One of the efficient producers was identified by morphological and biochemical tests were found to be belonging to genus *Arthrobacter***.** By analysis of 16srDNA sequencing showed that the isolated strain was *Arthrobacter creatinolyticus* and sequence was deposited GenBank with accessionnumberKP015744.

The isolate was able to produce 30U/ml keratinase over a wide range of pH from 5 to 9 and from temperature 10-45°c within 72h. Magnesium ions stimulated Keratinase production while copper, cobalt and zinc inhibited the activity. The enzyme production was strongly inhibited by EDTA, DMSO but not by PMSF. The isolate also showed its ability to produce collagenase caseinase and gelatinase.

Hence the present isolate having ability to produce diversity of enzymes can be used in leather waste treatment.

Introduction

Leather is an organic material that contains many nutrients for microorganisms. It is made up of 96.5% fibrous proteins and 3.5% are albumin and globulin. The fibrous proteins present in leather are collagen (98%), elastin (1%) and keratin (1%).Leather tanning is a general term use for numerous processing steps involve in converting animal hide and skins in to final leather (Tissier and Chensais, 2000). The tanning process converts the putrescible skin in to a durable, long lasting and versatile natural material for various use i.e. clothing, cricket ball, football, book binders, watch straps, foot-wares, briefcase etc.(Arvindhan et al., 2007) as well as in industrial sector. Traditional chemical leather processing generates huge amount of environmental pollutions (Thanikaivelan et al., 2004; Kanagraj et al., 2006).Whereas, enzyme biocatalysts were found to be effective in soaking, dehairing, bating operations of environmental friendly leather processing (Taylor et al., 1987).

Keratin is the most abundant insoluble structural protein of feathers, animal skin, horn ,hair and wool and is known for its high stability (Bradbury, 1973).Because of the high degree of cross-linking by disulphide bonds, hydrogen bonding and hydrophobic interactions, keratin is insoluble and shows high mechanical stability and resistance to proteolysis (Bradbury,1973; Parry and North,1998) and is poorly digested by common digestive enzymes, such as trypsin, papain and pepsin (Papadopoulos et al., 1986). Keratinolytic enzymes may have important uses in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the

development of nonpolluting processes (Shih, 1993; Onifade et al., 1998). These enzymes are produced by some species of *Bacillus* (Williams et al., 1990; Kim et al., 2001), and fungi (Ali, 2001; Awasti, 2011). Recently, keratinase activity was also reported for Gram positive novel rod coccus *Arthrobacter* (Jamile Queiroz Pereira et al., 2014).

The aim of this study was to isolate and identify a new efficient Keratin-degrading bacterium, which uses feather meal (keratin) as carbon and nitrogen sources, from deterioted leather samples.

Materials and Methods:

Chemicals and reagents

Nutrient broth (M002-100G), metal ions and organic solvents were obtained from Hi media Ltd., Mumbai. Phenylmethylsulphonylfluoride [PMSF], Dimethylsulphoxide [DMSO] was obtained from Sigma Chemical Co. USA. All other reagents used were of analytical grade.

Preparation of feather meal:

The feather meal was prepared from native chicken feathers as described by Tork et al., 2008 and Saibabu et al., 2013 with modifications. The feathers were cut with scissors in to small pieces of 3 to 4 cm long and washed several times with tap water. Defatting of feather pieces was done by soaking a mixture of chloroform: methanol (1:1) for 2 days followed by chloroform: acetone: methanol (4:1:3) for 2 days. The solvent was replaced every day. The feathers were finally washed several times with tap water to eliminate the solvent residual, dried at 60°C for 24 h(Han et al.,2012)' grinded using electrical mixer blender (Ken star Senator PCMG 0120) and used as feather meal.

Isolation and Identification of keratinolytic microorganisms:

Fifty deterioted leather samples were collected from different districts of Maharashtra state and processed for isolation keratinolytic microorganism using feather meal as a source of keratin.

