International Journal of Biotechnology and Biochemistry
ISSN 0973-2691 Volume 13, Number 1 (2017) pp. 95-110

http://www.ripublication.com

Partial characterization of keratinase from *Stenotrophomonas maltophilia* K279a and study of its dehairing potential

Malay Shah[1*] and Rajnish Vaidya[2]

[1] *Department of Microbiology, Vivekanand Education Society's College of Arts, Science & Commerce, Chembur, Mumbai, Maharashtra- 400071, India.*

[2] *Department of Microbiology, The Institute of Science, Colaba, Mumbai, Maharashtra- 400032, India.*
[*] *Corresponding author*

Abstract

In our present study keratinase from *Stenotrophomonas maltophilia* K279a was partially purified and characterized for certain parameters. The organism produced significant amount of extracellular keratinase having molecular mass between 30 to 40 K Da. Optimal enzyme activity was observed at pH 8.0 and 37 ºC. The enzyme was completely inhibited by phenylmethanesulfonyl fluoride (PMSF) indicating that it belongs to the serine protease family. The catalytic activity of enzyme increased to 138 % in presence of Ca^{2+}. Mg^{2+} and Mn^{2+} did not have any significant effect on enzyme activity. Heavy metal ions such as Cu^{2+} and Hg^{2+} inhibited enzyme activity by more than 80 %. In the presence of 0.5 % SDS and 0.5 % Triton X 100, the enzyme retained 75 % and 80 % of its activity respectively. The enzyme was slightly inhibited by the reducing agents β-mercaptoethanol and Dithiothreitol. The keratinase preparation caused easy epilation of goat skin with the recovery of intact hair. Thus, keratinase from *Stenotrophomonas maltophilia* K279a seems to have promising role in leather processing. This can be considered as a good alternative; not only to reduce pollution problems, but also reduce time and energy utilized in conventional processing.

Keywords: *Stenotrophomonas maltophilia* K279a, keratinase, leather processing.

INTRODUCTION:

Protease refers to a group of enzymes whose catalytic function is to break the peptide bond that joins amino acids together in proteins. Keratinase is a particular class of extracellular proteolytic inducible enzyme capable of degrading "Keratin". Keratins are insoluble and hard-to-degrade animal proteins that are ubiquitously present throughout the animal bodies for e.g. claws and scales of reptiles and the hooves, horns, hide hair, nails of mammals, feathers in case of birds, etc [1]. Keratins are made up of long chains of various amino acids; the two major types of keratin are alpha-keratin (e.g. α-helix of hair and wool) and beta-keratin (e.g. β-sheets of feather) [2]. Commonly known proteolytic enzymes like trypsin, pepsin and papain are unable to breakdown keratins due to their intensive cross-linkages.

Keratinase are produced by diverse groups of microorganisms like fungi, actinomycetes and several bacterial species [1, 3]. Keratinases have a variety of applications, mainly in waste management, leather processing industries, detergent industry, etc. Insoluble feather keratin from poultry industry may be converted by enzymatic hydrolysis to glues, feedstuffs, fertilizers, and films or used for the production of rare amino acids like serine, cysteine and proline [4, 5].

Keratinases are considered to have a promising role in the development of environmentally friendly leather processing technology. The tannery operation involves converting the raw skin, a highly putrescible material, into leather, a stable material, which can be used in the manufacture of a wide range of products. The conventional method of dehairing involves the use of lime and sodium sulphide. Presence of these chemicals in tannery waste is responsible for tremendous pollution, causing health hazards to the tannery workers. Lime produces a poisonous sludge while sodium sulphide is highly toxic and has obnoxious odor. Although enzyme assisted dehairing process reduces the pollution load to some extent, a technology based on enzyme alone, without the use of sulphide and other chemical inputs, has yet to be explored [6].

In view of the recent trend of developing environmental friendly technologies, the present study was undertaken to partially purify and characterize keratinase from *Stenotrophomonas maltophilia* K279a and check for its dehairing potential.

MATERIAL AND METHODS:

Organism and growth media: *Stenotrophomonas maltophilia* K279a was originally isolated in our laboratory from feather waste dumping site in Mumbai, India. The organism was grown in Feather basal medium (FBM) having following composition in gms/L: NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; Na_2SO_3, 0.5 and Feathers (cut into 1 - 2 cm), 10; pH 7.5; D/W, 1000 ml.

Keratinase assay:

Keratinase activity was determined by the method of Sigma Aldrich method with minor modification [7]. In this, the cell free supernatant (1 ml) was added to a tube containing 20 mg of Keratin Azure K 8500 (Sigma Aldrich chemicals) suspended in 4 ml of 50 mM Sodium Phosphate Buffer pH 7.5. The mixture was incubated at 37 ºC for 1 hour on shaker. The reaction mixture was centrifuged and absorbance of released azure dye was measured at 595 nm. Inactivated enzyme reaction mixture was used as a blank. A standard graph was generated using Proteinase K (Sigma Aldrich chemicals) solution of 25 - 100 U ml^{-1} enzyme activity. One Unit of keratinase activity is defined as the amount of enzyme that led to an increase of 0.01 unit absorbance at 595 nm in one hour.

