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Research Article

SOLID STATE FERMENTATION OF KERATINOLYTIC PROTEASE PRODUCTION USING *BACILLUS SPP* ISOLATED FROM WATER OF LEATHER PROCESSING PONDS IN NORTH GONDAR, ETHIOPIA

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ABSTRACT

The objective of this study was production of keratinolytic proteases by solid state fermentation using bacteria isolated from mud and surface water of leather processing stagnant ponds. Casein proteolytic bacteria were isolated. Partial purification of enzymes was carried out using 80% saturated ammonium sulfate and morphological and biochemical techniques were used to characterize the bacteria. Four isolated *Bacillus* species from two different regions showed highest proteolytic activity 10.21 U/ml (*Bacillus* strain Ss-2), 1.87 U/ml (*Bacillus* strain Ss-4), 9.2 U/ml (*Bacillus* strain Ms-1), 12.8 U/ml (*Bacillus* strain Ms-2). The optimum pH for protease production and stability of *Bacillus* species were 7 and 8 respectively, optimum temperature for isolates Ss-2 and Ss-4 was 37°C, whereas for isolate Ms-1 and Ms-2, it was 30°C. Maximum enzyme activity was observed at 0.2M NaCl. The optimum production time was 48 hours. *Bacillus spp.* grew best in wheat bran and rice bran carbon sources and at 1:3 ratios of media to moisture content showed highest enzymatic activity. Complete dehairing of cattle hide after 24h of incubation and complete removal of blood stains was only observed with *Bacillus spp* Ms-2 crude enzyme along with fruit juice. The study suggests that the two isolates should be further characterized and optimized for pure enzyme production.

Key words: *Bacillus spp.*, dehairing, leather industry, keratinolytic protease.

INTRODUCTION

Enzymes are important for thousands of metabolic processes that sustain life (Robinson 2015). Proteases represent one of the most important groups of industrial enzymes, because of their widespread use in detergents and dairy (Kumar *et al*., 2012). The proteases

play an important role in a wide range of industrial processes *viz*., baking, brewing, detergents, leather processing, pharmaceuticals, meat tenderization, cosmetics and medical diagnosis. Reduction in the production cost of enzymes could greatly reduce the cost of the enzyme. In submerged fermentation, up to 40% of the total production cost of enzymes is due to the cost of the growth substrate (Enshasy *et al.,* 2008). In this regard, solid state fermentation (SSF) which uses cheap agricultural residues has enormous potential in reducing enzyme production cost. Studies on protease that are produced in SSF by microorganisms are scarce in literature. As a result, it is of great importance to pursue such studies.

This study was focused on production of proteases through SSF using cheap substrate; the sources of bacteria for production of protease were stagnant pond used for dehairing of leather. Ethiopians have basic traditional leather processing knowledge using stagnant water as microbial source for dehairing purpose.

MATERIALS AND METHODS

Isolation of keratinolytic protease producing microbes

Samples were collected from hair of processing leather kept for couple of days and from bottom mud of the sixteen different traditional leather processing stagnant ponds of Sabaha, North Gondar. Collected samples were transferred to sterile labelled tubes and stored at 4°C until used. Keratinolytic protease producing microorganisms were isolated as per Sharma *et al*. (2017).

Morphological characterization

Macroscopic and microscopic characterization of isolates, Gram staining, endospore staining and motility of microorganisms were studied microscopically (Harley and Prescott, 2002).

Biochemical characterization

A loop-full of sample from an overnight culture was streaked on to nutrient agar plate and incubated for 24h at 37°C and the culture were used for different biochemical tests. Presence or absence of changes in the media was recorded as positive and negative, respectively and the results were interpreted using Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994). Catalase, starch, urea hydrolysis**,** urease, carbohydrate fermentation, gas production using triple sugar iron test**s** (Harley and Prescott, 2002) were done.

Screening for keratinolytic protease production

Bacterial colonies were screened for keratinolytic protease production using casein-yeast extract peptone (CYP) agar medium (Gessesse *et al.,* 2003) and the plates were incubated at 37°C for 48h. Colonies with halo zone were considered as positive for proteolytic activity and these colonies were isolated and repeated till single uniform colonies were obtained.

Optimization of the growth conditions

Effect of fermentation time

To determine the time for maximum production of keratinolytic protease, the culture in the medium containing wheat bran, peptone, yeast extracts, casein, $CaCl_2$, K_2HPO_4 and $MgSO_4$ was incubated at 37°C for 24-72h and the keratinolytic protease activity was determined at 12h intervals (Serin *et al*., 2012).

