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STREPTOMYCES SP. MM-3 FROM RHIZOSPHERE OF *PSIDIUM GUAJAVA:* A POTENTIAL CANDIDATE FOR PROTEASE WITH DEHAIRING PROPERTIES

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Abstract

Soil is the richest medium for isolation of microbial flora especially genus *Streptomyces* as potent source of extracellular enzymes. Soil samples from rhizosphere of guava plant (Psidium guajava) were screened for proteolytic bacteria. Among all isolates, *Streptomyces sp.* MM-3 showed best proteolytic activity by utilizing agro-based substrates like malt, soybean, wheat bran and peels of ground nuts as carbon/nitrogen sources. Optimum incubation time for enzyme production was 96h (420U/ml), while optimum age and size of inoculum was 24 h (95 U/ml) and 10% (482 U/ml), respectively. Maximum units of protease were observed with 0.5% starch (396U/ml) and 1% gelatin (439 U/ml). Application of partially purified protease on sheep and goat skins resulted in a highly promising dehairing efficiency as an alternative of lime-sulfide chemical unhairing process. Enzyme-treated dehaired leather was processed further through other pre-tanning steps like liming, fleshing, deliming and pickling. Final leather crust from chrome blue pelt showed high degree of tear strength (40.93 ± 4.21) N/mm k=2, good tensile strength (16.58 ± 2.31) N/mm k=2 and elongation (155.08 ± 5.97) N/mm k=2 comparing to lime-sulfide unhairing process. Other significant advantages of enzymatic dehairing were total recovery of hairs, simplification of pre-tanning process and reduction in use of sodium sulfide. Thus producer strain *Streptomyces* sp.MM-3 of soil origin emerged as a potential candidate for the production of protease using low-cost production medium to be utilized by leather industry.

Key words: *Streptomyces* sp. MM-3, Protease, Dehairing, Leather industry.

Introduction

Plant rhizosphere is a distinctive soil environment which is under the direct influence ofsoil microflora and different secretions of roots. This region of soil contains many microorganisms including bacteria, which feed on dead cells of plant as well as sugars and proteins released by roots of plant. All biological and biochemical activities in rhizosphere region of soil are overwhelmed by the interactions between plant roots, associated flora and root-microorganism (Gianfreda, 2015). Rhizosphere also known as "bio-influenced zone" (Hinsinger*et al*., 2008) is the richest environment for the isolation of industrially important microorganisms especially Actinomycetes, which are slow growing, branching filamentous and gram positive bacteria (Holt *et al*., 1994). Their presence with great diversity in particular soil depends on many factors like type of soil, geographical location, cultivation and organic matter (Arifuzzaman*et al*., 2010). Their industrial importance is based on their capabilities to produce biologically active compounds including enzymes, antibiotics and vitamins (De Boer *et al*., 2005). With the advent of modern technology and increased demand for microbial enzymes in international enzyme market, scientists are nowadays more interested in discovery of new enzymes with novel properties. Some of the reported extracellular enzymes produced by Actinomycetes are cellulase (Jang & Chenks, 2003; Arunachalam *et al*., 2010), amylase (Selvam *et al*., 2011; Reyad, 2013), protease and chitinase (Dahiya *et al*., 2006).

Proteases of microbial origin are among the most vital hydrolytic enzymes which have been extensively studied. Producer microorganisms are easy to grow in bulk quantities by sub-merged fermentation techniques for production of desired products within minimum time. Although many microorganisms have inherent capability of production of secondary metabolites, the strains producing extracellular enzymes have been employed for production of enzymes on industrial scale. Among hydrolytic enzymes, proteases are of most importance because of convenient methodologies of purification (Sudha *et al*., 2010).

