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Studies on potential application of crude keratinase enzyme from *Stenotrophomonas* sp. for dehairing in leather processing industry

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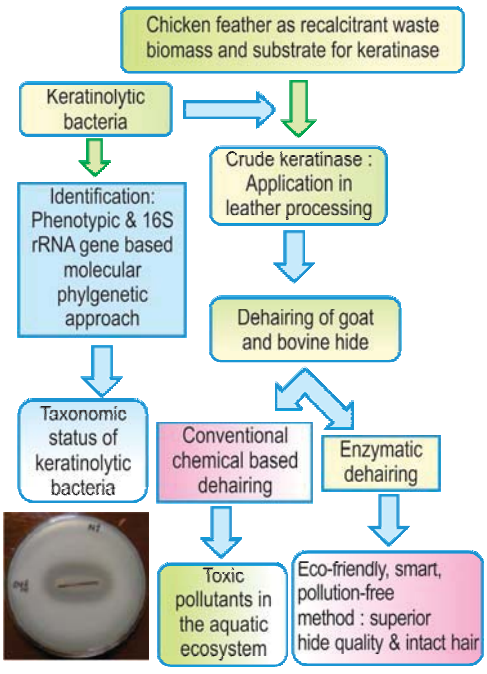
Abstract

Aim : Chicken feathers represent huge pool of waste biomass which can undergo microbial degradation to generate value added products. Chicken feathers can be used as cheap substrate for production of keratinase enzyme which can be effectively used in dehairing of hides. Such enzyme based process is superior over conventional chemical based dehairing process, known to be responsible for pollution of the aquatic ecosystems.

Methodology : Isolation, characterization and identification of keratin degrading bacteria in feather meal broth medium using 0.5% chicken feather as substrate. The effect of various parameters such as temperature; pH and substrate concentration on enzyme activity was assayed. The crude keratinase was evaluated comparatively with conventional chemical based process for dehairing activities with specific references to goat skin and cow hide.

Results : Application of bacterial crude keratinase enzyme towards eco-friendly dehairing process was highlighted from Norja-1, which was identified as *Stenotrophomonas* sp. by combination of 16S rRNA gene based molecular phylogenetic approach and phenotypic characterization. Keratinolytic activity was qualitatively demonstrated by scanning electron microscopic studies and quantitatively by using keratin azure as substrate. The crude keratinase enzyme showed optimum activity at 55°C, pH 9 and 5% substrate concentration. So, the enzyme was considered to be thermo-tolerant and alkaliphilic. Conventional chemical based process generated black spot on hide surface with broken hair while enzyme based process produced hides with better texture and intact hair which may be used further for making other value added products (such as wigs, doll's hair, carpets and mattresses).

Interpretation : The dehairing activities of crude keratinase enzyme were demonstrated to be better in comparison to conventional chemical methods and might be useful towards development of pollution free dehairing process for leather processing industries. Presence of enzyme activity at wide range of temperatures (10 to 60°C) and pH (7.4 to 10.7) makes it a potential candidate for possible biotechnological application towards development of eco-friendly cost effective dehairing process in leather industries.



Introduction

Human population on earth is possibly approaching towards its zenith period of development and to cater its daily activities, development of processes that needs less water (a scare but very important commodity) and generates less toxic effluents are in strong demand. Conventional chemical based dehairing process in leather industries generate effluents that are highly toxic for the environment, especially aquatic ecosystems (Bouacem *et al*., 2016). Replacement of toxic chemicals with efficient enzyme (non-toxic) based dehairing process is eco-friendly alternative approaches that not only requires less water but also generates pollution free effluents (Paul *et al*., 2016). Due to their ability to digest the tough, recalcitrant keratin protein, keratinase is currently considered as interesting, smarter protease of next generation which may find tremendous applications in several industries (Deivasigamani and Alagappan, 2008).

Keratinases are proteolytic enzymes involved in the hydrolysis of keratin and have been classified as serine and metallo-proteases or serine-metallo-proteases (Gupta and Ramnani, 2006). Keratinases of microbial origin is increasingly becoming useful in dairy, food, pharmaceutical and agricultural sectors (Chandel *et al*., 2007; Rai *et al*., 2009; Paul *et al*., 2014a).