Effect of temperature

The optimum temperature for keratinolytic protease production was determined by incubating the culture at different temperatures (i.e. 25, 30, 37, 40, 45 and 50°C), for 48h. At the end of incubation period, the cell free culture filtrate was tested for keratinolytic protease activity (Muthu and Christudhas, 2012).

Effect of pH

The effect of pH on the production of keratinolytic protease was investigated by adjusting the pH of the growth medium to pH 5.0, 6.0, 7.0, 8.0, and 9.0 and incubating at 37°C for 48h.

Effect of carbon source

Glucose, rice bran, wheat bran, and sucrose were used as carbon sources. The cultures were incubated at 37°C for 48h (Akcan, 2012).

Effect of different hair source

Cow skin, goat skin, human hair and feathers were used as carbon sources. Human hair was previously washed with distilled water. Feathers were washed with 0.1% (v/v) Triton X-100 and distilled water and then cut into small pieces to enhance the contact surface and the other substrates were not pretreated (Syed *et al.*, 2009).

Effect of nitrogen source

Two different sources of nitrogen, viz. organic nitrogen and inorganic nitrogen were tested. The production medium was initially supplemented with different organic nitrogen sources such as yeast extract, peptone, casein, each at 1% (w/v) and inorganic nitrogen sources such as, $(NH_4)_2SO_4$, and NH_4Cl at 1% (w/v) were tested after incubating the culture for 48h (Akcan, 2012).

Effect of NaCl concentration

NaCl was added at various concentrations, i.e. 0.0, 0.2, 0.4, 0.6 and 0.8M, into the keratinolytic protease production medium and crude enzyme activity was checked after 48h of incubation.

Effect of moisture

The effect of moisture level on keratinolytic protease production from the bacterial isolates (1% inoculum) were determined by adding moistening medium to wheat bran at level of 1:2, 1:3, 1:4 and 1:5 (w/v). SSF medium were incubated at 37°C and the crude enzymes were harvested after 48h of fermentation time using centrifugation at 10,000 rpm for 6 min. The activity of the crude enzymes was determined.

Effect of inoculum size

The effect of inoculum size on protease production from the selected bacterial isolates were checked by inoculating the SSF medium (wheat bran to moistening medium at 1:3 ratio) with inoculum size of 5%, 10%, 15% and 20% . After incubating the medium at 37°C for 48h, crude enzymes were harvested and enzyme activity was checked.

Solid state fermentation (SSF)

SSF medium with (g/g): wheat bran 10; K_2HPO_4 0.1; $MgSO_4.7H_2O$ 0.02; $CaCl_2$ 0.01; and casein 1.0 was prepared in a 250ml Erlenmeyer flask and the solid substrate moistened in 1:3 ratio and incubated at 37°C for 5 days. Then keratinolytic protease harvested by soaking the fermented solid with ten volumes of distilled water per gram solid substrate (wheat bran), under shaking at 121rpm for 30 min at room temperature. At the end of the extraction, the suspension was hand squeezed through a double layered muslin cloth and the particulate materials clarified by centrifugation at 10,000 rpm for 5 min.

Recovery efficiency was calculated using the formula:

Units/ml = $\frac{\mu \text{ mole of tyrosine X reaction vol}}{\text{Sample vol X reaction time X vol assay}}$

Effect of pH

The crude keratinolytic protease was incubated at different pH values of 5, 6, 7, 8, 9, 10 and 11 with phosphate buffer (pH 7.0). The effect on the activity was studied by incubating for 20 min and determining the remaining activity following the standard keratinolytic protease assay procedures described above. The effect on the stability was studied by pre-incubating for 12h and determined the enzyme activity.

Effect of temperature

It was performed by incubating keratinolytic protease at different temperatures viz.: 30, 40, 50, 60, 70, 80 and 90ºC. The effect on the activity was studied by incubating for 20 min and determined the enzyme activity. The effect on the stability was studied by pre-incubating for 12h and determined the enzyme activity.

Partial purification of crude enzyme

Partially purified enzymes were obtained by ammonium sulfate precipitation and dialysis using membrane tube.

Molecular weight determination

Molecular weights of the partially purified keratinolytic protease were determined by Sodium Doedcyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with 10% polyacrylamide gel (Annamalai *et al.*, 2011).

Enzymatic dehairing of cow hide and feather degradation

Three sets of cow hides were washed with distilled water and cut into 15x20 cm pieces. Control was treated with distilled water, the second piece was treated with enzyme solution alone and the third piece was treated with enzyme along with traditional fruit for dehairing (entelya) and fruit for softening (gulo fruit) at pH 7.0 and incubated under constant shaking at 121 rpm at room temperature. The skin pieces were examined at 12h and 24h and noted the percentage dehairing and amount of scud formation. Similar treatments were carried out for percentage feather degradation.