Lab scale production and partial purification:

The saline suspension of the isolate (absorbance adjusted to 0.1 at 540 nm) was inoculated into sterile FBM and incubated for 6 days in shaker incubator (90 rpm) at 32 ºC. After 6 days the culture broth was filtered. All enzyme purification procedures were carried out at 4 ºC. 2000 ml filtered broth was chilled from which enzyme was precipitated by the gradual addition of solid ammonium sulphate (70 % saturation) with gentle stirring. The cloudy solution was kept at 4 ºC overnight. The precipitate was collected by centrifugation at 7000 g for 30 minutes. Pellet obtained was dissolved in 50 ml of 100 mM Tris HCl buffer pH 8 and transferred into dialysis membrane - 110 (Himedia) and dialyzed using same buffer for 24 hours. The concentrated enzyme solution was diluted to 200 ml after dialysis and used in subsequent experiments.

Protein assay:

The protein content in the crude cell free and partially purified enzyme preparation was estimated by Lowry method using bovine serum albumin as standard protein [8].

Effect of pH and temperature on enzyme activity and stability:

The pH optimum of the partially purified keratinase was determined using Keratin Azure k-8500 as substrate suspended in 100 mM of different buffers (sodium acetate, pH 5 – 6; sodium phosphate, pH 7 – 8 and Tris HCl, pH 9 – 10) and at constant temperature of 37 ºC. In order to determine the pH stability, partially purified enzyme was pre-incubated with buffers of different pH for two hour followed by determination of keratinase activity as described previously. Likewise, temperature optimum was determined by incubating the substrate-enzyme reaction mixture from 8

ºC to 55 ºC at constant pH 7.5, followed by determination of keratinase activity. To check for the temperature stability, the partially purified enzyme was pre-incubated at different temperatures for two hour followed by measurement of keratinase activity as described earlier. All the experiments were conducted in three sets and data presented as mean value ± SD.

Effect of different metal ions, chelators, proteinase inhibitors, detergents, organic solvent and reducing agents:

To study the inhibition of keratinase, the enzyme was pre incubated with metal ions such as Ca^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (5 mM); Ethylenediamine tetraaceticacid (5 mM); Phenylmethylsulphonyl fluoride (5 mM); detergent such as Sodium dodecyl sulphate, and Triton X 100 (0.1 and 0.5 %); reducing agent such as Dithiothreitol and β-mercaptoethanol (0.1 and 0.5 %) and Dimethyl sulfoxide (0.1 and 0.5 %) for one hour at 37 ºC. Subsequently the enzyme assay was performed as described above. The residual activity was calculated with reference to the activity of the enzyme without these chemicals, which was taken as 100 % and expressed as percentage residual activity. All the experiments were conducted in three sets and data presented as mean value ± SD.

Zymogram analysis:

SDS – PAGE was performed with 10 % polyacrylamide gel containing 0.1 % casein. Enzyme mixture was mixed with sample buffer and loaded into the well without denaturation. The molecular weight of keratinase was determined by comparing with mobility of standard molecular weight protein markers (Bio-rad). After electrophoresis the gel slab was incubated at 37 ºC for 2 hours. The gel was stained with Coomassie Brillant Blue R-250 and then destained. Protease bands appeared as clear zones on a blue background.

Dehairing Application:

Goat skin was procured from a local slaughter shop. It was cut into 5 cm^2 pieces and transferred to three petri plates. 20 ml of keratinase enzyme preparation was added to the skin pieces in a petri plate. In second petri plate 20 ml distilled water was added as control. In the third petri plate, traditional tannery dehairing solution consisting of 8 % lime and 3 % sodium sulfide solution was added. All the petri plates were incubated at room temperature for 24 hours. At the end of the process, the skin pieces were gently scraped to remove loose hairs. The skin pieces were then visually analyzed [9, 10].

RESULT AND DISCUSSION:

Bacillus and *Stenotrophomonas* are considered to be the dominant keratinase producing taxa. These organisms have been frequently isolated from poultry and feather waste dumping sites. Their presence in such environments indicates their role in degradation and removal of feather wastes from the environment [11].

In-vitro studies on feather degradation have shown that the organism *Stenotrophomonas maltophilia* K279a has remarkable feather degrading potential (Figure 1). In the present study, keratinolytic protease from *Stenotrophomonas maltophilia* K279a was partially characterized. Keratinolytic proteases may have diverse properties depending on the producer microorganism [12]. Studying these enzymes and understanding their biochemical properties gives a useful insight which in turn can be exploited for diverse industrial application.