The conventional leather processing methodology includes a number of complex steps (such as curing, soaking, dehairing, bating and tanning) involving hazardous chemicals like sodium sulphide, calcium oxide as well as chrome, posing serious health hazards to the workers of the leather industry (Thanikaivelan *et al*., 2003; Arunachalam and Saritha, 2009). Moreover, the industrial effluents from the conventional leather processing industry have high BOD, COD, TDS and TSS posing serious threat to environment, as well as its components (both living and non-living). However, use of keratinases has reported to significantly decrease these pollution indices (Paul *et al*., 2014b). The use of organic chemicals such as aniline, benzene, toluene, sulphuric acid has deleterious effects on health (Rastogi *et al*., 2008). Due to high organic load and toxic chemicals, this effluent makes survival of various aquatic life forms difficult and often may lead to serious ecological problems. Keratinolytic proteases provide a better, eco friendly, safer alternative against chemical processes (such as those used in leather processing) and are a possible resource for tomorrow's smart green cleaner technology.

Isolation and characterisation of a keratin degrading bacterium *Stenotrophomonas* sp. strain Norja-1 was investigated in the present study. Application of crude keratinase (from this strain) towards efficient dehairing process was demonstrated as better pollution free, eco-friendly alternative over conventional chemical based processes.

Materials and Methods

Isolation, characterization and identification of keratinolytic bacterium : The keratinolytic bacterium was isolated by enrichment culture of soil samples, collected from keratinous waste dumping site located at Norja village in Burdwan district of West Bengal, India, using chicken feathers as substrate (Agrahari and Wadhwa, 2010). Feather meal media (FMM) with composition, (g l^{-1}): NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2.6H_2O$, 0.1; chicken feather, 5.0; pH 7.5, was used for enrichment followed by its isolation and maintenance on skim milk agar (SMA) (HiMedia, India) at 37°C.

Morphological and biochemical characterization of the bacterial isolate was carried out according to established methods (Smibert and Krieg, 1994). The strain was checked for its ability to grow at different temperature, pH and salt concentrations following standard methods reported by Saha and Chakrabarti (2006). Phylogenetic affiliation of the strain was determined by using 16S rRNA gene based sequence analysis carried out at EzTaxon server and at RDP site.

Keratin degradation study by scanning electron microscopy: Qualitative evaluation of keratin degradation using chicken feather was carried out by scanning electron microscopy. For this, feathers were taken from test samples (FMM with culture grown for 3 days at 37°C under shaking conditions) and control sample (FMM without bacterial culture incubated under similar condition) and washed extensively with water. The feather samples were dried in hot air oven at 60°C and were mounted on stubs and gold coated using IB-2 ion counter, followed by their observation by scanning electron microscope.

Production of crude enzyme : The strain Norja-1was grown in FMM broth supplemented with 0.5% feather and incubated at 37°C under shaking conditions (120 rpm) for 4 days. In order to collect crude extracellular enzyme from culture supernatant, after growth, the broth culture was centrifuged at 10,000 rpm for 15 mins, followed by decantation (without disturbing the pellet) of supernatant (containing the enzyme) and its subsequent storage at 4°C until further used.

Total protein estimation and keratinase assay : Total protein in the culture supernatant was quantified by Lowry method (Lowry *et al*., 1951). Bovine serum albumin was used as standard for calibrating the amount of total protein in the culture filtrate. Assay for keratinase enzyme was carried out with keratin azure (Sigma-Aldrich) as substrate (0.5%), following existing method (Bressollier *et al*., 1999) with slight modification where pH 9 for 50 mM Tris-HCl buffer was used. Reaction mixture contained 1ml of enzyme extract and 1ml substrate suspension. One unit of keratinase activity was defined as the amount of enzyme causing 0.01 increase in absorbance between sample and control at 595 nm for 1 hr. Release of blue colour azo dye in the test was

considered as positive result as compared to the control (where no such colour was released). Estimation of total protein and enzyme activity were determined from day 1 to day 7.