Enzymatic degradation of blood stains

Blood drop was taken on cloth and allowed to dry and blood stain was treated with distilled water/ enzyme/ detergent/ enzyme along with gulo/ enzyme along with gulo and entelya at room temperature for five min, then washed with tap water and noted the percentage blood stain removal.

Data Analysis

All the experiments were performed in triplicates, data was tabulated and ANOVA test was performed using SPSS (version 20.0) statistical software at 95 significance level ($P \leq 0.05$).

RESULTS AND DISCUSSION

Initially 235 colonies were isolated from 20 different samples and out of that, 165 colonies (70.2%) were keratinolytic protease positive (Table 1). The isolates showed great variation in clear zone of hydrolysis on casein agar plates ranging from minimum 1mm to the maximum 20mm. Our results is in agreement with earlier studies (Akpomie *et al*., 2012). Among the total of 165 positive isolates, 10 isolates with relatively large clear zone of hydrolysis were selected for further investigation. The selection of potent bacteria was done by corresponding the isolates with each other in terms of both their diameter of clear zone of hydrolysis and their keratinolytic protease activities. The results showed that the isolates with higher clear zone of hydrolysis

also give higher keratinolytic protease production. This step resulted in selection of four potentially potent isolates, named as **Ss-2** and **Ss-4** from mud, **Ms-1** and **Ms-2** from hair. The isolates Ss-2, Ss-4, Ms-1 and Ms-2 were identified as spore-forming bacterial species genus *Bacillus* based on the Bergey's manual classification of determinative bacteriology.

It was observed that the growth medium containing casein yielded highest activity in all isolates (24 U/ml, 19.1 U/ml, 18.5 U/ml and 27.5 U/ml for Ss-2, Ss-4, Ms-1 and Ms-2 respectively). This was followed by peptone, yeast extract, ammonium sulphate and ammonium chloride. Within the isolates enzymes, significant difference ($P<0.05$) in keratinolytic protease activity were shown. Therefore, casein was selected as substrate for further optimization. Among the various carbon sources, complex carbon sources like wheat bran and rice bran were found to be

Table 1. Keratinolytic protease producing isolates.

Sample source	Positive isolates	Zone of clearance (mm)	Protease activity (U/ml)
Surface	Ss-1	10	1.25
	Ss-2	13	10.21
	Ss-3	07	1.65
	Ss-4	11	1.87
	Ss-5	09	1.48
	Ms-1	12	9.2
Medium	Ms-2	15	12.8
	Ms-3	08	2.1
	Ms-4	10	1.13
	Ms-5	9	1.26

the best and easily available substrates. Wheat bran showed maximum enzyme production even better than glucose for isolates Ss-2(19 U/ml) Ss-4 (10 U/ml), Ms-1 (14.75 U/ml) and Ms-2 (18.18u/ml) (Fig 1). In all isolates, maximum keratinolytic protease activity was shown at moisture content 1:3 (i.e. 9 U/ml, 12.5 U/ml, 14.3 U/ml and 16.75 U/ml for isolates Ss-2, Ss-4, Ms-1 and Ms-2 respectively).

The organic nitrogen sources (casein, yeast extract and peptone) were better support for keratinolytic protease production compared to inorganic nitrogen sources (ammonium sulphate and ammonium chloride).

