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Article

Partial purification and characterization of alkaline protease enzyme from *Pseudomonas aeruginosa* for tannery in Bangladesh

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Abstract: An extracellular protease producing bacteria was isolated from poultry waste deposited site at Dhamrai, Dhaka. The isolated bacteria was confirmed as *Pseudomonas aeruginosa* after performing different morphological and biochemical tests The extracellular protease from the isolated *P. aeruginosa* was partially purified and characterized. The enzyme was purified by ammonium sulphate precipitation and DEAE cellulose column chromatography. SDS-PAGE was performed and the presence of protease enzyme was confirmed and the molecular weight of protease was determined as 66 KDa. Dehairing treatment with the isolated protease enzyme showed higher activity comparing with some available protease of other bacterial sources as well as conventional chemical method. Effect of pH, temperature, salt-ions and other effector molecules on protease from isolated *P. aeruginosa* was also examined. The enzyme showed higher activity at pH 8.0 and optimum temperature was found as 35-45˚C. The activity of the enzyme slightly decreased in presence of salt-ions (Mg^{++}, Ca^{++}, Zn^{++}, Mn^{++}, Na^{+}, K^{+}) and inhibitors (EDTA, 2-mercaptoethenol, DMSO, SDS, Ethanol, Methanol). The deactivating effect of 2-marceptoethanol indicated the enzyme as cystine protease.

Keywords: *Pesudomonas aeruginosa*; protease enzyme; SDS-PAGE; tannery

1. Introduction

Leather industry is one of the oldest industries in Bangladesh and plays substantial role in the national economy with a good reputation worldwide. The leather industry is well established and ranked fourth in terms of earning foreign exchange in Bangladesh. The leather products sector have huge opportunities in generating employment, entrepreneurship and investment by increasing export of higher value added products rather than finished leather and by utilizing locally made raw material (finished leather) to convert into more value added leather products (Sarath *et al.,* 1996).

Enzymes are the well known biocatalysts that perform a multitude function of chemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics, fine chemical, leather and poultry processing industries. Over the last few decades leather industry is based on large scale chemicals treatment that causes worldwide environmental hazards. Leather manufacturing is one of the most industrial activities globally wide spread, which involves the use of wide range of chemicals many of which are hazardous, highly toxic and obnoxiously odorous(De Aquim *et al.,* 2010; Sneath *et al.,* 1986). Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides (Song *et al.,* 2011). Use of enzymes for industrial processing has received considerable attention in recent years owing mainly to environmental concerns (Ibrahim and Al-Salamah, 2009).

Proteases help in breakdown of proteins into simpler form that exist between two amino acids of a polypeptide chain by the process of hydrolysis (Brandelli and Riffel, 2005).

Most of the tannery industries in Bangladesh use chemicals for dehairing that led great environmental hazards and health problem. However, leather industries are one of the most promising fields for export to earn foreign currency in Bangladesh. Recently government of People's Republic of Bangladesh has taken initiative to develop the modernize industry from outside the city. Enzymatic dehairing is suggested as an environment friendly alternative to the conventional chemical process (Uddin *et al.*, 2014). Enzymes have been pursued as one of the promising alternates to lime and sodium sulfide (Dettmer *et al.,* 2013). Enzymes display a high capability of degrading insoluble keratin substrates of their several potential uses associated to the hydrolysis of keratinous substrates and other applications (Brandelli *et al*. 2010). In recent years proteases find application in leather making among the different industrial proteases the most widely used enzymes in leather manufacturing (Choudhary *et al.*, 2004). Alternatively, feather biodegradation processes have been proposed as viable substitutes (Xie *et al.,* 2010).