Influence of temperature, pH, incubation time and substrate concentrations on enzyme activity : To determine the influence of pH on keratinase activity, the crude enzyme extract was incubated with the substrate keratin azure for 1 hr at different pH using the following buffers: citrate-phosphate buffer (for pH 3 and 5), phosphate buffer (pH 7), Tris-HCl buffer (pH 7.4, 8 and 9) and carbonate-bicarbonate buffer (pH 10 and 10.7). To determine the effect of temperature on enzyme activity the enzyme assay mixture was incubated at a wide range of temperatures: 10ºC, 28 ºC, 37 ºC, 45 ºC, 48 ºC, 50 ºC, 55 ºC and 60 ºC. The optimum enzyme activity was determined over a wide range of substrate concentrations ranging from 1 to 7%.

Dehairing of goat and bovine hide by crude enzyme : Goat and cow hides were obtained from local butcher. The hides were washed with water repeatedly to remove the surface contamination, followed by immersion in detergent solution to remove the impurities. These were next cut into small pieces (2cm x 4cm) to evaluate the dehairing activity. Hide pieces were next immersed in 30ml crude keratinase enzyme extract (culture supernatant) having keratinase activity (13.88 U ml^{-1}) and incubated at room temperature. Hides immersed in distilled water were used as control. The dehairing ability was checked at 6 hrs and 12 hrs for goat hide, while the same for cow hide was evaluated at 18 hrs and 24 hrs of treatment with crude enzyme. After incubation, the pieces of hides were taken out and the hairs loosely bound to the skin were scraped with fingers.

Comparison and evaluation of enzymatic versus conventional chemical methods of dehairing: Comparison of conventional chemical treatment versus enzymatic treatment for dehairing was performed by subjecting hides to each of these treatments separately. Enzymatic treatment was carried out as mentioned above. While, chemical treatment was carried out by treating the hide with 10% w/v lime and 2% w/v sodium sulphide. In both the treatment conditions dehairing activity were checked after 24 hrs of incubation. After incubation, the hides were cleaned to remove the loosely bound hairs in case of enzymatic treatment. While, in case of chemical treatment, the slurry of chemicals was washed and the distorted hairs were removed. The characteristic grain structures of depilated area of hide (enzymatic versus chemical) were observed under stereo microscope at 4X magnification. The surface views of hides were captured by using a camera and a thorough comparative evaluation was carried out.

Results and Discussion

Several keratinase producing bacterial cultures were isolated by enrichment culture (using minimal medium supplemented with chicken feathers as sole source of carbon) followed by screening on SMA plates. Among these, one isolate, designated as Norja-1 was selected for further study based on its ability to degrade chicken feathers completely within 72 hrs. The strain Norja-1 showed cream colonies on skim milk agar plates. It could grow over a wide range of temperatures (10°C to 47°C; optimum 37°C), pH (5 - 10) and could tolerate up to 5% sodium chloride. Since, it could grow well at high alkaline pH, it could be considered as alkali tolerant. Its phenotypic characteristics are summarised in Table 1. Results of 16S rRNA gene based

Table 1 : Profile of phenotypic properties of the strain Norja-1

Phenotypic trait	Norja-1
Cell Morphology	Small single rods
Gram's Nature	Gram negative
Endospore formation	-
Motility test	+
Catalase activity	+
Oxidase activity	-
Amylase activity	-
Casein hydrolysis test	+
Cellulose hydrolysis	-
Methyl Red test	+
Indole production	-
Voges Proskauer test	-
Growth on MacConkey Agar	+
DNase activity	-
Lipase test	-
Carbohydrate utilization pattern:	
Dextrose	+
Melibiose	+
Esculin hydrolysis	+
Citrate utilization	+
Malonate utilization	+
Growth between Temperatures 10ºC to 47ºC	+
Growth between pH 5 to 10	+
Growth in presence of NaCl:	
2% to 5%	+
7% & 10%	-

- = Negative; + = Positive

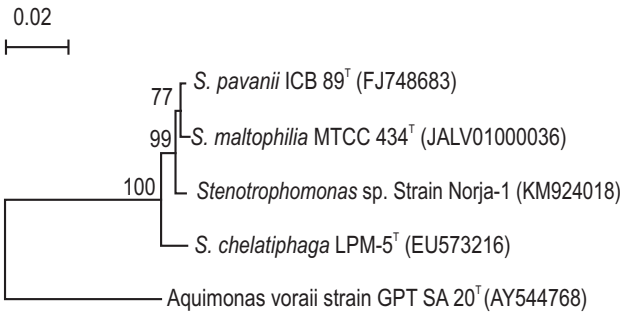


Fig. 1 : Phylogenetic tree based on 16S rRNA gene sequences showing relative position of Norja-1 among closely related spp. of the genus *Stenotrophomonas*. Number at the node indicates bootstrap values of 100 replications. Sequence of 16S rRNA gene from *Aquimonas voraii* was taken as out group. Bar 0.02 substitutions/site