The role of proteases is vital in tannries starting from soaking of skin/hides to finished products (Mukhtar & Haq, 2007). Protease produced in the current study was tested for its dehairing ability to be fairly utilized in leather industry. In the developing industrial era, leather industry is one of the important economic sector that causes oppressive environmental hazards due to the use of harmful chemicals hence resulting in generation of toxic effluents from tanneries. The use of high amount of lime and sulfide in conventional leather processing contributes 80-90% in the total pollution load of effluent generating toxic gases including hydrogen sulfide (Thanikaivelan *et al*., 2004). Multiple studies have reported death of workers and inhabitants due to discharge of toxic effluents (Balasubramanian & Pugalenthi, 2000; Gupta *et al*., 2002). Beamhouse operation (soaking, liming, deliming, bating, pickling, etc.) results secretion containing 75% of organic wastes having abundant quantity of nitrogen containing compounds (Purushotham *et al*., 1994). Environmental pollution caused by the use of harmful processing agents has necessitated the development of enzyme technology as an alternative (Gupta *et al*., 2002). The use of proteases in leather processing can help in partial or complete replacement of

toxic conventional methods (Asokan & Jayanthi, 2010). About 50 % of the total microbial enzymes are employed in industries, of which 40% are proteolytic enzymes used for dehairing purpose (Pepper & Wyatt, 1963). In dehairing, protease degrades the hair root sheath which causes the hair to become loose followed by subsequent digestion of non-keratinized part of hair (Kuntzel & Stirtz, 1958). The main benefits of enzymatic dehairing includes replacement of sodium sulfide, recovery of hair as value added product, providing ecologically friendly environment for workers and reduction in pre-tanning operations by cutting down liming and de-liming steps (Kamini *et al*., 1999). The purpose of current study was to optimize physico-chemical conditions for producer strain during fermentation, by utilizing agriculture based substrates and examining the characteristics of partially purified protease for its dehairing application in tanneries.

Materials and Methods

Isolation of the microorganisms: Soil sample for the isolation of bacterial strains was collected from rhizosphere of *Psidium guajava* (guava) and isolation of microbial strains was done by spread plate technique in Applied Microbiology and Biotechnology Laboratory, FUUAST, Karachi, Pakistan.

Screening and identification for protease producers: Isolates were screened for production of protease on skim milk agar (SMA) by streaking and stabbing methods. The isolate showing maximum ratio of diameter of zone of hydrolysis to colony diameter was selected and identified using microscopic, cultural and biochemical characteristics (Holt *et al*., 1994).

Preparation of inoculum: Seed medium containing 0.1% glucose, 0.1% casein, 1% peptone, 0.01% $MgSO_4$, 0.01% $CaCl_2$ and 0.05% $K_2HPO_4$was inoculated with a loopful culture (24 h old) from Skim milk agar plate and kept in rotary shaker (150rpm) for 24 h at 37°C.

Fermentation for protease production: Production of protease was carried out in a medium containing 0.5% glucose, 0.1% casein, 1.0% peptone, 0.01% $MgSO_4$, 0.01% $CaCl_2$ and 0.05% K_2HPO_4 at pH 7. The flasks were inoculated with 10% of 24 h fresh seed culture at 150 rpm and 37°C. After fermentation the broth samples were harvested by centrifugation at 4,000 rpm for 20 min followed by filtration to obtain cell free supernatant.

Assay for proteolytic activity: Proteolytic units were assessed by colorimetric method using azocasein as substrate (Caldas *et al.,* 2002). One unit of enzyme activity is defined as the amount of enzyme which yields an increase in absorbance of 0.001 at 440nm in 30 minutes at 37ºC.

Effect of incubation time on protease production: Effect of incubation period was studied by taking samples from production medium at different time intervals (24, 48, 72, 96 and 120). After centrifugation and filtration, the enzyme activity of cell free filtrate was measured to assess units of protease at different time intervals.

Effect of age and size of inoculum on protease production: Effect of age of inoculum was observed by inoculating the production medium with 24, 48 and 72 h old inocula.Similarly, effect of size of inoculum was studied by adding 10, 15 and 20% of inoculum to the production media. Broth samples were taken aseptically from each flask and proteolytic units were assessed.

Effect of carbon sources on protease production: Glucose (0.5%) was replaced with other carbon sources (glycerol, fructose, lactose maltose, and soluble starch) in basal medium to identify optimum carbon source for production of protease. Fermentation flasks were incubated with same parameters mentioned above. Effect of multiple carbon sources on production of protease was studied at 37°C. More than two sources of carbon (glucose, fructose and starch) having 0.5% concentration were used in the production media.