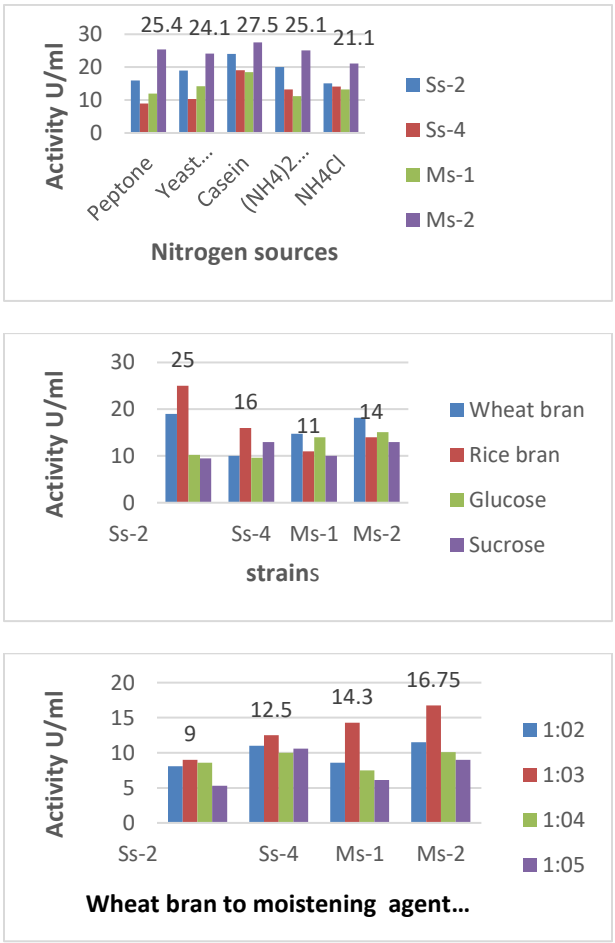
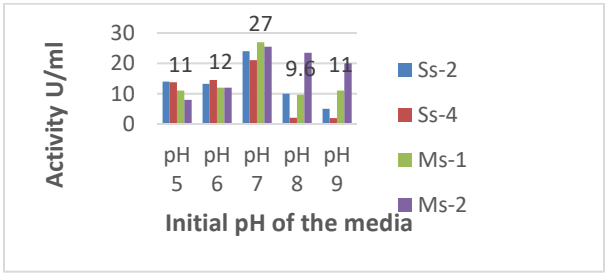
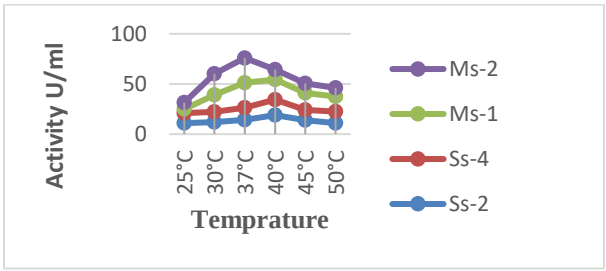


Fig.1. Nitrogen, carbon sources, moisture ratio on keratinolytic protease production.

This maximum keratinolytic protease production by casein, peptone and yeast extract might be due to the presence of high nutritional amino acids and compatible nitrogen source in these organic nitrogen sources. By contrast, least production of keratinolytic protease was observed in SSF medium supplemented with, ammonium sulphate and ammonium chloride respectively. This might be due to the inability of the bacteria to utilize these nitrogen sources due to difficulty in degrading these nitrogen sources into utilizable forms, which was in agreement with results of Shyam *et al.* (2013). Microbial growth medium for enzyme production at industrial scale takes about 30-40% production cost (Enshasy *et al.*, 2008). By using wheat bran alone, appreciable amount of keratinolytic protease production can be achieved which was in agreement with previous studies which suggested that larger amount of enzyme was synthesized when carbon sources were poorly utilized for growth purposes (Tambekar and Tambekar, 2013). The moisture level is one of the most critical factors (Mrudula *et al*., 2011). In our study, high enzyme activity was obtained when the substrate to moisture ratio was maintained at 1:3. In all isolates, any further increase or decrease of moisture ratio from the optimum (1:3) resulted in a slight decline of enzyme production which might be due to clumping of solid particles, reduction in solubility of the nutrients of the substrate, low degree of swelling and higher water tension (Mrudula *et al*., 2011). The optimum pH for keratinolytic protease production for the four isolates was 7.0 although the enzyme was active in the pH range of 7-11 (Fig. 2). At pH 7, the keratinolytic protease activities for Ss-2, Ss-4, Ms-1 and Ms-2 were 24 U/ml, 21 u/ml, 27 U/ml and 25.44 U/ml respectively which corroborates report of pH 7 and 9 by Josephine *et al*. (2012). The optimum keratinolytic protease production time for the four isolates was found to be 48 h corresponding to keratinolytic protease activity of 6.4 U/ml for Ss-2, 24 U/ml for Ss-4, 19 U/ml for Ms-1 and 27 U/ml for Ms-2 (Fig. 2). After 72 h, no further increase or decrease in keratinolytic protease production was observed. This might be due to the decrease in microbial growth associated with the depletion of available nutrient, loss of moisture content, production of toxic metabolites and autolysis of produced keratinolytic protease (Sumantha *et al.*, 2006). The optimum temperature for isolates Ss-2 and Ss-4 was found to be 40°C, corresponding to keratinolytic protease activities of 19 U/ml and 15.45 U/ml, respectively whereas for isolate Ms-1 and Ms-2 the maximum activities 25 and 24.75 were obtained at 37°C. However, considerable decreases in activity were observed with further increase in temperature beyond the maximum for the respective isolates (Fig. 2). It might be due the fact that at high temperature, the growth of the bacteria was hindered.