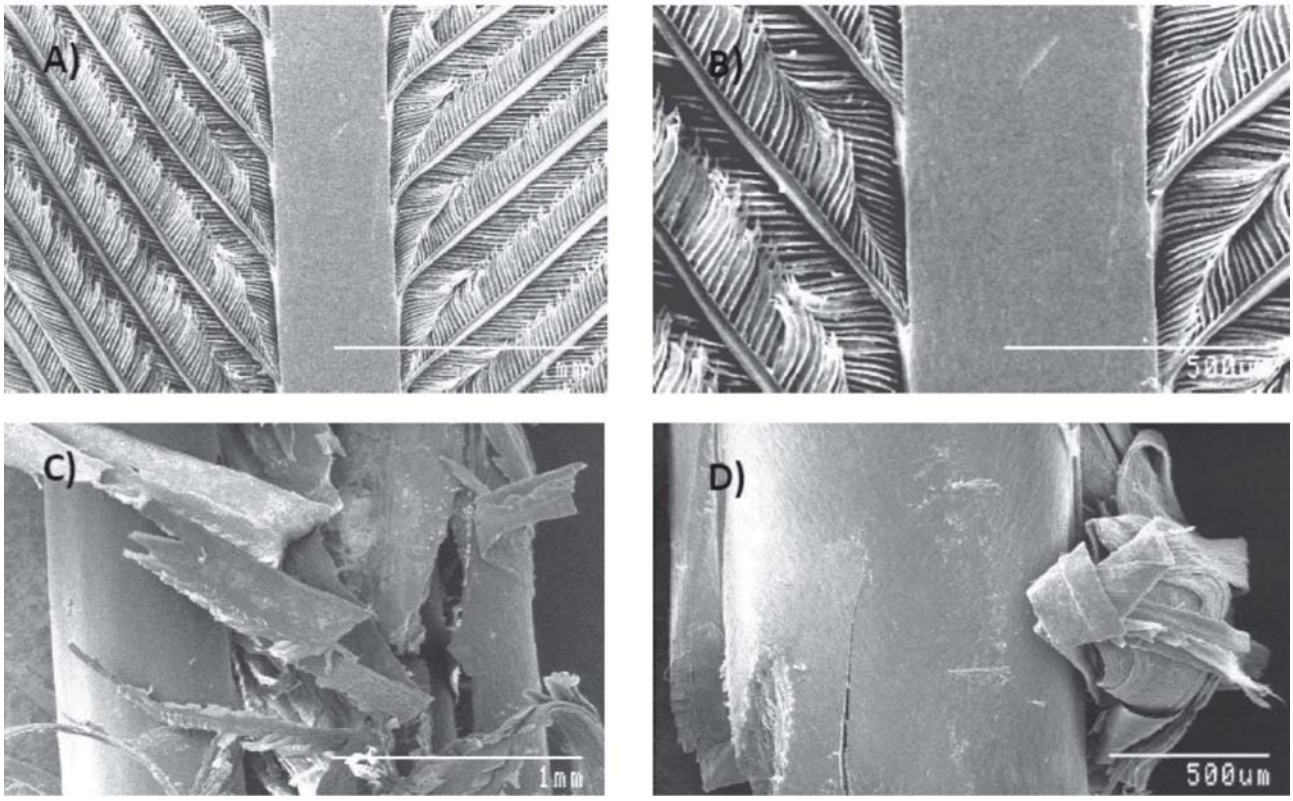


Fig. 2 : Scanning electron microscopy (SEM) images of chicken feather, control at (A) 30X (B) 50X magnifications and degradation of chicken feather by isolate Norja-1 after 48 hrs at 50X magnification (C) after 72 hrs at 60X magnification (D)