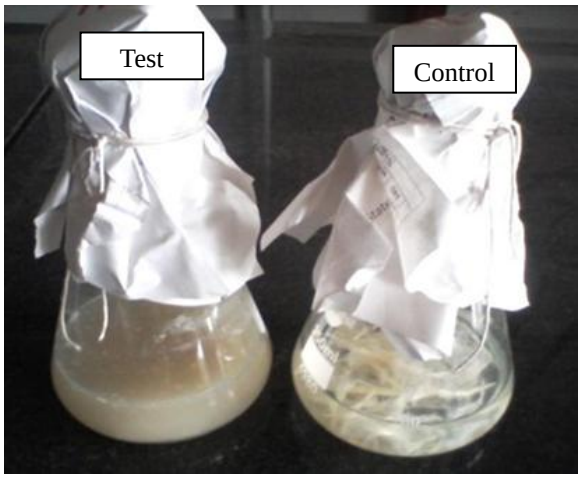

Figure 1: Degradation of feather by *Stenotrophomonas maltophilia* K279a in submerged condition at 32 °C after 6 days of incubation.

Production and Partial Purification of keratinase:

Keratinase are inducible enzymes produced by the microorganism in response to the presence of keratinolytic substrates. Thus, feather basal broth media was employed for laboratory scale production of keratinase. The media contained feather as a sole source of carbon, nitrogen and energy. Partial purification of enzyme from cell free supernatant was carried out using ammonium sulphate precipitation (70 % saturation), followed by dialysis. Specific activity of the crude and partially purified enzyme is summarized in Table 1.

Table 1: Purification of keratinase enzyme from *Stenotrophomonas maltophilia*

Purification Step	Volume (ml)	Total Protein (mg)	Total activity U	Specific activity U/mg	Purification fold	Yield (%)
Crude cell free supernatant	2000	1280.8	86080.00	67.20	1	100
Ammonium sulphate	200	52.86	7356.66	139.17	2.1	85.5

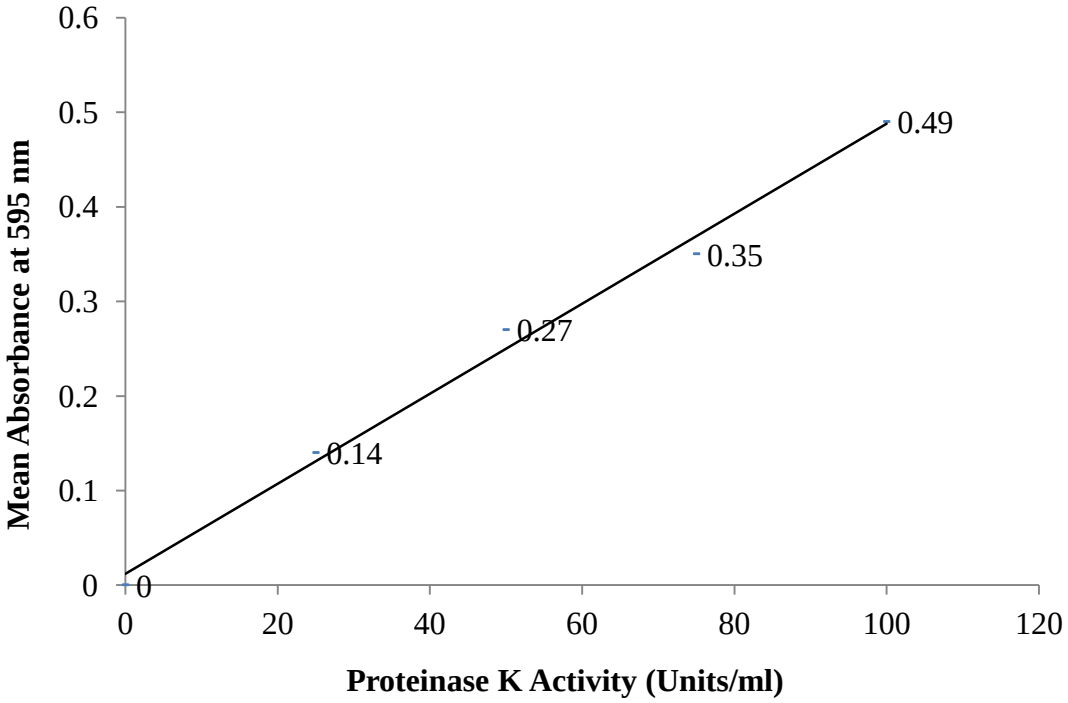

Figure 2: Standard graph for proteinase keratinase activity in U/ml^{-1} using Keratin Azure k 8500 as a substrate.

Effect of pH and Temperature:

The keratinase from *Stenotrophomonas maltophilia* was active in pH range of 6.0 to 10.0, with optimum activity at pH 8.0. Further, the enzyme was stable over a broad range of pH for two hours and retained 95 % of its activity at all pH except pH 6.0 and pH 5.0 where it retained around 70 % and 45 % of its activity respectively (Figure 3). Keratinase are generally active and stable over a wide range of pH from 5.0 to 13.0 and have pH optima in neutral to alkaline range [13]. *Microbacterium* kr10

keratinase has an optimum activity at pH 7.0, *B. pumilus* FH9 at pH 8.0, *Fervidobacterium islandicum* AW-1 at pH 9.0 [14].