Higher cost of enzyme is one of the major factors for the system not being practiced through found environmentally friendly. It is essential to develop a cost effective and eco-friendly technology by screening for efficient enzymes from microbial sources and producing them in large quantities by applying recombinant DNA technology. Enzymes found in nature are quite often not readily available in quantities sufficient for industrial use, so use of gene expression methods to express recombinant proteins in suitable heterologous expression systems is required (Araujo *et al.*, 2008). Genetic engineering could be used to increase the gene copy number as an effective method for improving enzyme productivity (Jorgensen *et al.,* 2000). Genes coding for microbial proteases have been cloned and expressed in a broad range of microorganisms in order to improve their properties or their expression levels. Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of Enzymology. These enzymes can be used in various industries such as tannery industries to degrade complex proteins on such organic pollutents present in ebvironment. The aim of this research effort was carriedout screening of elevated protease producing *Pseudomonas aeruginosa* from soil sample and observed the de-hairing activity so that it can be used used for deharing the hides and skins of cattle in the tannery industries

2. Materials and Methods

2.1. Sample collection, Isolation and culture conditions

The experiment was conducted at the vaccine research laboratory under the department of Biochemistry and Microbiology of the Gono University, Dhaka, Bangladesh, with the objective to screening, optimization and exploration of microbial enzymes from local soil samples with special characteristics for biotechnological applications. Chemicals used in the experiment were from Oxoid Ltd. (Basingstoke, UK), Merck AG (Darmstadt, Germany) and Sigma (USA). Azokeratin was synthesized based on the method described in a previous study (Puvanakrishnan. and Dhar, 1986).

The soil sample was collected from the poultry wastes in Savar, after serial dilution, culture were inoculated in LB broth media and incubated for 16 h at 37°C. After incubation few single colonies were identified on the basis of different colony morphology. Each colony was inoculated into screw capped test tubes containing autoclaved feather with liquid broth media and incubated overnight at 37°C with shaking at 160 rpm. Media without inoculation used as negative control.

2. 2. Screening of soil sample for Identification of protease producing microbial strains

A rapid bacterial identification test kit for Bacteria, API 50 CHB (BioMerieux, France), was used to identify species of bacteria. Gram's staining; morphological studies, physiological and biochemical characteristics of the isolate were investigated according to Bergey's Manuals (Sneath *et al.,* 1986). To identify the biochemical properties of the organism different tests were performed. For correct interpretation of the results in every test *Escherichia coli* was taken as control. Fermentative capabilities of the isolated organisms were tested under anaerobic conditions in Durham tube. The carbohydrate such as Glucose, Lactose, Ribose, Sucrose, Mannitol, Adonitol, Arabinose, Sorbitol, and Maltose utilization tests that were performed. Others Biochemical tests that were performed are the hydrogen sulfide test, motility test, indole production test, citrate utilization test, nitrate reduction test, oxidase test (young culture), catalase test, urease test, indole (SIM) test, methyl Red (MR) test, Voges-Proskauer (VP) Test, starch hydrolysis test and gelatin liquefaction test. Some microbiological tests such as Gram's staining, spore staining, colony morphology and growth curve determination also performed.

2.3. Assessment of protein content and evaluation of proteolytic activity

The microorganism was cultivated in sterile nutrient broth medium. The culture was grown overnight at 37°C for 15-20 hours on a rotary shaker at 150 rpm. The culture was then centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and used as crude enzyme sample. Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981). Here azocasein is used as a substrate. Optical density was measured at 440 nm. One unit of proteolytic activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.01 at 440 nm.

2.4. Exploration of the effect of temperature on bacterial growth and protease synthesis

The bacterial culture was grown in nutrient broth at 25ºC, 30ºC, 35ºC, 40ºC, 50ºC and 60ºC was incubated for 48 hours to measure its growth profile. For the determination of the effect of temperature, the culture medium was incubated at temperature ranging from 25-60˚C and the protease activity was determined at 37˚C using the usual methods.

2.5. Determination of effect of pH and temperature on protease activity and stability

For determining the effect of pH on protease activity and stability different buffer system with different pH ranging from 4.0 to 10.5 were used. Azocasein was dissolved in different buffer solution and the enzyme assay was carried out within pH range (4.0 to 10.5) by azocasein assay method. All of them were used at 0.05M concentration.