molecular phylogenetic analyses revealed the strain Norja-1 to be a member of the genus *Stenotrophomonas*, with *S. maltophilia*^{T} as its closest relative (with 99.41% sequence identity), followed by *S. pavanii*^{T} (99.27% identity) and *S. chelatiphaga*^{T} (98.53% identity), respectively. The phylogenetic tree (Fig. 1) showed clade of strain Norja-1 being placed close to *S. maltophilia* with high bootstrap value of confidence. Although, the strain Norja-1 showed high degree of sequence identity to their respective closest species (represented by the type strains), in absence of detail polyphasic characterization and over all genome relatedness data, no specific conclusions were made about its species status (Stackebrandt and Goeble, 1994) and it was conclusively identified as *Stenotrophomonas* sp. The strain Norja-1 was made available to public domain (for research purpose) with its accession numbers MCC2174 (deposited at MCC, NCCS, Pune), while accession number of its 16S rRNA gene was KM924018 (deposited at GenBank database).

As evident, from qualitative studies based on scanning electron microscopy (Fig. 2A, 2B, 2C and 2D); compared to intact feather in control condition; the barbs and barbules were disintegrated completely leaving behind the fragile rachis only in test condition. These confirmed the presence of strong keratin degradation activity of the bacterial isolate. Although, keratinolytic

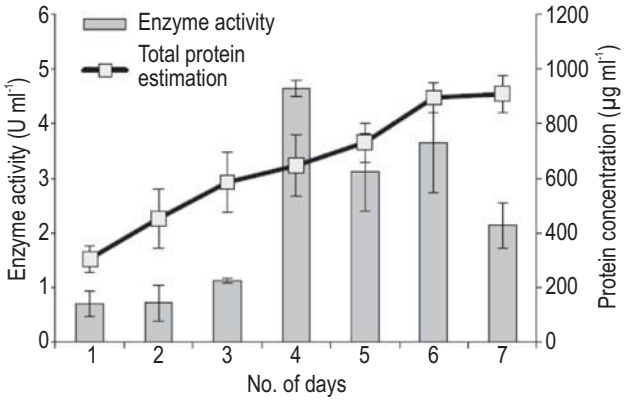


Fig. 3 : Total protein estimation and keratinase enzyme activity for Norja-1on different days of incubation

bacteria belonging to several genera has been isolated from diverse sources, very few are known to be affiliated to the genus *Stenotrophomonas.* There are only few reports of keratinolytic bacteria belonging to the genus *Stenotrophomonas*. These are *Stenotrophomonas nitritireducens* isolated from soil containing deer fur (Yamamura *et al*., 2002) and *Stenotrophomonas maltophilia* isolated from poultry decomposing feathers in China (Cao *et al*., 2009) .