Primary isolation was carried out on nutrient agar. Among the different organisms isolated, one of colony occurring with higher frequency was selected for keratinase production by using feather meal agar containing 10g/L feather meal. (Onuoha et al., 2011)

Keratinase production was confirmed by the presence of a clear zone around the bacterial colony. The isolate was identified by morphological, biochemical characteristics as described in the Bergey's manual of systematic bacteriology and by 16srDNA sequencing.

Crude enzyme preparation:

One of the efficient producers was selected for further study. Submerged fermentation was performed by inoculating pure culture of the isolate into the production medium containing feather meal (1%), yeast extract (0.01%), NaCl (0.05%), KH_2PO_4 (0.03%), K_2HPO_4 (0.04%) and $MgCl_2$ (0.01%) of pH 8 which is the optimum pH for the growth of the isolate. The incubation was carried out at 37ºC for 3 days at 180 rpm (Kainoor et al., 2010). The broth was centrifuged (REMIC-30 BL centrifuge, India) at 10,000 rpm for 10 min and the supernatant was used as crude enzyme (Saibabu et al., 2013).

Keratinase assay:

Keratin from feather meal was used as a substrate. The 5 mg keratin was suspended in 1 mL 50 mmol/L Tris-HCl buffer (pH 8.0). The reaction mixture contained 1 mL keratin suspension and 1 mL appropriately supernatant. The reactions were carried out at 50^{o}C with constant agitation of 200 rpm/min for 1 hr. After incubation, the reactions were stopped by adding 2 mL 0.4M trichloroacetic acid (TCA) and followed by filtration to remove the substrate. The filtrate was spectrophotometrically measured at 595 nm. One unit (U) of keratinase activity was defined as the amount of enzyme causing 0.01 increases in absorbance between sample and control at 595 nm after one hour under the given conditions. The result was taken as an average of three replicates (Augstskola et al., 2009).

Effect of physicochemical parameters on keratinase production:

The effect of pH and temperature on keratinase production was individually tested by taking the production media at different pH and temperature. The effect of substrate concentration on the production of keratinase was tested by adding different concentrations of feather meal as a substrate in the production medium. The fermentation media were assayed every day for keratinase production till a decline was observed in the enzyme activity. For time course analysis of keratinase production, the isolate was grown in the optimized growth medium and the activity was measured every day for a period of four days (V. Saibabu et al., 2013).

Effect of metal ions and chemicals on enzyme production:

The effect of different metal ions, organic solvents and inhibitors was studied by adding 1mM MnCl2, ZnCl2, HgCl2, CuSO4, MgCl2, sodium sulphite, cysteine, PMSF, EDTA, lead acetate, FeCl3, COCl2, CaCl2and 1% concentration of mercaptoethanol, glycerol, DMSO in the fermentation media .The effect of them was measured by assaying activity every day for a period of three days at 50°C for 1h. (S. Kainoor et al., 2010; V. Saibabu et al., 2013).

Result and Discussion:

Isolation and Identification of keratinolytic microorganisms:

From fifty deterioted leather samples forty-eight keratinolytic microorganisms were isolated. One of efficient Gram positive bacterium from isolates was used for further study. The isolate showed typical rod coccus cycle. From morphological, biochemical and 16srDNA analysis, the isolate was identified as *Arthrobacter creatinolyticusKP015744*.

Keratinase assay:

Supernatant obtained after three days of incubation was subjected to keratin assay by using feather meal as a substrate. The isolate was able to produce 35±0.98U/ml of keratinase after 72h.One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 595nm for 1h at 50°C (Augstskola et al., 2009).*Bacillus TS2* shown maximum keratinase activity of 41u/ml after 96h at 37°C (T. Shivkumar, 2012).*Arthrobactersp108* showed 5U/ml keratinase activity after 7days of incubation period on feather meal agar. In a present study, the isolate showed efficient keratinase production than the reports cited until.