Effect of nitrogen sources on protease production: Protease production from isolate MM-3 was investigated using agro based (groundnut peels, malt, soybean meal, wheat bran) and other nitrogen sources (gelatin and skim milk) during fermentation experiment. Different substrates (1% w/v) were taken by replacing peptone in the production media under submerged conditions. The agro-industrial residues were sieved through standard mesh sieve. Multiple nitrogen sources (yeast extract and peptones) were added to the production media to investigate their effect on production of protease. At the end of cultivation period, cell free filtrate was collected and analyzed for proteolytic activity.

Effect of pH on protease activity: Proteolytic activity of crude protease was assessed using standard procedure at various pH (6, 7, 8, 9 and 10) using different buffers (0.05 M): sodium citrate (pH 5-6), potassium phosphate (pH 7) Tris–HCl (pH 8-9) and glycine-NaOH (pH 9-10).

Effect of temperature on protease activity: Effect of temperature on proteolytic activity was observed by carrying out the enzyme assay at varied temperatures. To determine the thermostability, reaction mixture was pre-incubated at different temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85°C) in a water bath for 30 minutes and assayed for proteolytic units.

Partial purification of protease enzyme: Ammonium Sulfate precipitation technique was used for the partial purification of extracellular enzyme. Cell-free filtrate was subjected to gradual addition of ammonium sulfate with continuous stirring in magnetic stirrer at 4°C for 7-8 h. Precipitation was checked at different saturation level (10-60%) by increasing concentration of ammonium sulfate gradually. Proteins precipitated at 60-70% saturation levels were collected by centrifugation at 4000 rpm for 30 minutes, redissolved in phosphate buffer (pH 7.9) and checked for protease units.

Application of the protease for dehairing

At laboratory scale: Salted goat and sheep skin pieces (7x7cm^2 sizes) were dipped in partially purified protease preparations (350 U/ml) and incubated on rotary shaker (150 rpm) at 37°C for 18-20 h. After treatment, skin pieces were gently scraped with fingers to remove loose hairs. This was important because rubbing at lab scale was not as forceful as in industrial drum.

At pilot scale: At beam house of Leather Research Center-PCSIR, Karachi, salted sheep skin piece (~900 g) was engrossed in protease preparation (370 U/ml) and incubated for 20-22 h at 37ºC. The tests were conducted in experimental drum rotating reactor with 22-30 rpm. After enzymatic treatment, the sheep skin was examined for the presence of depilated areas. Enzyme-treated dehaired leather was further processed through other pre-tanning steps like fleshing, de-liming and pickling. Finally, hair removal efficiency was compared against the conventional lime-sulfide unhairing method.

Physical and mechanical analyses for processed leather

Qualitative tests: For thumb impression test, thumb was pressed on the dehaired side of skin; intensity of thumb impression was noted by time taken by treated pelt to regain its previous shape. The degree of smoothness of the enzyme treated pieces was also observed.

Quantitative test: Sample for physical testing of sheep crust leather were cut using cutting dies and then conditioned standard atmospheric conditions i.e. temperature 23±2 and relative humidity 50±5 % for 24 to 48 hours. Physical properties such as tensile strength, elongation at break and tear strength were measured using their standard test methods, Universal Testing Machine Tinius Olsen model H5KS .UK, England. Softness of leather was measured using official method of analysis and softness tester ST300 MSA engineering UK.

Processed sheep leather from resulting pelts after application with enzyme preparations were tested for tensile strength, % elongation, tear load and softness at quality testing lab of Leather Research Centre-PCSIR. For physical testing, leather cutting was carried out (BS-3144 IUP-1/ EN ISO 2419:2006), conditioning of leather using (SLP3, IUP 3; BS 3144: method 2, 2001), thickness (SLP4, IUP4; BS 3144: method 3), tensile strength and percentage elongation (EN ISO 3376: 2002), tear strength (EN ISO 3377: method 1, 2002) and softness (IUP 36, SLP 37).

Analysis of data: The resulting data was analyzed for its standard deviation and arithmetic mean using GraphPad PRISM version 5.01.

Results and Discussions

Isolation, screening and identification for protease producers: The soil samples from rhizosphere of *Psisdium guajava* were collected and screened for protease producer strains. The use of SMA is an established method for qualitative screening of proteolytic bacteria (Durham *et al.,* 1987). All bacterial strains showed hydrolysis of casein by exhibiting zone of clearance around the colonies. However, isolate MM-3 was chosen based on maximum zone of hydrolysis (15mm) on SMA.