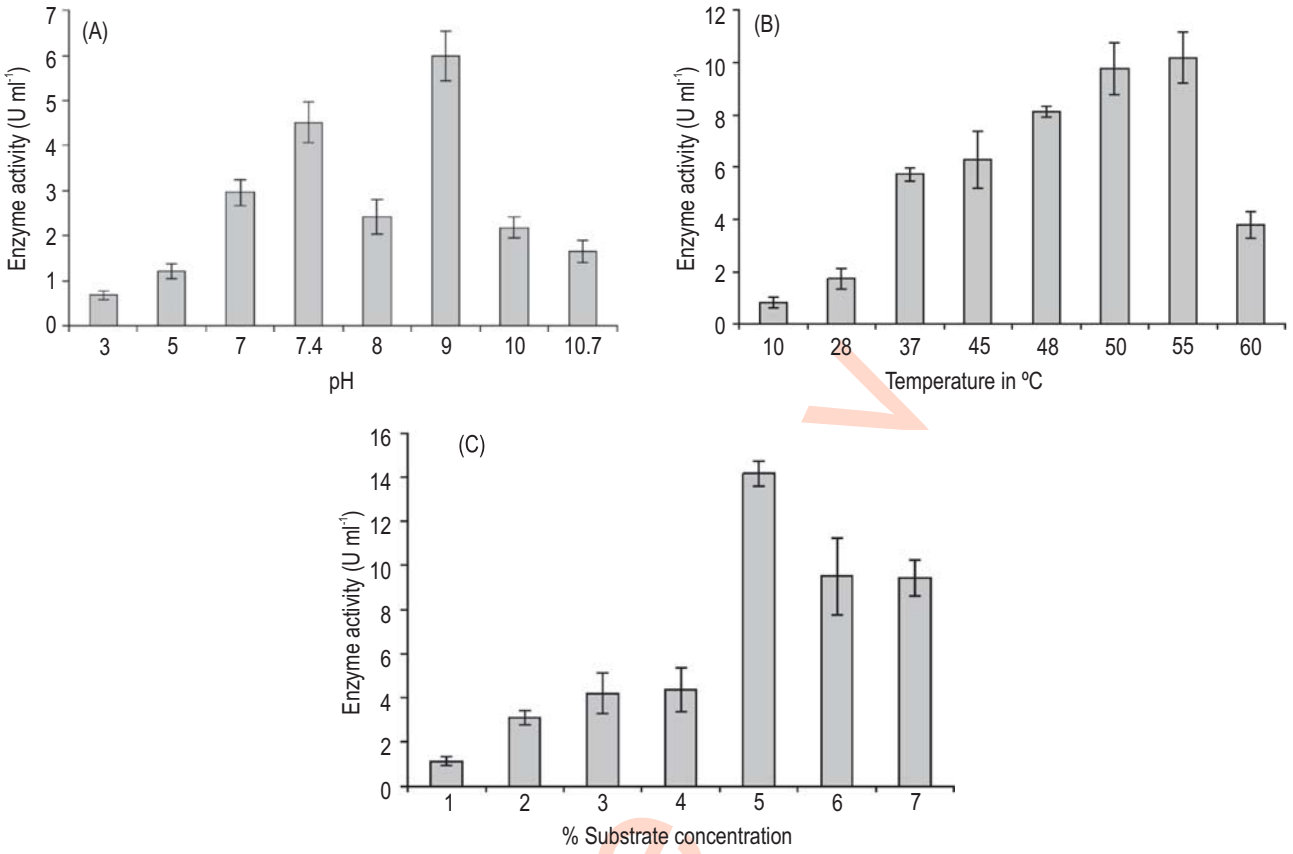


Fig. 4 : Influence of (A) pH; (B) temperature and (C) substrate concentration on enzyme activity of isolate Norja-1

Analysis carried out with the crude enzyme extract (culture supernatant) indicated maximum keratinase activity to be on day 4, which was 4.64 ± 0.15 U ml^{-1} (Fig. 3). The optimum pH, temperature and substrate concentration for keratinolytic enzyme activity were recorded to be at pH 9.0 (5.98 ± 0.54 U ml^{-1}; Fig. 4A), 55ºC (10.18 ± 0.96 U ml^{-1}; Fig. 4B) and 5% chicken feather (14.17 ± 0.57 U ml^{-1}; Fig. 4C), respectively. Previously, Cao *et al*. (2009) reported a *Stenotrophomonas* strain (from China) having optimum keratinase activity at 40°C and pH 7.8, respectively. So, crude keratinase enzyme obtained from strain Norja-1 in this study may be considered superior.

The crude enzyme from Norja-1 was evaluated for dehairing properties and was demonstrated to be a potential candidate. Demonstrative evidence indicated that crude enzymatic treatment of goat hide lead to removal of more than 50% of the hairs after 6 hrs (Fig. 5A), while its complete removal was recorded after 12 hrs (Fig. 5B). For bovine hide, partial removal of hairs were observed after 18 hrs (Fig. 5C) and for complete removal 24 hrs (Fig. 5D). For both goat and bovine hides gentle rubbing removed the loosened hairs revealing their better, shiny texture during such treatment process. Therefore, enzymatic dehairing process provided better texture to both the hides (goat and bovine).

A thorough comparative evaluation of quality of bovine hide after both chemical and enzymatic treatments was carried out by careful microscopic observation. The study revealed that the chemically treated hides showed black spots and disintegrated broken hairs (Fig. 5E), while enzymatically treated hide, on the other hand, showed perfect grain structure with no black patches (Fig. 5F). This indicated superiority of crude enzyme of strain Norja-1 for dehairing activities over conventional chemical treatment methods. The significance of intact hairs obtained as by-product from goat skin or bovine hide lies in the fact that these hairs can be spun into yarns for making other value added products (such as wigs and doll's hair; coarse yarns, carpets or felts and for padding or stuffing purposes), which may serve as means of earning livelihood for unemployed rural youth, (especially village women folk) thus, may contribute to scale up societal economy of rural India.

