Screening and optimization of candidate alkaline protease for dehairing potential from marine Bacillus paramycoide M2

Mandragutti Teja1*, Dokka Muni Kumar2, Sanapala Pavan3, Karrotu Chandana Vineela3 and Godi Sudhakar4

1. Department of Biotechnology, Andhra University, Visakhapatnam, 530 003, Andhra Pradesh, INDIA
2. Department of Biochemistry, Andhra University, Visakhapatnam, 530 003, Andhra Pradesh, INDIA
3. Department of Microbiology, Andhra University, Visakhapatnam, 530 003, Andhra Pradesh, INDIA
4. Department of Human Genetics, Andhra University, Visakhapatnam, 530 003, Andhra Pradesh, INDIA
*tejaaron75@gmail.com

Abstract
Alkaline proteases are active from neutral to alkaline pH range and have extensive applications in detergent and leather industries. In the present research, bacteria isolated from marine water samples were screened for proteolytic activity. Among the isolates, M2 showed maximum proteolysis with a clear zone when cultured on skim milk agar plates at 37°C for 24 h. Molecular identification using 16S rRNA sequencing and phylogenetic analysis revealed that M2 has sequence identity (99.93%) to Bacillus paramycoide. SEM analysis was carried for determining the morphology of M2 and also for enzyme treated skin. FAME analysis using GCMS was performed for the determination of fatty acids in the strain.

The selected isolate was inoculated into protease production medium under submerged fermentation conditions at 37°C for 48 h with a constant agitation of 120 rpm. Protease activity was determined under varying conditions of pH, incubation temperature, carbon and nitrogen sources, metal ions and NaCl (1-5%) using casein as substrate. The isolate M2 utilized molasses and peptone as carbon and nitrogen sources for better alkaline protease production at 40°C and pH 10 under optimal conditions. The dehairing experiments with M2 alkaline protease revealed dehairing efficacy of protease over chemical treatment. Hence, extracellular alkaline protease from M2 isolate could find potential application in leather processing industries and can be exploited commercially.

Keywords: Proteolysis, GC-MS, Phylogeny, SEM analysis, Dehairing.

Introduction
Proteases are the important hydrolytic enzymes which break the peptide bonds in proteins and degrade them into small peptides and amino acids and account for about 65% of the total industrial enzyme market44. Proteases are the major categories of industrial enzymes produced by a broad range of microbes such as bacteria, molds, yeasts and are also present in plants and various animal tissues46. Ever since the initiation of enzymology, one of the most significant classes of hydrolytic enzymes widely studied is the microbial proteases49. Furthermore, proteases of microbial origin constitute approximately 40% of the total global production of enzymes. Bacteria produce extracellular thermostable proteases in large amounts which are active at high pH.

Proteases from bacteria are significant enzymes in industries such as leather, textile, pharmaceuticals and detergent pharmaceuticals and also play an important part in developing protein hydrolysates, flavor, color to food, meat tenderization process and silver retrieval from X-ray films12,31. The major bacterial strains employed in enzyme production are still Bacillus species used primarily to produce alkaline serine and neutral proteases33.

However, the main applications of alkaline protease exist to be in detergent industry, accounts for about 30% of global enzyme production because the pH of laundry detergents lies in 9.0–12.010. Industries of food, agricultural, pharmaceutical and medical have been taking benefit of using Bacillus sp. because of its widespread range of physiological characteristics and capability to produce enzymes like proteases and other metabolites18. Enhanced production of protease from marine bacterial strain Pseudoalteromonas sp. CSN423 in larger amounts by random mutagenesis was reported13.

In leather manufacturing industries, proteolytic enzymes are convenient in dehairing of animal skin. Ever since the commencement of human advancement, regular process of unhairing involves the usage of calcium carbonate and Na2S and this process accounts for about 80%–90% of overall pollution caused in the pelt industry and releases certain obnoxious gases besides tough waste materials42. Therefore, enzymes linked dehairing methods with proteolytic enzymes assist to lower or evade chemicals and provide immense ecofriendly benefits18.

But at present, substitution of chemical unhairing methods with alkaline proteolytic enzymes has been established. Breakdown of undesired proteins by ecofriendly and economical process is the key requirement in industries especially pelt and tanning. Proteolytic enzymes which show high activity between 8-12 pH and steady at basic pH are identified to be potent sources for unhairing of skins21,37. Proteolytic enzymes could find uses at different phases in treating of animal skin i.e. alkaline enzymes in dehairing,
neutral enzymes in soaking and acidic enzymes during fermentation of pelts with excrements. Proteases used for dehairing are also identified among Bacillus sp. Nevertheless, several enzymes are not appropriate for dehairing process because use of proteases resulted in considerable degradation of collagen resulting in the alteration of collagen native form.