A large number of species of bacteria produce alkaline proteases as it is their inherent ability (Gupta *et al*., 2002). Furthermore, proteases from microbial sources have significant dominance over animal or plant based proteases due to the presence of characteristics essentially required for industrial and biotechnological processes. The luxuriance diversity of microorganisms, always creates an opportunity for exploration of novel organisms for enzyme production with superior properties and suitability for commercialization.

Based on microscopic, macroscopic and biochemical characteristics, the selected strain was identified to be *Streptomyces* sp. MM-3. (Fig. 1). (Table 1). The color of the colony of selected strain MM-3 was whitish grey. Vanajakumar*et al.,* (1991) also reported that Actinomycetes were the dominant forms of soil flora with white color series.

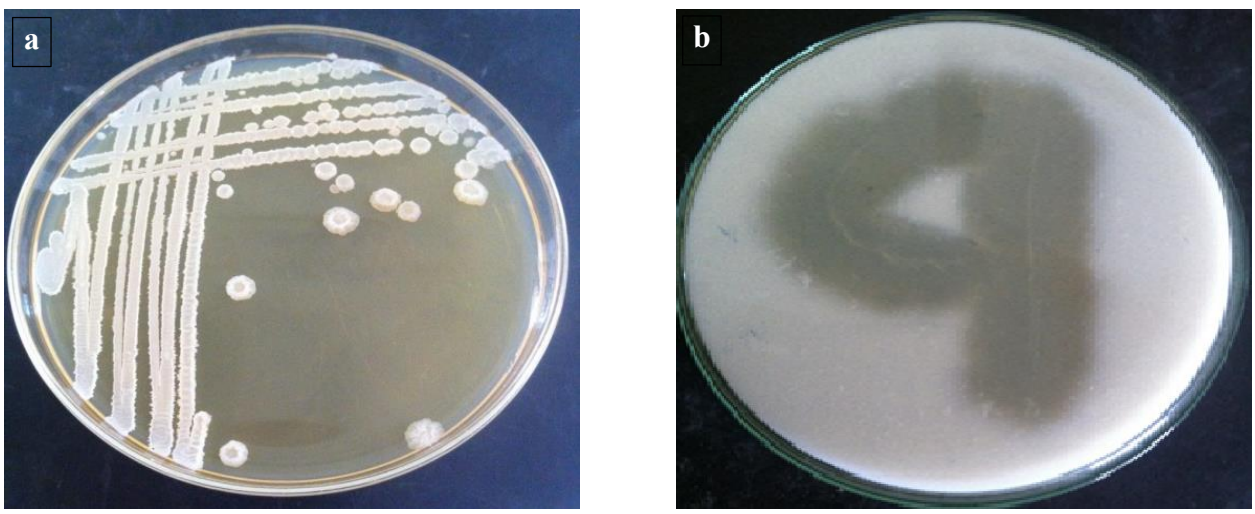

Fig. 1. Pure culture of soil isolate MM-3 on nutrient agar plate (a), Proteolytic activity of MM-3 on Skim Milk agar plate (b).

Table 1. Colonial, morphological and biochemical characteristics of Streptomyces sp. MM-3.

S. No.	Colonial characters	Isolate MM-3
1.	Color	Off-white
2.	Elevation	Flat
3.	Margin / Edge	Rough
4.	Shape/ Form	Irregular
5.	Texture / Surface	Wrinkled
6.	Transparency	Opaque
S. No.	**Cell morphology**	**Isolate MM-3**
1.	Gram reaction	Gram positive
2.	Shape	Filamentous rods
3.	Arrangement	Diplo , chains
S. No.	Biochemical test	Results
1.	Catalase	Positive
2.	Coagulase	Negative
3.	Citrate	Negative
4.	Sugar fermentation on TSI	Positive
5.	H_2S production on TSI	Negative