Effect of physicochemical parameters on keratinase production:

Effect of temperature:

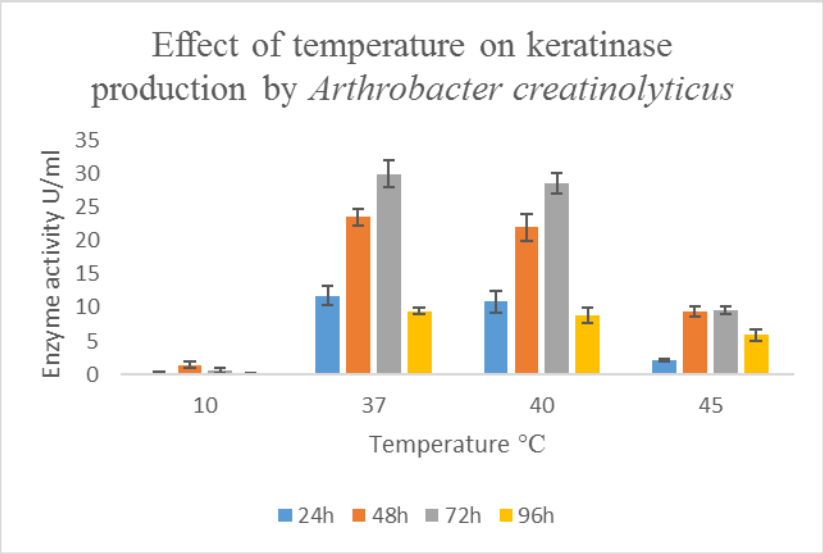

Figure 1 Effect of temperature on Keratinase production

The isolate showed keratinase production after 24h at a temperature range of 37-45°C.Maximum keratinase production of was 30U/ml at 37°C after 72h was detected indicating 37°C as an optimum temperature for given isolate.(**Figure 1.)***Bacillus thuringiensis TS2* showed keratinase production of 41U/ml at 37ºC after 96 h (T .Shivkumar et al., 2012) whereas *Pseudomonas sp. MS21*produced 0.43keratinase U/ml at 37ºC after 72h. (Tork et al., 2008).

Effect of pH:

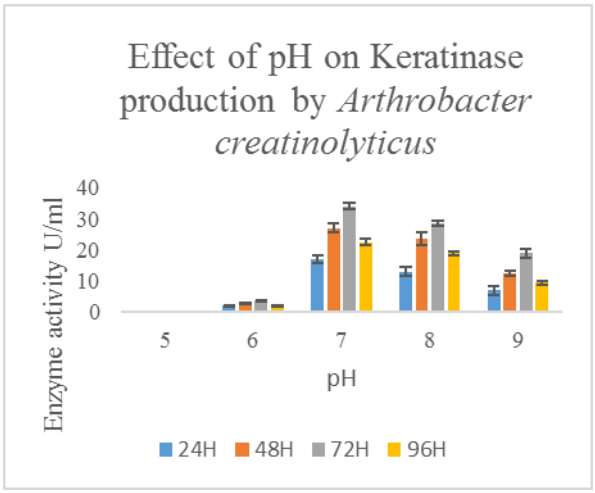

Figure 2 Effect of pH on keratinase production

The isolate showed keratinase production from pH 7-9 (**Figure 2).** Highest keratinase production of 34U/ml was obtained at pH 7 within 3 days. Lowest production of 0.09U/ml was found at pH 5.*Arthrobacter sp. 108* also showed maximum enzyme activity of 1-2U/ml at pH 8-8.5(Jamile Queiroz Pereira et al., 2014) while *Bacillus licheniformis* gave10.76U/ml of keratinase at pH7(C. Vigneshwaran et al., 2010).

Effect of Substrate:

Effect of substrate concentration was studied by using feather meal as a substrate in the range of 0.5 to 2.5%. The isolate showed maximum keratinase production of 30.6±1.15U/ml at 1% concentration feather meal after 72h. (**Figure3**).