For the determination of the effect of temperature, the reaction medium was incubated at varied temperature and the protease activity was determined. For this purpose the enzyme preparation was added to a mixture of 1 mg 1 % azocasein solution, 0.1 ml of 0.06 M $CaCl_2$ and buffer (0.2 M Tris-HCI buffer, pH 8.0) and incubated at 37°C, 40°C, 50°C, 60°C, 65°C temperatures.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein patterns of the selected fractions were determined by 10% SDS-PAGE according to the method of Laemmli (1970) as modified by Smith (1984).

Electrophoresis is the study of the movement of charged molecules in an electric field. The generally used support medium is cellulose or thin gels made up of either polyacrylamide or agarose. Cellulose is used as support medium for low molecular weight biochemicals such as amino acid and carbohydrates whereas agarose and polyacrylamide gels are widely used for larger molecules like proteins. The general electrophoresis techniques cannot be used to measure the molecular weight of the biological molecules because the mobility of a substance in the gel is influenced by both charge and size. In order to overcome this, if the biological samples are treated so that they have a uniform charge, electrophoretic mobility then depends primarily on size. The molecular weight of protein may be estimated if they are subjected to electrophoresis in the presence of a detergent sodium dodecyl sulfate (SDS). SDS disrupts the secondary, tertiary and quaternary structure of the protein to produce a linear polypeptide chain coated with negatively charged SDS molecules. 1.4 grams of SDS binds per gram of protein. SDS-Polyacrylamide Gel Electrophoresis (PAGE) Polyacrylamide gels are prepared by the free radical polymerization of acrylamide and the cross linking agent N, N' methylene bis acrylamide.

2.7. Determination of effect of other effectors on protease activity

The activity of the isolated protease was tested in the presence of various known protease effectors *i.,e.*, EDTA, 2-mercaptoethanol, DMSO, SDS, Methanol, and Ethanol. The azocasein assay was used with the addition of these effectors solution to achieve a final desired effectors concentration of 5mM. Control was taken where azocasein assay without these effectors were carried out.

3. Results and Discussion

3.1. Characterization and identification of the isolated bacterial stains

Tannery is one of the important export oriented industries in Bangladesh. The main object of this study was to isolate and characterize thermophilic enzyme that might be used for deharing the hides and skins of cattle in the tannery industries. In this study extracellular protease producing thermophilic bacteria isolated from different natural sources of Dhamrai, Dhaka were identified and characterized the growth and some biochemical characteristics of the organism was determined. This organism was characterized and identified as a member of gram negative *Pseudomonas* family by several test but the species was not identified. So this bacteria is named here as a *Pseudomonas aeruginosa* by several test. The features agreed with the description of *P. aeruginosa* in Bergey's Manual of Systematic Bacteriology (Sneath *et al.*, 1986).

3.2. Gram's staining

The Gram's staining showed that *P. aeruginosa* were Gram negative rod because the cells had a pink color under the microscope using a 100X (oil immersion) lens. The cellular arrangements of this bacteria were in chains.

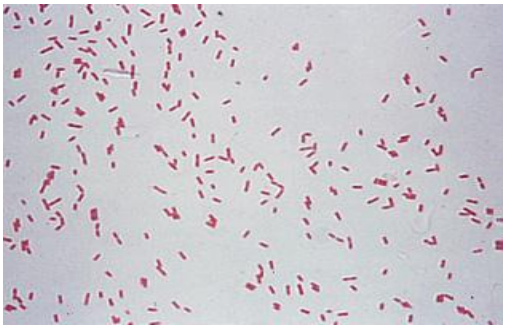

Figure 1. Gram's staining result of small rod gram negative *Pseudomonas aeruginosa* (100X objective lens).

3.3. Azocasein test for proteolytic activity of the enzyme

Proteolytic activities were assayed by Azocasein test, described by Kreger and Lockwood (1981) was done. Here azocasein is used as a substrate. The proteolytic activity was found as 21.13 units for the sample. One unit of proteolytic activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.01 at 440 nm. Azocasein assay developed by Kreger and Lockout is a well accepted method for the assay of wide variety of protease having overlapping specificity. The enzyme hydrolyses a number of proteins including Azocasein which suggest that it is an extracellular protease (Dhar and Sreenivasulu, 1984). *P. aeruginosa* have been reported to produce proteases (Tatinenin and Mangamoori, 2007). Therefore, it may be called a very good method for the large scale screening of bacterial protease (Ishikawa and Fujiwara, 1993).