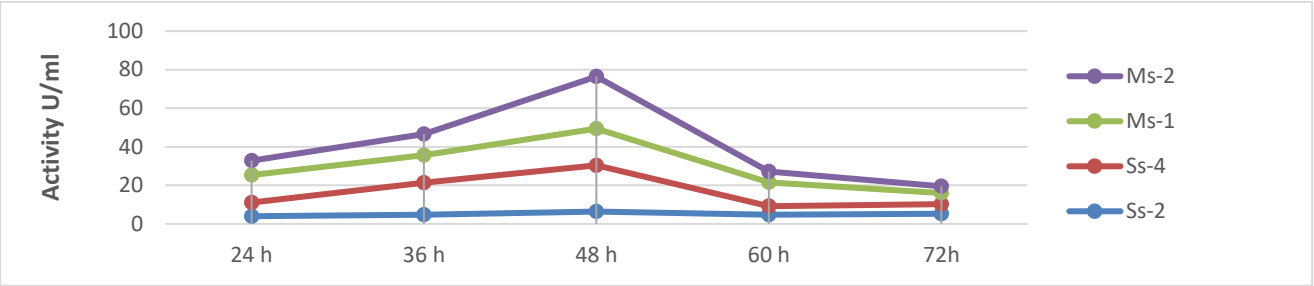
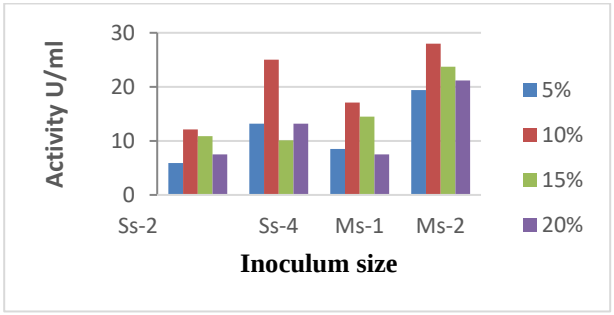


Fig. 2. Effect of pH, temperature, time on keratinolytic protease production.

Supplementary increase in initial pH values resulted in the decrement of keratinolytic protease production. This might be the isolates prefer neutral pH for optimum growth (Gangadharan *et al*., 2006). For bacteria isolated from mesophilic environments, an optimum pH for keratinolytic protease production was pH 7 (Ashwini *et al.*, 2011). The keratinolytic protease activity within the enzymes of the isolates showed significant difference ($P<0.05$). Several reports indicate that maximum keratinolytic protease production was achieved at 35-40°C for certain *Bacillus* spp. (Josephine *et al*., 2012; Kumar *et al*., 2012). These results suggest that isolates Ss-2, Ss-4, Ms-1 and Ms-2 belong to the mesophilic keratinolytic protease group. In the present study, 10% was found to be an optimum inoculum size for keratinolytic protease production in all isolates (12.1 U/ml, 25 U/ml, 17.1 U/ml and 28 U/ml for isolates Ss-2, Ss-4, Ms-1 and Ms-2 respectively). The size of inoculum plays an important role in the production of high keratinolytic protease (Shyam *et al*., 2013).

According to this study, inoculum size higher or lower than 10% has been shown to decrease keratinolytic protease production. The decreased keratinolytic protease yield at lower inoculum size might be due to the need of longer time by the bacteria isolates to grow to an optimum number to utilize the substrate and form the desired product. On the other hand, the low keratinolytic protease production at higher inoculums size (>20%) might be due to the stressful conditions created by the microbial cells such as depletion of nutrients, pH fluctuation, change in availability of oxygen and competition to limited resources, which were in line with the results reported by Kumar *et al.* (2012) and Shyam *et al.* (2013). The keratinolytic protease activity within the enzymes of the isolates did not show significant difference ($P>0.05$) except Ms-1.

It was observed that the growth medium containing 0.2M NaCl yielded the maximum activity in all isolates (19 U/ml, 15 U/ml, 21 U/ml and 28 U/ml for Ss-2, Ss-4, Ms-1 and Ms-2 respectively) (Fig. 3). The same work also has been reported in alkali-tolerant *Bacillus patagoniensis* (Oliver *et al.*, 2006), and by other halophilic and alkaliphilic bacterial isolates at 4M NaCl (Patel *et al*., 2006). The keratinolytic protease activity within the enzymes of the isolates were shown significant difference ($P<0.05$). The results showed that the stability of keratinolytic protease was higher at pH values ranging from 7.0 to 10.0 than at lower pH values exhibiting maximum stability at pH 8.0 in Ss-2,Ms-1 and Ms-2 and Ss-4 at pH 9 (Fig. 4).