Alkaline protease enzyme based, leather processing technique is considered superior, eco-friendly, cost effective, energy saving, smarter alternative to conventional chemical based formulations and processes (Paul *et al*., 2014b). Animal hide dehairing activity of keratinase KERUS from *Brevibacillus brevis* strain US575 has been previously reported (Jaouadi *et al*.,

Fig. 5 : Dehairing properties of crude enzyme extract from Norja-1 on goat and bovine hide. The goat skin (A) after 6 hrs; (B) 12 hrs and bovine hide; (C) after 18 hrs; (D) after 24 hrs. Comparative evaluations of dehairing process; (E) Conventional chemical treatment method and (F) enzymatic treatment method

2013). So far, extensive demonstrative evidence of crude keratinase enzyme from *Stenotrophomonas* sp. in effective dehairing of goat skin and bovine hide is lacking and recently in a report, (Fang *et al*., 2013) documented that the co-operative action of two keratinolytic enzymes K1 and K2 from *Stenotrophomonas maltophilia* BBE11-1 to wiped out cuticle layers of wool without damaging the internal fibres. This indicates potentiality of keratinase enzymes towards effective dehairing process.

Annually, 700,000 t of animal hides and skins are processed through 3000 tanneries in India, most of which are based on the banks of Ganges river system in the north and Palar river in the south (Joseph and Nithya, 2009). The toxic chemicals released from leather processing units (lime, sulphide, chrome etc.) are not only hazardous to environment but also to those who are directly exposed (tannery workers, sewer men) as well as to different life forms (macroscopic and microscopic) of contaminated water bodies and other ecosystems. Regular discharges of toxic effluents from these tanneries to the rivers are responsible for pollution of river. Through conventional method, usually, 1 kg of leather processing generates 30 to 40 l of liquid effluents mixed with toxic chemicals, that up on release ultimately pollutes ecosystems and in fact in 2002, Rs. 46 million was imposed as fine on different tanneries to compensate loss of food production by agriculturists and farmers (Saran *et al*., 2013; Paul *et al*., 2016). Since, pure water is becoming a rarer essential life throbbing commodity, day by day; it is right and urgent stage that we take measures to stop this water pollution. Therefore, it is very essential to look for a greener, safer eco-friendly alternative as possible replacements for these toxic chemicals used in leather industry. In fact, keratinolytic proteases are such alternatives. These enzymes are non-toxic, bio-degradable and do not generate any toxic residues (Paul *et al*., 2016**).**

To conclude, in a nutshell, crude extracellular keratinase enzyme (from culture supernatant) of strain Norja-1 was demonstrated for effective dehairing of animal hides (goat and bovine). This enzymatic method produced better hide texture, intact hair and was pollution free, eco-friendly alternative over conventional chemical based method which causes environmental pollution, thus, making it a potential candidate for possible biotechnological applications towards development of smart, clean, pollution free, green leather processing technology.

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References

Agrahari, S. and N. Wadhwa: Degradation of chicken feather a poultry waste product by keratinolytic bacteria isolated from dumping site at Ghazipur poultry processing plant. *Int. J. Poult. Sci.*, **9**, 482-489 (2010).

Arunachalam, C. and K. Saritha: Protease enzyme: an eco-friendly alternative for leather industry. *Indian J. Sci. Technol.*, **2**, 29-32 (2009).

Bouacem, K., A.B. Darenfed, N.Z. Jaouadi, M. Joseph, H. Hacene, B. Ollivier, M.L. Fardeau, S. Bejar and B. Jaouadi: Novel serine keratinase from *Caldicoprobacter algeriensis* exhibiting outstanding hide dehairing abilities. *Int. J. Biol. Macromolec.*, **86**, 321-328 (2016).

Bressollier, P., F. Letourneau, M. Urdaci and B. Verneuil: Purification and characterization of a keratinolytic serine proteinase from *Streptomyces albidoflavus*. *Appl. Environ. Microbiol.*, **65**, 2570-2576 (1999).

Cao, Z.J., Q. Zhang, D.K. Wei, L. Chen, J. Wang, X.Q. Zhang and M.H. Zhou: Characterization of a novel *Stenotrophomonas* isolate with high keratinase activity and purification of the enzyme. *J. Ind. Microbiol. Biotechnol.*, **36**, 181-188 (2009).