The enzyme was active in the temperature range of 27 - 55 °C with optimum activity at 37 °C. After two hours of incubation at 45 °C and 55 °C, the enzyme retained 75 % and 40 % of its activity respectively (Figure 4). *Stenotrophomonas maltophilia* DHHJ keratinase was active and stable from pH 5.0 to 9.0, and had an optimum activity at the pH of 7.8 [15]. The optimum temperature for this keratinase was observed as 40 °C. Majority of the keratinase have activity in range of 30 to 80 °C. Enzyme from *F. pennavorans* has optimum activity at 80 °C. Temperature optima of keratinase may also be variable depending on the source and origin of the isolate [12].

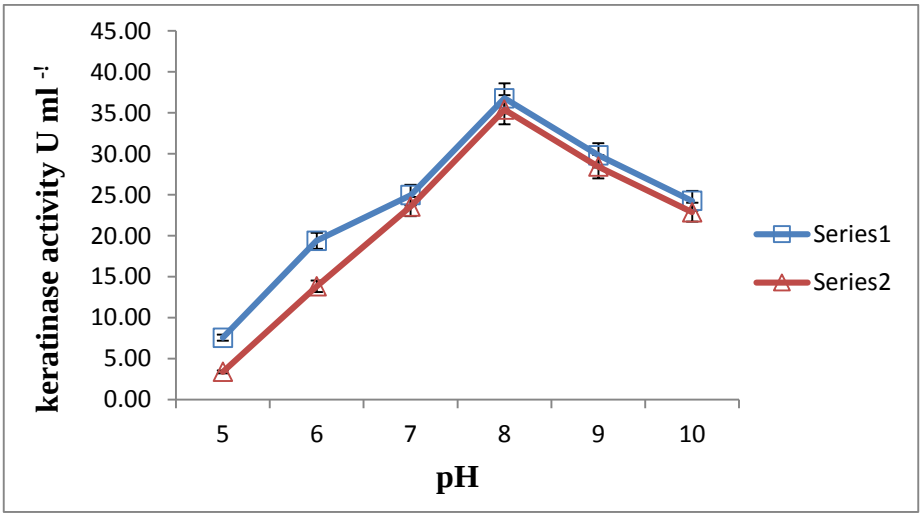

Figure 3: Effect of pH on enzyme activity. Series 1 indicates effect of pH and series 2 indicates pH stability

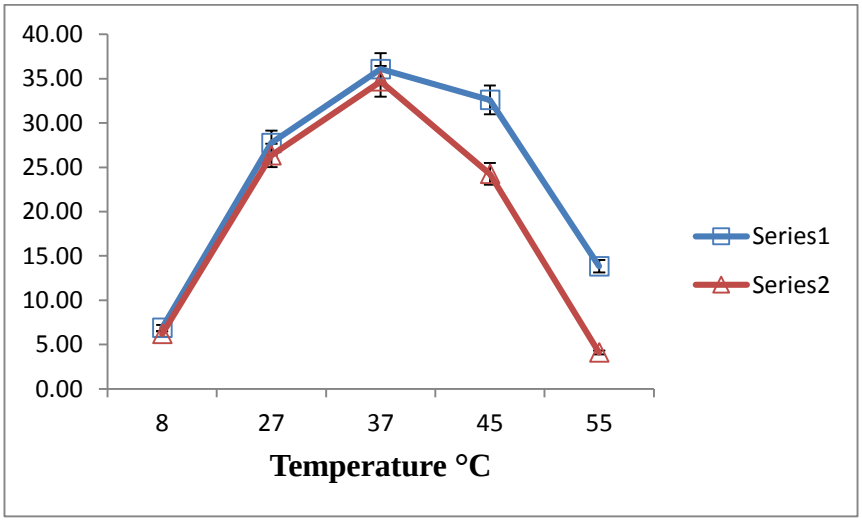

Figure 4: Effect of temperature on enzyme activity. Series 1 indicates effect of temperature and series 2 indicates temperature stability

Biochemical characterization:

The effect of different metal ions, chelators, proteinase inhibitors, detergents, organic solvent and reducing agents on partially purified enzyme is represented in Table 2. The keratinase activity was inhibited by PMSF, indicating that it is a serine protease. Similar inhibitory response to serine group specific inhibitor PMSF was seen in *Microsporum canis* and *D. Microspores* [16]. The enzyme retained 60 % activity in presence of 5 mM EDTA. Several reports have shown serine proteases to be slightly affected by metalloprotease inhibitors. However, its substrate specificity and amino acid sequence will be evaluated to confirm this conclusion [17].