Effect of incubation time on protease production: A typical growth curve was observed on the basis of optical density at 600 nm where less extracellular proteolytic activity was noted during early- and mid-exponential growth phase i.e. 66 U/ml and 83 U/ml at 24 and 48 h, respectively. The enzyme production started with the onset of stationary phase and reached its maximum (420 U/ml) after 96 h. The enzyme production further decreased to 322 U/ml at 120 h (Fig. 2a). It is reported that, incubation for longer periods trigger enzyme degradation and digestion by other enzymes which results into down fall of enzyme activity (Priest, 1977). Production of extracellular proteases is influenced by various physical factors, such as composition of medium, age of inoculum, size of inoculum and incubation time. The proficient production of enzyme largely depends upon the conditions of growth and nutrients provided in the media (Gupta *et al.,* 2002). Previous studies have reported similar results for growth and maximum production of protease during early log phase following a decline with increase in incubation period due to depletion of nutrients (Jignasha *et al.,* 2007; Lazim *et al.,* 2009).

Effect of age and size of inoculum: Maximum production (95-91U/ml) of protease was observed with 24-48 h old inoculum while production was decreased to 27 U/ml with 72 h old inoculum (Fig. 2b). Thus, in subsequent experiments, *Streptomyce*s sp. MM-3 was grown for 24 h and it was used as starting culture for inoculation in different sizes (10, 15 and 20%). Protease activity was increased using 10% inoculum with 482 U/ml (Fig. 2c)while decreased to 53 U/ml with 15% inoculum and further declined to 29 U/ml with 20% inoculum. The best size of inoculum was found to be 10% after 24h. Similar results had been previously reported (Ohta *et al.,* 1995; Kaur *et al.,* 2001; Kumar *et al.,* 2010*)* where maximum protease units were observed using 24-48 h old inocula for protease production.

Effect of single and multiple carbon sources: Maximum units of proteolytic enzyme were observed with starch (396 U/ml) and glucose (370 U/ml) when taken as carbon source while fructose, glycerol, lactose and maltose produced 362, 339, 350 and 322 U/ml, respectively (Fig. 2d). A decline in protease activity was observed after 96 h in the production medium containing the combination of glucose, fructose and starch whereas the combination of starch and glucose yielded increased production of protease (Fig. 2e).

Carbon sources and other growth conditions are significant parameters for production of microbial enzymes (Nascimento & Martins, 2004). When glucose was replaced with five other sources (glycerol, fructose, lactose, maltose and starch) taken separately, starch was found to be the ideal source for production of protease followed by glucose. Similar results had been reported (Gibb & Strohl, 1988) when glucose was used as a carbon source for producing protease by *Streptomyces peucetius.*

Effect of single and multiple nitrogen sources: Optimum nitrogen source for production of enzyme was gelatin with 439 U/mL while comparable enzyme units 401, 373, 330, 398 and 385 U/ml were observed with ground nuts, malt, skim milk, soybean and wheat bran (Fig. 2f). However, protease activity was found decreasing to 333 U/ml in the medium with gelatin and peptone and 325 U/ml in production medium with gelatin-yeast extract as nitrogen sources comparing to the production medium supplied with one substrate i.e. gelatin (Fig. 2g).

Our results suggests that agro-based nitrogen sources are better option being economical and easily available to reduce the over all cost of production methodologies as about 30-40 % of the cost of industrial enzymes depends on the growth medium (Joo *et al.,* 2003). For production of alkaline protease complex nitrogen sources are required, however, optimum nitrogen sources vary with different microorganism (Kumar & Takagi, 1999). Protease production by *Streptomyces sp.*MM-3 was found to be maximum with the addition of gelatin in basal medium replacing peptone as nitrogen source beyond a certain level repressed the rate of protease production (Kanekar *et al.,* 2002). Moreover, significant production of protease was observed with soyabean meal, wheat bran and malt when used as sources of nitrogen.

Effect of pH on protease activity: Effect of pH on activity of protease was observed, optimum pH was 9 with 302 U/ml while protease activity rapidly declined to 11 U/ml at pH 10. Comparable activity of 204 U/ml was observed at pH 8. Enzyme activity of 108 and 105 U/ml was observed at pH 5 and 6, respectively (Fig. 3a). Moreover, the activity was decreased under neutral conditions (pH 7) these results indicated that enzyme was alkaline in nature. Previous studies on *Bacillus brevis* (Banerjee *et al.,* 1999) and *Bacillus clausii* I-52 (Joo *et al.,* 2003) have reported similar findings.