3.4. Evaluation of effect of temperature on bacterial growth and enzyme synthesis

The aim of this experiment was to monitor the effect of temperature on the bacterial growth. For this purpose this organism was grown in nutrient agar medium at various temperatures (25ºC, 30ºC, 35ºC, 40ºC, 45ºC, 50ºC, 60ºC) for 48 hours and observed the growth profile of the bacteria. There was a significant increase in enzyme activity between 35ºC to 45ºC. After 50ºC the activity of the enzyme decrease gradually. The enzyme showed its maximum activity from 35ºC to 45ºC. There was a significant increase in enzyme activity between 20°C to 55°C.There was no effect of other temp.such as 4ºC and 70ºC.

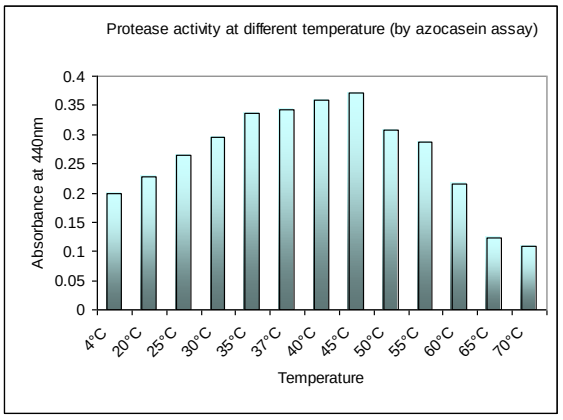

Figure 2. Graphical presentation of protease activities at different temperature. There were two replicable found in this experiment and no SE (standard error) in the graph. Two times experiment were done.

3.5. Effect of p^H and temperature on protease activity from the organism

The pH of the reaction media can affect the protease activity. For this purpose the enzyme activity over a pH range between 4 and 11 was studied. The enzyme shows its maximum activity at pH 8.5. The activity declined at pH 8.0 or above 8.5. Therefore pH 8.5 might be the optimum pH for enzyme activity. Additionally, its

optimum pH was similar to that of previous reports (Rozs *et al.*, 2001). *Pseudomonas aeruginosa* are strains had been widely utilized for enzyme production, including the proteases (Madern *et al.*, 2000). The activity of the enzyme was measured over a range of temperature (0°C, 4°C, 20°C, 30ºC, 37ºC, 40ºC, 50ºC, 60ºC, 65ºC, 80°C). The enzyme activity is increased with the increase of temperature. The experiment was reported 2 times and the result is reproducible. There was a significant increase in enzyme activity between 20°C to 55°C. This suggests that the enzyme might be a thermostable enzyme. The protease was active over a temperature range of 4°C~80°C. For example, the activity optimum of protease from *Mycobacterium* kr10 is pH 7.0, *B. pumilus* FH9 of pH 8.0 (Gessesse *et al.*, 2003*), Fervido bacterium islandicum* AW-1 of pH 9.0 (Nam and Pyun, 2002).

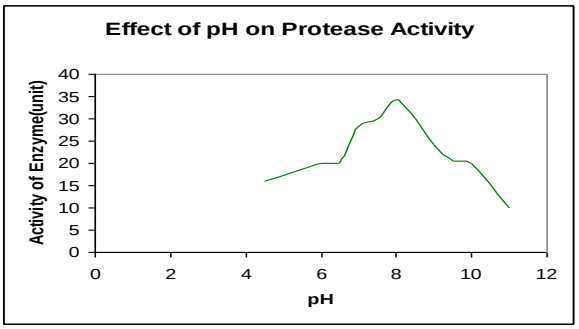

Figure 3. Graphical presentation of effect of pH on protease activity. There were two replicable found in this experiment and no SE standard error in the graph. Three times experiment were done.