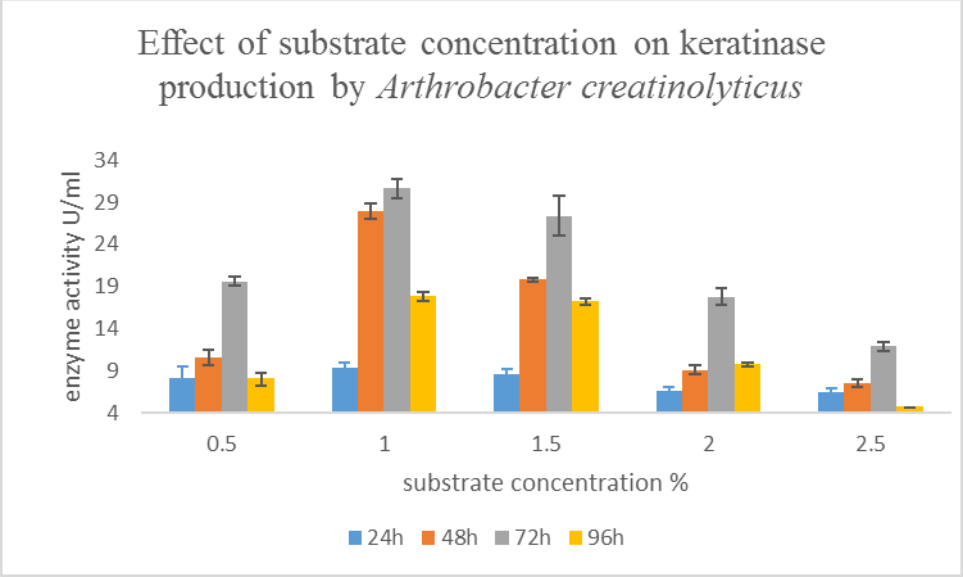

Figure 3 Effect of substrate concentration on keratinase production

In present studies, it was observed that increased concentration of feather meal decreased the enzyme production. High substrate concentration may cause repression of keratinase production. Similar results was reported by Kainoor and Naik (2010) by using 1% feather powder giving maximum keratinase activity of 52.4ug/ml/min for *Bacillus sp.*

Cheng S.W.et al., (1995) achieved highest keratinase production in presence of 0.5% feather meal and 0.1% yeast extract.

Effect of incubation period: The isolate was inoculated in to fermentation media and incubated at different time intervals ranging from 8 hours to 96 hours. Keratinase production was noticed after 12h of incubation period. Maximum keratinase production of 34.6 ±1.5U/ml was shown after 72h. (Figure 4) Tork et al., 2008 reported maximum keratinase production of 0.64and 0.89 U/ml by *Kera MS2, Kera MS21* respectively, after five days of incubation.