Keratinase activity was activated by Ca^{2+}, while $Mg^{2+}$and Mn^{2+} did not have any significant effect of its activity. Majority of the keratinases are stimulated in the presence of divalent metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+} [18]. Calcium ions are known to be stabilizers of many enzymes, protecting them from conformational changes [19]. *Stenotrophomonas maltophilia* keratinase was inhibited by heavy metal ions such as Cu^{2+} and Hg^{2+}. Hg^{2+} is recognized as an oxidant agent of thiol groups, and the enzyme inhibition by this ion could suggest the presence of important -SH groups (such as free cysteine) at or near the active site [20, 21]. However, Hg^{2+} might also react with tryptophan residues and carboxyl groups in amino acids of the enzyme [22]. On contrary to the previous reports, the keratinase enzyme from this isolate retained 85 % activity in the presence of Zn^{2+} ions. The inhibitory effect of Zn^{2+} is reported in some metalloproteases, resulting from bridges formed between zinc monohydroxide ($ZnOH^{+}$) and catalytic zinc ions at the active [23]. Activation by Ca^{2+} and inhibition by Zn^{2+} is also a feature of keratinase of *Vibrio* kr2 [24].

Presence of 0.1 % SDS did not affect the activity of enzyme. However, 0.5 % SDS reduced the enzyme activity by 25 %. The enzyme retained around 80 % of activity in presence of Triton X 100, a non-ionic detergent. SDS is reported to inhibit some microbial keratinases [23, 25]. *Bacillus* sp. P7 keratinase retained 63 % and 83 % activity in presence of 0.5 % SDS and Triton X 100 [6]. Keratinase having significant activity in presence of detergents is considered to be an important property when they have to be used as additive in detergent formulation [13].

In presence of 0.5 % Dithiothreitol more than 90 % activity was retained, whereas in presence of 0.5 % β-mercaptoethanol around 85 % activity was retained, indicating that reducing agent has minimal effect on enzyme activity. *Bacillus* sp. P7 keratinase was only slightly inhibited by the reducing agent β-mercaptoethanol. The relative resistance of the enzyme to β-mercaptoethanol is interesting for industrial purposes, since the tight structure of keratin can be weakened through the disruption of disulfide bonds by reducing agents without significant loss of enzyme activity [22].

The keratinase showed good stability in presence of DMSO and retained more than 100 % activity. Keratinases are generally stable in the presence of organic solvents

[13]. Keratinase from *Streptomyces pactum* DSM 40530 showed slight increase in activity in presence of DMSO [26]. *Serratia* sp. HPC 1383 had shown similar increased activity in presence of DMSO [27].

Table 2: Effect of various chemicals on keratinase activity of *Stenotrophomonas maltophilia*

Chemicals	**Concentration**	**% Residual activity**
Control	-	99.99 ± 1.59
Ca^{2+}	5 mM	138.07 ± 2.76
Cu^{2+}		19.82 ± 2.62
Fe^{2+}		77.95 ± 3.13
Hg^{2+}		1.93 ± 0.58
Mg^{2+}		95.98 ± 2.09
Mn^{2+}		91.98 ± 2.17
Zn^{2+}		85.96 ± 2.62
EDTA		59.91 ± 2.76
PMSF		5.87 ± 1.03
SDS	0.10%	97.99 ± 1.59
	0.50%	73.94 ± 2.17
Triton X 100	0.10%	83.96 ± 1.04
	0.50%	81.95 ± 3.19
DMSO	0.10%	102.00 ± 2.76
	0.50%	97.98 ± 2.62
DTT	0.10%	97.98 ± 2.17
	0.50%	93.98 ± 1.59
β-mercaptoethanol	0.10%	91.97 ± 1.59
	0.50%	85.97 ± 1.59

Data represents mean value and standard deviation (n = 3). The 100 % activity corresponds to 34.7 U ml^{-1}

Abbreviation: EDTA - Ethylenediamine tetraaceticacid, PMSF – Phenylmethylsulphonyl fluoride, SDS – Sodium dodecyl sulphate, DTT – Dithiothreitol, DMSO – Dimethyl sulfoxide.

Zymogram studies:

Single major protease band appeared as a clear zone in blue background of the unhydrolysed casein that retained the coomassie blue stain (Figure 5). These results indicate that *Stenotrophomonas maltophilia* K279a, produces large quantities of extracellular protease. The apparent molecular mass of the major band was between 30 to 40 kDa, which correlates with most of the reported serine proteases whose mass ranges from 18 to 35 kDa [10]. *Stenotrophomonas maltophilia* DHHJ keratinase seemed to be a monomer, and molecular mass was estimated as 35.2 kDa [15].

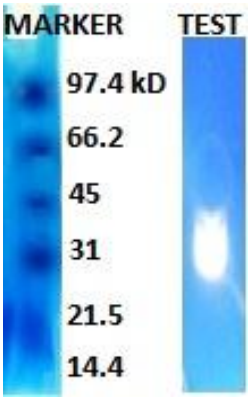

Figure 5: Zymogram of keratinase from *Stenotrophomonas maltophilia* (test) along with molecular mass marker proteins.