Chandel, A.K., R. Rudravaram, L.V. Rao, P. Ravindra and M.L. Narasu: Industrial enzymes in bioindustrial sector development: An Indian perspective. *J. Commer. Biotechnol.*, **13**, 283-291 (2007).

Deivasigamani, B. and K.M. Alagappan: Industrial application of keratinase and soluble proteins from feather keratins. *J. Environ. Biol.*, **29**, 933–936 (2008).

Fang, Z., J. Zhang, B.H. Liu, G.C. Du and J. Chen: Biochemical characterization of three keratinolytic enzymes from *Stenotrophomonas maltophilia* BBE11-1 for biodegrading keratin wastes. *Int. Biodeter. Biodegr.*, **82**, 166-172 (2013).

Gupta, R. and P. Ramnani: Microbial keratinases and their prospective applications: an overview. *Appl. Microbiol. Biotechnol.*, **70**, 21-33 (2006).

Jaouadi, N.Z., H. Rekik, A. Badis, S. Trabelsi, M. Belhoul, A.B. Yahiaoui, H.B. Aicha, A. Toumi, S. Bejar and B. Jaouadi: Biochemical and molecular characterization of a serine keratinase from *Brevibacillus brevis* US575 with promising keratin-biodegradation and hide-dehairing activities. *PlosOne8*, e76722 (2013).

Joseph, K. and N. Nithya: Material flows in the life cycle of leather. *J. Clean. Prod.*, **17**, 676-682 (2009).

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

Paul, T., A. Das, A. Mandal, S.K. Halder, P.K. Das Mohapatra, B.R. Pati and K.C. Mondal: Biochemical and structural characterization of a detergent stable alkaline serine keratinase from *Paenibacillus woosongensis* TKB2: A potential additive for laundry detergent. *Waste Biomass Valor.*, **5**, 563-574 (2014a).

Paul, T., A. Das, A. Mandal, A. Jana, C. Maity, A. Adak, S.K. Halder, P.K. Das Mohapatra, B.R. Pati and K.C. Mondal: Effective dehairing properties of keratinase from *Paenibacillus woosongensis* TKB2 obtained under solid state fermentation. *Waste Biomass Valor.*, **5**, 97-107 (2014b).

Paul, T., A. Jana, A.K. Mandal, A. Mandal, P.K. Das Mohapatra and K.C. Mondal: Bacterial keratinolytic protease, imminent starter for NextGen leather and detergent industries. *Sustainable Chem. Pharma.*, **3**, 8-22 (2016).

Rai, S.K., R. Konwarh and A.K. Mukherjee: 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. *Biochem. Eng. J.*, **45**, 218-225 (2009).

Rastogi, S.K., A. Pandey and S. Tripathi: Occupational health risks among the workers employed in leather tanneries at Kanpur. *Indian J. Occup. Environ Med.*, **12**, 132-135 (2008).

Saha, P. and T. Chakrabarti: *Emticicia oligotrophica* gen. nov., sp. nov., a new member of the family 'Flexibacteraceae' phylum Bacteroidetes. *Int. J. Syst. Evol. Microbiol.*, **56**, 991-995 (2006).

Saran, S., R.V. Mahajan, R. Kaushik, J. Isar and R.K. Saxena: Enzyme mediated beam house operations of leather industry: A needed step towards greener technology. *J. Clean. Prod.*, **54**, 315-322 (2013).

Smibert, R.M. and N.R. Krieg: Phenotypic Characterisation. In: Methods for General and Molecular Bacteriology (Eds.: P. Gerhard, R.G.E. Murray, W.A Wood and N.R. Krieg). American Society for Microbiology, Washington DC, pp. 607-654 (1994).

Stackebrandt, E. and B.M. Goebel: Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.*, **44**, 846-849 (1994).

Thanikaivelan, P., J.R. Rao, B.U. Nair and T. Ramasami: Approach towards zero discharge tanning: role of concentration on the development of eco-friendly liming-reliming processes. *J. Clean. Prod.*, **11**, 79-90 (2003).

Yamamura, S., Y. Morita, Q. Hasan, S.R. Rao, Y. Murakami, K. Yokoyama and E. Tamiya: Characterization of a new keratin-degrading bacterium isolated from deer fur. *J. Biosci. Bioeng.*, **93**, 595-600 (2002).