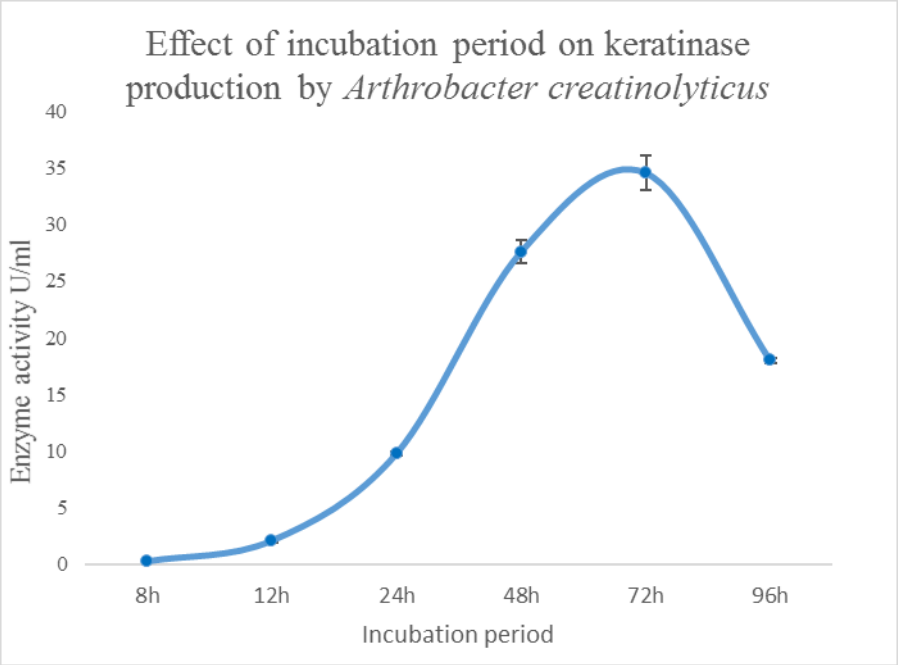

Figure 4 Effect of incubation period on keratinase production

Effect of metal ions and chemicals: The isolated keratinolytic protease was found to be metaloprotease, Magnesium ions and Mn^{2+} stimulated keratinase production while copper, cobalt, lead, mercury and zinc inhibited the activity. The enzyme activity was strongly inhibited by EDTA, DMSO but not by PMSF as (**Figure 5 and 6).**Lin et al.,(2009) reported that Ba2+, Ca2+, Mg2+, Mn2+, Zn2+, dithiothreitol, glutathione and β-mercaptoethanol, activated, while Cu2+, Fe2+, Hg2+,Fe3+, ethylene glycol, tetra acetic acid, EDTA and p-chloromercuribenzoate inhibited keratinase activity whereas Zn2+greatly inhibited its activity .Sangali et al., 2000; Riffel et al., 2003, 2007 also reported inhibition of keratinase by Zn2+ from Gram negative bacteria.

Effect of metal ions:

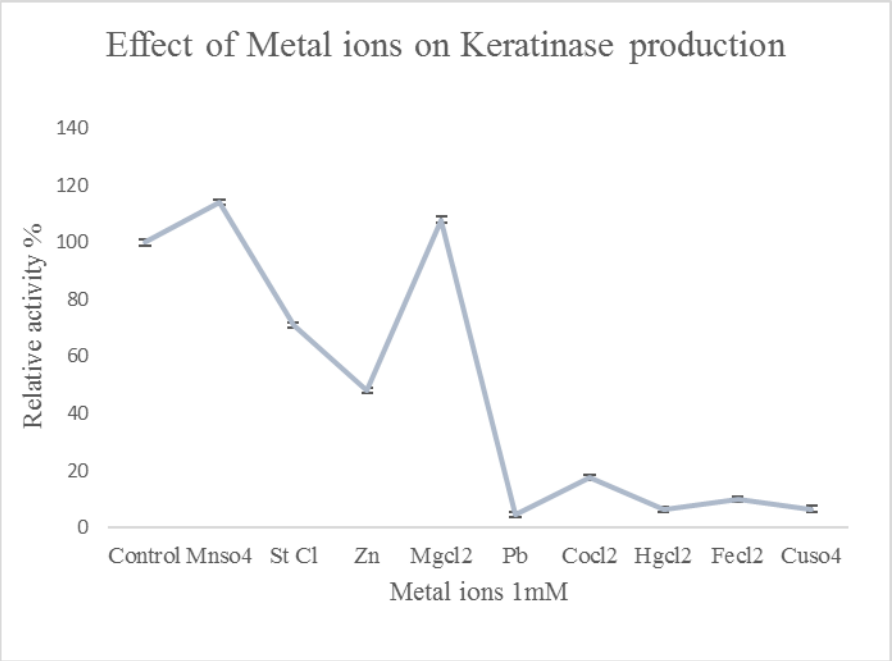

Figure 5 Effect of metal ions on keratinase production

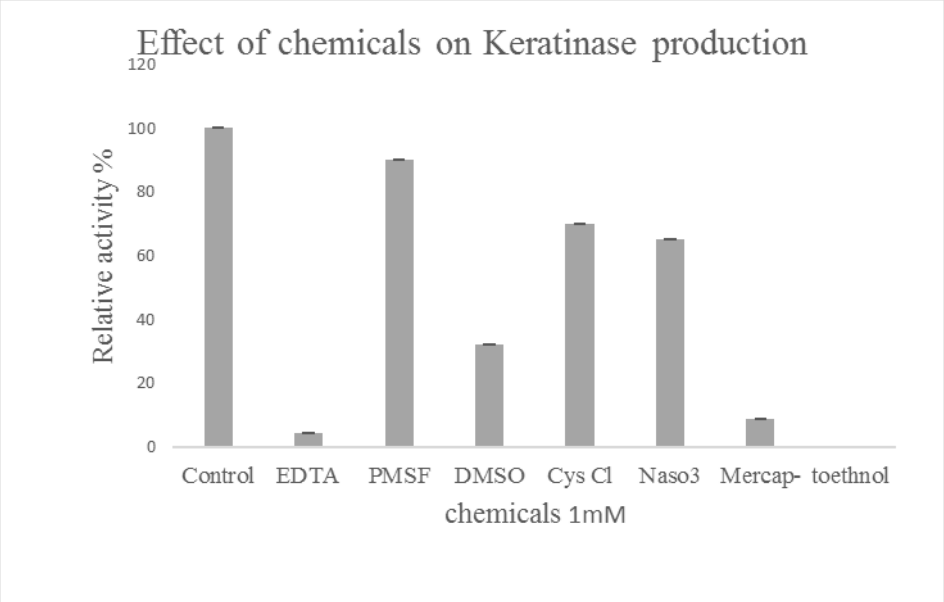

Figure 6 Effect of chemicals on keratinase production

Conclusion:

From morphological, biochemical and molecular characterization, the isolated organism was identified as an *Arthrobacter creatinolyticusKP015744***.**The isolate was able to produce maximum keratinase of 30U/ml at pH 7 and also showed keratinase production at pH 7 to 9 within 72h at 37°C. Mg2+ and Mn2+ were found to be best activators among the selected activators. The enzyme production was strongly inhibited by EDTA. The isolate was able to produce diverse types of enzyme such as keratinase, collagenase, gelatinase and caseinase, which play an important role in leather effluent treatment and also in de-hairing process in leather industry.

References:

- Alessandro, Riffel and Adriano, Brandelli. (2006): Keratinolytic bacteria isolated from feather waste. Brazilian Journal of Microbiology. , 37:395-399.