3.5. Effect of salts and other effectors on the protease activity

The effect of different salts ($MgSO_4$, $ZnSO_4$, $CuSO_4$, NaCl, KCl) and other effectors (EDTA, 2-mercaptoethanol, sodium thiosulfate) at different concentration was measured. SDS increased the activity and β-Mercaptoethanol decreased the activity of the enzyme. NaCl didn't change the activity. Others had little deactivating effect. The effect of a number of ions on the activity of the enzyme was observed.

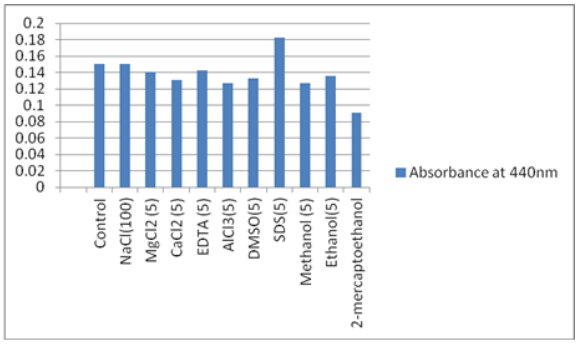

Figure 4. Graphical presentation of effects of salts and other chemicals on the activity on the protease activity.

The result shows that 5mM Na^{++} ion slightly increased the activity of the enzyme while Ca^{++} showed slightly decrease. Other elements had no effect on the enzyme. EDTA showed no effect on the protease activity suggested that the enzyme might not be metallo protease. The enzyme activity was significantly reduced by β-Merceptoethanol. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins (Scopes, 1982). But β-Mercaptoethanol will cause alteration of structure of enzyme by reducing disulfide bonds of the enzyme. Thus the protease could contain disulfide bonds. No effect of EDTA was detected on enzyme activity suggesting that the metal might not be involved in enzyme activity (Madern *et al.*, 2000).

3.6. Optimization of media formulation for protease production by *Pseudomonas aeruginosa*

In the preliminary screening studies on the development of the production medium, various industrially low-cost carbon and nitrogen sources and elements were screened and as a result, maltose, soybean meal, Tween 80 and pH of the medium were found to be important factors in enhancing the alkaline protease formation. Different carbon sources obtained from a Cargill Sweeteners and Starches Inc. were screened as main industrial carbon sources in the formulation of the reference labeled F1 medium. As it is seen except for whey, molasses and

potato starch, the rest of the carbon sources gave satisfactory specific protease activity results if compared with the control. However if total protease enzyme activities were considered Glucose–Fructose (35%) and Maltose (55%) were taken as best carbon sources. These two sources were also very much satisfactory with respect to their specific protease activities.

Different nitrogen sources were screened which were obtained from Cargill Soy Protein Solution Inc. as main nitrogen sources used industrially. As it is seen the lowest total and specific activities were obtained with casein, corn meal and whey. Corn steep liquor, yeast extract and soybean meal gave much more satisfactory result compared to the other sources used. In these experiments the carbon source was taken as Maltose (55%) at a constant level of 10 g/L. According to these results, soybean meal or corn steep liquor could be used as a single nitrogen source, provided that there is Tween 80 at 0.15 g/L and $CaCl_2$ at 0.7 g/L concentrations. Therefore since soybean meal resulted in slightly higher activity than corn 7steep liquor, we decided on using soybean meal as the nitrogen source in the experimental design set up for response surface method analysis.

In order to study the effects of different elements on the protease activity, Tween 80 was used in these experiments in order to determine if it had a promoting or inhibiting effect on the enzyme synthesis. The positive effect of Tween 80 and $CaCl_2$ at 0.1g/L concentration was pronounced with respect to the specific protease activities as well. The higher $CaCl_2$ concentration (1 g/L) however had inhibitory effect. The presence of $CaCl_2$ (0.7 g/L) at its maximum concentration together with Tween 80 (0.17 g/L) at minimum concentration gave satisfactory result. Using Tween 80 at its maximum concentration with CaCl2 at minimum level decreased the protease activity by at least 17.8 %. Therefore according to this preliminary study, we decided to keep the $CaCl_2$ concentration constant at the maximum concentration of 0.7 g/L and use Tween 80 as the main variable to be used in the response surface.