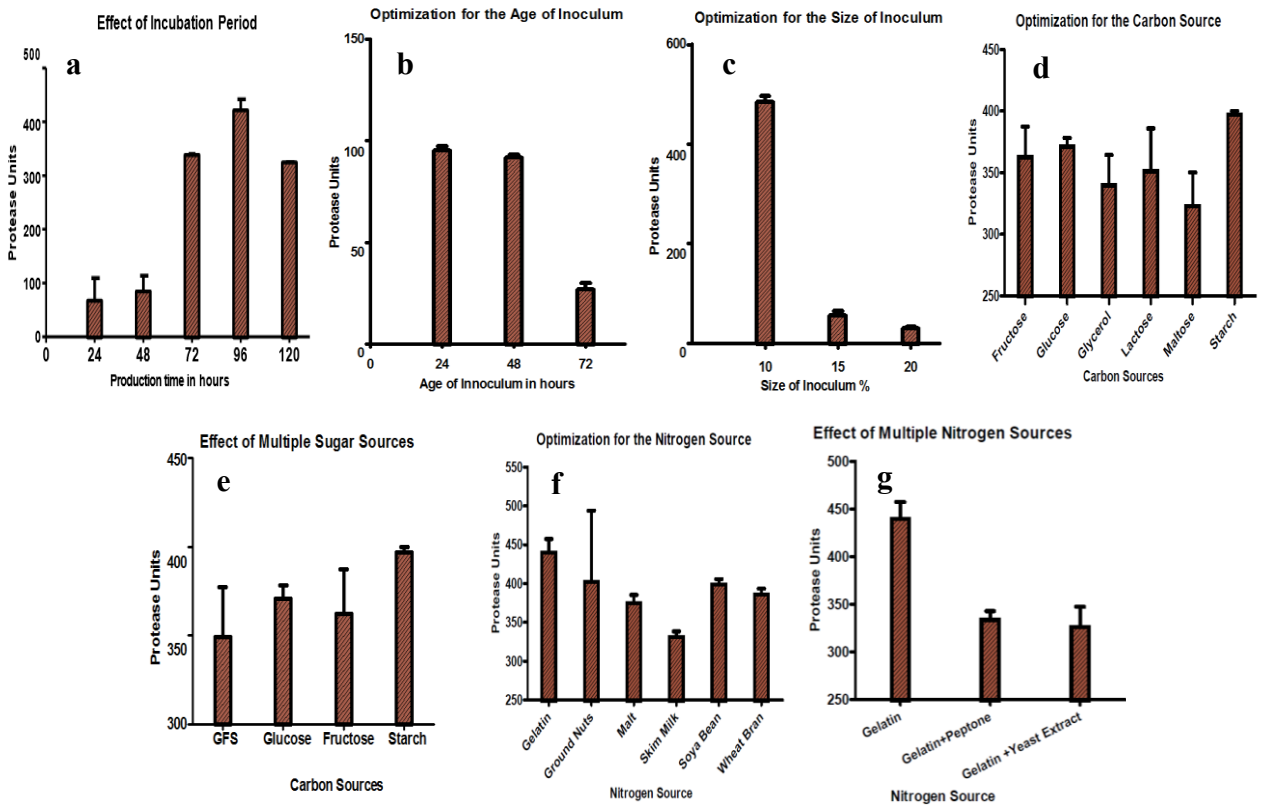

Fig. 2. Optimization of protease production: effect of incubation period (a), effect of age of inoculum (b), effect of size of inoculum (c), effect of different carbon source (d), effect of Multiple Carbon source (e), effect of Nitrogen Source (f), effect of multiple nitrogen sources (g).