Dehairing of goat skin:

Complete dehairing with clean white pelt was achieved after 24 hours of treatment with partially purified keratinase (34.7 U ml^{-1}) from *Stenotrophomonas maltophilia* K279a. No dehairing was observed in the specimen treated with D/W only. Traditional chemical process of hide dehairing using lime and sulphide resulted in complete dehairing (Figure 6). Alkaline protease from *Aspergillus tamarri*, dehaired the goat skin at pH 9–11, temperature 30–37 °C with 1% enzyme concentration and incubation period of 18–24 hour [28]. Similarly, an enzyme isolated from *Bacillus* sp. was used for dehairing of goat skin with 2 –3% concentration and was active in pH

7.5 and 9.0 at 37 °C [29]. In general, the dehairing process required activity of enzyme under alkaline condition; this criterion was satisfied by protease from *B. cereus* MCM-B326 and thus suitable for dehairing [6, 23]. The keratinase enzyme obtained from *Stenotrophomonas maltophilia* K279a was effective in dehairing of goat skins. Keratinase possessing offers an effective bio-treatment of leather, especially dehairing and bating of skins and hides. This process is pleasant and safer than traditional methods using sodium sulphite treatment, which contributes to 100 % of sulphite and over 80 % of the suspended solids in tannery effluents [30]. Enzymatic dehairing process has been gaining importance as an alternative chemical methodology in present day scenario as this process is significant in the reduction of toxicity.

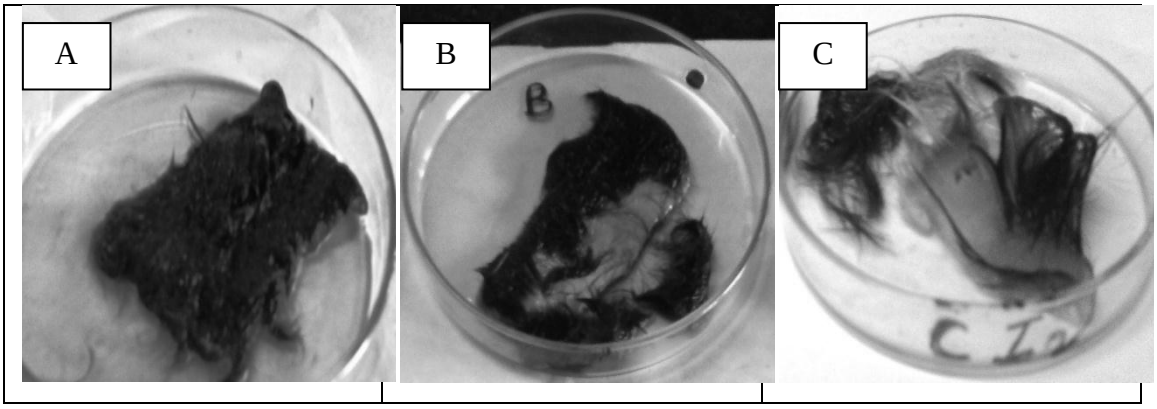

Figure 6: Goat skin dehairing using keratinase preparation from *Stenotrophomonas maltophilia* K279a. A: Control: with D/W treatment, B: Chemical treatment and C: keratinase enzyme treatment only.

CONCLUSION

Keratinolytic protease from *Stenotrophomonas maltophilia* K279a was partially purified. A single large band having molecular mass between 30 and 40 K Da was obtained on zymogram gel. The enzyme had an optimum activity at pH 8.0 and 37 °C. The enzyme was inhibited by PMSF, indicating that it belonged to serine protease. Its activity increased in presence of Ca^{2+}. The enzyme activity was inhibited by heavy metal Hg^{2+} and Cu^{2+}. Presence of Mn^{2+}, Mg^{2+} and Zn^{2+} did not have any significant effect on enzyme activity. The enzyme retained most of its activity in presence of detergents, reducing agents and DMSO. The enzyme was effective in dehairing goat skin. These results indicate that the enzyme may have a promising role in leather processing.

ACKNOWLEDGEMENTS:

The authors would like to thank UGC, India for providing financial assistance towards the project work (Project No: 47- 578 / 13 - WRO).

REFERENCES

[1] Adriano Brandelli, 2008, "Bacterial Keratinases: Useful Enzymes for Bioprocessing Agroindustrial Wastes and Beyond", Food Bioprocess Technology, 1: 105–116.

[2] Subbiah Poopathi and S. Abidha, 2008, "Biodegradation of poultry waste for the production of mosquitocidal toxins", International Biodeterioration & Biodegradation, 62: 479–482.

[3] Savitha G. Joshi, M.M. Tejashwini, N. Revati, R. S. and D. R., 2007, "Isolation, identification and characterization of feather degrading bacteria", International Journal of Poultry Science, 6(9): 689–693.

[4] Cheng-Gang CAI, Bing-Gan LOU and Xiao-Dong ZHENG, 2008, "Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis*", Journal of Zhejiang University, Science B, 9(1): 60–67.

[5] R.R. Chitte, V.K. Nalawade and S. Dey, 1999, "Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaceus* strain SD8", Letters in Applied Microbiology, 28: 131–136.

[6] Nilegaonkar, S. S., Zambare, V. P., Kanekar, P. P., Dhakephalkar, P. K., & Sarnaik, S. S., 2007, "Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326", Bioresource Technology, 98(6): 1238–1245.