3.7. Purification of protease enzyme

To remove unwanted proteins from the crude enzyme solution, 40–80% saturation of $(NH_4)_2SO_4$ had the best effect on enzyme purification. Most of the protein in bacterial culture filtrate precipitated at 60% saturation. The most active enzyme protein preparation could be obtained at the ammonium sulphate level of 60%. This result was in complete accordance with other workers (Kim, 2004). The overall purification factor was about 22.6 fold and the final yield was 51%. The final product had a specific activity of about 839.41 U/mg. Protein purification and different enzymetic properties of the protease. Ion-exchange DEAE cellulose column chromatography was for protein purification. The desired enzyme was found in 53-55 numbers tube by Azocasein test. The result is presented in figure 5 that shows the desired enzyme was found in 53-55 numbers of tubes/fractions and it was also found that 54 numbers of tube/fraction contains large amount of desired enzyme. A trial was given to obtain the partially purified proteases from the culture supernatant of *P. aeruginosa* from one hand to create an interesting comparative study of the characteristics of the purified enzyme preparations from the other hand. This microbial enzyme was partially purified by ammonium sulphate fractionation, dialysis, DEAE cellulose chromatography and electrophoretic analysis. The protease precipitated by the ammonium sulphate had been reported in many previous studies The precipitates were found to be very active after the dialysis. This gave 2.9 fold purification of the proteins. After ultra filtration protein was further purified by gel filtration chromatography using DEAE cellulose. This method (Tatineni and Mangamoori, 2007) is very laborious and time consuming but separation of protein is very reliable. Ultra filtration is another method for the separation of proteins of different molecular weight (Song *et al.*, 2011). Proteins having molecular weight higher than or equal to 100 kDa were used. In this process the protein were purified to 4.9 fold.

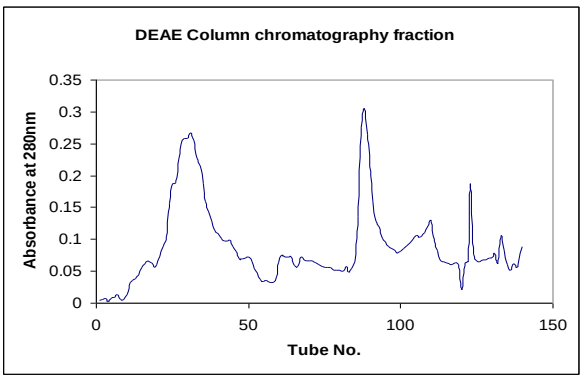

Figure 5. Graphical presentation of OD of collected fractions from DEAE cellulose column chromatography.

Three different protein picks of different molecular weight was found and one of the pick showed considerable enzyme activity (Cappuccino and Sherman, 2001). In this process the protein was purified to 11.5 fold. Enzyme purity was tested by SDS-PAGE according to Laemmli (1970) and operated at 4°C. It was found that a single band is appeared in the gel. It proves that the enzyme has purified and separated. The subunit molecular mass of the protease was estimated by comparing the electrophoretic mobility of the protease with the electrophoretic mobilities of marker proteins. The level of purification is higher than those reported in other similar papers (Kim, 2004).

3.8. SDS-PAGE

The single band in the figure indicate purification of the protease. Incomparism with the marker on the left side of the figure, it is clear that the molecular weight of the protein is approximately 66 kD.

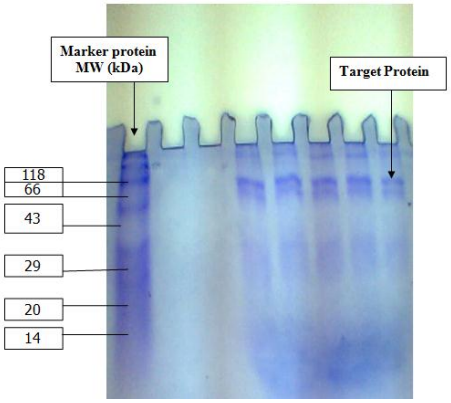

Figure 6. Determination of protease by SDS-PAGE.