- Ali, T., Ali, N., and Latifa, M. (2011): Production, purification and some properties of extracellular keratinase from feathers-degradation by *Aspergillus oryzaenrrl-447*.Journal of Applied Sciences in Environmental Sanitation., 6 (2): 123-136.
- Arvindhan, R., Saravanabhavan, S., Thanikavelan, P., Raghav Rao, J. and Unni Nari, B. (2007): A chemo-enzymatic pathway leads towards zero discharge tanning. J. Clean. Prod. , 15: 1217-1227.
- Awasthi, P., and Kushwaha, R. K. S. (2011): Keratinase Activity of Some Hyphomycetous Fungi from Dropped Off Chicken Feathers. International Journal of Pharmaceutical & Biological Archives. , 2(6):1745-1750.
- Bradbury, J.H. (1973): The structure and chemistry of keratin fibers. Advances in Protein Chemistry. , 27: 111–211.
- Cheng, S.W., Hu, M.N., Shen, W., Takagi H., Asano, M., and Tsai, Y.C. (1995) Production and characterization of keratinase of a feather degrading Bacillus *licheniformis PWD-1* .Biosci. Biotech. Biochem. , 59:2239-2243.
- Han, M., and Luo, W. (2012): Isolation and characterization of a keratinolytic protease from a feather-degrading bacterium *Pseudomonas aeroginosa* C11.African Journal of Microbiology Research. , 6(9): 2211-2221.
- Kainoor, P.S., and Naik, G.R. (2010): Production Characterization of feather degrading keratinase from Bacillus sp. JP99, Indian Journal of Biotechnology., 9:384-390.
- Kanagraj, J., Velappan, K.C., Chandra, Babu, N.K., and Sadulla, S. (2006): Solid waste generation in the leather industry and its utilization for cleaner environment- a review. Journal of Scientific and Industrial Research. 65:541- 548.
- Kim, J.M., Lim, W. J., and Suh, H.J. (2001): feather degrading Bacillus species from poultry waste Process Biochemistry., 37:287–291.
- Lin, H. H., Yin, L.J., and Jiang. S. T. (2009a): Cloning, expression, and purification of *Pseudomonas aeruginosa* keratinase in *Escherichia coli AD494* (DE3) pLysS expression system. J. Agric. Food Chem., 57: 3506-3511.
- Lin, H.H., Yin, L .J. and Jiang, S.T. (2009b): Functional expression and characterization of keratinase from *Pseudomonas aeruginosa* in Pichiapastoris. J. Agric. Food Chem., 57: 5321-5325.
- Matikeviciene V. and Masiliuniene D. (2009): Degradation of keratin containing waste by Bacteria with keratinolytic activity .Environment Technology, Resources. , 1: 284-289.
- Onifade, A.A., Al-Sane, N.A., Al-Musallam, A.A., and Al-Zarban, S. (1998): Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresour. Technol., 66: 1-11.
- Papadopoulos, M.C., El Boushy, A.R., Roodbeen, A.E., and Ketelaars, E. H. (1986): Effects of processing time and moisture content on amino acid composition and nitrogen characteristics of feather meal. Animal Feed Science and Technology. , 14:279–290.
- Parry, D.A.D., and North, A.C.T. (1998): Hard a-keratin intermediate filament chains: substructure of the N-and C-terminal domains and the predicted structure and function of the C-terminal domains of type I and type II chains. Journal of Structural Biology. , 122: 67–75.

- Riffel, A., F. Lucas, P. Heeb and A. Brandelli, (2003a):A.P.F., D.J. Dariot and A. Brandelli, 2010. Characterization of a new keratinolytic bacterium that completely degrades native feather keratin. Archives of microbiology. 179: 258-265.
- Saibabu, V., and Niyongabo, N. (2013): Isolation Partial purification and characterization of keratinase from *Bacillus megaterium.* International Research Journal of Biological Sciences. , 2(2), 13-20.
- Shih, J.C.H. (1993): Recent development in poultry waste digestion and feather utilization-a review. Poultry Sci., 72: 1617-1620.
- Taylor, M. M., Bailey, D. G. and Feairheller, S. H. (1987): Review of the uses of enzymes in the tannery. Journal of the American Leather Chemists. Association, 82(6)153-165.
- Thanikaivelan, P., Rao, J. R., Nair, B. U. and Ramasami, T. (2005): Recent trends in leather making: Processes, problems, and pathways. Critical Reviews in Environmental Science and Technology. , 35 (1): 37-79.
- Tissier, C., and Chesnais, M. (2000): Biocides used as preservatives in the leather industry. Product type 9: fiber, leather, rubber and polymerized materials preservatives. Emission scenario Documents, Pp. 1-14.
- Tork, S., Aly, M. M., and Nawar, L. (2008): Molecular Characterization of a New Keratinase Producing *Pseudomonas sp.MS21* Journal of Genetic Engineering and Biotechnology. , 6(1): 37-46.
- Vigneshwaran, C., Shanmugam, S., and Sathish Kumar, T. (2010): Screening and characterization of keratinase from *Bacillus licheniformis* isolated from NAMAKKAL POULTRY FARM. Researcher. , 2(4):89-96.
- Willams, C. M., Richter, C.S., Mackenzie, J. M., Shih, C.H. (1990): Isolation, Identification and Characterization of a feather degrading Bacterium. Applied and Environmental Microbiology. , 56(6):4509-1515.