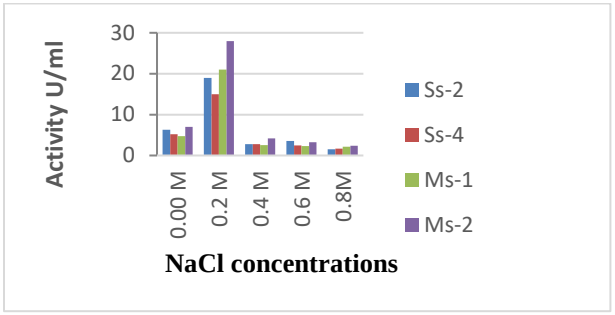


Fig. 3. Effect of inoculums size and NaCl on keratinolytic protease production.

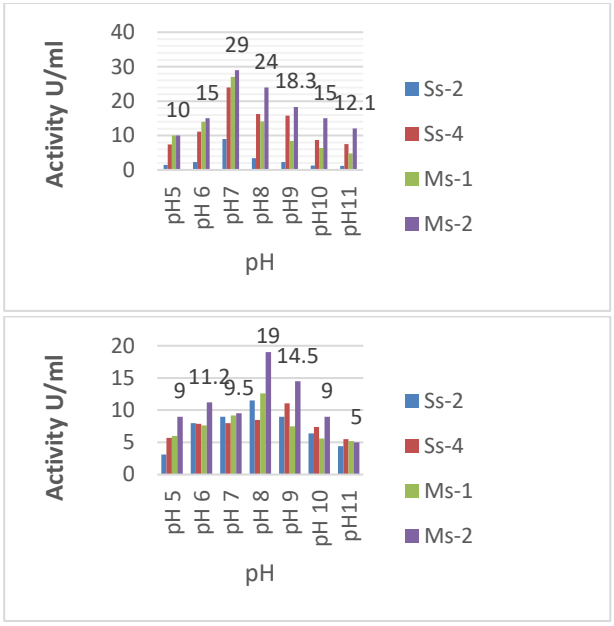


Fig. 4. Effect of pH on the activity and stability of keratinolytic protease.

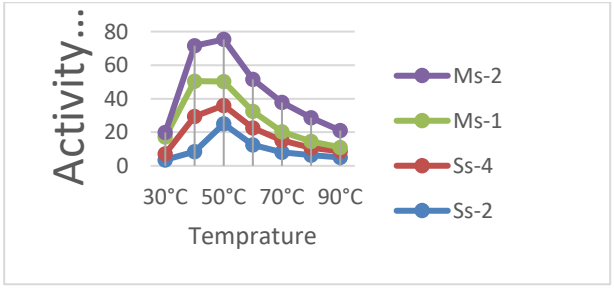


Fig. 5. Effect of temperature on the activity of keratinolytic proteases.

The optimum pH of the enzymes was 7.0 with more than 75% enzyme activity. This suggests the enzymes would be useful in processes that require a wide pH range from slight acidic to alkaline medium. These were in line with activity of keratinolytic protease produced from *Bacillus cereus* at wide range of pH and maximum activity at pH 7.0 (Mrudula *et al*., 2011; Salwa *et al.*, 2012). As shown in Fig.5 highest stability was obtained when held at 50°C for Ss-2, Ms-2 and at 40°C Ss-4 and Ms-1 for 12 h.

After partial purification, enzymes of isolates Ss-2, Ss-4, Ms-1 and Ms-2, purity of the enzymes were increased by 1.88, 2.6, 2.54 and 2.2 folds, respectively. Moreover, after dialysis, purity of the enzymes of the isolates Ss-2, Ss-4, Ms-1 and Ms-2 were increased by 2.87, 3.0, 5.3 and 6.2 folds, respectively (Table 3). The results indicate that the optimum temperature of proteolytic activity is in the range of the optimum temperature of enzyme production. The keratinolytic protease activity within the enzymes of the isolates showed significant difference (Fig. 5), the enzyme is active at temperatures between 30 and 80°C, with a reported 20h for *Aspergilus flavus* protease by Malathi and Chakraborty (1991). Thus, keratinolytic protease has a potential to substitute environmentally objectionable dehairing chemicals for hide/skin de-haring in leather industries and for production of quality

leather products.

Table 2. Partial purification of keratinolytic protease.

Bacterial Isolates	Purification steps	Vol (ml)	U/ml)	Purification (fold)
Ss-2	Crude	50	6.8	1.0
	(NH3)2SO4	20	12.8	1.88
	Dialysis	9	19.5	2.87
Ss-4	Crude	50	10.2	1.0
	(NH3)2SO4	21	26.5	2.6
	Dialysis	8	30.4	3.0
Ms-1	Crude	50	6.5	1.0
	(NH3)2SO4	18	16.5	2.54
	Dialysis	11	34.3	5.3
Ms-2	Crude	50	6.3	1.0
	(NH3)2SO4	25	13.8	2.2
	Dialysis	10	39.2	6.2

In relation to migration of the reference marker protein, the approximate molecular weights of the partially purified enzymes were found to be 80 kDa.