[7] Sigma Aldrich, 2008, "Sigma quality control test procedure - Suitability Assay for Keratin Azure as a Substrate for Proteinase K", 1- 2.

[8] Oliver H. Lowry, Nira J. Rosebrough, A Lewis Farr and Rose J. Randall, 1951, "Protein Measurement with the Folin Phenol Reagent", The Journal of Biological Chemistry, 265 – 275.

[9] Sudhir. K. Rai and Ashis 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", Biochemical Engineering Journal, 48(2): 173–180.

[10] Priya Pillai and G. Archana, 2008, "Hide depilation and feather disintegration studies with keratinolytic serine protease from a novel Bacillus subtilis isolate", Appl. Microbiol. Biotechnol., 78: 643–650.

[11] Bastian Herzog, David P. Overy, Bradley Haltli and Russell G. Kerr, 2016,"Discovery of keratinases using bacteria isolated from marine environments", Systematic and Applied Microbiology, 39: 49–57.

[12] Adriano Brandelli, Daniel J. Daroit and Alessandro Riffel, 2010, "Biochemical features of microbial keratinases and their production and applications", Applied Microbiol. Biotechnology, 85:1735–1750.

[13] Rani Gupta and Priya Ramnani, 2006, "Microbial keratinases and their prospective applications", Appl. Microbiol. Biotechnol., 70: 21–33.

[14] Cheng-gang CAI, Ji-shuang CHEN, Jiong-jiong QI, Yun YIN, Xiao-dong ZHENG, 2008, "Purification and characterization of keratinase from a new Bacillus subtilis strain", Journal of Zhejiang University, Science B, 9(9): 713-720.

[15] Zhang-Jun Cao, Qi Zhang, Dong-Kai Wei, Li Chen, Jing Wang, Xing-Qun Zhang and Mei-Hua Zhou, 2009, "Characterization of a novel Stenotrophomonas isolate with high keratinase activity and purification of the enzyme", J. Ind. Microbiol. Biotechnol., 36: 181–188.

[16] P. Anbu, S.C.B. Gopinath, A. Hilda, T. Lakshmi Priya and G. Annadurai, 2005, "Purification of keratinase from poultry farm isolate-*Scopulariopsis brevicaulis* and statistical optimization of enzyme activity", Enzyme and Microbial Technology, 36: 639–647.

[17] Worapot Suntornsuk, Junthip Tongjun, Poranee Onnim, Hiroshi Oyama, Kanok Ratanakanokchai, Thanit Kusamran and Kohei Oda, 2005, "Purification and characterisation of keratinase from a thermotolerant feather-degrading bacterium", World Journal of Microbiology & Biotechnology, 21: 1111–1117.

[18] J. Peddu, C. Chitturi and V. Lakshmi, 2009, "Purification and Characterization of Keratinase from Feather Degrading *Bacillus* sp.", The Internet Journal of Microbiology, Volume 8, Number 2: 1 – 9.

[19] Fernanda Sousa, Susana Jus, Anita Erbel, Vanja Kokol, Artur Cavaco-Paulo and G.M. Gubitz, 2007, "A novel metalloprotease from *Bacillus cereus* for protein fibre processing", Enzyme and Microbial Technology, 40: 1772–1781.

[20] R.C.S. Thys and A. Brandelli, 2006, "Purification and properties of a keratinolytic metalloprotease from *Microbacterium* sp.", Journal of Applied Microbiology, 101: 1259–1268.

[21] Daniel J. Daroit, Ana Paula F. Correˆa and Adriano Brandelli, 2009, "Keratinolytic potential of a novel *Bacillus* sp. P45 isolated from the Amazon

basin fish *Piaractus mesopotamicus*", International Biodeterioration & Biodegradation, 63: 358–363.

[22] Ana Paula F. Correˆa, Daniel J. Daroit and Adriano Brandelli, 2010, "Characterization of a keratinase produced by *Bacillus* sp. P7 isolated from an Amazonian environment", International Biodeterioration & Biodegradation, Volume 64, Issue 1: 1–6.

[23] Alessandro Riffel, Adriano Brandelli, Cl´audia de M. Bellat, Gustavo H.M.F. Souza, Marcos N. Eberlin and Flavio C.A. Tavares, 2007, "Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium* sp. kr6", Journal of Biotechnology, 128: 693–703.

[24] S. Sangali and A. Brandelli, 2000, "Feather keratin hydrolysis by a *Vibrio* sp. strain kr2", Journal of Applied Microbiology, 89: 735 – 743.

[25] Radhika Tatineni, Kiran Kumar Doddapaneni, Ravi Chandra Potumarthi, Ravi Nagaraj Vellanki, Manjusha Thomas Kandathil, Nilima Kolli and Lakshmi Narasu Mangamoori, 2008, "Purification and characterization of an alkaline keratinase from *Streptomyces* sp.", Bioresource Technology, 99: 1596–1602.

[26] Brigitte Bockle, Boris Galunsky and Rudolf Muller, 1995, "Characterization of a Keratinolytic Serine Proteinase from *Streptomyces pactum* DSM 40530", Applied and Environmental Microbiology, 3705–3710.