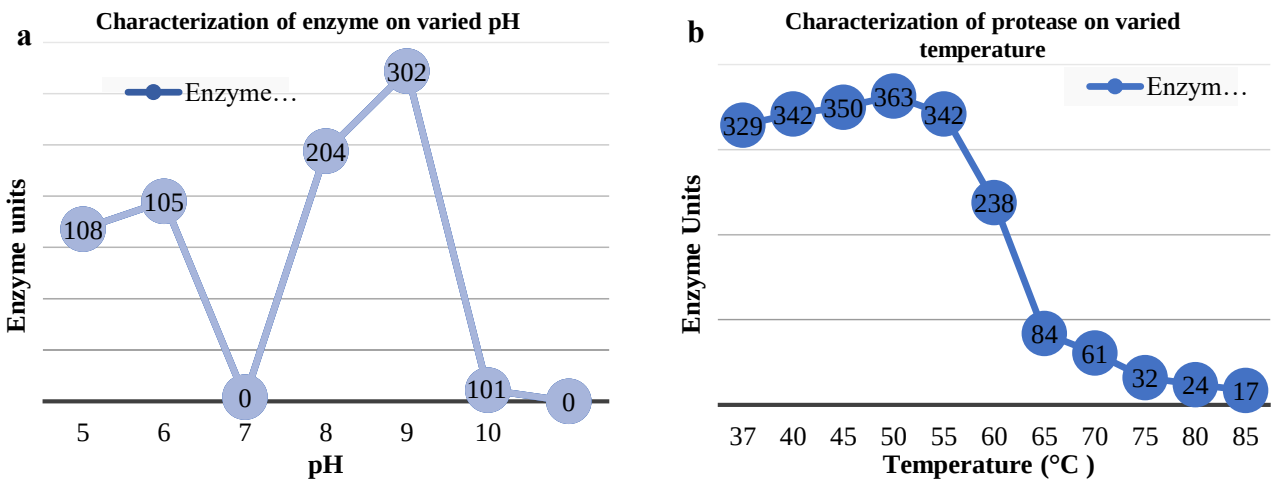

Fig. 3. Characterization of Protease: Stability of protease on varied pH (A), Stability of protease on varied temperature (B)

Effect of temperature on protease activity: Protease activity gradually increased by increasing the temperature from 329 to 342, 350, 363 U/ml at 37, 40, 45 and 50ºC, respectively (Fig. 3b). A drastic decrease in proteolytic units was observed from 342 to 17 U/ml at values above 55ºC. Results suggested that protease was active over a wide range of temperature, however, optimum temperature observed was 50ºC. Previous researchers also reported 50ºC as the best suitable temperature for maximum activity of alkaline protease (Beg *et al.*, 2003; Joo *et al.*, 2003).

Partial purification of protease: Maximum precipitation was achieved at 60% saturation level of ammonium sulfate. Furthermore, it was observed that ammonium sulfate precipitation increased the protease activity by 50-60 % as compared to the activity in the cell free filtrate.

Application of the protease for dehairing: Leather industry is one of the leading industries of Pakistan, where unhairing is significant and inevitable stage during processing of leather. In dehairing process use of toxic chemicals result in drastic effects to environment and health of workers(Chandrashekharan & Dhar, 1985). Hence the present study was focused on reduction in use of chemicals for leather processing

and development of ecofriendly and safe alternatives. After characterization, the enzyme preparation was applied in the leather unhairing process to check its dehairing capability. The enzymatic treatment of the sheep skins resulted in the noticeable improvement as compared to the conventional lime-sulfide treated leather as control. Complete denhairing was achieved after 20 hours of treatment. Complete enzymatic removal of hairs without chemical aid has been previously emphasized by various reporters (Thangam, 2001; Macedo *et al.,* 2005). In present study, crude preparation of protease was employed for dehairing purpose. Significant findings of enzyme treatment were observed i.e., unlike lime and sulfide treatment which destroy hair at the skin surface, enzyme treatment removed hairs from the root level which can be utilized as a source of keratin protein. Leather processing time was reduced as soaking, unhairing and bating was achieved simultaneously. Furthermore, processing cost was cut down as partially purified enzyme was applied for dehairing saving the purification cost.

Leather- physical and mechanical test for quality analysis: Physical analysis of the finally prepared leather showed that the tear strength, tensile strength, percentage elongation at break and softness were 40.93 N/mm, 16.58 N/mm, 155.08 N/mm and 4.23 N/mm, respectively. Physical evaluation data showed that all the values of physical parameters of enzyme treated leather were also better than conventional lime and sulfide treated leather. As far as the quality of finally prepared leather is concerned, it was of good quality showing brown color, smooth surface, good stretching, no scud and having a normal appearance (Fig. 4). The physical quality test results of the pelt obtained after enzymatic treatment were better than results of the pelt treated with conventional chemical methods.