3.9. Dehairing capability of the isolated protease enzyme

The cell-free supernatants were used as sources of crude enzyme. The treated skins and controls showed visible differences after 9 h incubation. No color alteration was observed, although the presence of depilated areas was noticed in the skins treated with enzymes. Enzymatic dehairing may be the ideal process. Quantitative estimation has shown that 40 mL of culture supernatant could dehair 2×1 cm of leather completely in a 9 hours. After 9 h incubation intact hairs could be taken out of the skins easily by simple scraping. In controls, hair loosening was not observed, even by the mechanical action of a forceps. This shows that the bacterial isolate moderate to high amount of enzyme for dehairing. Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides (Puvanakrishnan and Dhar, 1986).This result was much better than other different bacteria that also caused dehairing. Proteases have been used in the hide dehairing process, where dehairing is carried out at pH values between 8-10 (Kembhavi *et al.*, 1993). In most cases the enzymes work and bring about efficient dehairing within 6-20h (Uddin *et al.*, 2015).

Figure 7. Direct dehairing activity of the enzyme - A is Control (Left) and B (100% Hair removed).

3.10. Screening of storage stability of protease from *Pseudomonas aeruginosa*

There was no significant decrease in enzyme activity when stored at 4°C within the above mentioned period of investigation. After fourth week the enzyme activity was found for 562U/ml (100%) at 4°C, room temperature 492U/ml (87.5%) and room temperature with chemical 542 U/ml (96.4%) respectively.

4. Conclusions

The results showed that the *Pseudomonas* proteases enzyme can be utilized in poultry processing industry and enzymatic dehairing of skin in tannery industry to control the environment from pollution, which is a prerequisite for biotechnological applications. Finally, it plans to clone and over-express the genes encoding enzymes for large scale industrial production and commercial use for pretreatment of industrial residues from leather and poultry processing industry.

Acknowledgements

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Conflict of interest

None to declare.

References

Araujo RM Casal and PA Cavaco, 2008. Application of enzymes for textile fibers processing, Biocatal Biotransfor, 26: 332-349.

Brandelli A and Riffel A, 2005. Production of an extracellular keratinase from Chryseobacterium spp. growing on raw feathers. Electronic Journal of Biotechnology, 8: 35-42.

Brandelli A, DJ Daroit and A Riffel, 2010. Biochemical features of microbial keratinases and their production and applications. Appl. Microbiol. Biotechnol., 85: 1735-1750.

Cappuccino JG and N Sherman, 2001. Microbiology: A Laboratory Manual.6th Edition. Benjamin Cummings. CA.

Choudhary RB, AK Jana and MK Jha, 2004. Enzyme technology applications in leather processing, Indian Journal of Chemical Technology, 11: 659-671.

Dettmer AE Cavalli and M Gutterres, 2013. Environmentally friendly hide unhairing: enzymatic hide processing for the replacement of sodium sulfide and delimig. Journal of Cleaner Production, 47: 11-18,

Dhar SC and S Sreenivasulu, 1984. Studies on the use of dehairing enzyme for its suitibilty in the preparation of improved animal feed. Leather Sci., 31: 261-267.

Gessesse A, HK Rajni and BA Gashe, 2003. Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enzyme Microb. Technol., 32: 519-524.

Ghorbel-Frikha B and M Nasri, 2005. Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1. J. Ind. Microbiol. Biotechnol., 32: 186-94.

Ibrahim ASS and AA Al-Salamah, 2009. Optimization of media and cultivation conditions for alkaline protease production by alkaliphilic *Bacillus halodurans*. Res. J. Microbiol., 4: 251-259.

Ishikawa and N Fujiwara, 1993. Kinetics and mechanism of enzymatic hydrolysis of gelatin layers of X-ray film and release of silver particles. J. Ferm. Bioeng., 76: 300-305.

Jorgensen PL, M Tangney and ST Jorgensen, 2000. Cloning and sequencing of an alkaline protease gene from *Bacillus lentus* and amplification of the gene on the *B. lentus* chromosome by an improved technique, Appl. Environ. Microbiol., 66: 825-827.