Hence, it is essential to search a novel dehairing proteolytic enzyme which does not result in collagen breakdown. As a consequence, unhairing methods using proteolytic enzymes facilitate to lower or avoid the chemical usage and offer vast ecological profits.

Hence, the present investigation has focused on the isolation of novel alkaline protease producing bacteria from marine water samples and optimization of protease production by studying the effect of several physico-chemical properties. In addition, efficiency of alkaline protease of M2 isolate as dehairing agent was studied in the present study.

Material and Methods
Isolation and Screening of bacteria: Sea water was collected in sterilized flasks along the Visakhapatnam coast, India and stored at -4°C. Bacteria were isolated by using serial dilution method. The isolates were primarily isolated on nutrient agar medium by spread plate method and incubated at 37°C for 24 hours. Based on the morphological characteristics, single bacterial colonies were selected and further sub-cultured to maintain purity.

Phenotypic characterization of isolates: Morphological characteristics of the isolates were studied following Differential staining and Scanning electron microscopic techniques.

Screening of proteolytic bacteria: The isolated colonies were cultured on SMA (skim milk agar) medium composed of peptone (0.5%), powdered skimmed milk (1.0%), NaCl (5%) and agar-agar (2.5%), pH 10 and the plates were incubated at 37°C for 24h for a clear hydrolysis on skim milk agar. Further selection of proteolytic organisms was done based on maximum degree of hydrolysis.

Metabolic profiling of proteolytic isolate: IMViC, sugar fermentations, hydrolytic ability of M2 on different substrates and other tests were done for the identification of bacterial species.

Molecular characterization: Bacterial cells were obtained from late-exponential cultures shaken in LB broth at 37°C. Pure genomic DNA was isolated following the method of Ausubel et al. The DNA was confirmed on 1% agarose gel electrophoresis. 16S rRNA gene was amplified by universal primers 27F and 1492R. The PCR product was purified and analyzed using ABI 3730xl genetic analyzer. The obtained 16S rRNA gene sequence has been deposited in GenBank, NCBI for authenticated accession number and subjected for BLAST analysis. Multiple alignment and phylogram were constructed using Clustal W and MEGA7 softwares.

FAME analysis by GC-MS: Dodds et al. technique was followed for the identification of fatty acids (FA) in tissues and lipids. This is refined by a dissolvable extraction driven by high temperature and pressure factor in an idle air of nitrogen. The analytes were derivatized into free fatty acids by a base catalyzed reaction and esterified to form fatty acid methyl esters (FAMEs) in acid catalyzed reaction utilizing the Lewis acid boron trifluoride in methanol.

Production of alkaline protease: Production of alkaline protease from the isolate was carried out in a production medium, pH 8.0 containing glucose, 0.5%(w/v); peptone, 0.75%(w/v); salt solution-5%(w/v) \{(MgSO_4\cdot7H_2O, 0.5%(w/v); KH_2PO_4, 0.5%(w/v)\}) and FeSO_4\cdot7H_2O, 0.01%(w/v) at 160rpm for 48 hrs in a shaking incubator. The broth was centrifuged at 10,000 rpm, 4°C for 20 min and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies.

Assay of alkaline protease activity: The supernatant was assayed for proteolytic activity following the method of Tsuchida et al. 200 μl of crude enzyme extract was incubated with 0.5ml of 1% (w/v) of casein in 50 mM phosphate buffer, pH 7.8 at 40°C for 20 min. After incubation, reaction was arrested by adding 1 ml 10% (w/v) TCA and left for 15 min at 37°C. The unreacted casein was separated at 10,000 rpm for 5 min. 2.5 ml of 0.44M Na_2CO_3 and 1 ml of 2-fold diluted FC reagent were added to the supernatant, incubated for 30 min and the absorbance was read at 660 nm against a reagent blank. Standard graph was plotted by taking tyrosine (0–100 μg/ml). One enzyme unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per min per ml under the experimental conditions.

Estimation of total protein: Protein content was determined following the method of Lowry et al with Bovine serum albumin (BSA) as the standard.