Enzymatic evaluations of cow hide dehairing

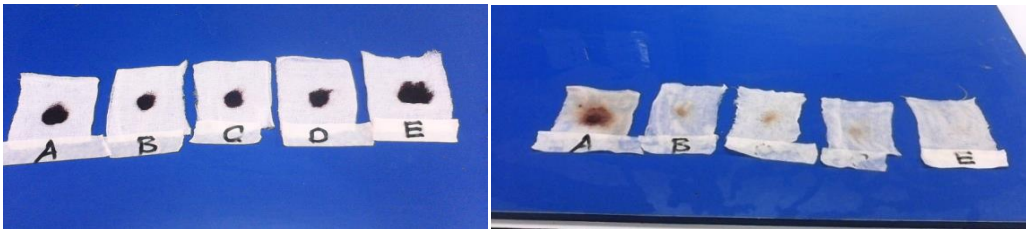
Complete dehairing of cow hide with fruit was achieved at12 h (Fig. 6). Because of specificity to hydrolysen on-collagen protein part at hair roots in hide, keratinolytic proteases are very important in shortening hide dehairing time and in production of high quality full gain leather having natural hair poreson the surface (Sivasubramanian *et al.,* 2008). Cow hide usually treated with dehairing chemicals in a drum for 24h (Thanikaivelan *et al*., 2004). Shortening of dehairing time was also reported, 20h for *Aspergilus flavus* protease by Malathi and Chakraborty (1991). Thus, keratinolytic protease has a potential to substitute environmentally objectionable dehairing chemicals for hide/skin de-haring in leather industries and for production of quality leather products.

Washing performance of Ms-2 Enzyme

The blood stain removal levels achieved with the use of Ms-2 with the fruit were more effective than the ones obtained with detergent alone. In fact, Ms-2 facilitated the release of proteinacious materials in a much easier way than the commercialized SB 309 protease (Jaouadi *et al*., 2009). Furthermore, the combination of Ms-2 and the detergent resulted in complete stain removal (Fig.7).

(A). Control (B). Enzyme only (C) both fruit with Enzyme (D).Total hair

Fig. 6. Washing performance analysis test of Ms-2. Stained cloth pieces with blood. (A) Control hair washed with distilled water, B. Enzyme only C. Enzyme with gollo fruits



A. Before Washing B. After Washing

Fig. 7. Washing performance test of Ms-2. Stained cloth pieces with blood. (A) Control stained cloth pieces washed with distilled water, (B). Detergent (7mg/ml). (C). Enzyme only, (D) Enzyme with gollo, (E).Enzyme with entelya.

CONCLUSION

Out of 235 pure bacterial colonies, 165 (70.2%) were keratinolytic protease positive, *Bacillus spp*. Keratinolytic protease production increased when the percent of inoculums increased up to the optimum and decreased beyond the optimum size. In all isolates maximum keratinolytic protease were harvested in 10% v/v inoculums. Keratinolytic protease production increased with increased bran to moistening agent till optimum, decreased beyond the optimum and all isolates gave maximum protease at 1:3 v/w bran to moisture ratio. On the other hand, production of keratinolytic protease is also influenced by the concentration of NaCl in the growth media which was found to be 0.2 M for all four isolates. The new source of keratinolytic protease producing bacteria from the soil and water samples collected from traditional leather processing ponds might be an alternative source for the potential industrial applications.

CONFLICT OF INTERESTS

We declare that we have no conflict of interest.

REFERENCES

Akcan N (2012). Production of extracellular protease in submerged fermentation by *Bacillus licheniformis* ATCC 12759. African J Biotechnol. 11: 1729 -1735.

Akpomie OO, Akponah E, Okorawhe P (2012).Amylase production potential of bacterial isolates obtained from cassava root peels. Agri Sci Res J. 2: 95-99.

Annamalai N, Thavasi R,Vijayalakshmi S, Balasubramanian T(2011). Extraction purification, and characterization of thermostable, alkaline tolerant amylase from *Bacillus cereus*.Indian J Microbiol. 51: 424-429.

Ashwini K,Kumar G,Karthik L,Rao BKV(2011).Optimization, production and partial purification of extracellular

protease from *Bacillus spp Marini.* Arch Appl Sci Res. 3:33- 42.