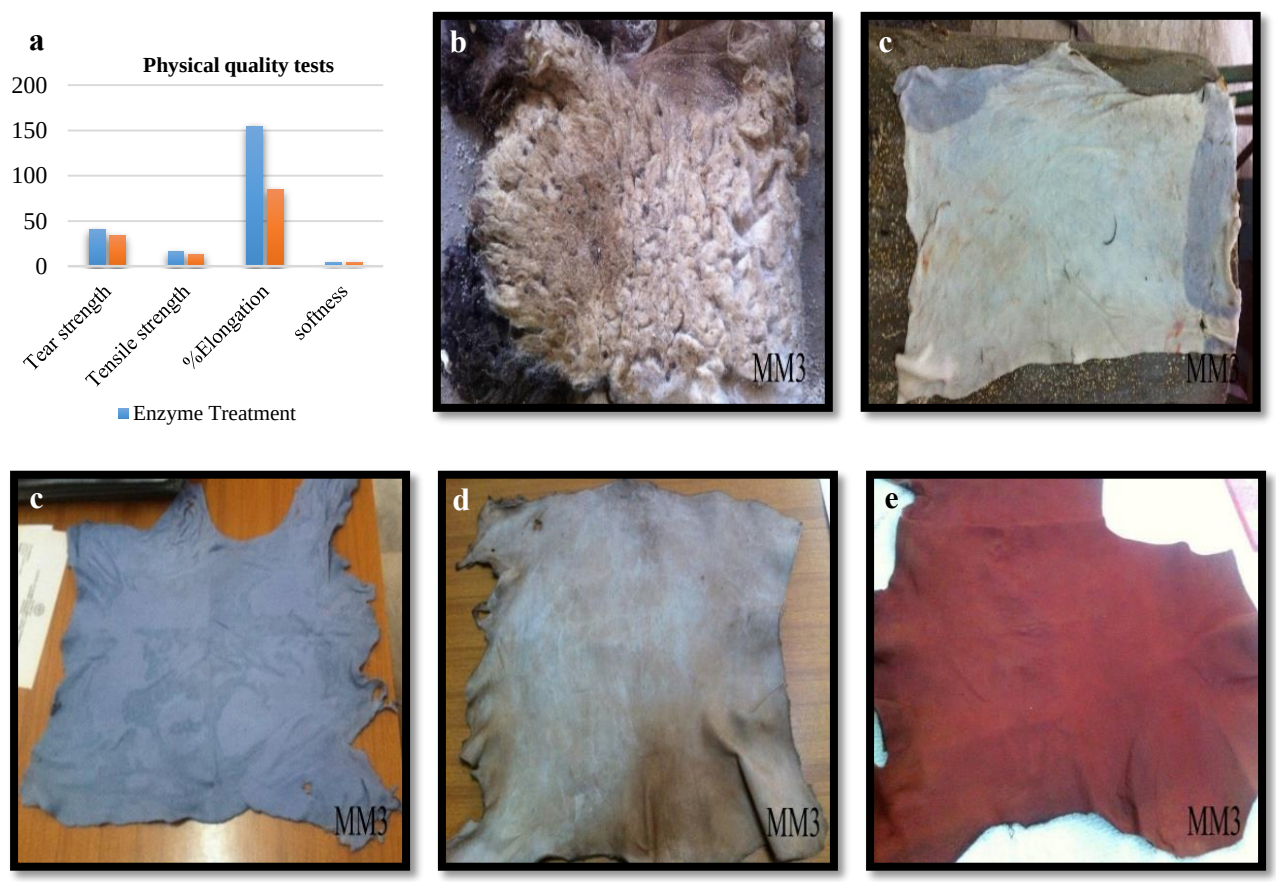

Fig. 4. Physical quality test for enzyme treated and conventional lime-sulfide treated leather (a), Sheep skin (b), Dehaired sheep skin (c), Chrome blue Pelt (d), Crust Leather (e), Finished dyed leather (f).

Conclusions

Streptomyces sp. MM-3 was found as potent producer of high titres of protease with dehairing properties and appeared to be highly efficient for its application in dehairing of animal skin as compared to conventional lime-sulfide unhairing method. Thus the successful lab scale production of protease opens a gateway for the production of protease enzyme at industrial scale in bulk quantities.

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