Kembhavi AA, Kulkarni A and Pant A, 1993.Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM no 64. Appl. Biochem. Biotechnol., 38: 10-22.

Kojima M, M Kanai and K Horikoshi K, 2006. Isolation and characterization of a feather degrading enzyme from *Bacillus pseudofirmus* FA30-01. Extremophiles, 10: 229-235.

Kim JD, 2004. Purification and Characterization of a Keratinase from a Feather-Degrading Fungus, *Aspergillus flavus* Strain K-03. Microbiology, 35: 219-225.

Madern D and G Zaccai, 2000. Halophilic adaptation of enzymes. Extremophiles, 4: 91-98.

Monteiro De Aquim P, M Gutterres and J Trierweiler, 2010. Assessment of Water Management in Tanneries. Journal of the Society of Leather Technologists and Chemists, 94: 253-258.

Madern D, C Ebel C, and G Zaccai, 2000. Halophilic adaptation of enzymes. Extremophiles, 4: 91-98.

Nam and YR Pyun, 2002. Native-feather degradation by Fervido bacterium islandicum, a newly isolated keratinase-producing thermophilic anaerobe, Arch. Microbiol., 178:538–547.

Puvanakrishnan R and SC Dhar, 1986. Leather Sci., 33: 177–191.

Roja RM, KB Lalitha and DS Prasad, 2012. Isolation and screening of alkaline protease producing bacteria and induction of overproducing Bacillus licheniformis mutants through UV irradiation, IOSR Journal of Pharmacy, 1: 1-14.

Rozs M, L Manczinger, CVágvölgyi, F Kevei F, 2001. Secretion of a trypsin- like thiol protease by a new keratinolytic strain of *Bacillus licheniformis*. FEMS Microbiol. Lett., 205: 221-224.

Sarath G, RSDL Motte, FW Wagner, 1996. In: Beynon, R.J., Bond, J.S. (Eds.), Proteolytic Enzymes-A Practical Approach. Oxford IRL Press, New York:7.

Scopes RK, 1982. Protein Purification: Principle and practice, Springer-Verlag, New York: 195-196.

Sneath PHA, Mair NS, Sharpe ME and Holt JG, 1986. Bergey's Manual of Systematic Bacteriology, Vol. 2 Baltimore: Williams and Wilkins. ISBN 0-683-07893-3.

Song J, W Tao and W Chen, 2011. Kinetics of enzymatic unhairing by protease in leather industry, Journal of Cleaner Production, 19: 325-331.

Suntornsuk W and K Oda, 2005. Purification and characterization of keratinase from a thermotolerant feather degrading bacterium. J. Ind. Microbiol. Biotechnol., 21: 1111-1117.

Sztajer H and E Zboinska E, 1988. Acta Biotechnol., 8:169–175.

Tatineni R and LN Mangamoori, 2007. Optimization of keratinase production and enzyme activity with *Streptomyces* spp. Appl. Biochem. Biotechnol., 141: 187-201.

Thys RCS, Lucas FS, Riffel A, Heeb P and Brandelli A, 2004. Characterization of a protease of a feather-degrading Microbacterium species. Letters in Applied Microbiology, 39:181-186.

Uddin ME, T Ahmad, MKI Sarkar, DA Azim, SS Rahman, MS Islam, MR Karim, MM Rahman, MR Islam. R, 2015. Microbial production of alkaline proteases and Evaluation of its performances for pretreatment of leather industry. Journal of Multidisciplinary Engineering Science and Technology, 2: 3370-3377.

Uddin ME, P Maitra, MF Hossain, MF Alam, 2014. Isolation and Characterization of Proteases Enzyme from Locally Isolated *Bacillus* spp. American Journal of Life Sciences, 2: 338-344.

Xie F, Y Chao, X Yang, J Yang, Z Xue, Y Luo and S Qian, 2010. Purification and characterization of four keratinases produced by *Streptomyces* spp. strain 16 in native human foot skin medium. Bioresour Technol., 101: 344-350.