Enshasy EH, Abuol-Enein A, Helmy S,Azaly E (2008). Optimization of the industrial production of alkaline protease by *Bacillus licheniformis* in different production scales. Australian J Basic Appl Sci. 2:583-593.

Folin O, Ciocalteu V (1929). Enzymatic assay of protease casein as a substrate. J Biol Chem. 73:627.

Gangadharan D, Sivaramakrishnan S, Namboothiri K M, Pandey A (2006). Solid culturing of *Bacillus amyloliquefaciens* for α-amylase production.Food Technol Biotech. 44: 269-274.

Gessesse A,Hatti-Kaul R,Gashe BA, Mattiasson B (2003).Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enzyme and Microbial Technology. 32: 519-524.

Harley JP, Prescott LM (2002). Laboratory exercise in microbiology, 5th ed, McGraw−Hill.

Hema, TA and Shiny, M (2012). Production of protease enzyme from *Bacillus Clausii* Sm3. IOSR J Pharmacy Biol Sci. 1: 37-40.

Holt JG, Krieg NR, Sneath PHA, Staley JT (1994).Bergey's manual of determinative bacteriology. 19th ed, Williams and Wilkins, Josephine S, Ramya V, Devi N, Ganapa B, Siddalingeshwara KG, Venugopal N, Vishwanatha T (2012). Isolation, production and characterization of protease from *Bacillus Sp* isolated from soil sample. J Microbiol Biotech Res. 2:163-168.

Kumar DJM, Venkatachalam P, Govindarajan N, Balakumaran MD, Kalaichelvan PT (2012). Production and purification of alkaline protease from *Bacillus* sp. MPTK 712 isolated from dairy sludge. Global Veterinaria. 8: 433-439.

Malathi S, Chakraborty R (1991). Production of alkaline protease by a new *Aspergillus flavus* isolate under solidsubstrate fermentation conditions for use as a depilation agent. Appl Environ Microbiol. 57:712–716.

Mrudula S, Gopal R,Seenayya G(2011).Effect of substrate and culture conditions on the production of amylase and pullulanase by thermophilic *Clostridium Thermo sulfurogenes* SVM17 in solid state fermentation. Malaysian J Microbiol.7: 15-21.

Muthu P, Christudhas W (2012). Purification and characterization of neutral protease from *Bacillus Subtilis.* J Microbiol Biotech Res. 2: 612-618.

Oliver N, Sequeiros C, Sineriz F, Breccia J (2006). Characterization of alkaline proteases from novel alkali-tolerant bacterium *Bacillus patagoniensis*.World J Microbiol Biotechnol. 22:737-743.

Patel RK, Dodia MS, Joshi RH, Singh SP(2006).Production of extracellular halo-alkaline protease from newly isolated halophilic *Bacillus* sp. Isolated from sea water in western India. World J Microbiol Biotechnol. 22: 375-382.

Robinson PK (2015). Enzymes: principles and biotechnological applications. Essays Biochemistry. 59: 1-41.

Salwa E I, Hassan B E, Elmutaz N H, Elhadi S (2012). Amylase production on solid-state fermentation by *Bacillus s*pecies. Food Public Health. 2: 30-35.

Serin B,Akcan N, Uyar F(2012). Production and optimization of α-amylase from *Bacillus circulans* ATCC 4516 with solid-state fermentation. Hacettepe J Biol Chem.40: 393-400.

Sharma, Miglani K, Kumar R, Panwar S, Kumar A (2017). Microbial alkaline proteases: optimization of production parameters and their properties. J Genetic Eng Biotechnol. 15: 115-126.

Shyam SA, Sonia S S, Lal G (2013). Amylase activity of a starch degrading bacteria isolated from soil. Arch Appl Sci Res. 5: 15-24.

Sivasubramanian S, Manohar BM, Puvanakrishnan R (2008). Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. Chemosphere.70:1025-1034.

Sumantha A, Larroche C, Pandey A (2006). Microbiology and industrial biotechnology of food-grade proteases: A perspective. Food Technol Biotechnol. 44:211–220.

Syed DG, Lee JC, Li W-J, Kim C-J, Agasar D (2009). Production, characterization and application of keratinase from Streptomyces gulbargensis. Bioresource Technol.100:1868-1871.

Tambekar DH,Tambekar SD (2012). Partial Characterization and optimization of Alkaline protease production of Bacillus pseudofirmus from Lonar Lake. Internat J Adv Pharmaceutical Biol Sci. 2: 130-138.

Thanikaivelan P, Rao JR, Nair BU, Ramasami T (2004).Progress and recent trends in biotechnological methods for leather processing. Trends Biotechnol. 22**:**181-188.