[27] Anshuman A. Khardenavis, Atya Kapley, Hemant J. Purohit, 2009, "Processing of poultry feathers by alkaline keratin hydrolyzing enzyme from *Serratia* sp. HPC 1383", Waste Management, 29: 1409–1415.

[28] A. Dayanandana, J. Kanagarajb, Lesley Sounderraja, R. Govindarajua and G.Suseela Rajkumara, 2003, "Application of an alkaline protease in leather processing: an eco-friendly approach", Journal of Cleaner Production, Volume 11, Issue 5: 533–536.

[29] Annapurna, R.A., Chandrababu, N.K., Samivelu, N., Rose, C. and Rao, N.M., 1996, "Eco-friendly enzymatic dehairing using extracellular protease from *Bacillus* species isolate", J. Am. Leath. Chem. Assoc., 91: 115–119.

[30] Pathange Prakash, Senigala K. Jayalakshmi & Kuruba Sreeramulu, 2010, "Production of Keratinase by Free and Immobilized Cells of *Bacillus halodurans* Strain PPKS-2: Partial Characterization and Its Application in Feather Degradation and Dehairing of the Goat Skin", Appl. Biochem. Biotechnol., 160: 1909–1920

[31] Brigitte Bockle and Rudolf Muller, 1997, "Reduction of disulfide bonds by *Streptomyces pactum* during Growth on Chicken Feathers", Applied and

Environmental Microbiology, 790–792.

[32] A. R. Birari, K. P. Narkhede and R. C. Salunkhe, 2013, "Feather Keratin Degradation by *Stenotrophomonas* sp. AB20 Screened from Poultry Waste", Journal of Pure and Applied Microbiology, Vol. 7 (4): 2973-2979.

[33] Dastager G. Syed, Jae Chan Lee, Wen-Jun Li, Chang-Jin Kim and Dayanand Agasar, 2009, "Production, characterization and application of keratinase from *Streptomyces gulbargensis*", Bioresource Technology, 100: 1868–1871.

[34] C. Bernal, J. Cair´o, N. Coello, 2006, "Purification and characterization of a novel exocellular keratinase from *Kocuria rosea*", Enzyme and Microbial Technology, 3849–3854.

[35] Chao Ya-Peng, Xie Fu-Hong, Yang Jing, Lu Jing-Hua and Qian Shi-Jun, 2007, "'Screening for a new *Streptomyces* strain capable of efficient keratin degradation", Journal of Environmental Sciences, 19: 1125–1128.

[36] Fuhong Xie , Yapeng Chao, Xiuqing Yang, Jing Yang, Zhiquan Xue, Yuanming Luo and Shijun Qian, 2010, "Purification and characterization of four keratinases produced by *Streptomyces sp.*strain 16 in native human foot skin medium", Bioresource Technology, 101: 344–350.

[37] H. Korkmaz, H. Hür and S. Dinçer, 2004, "Characterization of alkaline keratinase of *Bacillus licheniformis* strain HK-1 from poultry waste", Annals of Microbiology, 54 (2): 201-211.

[38] Khushboo Bhange, Venkatesh Chaturvedi and Renu Bhatt, 2016, "Feather degradation potential of *Stenotrophomonas maltophilia* KB13 and feather protein hydrolysate (FPH) mediated reduction of hexavalent chromium", Biotech 6, 42: 1-9.

[39] P. Anbu, Azariah Hilda, Hwal-Won Sur, Byung-Ki Hur and S. Jayanth, 2008, "'Extracellular keratinase from *Trichophyton sp.* HA-2 isolated from feather dumping soil", International Biodeterioration & Biodegradation, 62: 287–292.

[40] Sanaa Tork, Magda M. Aly and Lubna Nawar, 2008, "Molecular Characterization of a New Keratinase Producing *Pseudomomanas* sp. MS21", Journal of Genetic Engineering and Biotechnology, 6 (1): 37-46.

[41] Shinji Mitsuiki, Misato Ichikawa, Takuji Oka, Masashi Sakai, Yasushi Moriyama, Yuka Sameshima, Masatoshi Goto and Kensuke Furukawa, 2004, "Molecular characterization of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis* sp. TOA-1", Enzyme and Microbial Technology, 34: 482–489.

[42] Sudhir K. Rai, Rocktotpal Konwarh, Ashis K. Mukherjee, 2009, "Purification, characterization and biotechnological application of an alkaline

β-keratinase produced by *Bacillus subtilis* RM-01 in solid-state fermentation using chicken-feather as substrate", Biochemical Engineering Journal, 45: 218–225.

[43] The Ministry of Environment and Forest, Govt. of India, 2010, "The Technical EIA guidance manual for leather / skin / hide processing industry".

[44] Zhen Fang, Juan Zhang, Baihong Liu, Guocheng Du and Jian Chen, 2013, "Biodegradation of wool waste and keratinase production in scale-up fermenter with different strategies by *Stenotrophomonas maltophilia* BBE 11-1", Bioresource Technology 140, 286–291.