Optimization of parameters influencing protease production
Effect of medium pH: Initial pH of the medium necessary for obtaining maximum production depends not only on the bacterium but also upon the ingredients of the medium. To study the effect of pH on protease production, the medium was prepared at varied range of pH 2.0-12.0. The effect of pH on the production of extracellular alkaline proteases was studied by assaying the enzyme activity after 48 h of incubation period.

Effect of incubation temperature: Production of alkaline proteases was studied by incubating the inoculated broth at different temperatures (20°C - 70°C) for 48 h and assayed for protease activity.
Effect of incubation period: The effect of incubation time on alkaline protease production was studied at different incubation periods, 24 h to 120 h and protease activity was assayed at every 6 h interval.

Effect of carbon and nitrogen sources: In testing the best carbon sources facilitating protease production, standard production media were supplemented with carbon sources (0.5% w/v) such as maltose, glucose, mannose, wheat flour, molasses, rice bran, soybean meal, rice husk and sugarcane bagasse. Production media were also optimized with different organic and inorganic nitrogen sources (0.75% w/v) such as NaNO₃, KNO₃, (NH₄)₂SO₄, NH₄Cl, peptone, skim milk powder, casein, yeast extract and beef extract. After incubation, the cell free supernatant obtained by centrifugation at 10,000 rpm was quantified for protease production by determining the enzyme activity.

Effect of NaCl: The effect of NaCl on protease production by the isolates was studied by varying the salt concentration from 1% - 5% (w/v) in the medium.

Effect of metal ions: The effect of metal ions such as MgCl₂, MnCl₂, ZnCl₂, CaCl₂, CuCl₂, CoCl₂, FeCl₂ and BaCl₂ (5 mM) on the protease activity in the production medium was studied. Residual alkaline protease activity (%) was calculated and reaction medium without metal ion was taken as control (100%).

Dehairing efficacy studies of M2 alkaline protease: Dehairing potential of alkaline protease was determined following the method of Rajkumar et al. Goat skins were bought from slaughter house and were made into small pieces. The skins were drenched with water till the skins were free from blood and other contaminants. After drenching, the pelts were stacked to remove water before the application. Chemical dehairing using lime-sulphide was done with one of the piece as control and the other piece for enzyme application. Control skin was applied homogenously with a mixture of calcium carbonate (10%) and Na₂S (3%) and the other portion was soaked in 50 ml of glycine-NaOH buffer (pH 10.0) augmented with 1ml of crude enzyme.

The skins were left for overnight for about 20 h and the pelts were unhaired physically on a wooden platform with a scalpel and dehairing potential of the enzyme was determined by detecting the depilated area of the skin visually by eyes. All the experiments were performed in triplicate and ±SD was calculated using SPSS 14.0. The standard deviations (n = 3) are specified as error bars.

Results and Discussion

Screening of protease producing isolates: Based on distinct colonial characteristics like size, shape, colour, elevation and margins, ten bacterial isolates (M1- M10) were identified. Differential staining was performed for the isolates. The bacterial isolates were then screened for protein hydrolysis ability on skim milk agar plates. The isolate M2 (Figure 1a) was identified as the potent alkaline protease producer based on the appearance of a clear hydrolysis on skim milk agar plate (Figure 1b). Scanning electron microscope (SEM) analysis revealed the morphology of isolated strain M2 as uniform rods (Figure 2). Table 1 depicts the colonial features of isolate M2.

Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>Gram positive</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Texture</td>
<td>Dry</td>
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<tr>
<td>Spreading</td>
<td>Yes</td>
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<tr>
<td>Margin</td>
<td>Lobate</td>
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<tr>
<td>Elevation</td>
<td>Flat</td>
</tr>
<tr>
<td>Colony size</td>
<td>Small</td>
</tr>
<tr>
<td>Colony shape</td>
<td>Circular</td>
</tr>
<tr>
<td>Pigment</td>
<td>No</td>
</tr>
<tr>
<td>Opacity</td>
<td>Opaque</td>
</tr>
</tbody>
</table>

Figure 1: (a) Colonies of M2 on Agar plate (b) Protein hydrolysis of M2 on Skim milk agar plate
Metabolic profiling of isolate M2: The biochemical tests were done to characterize the isolate M2 and the results were tabulated in Table 2. M2 showed negative for urease, vogues proskauer and nitrate reduction tests whereas acid production was observed with all the five sugars used during fermentation. The obtained results were correlated with the data in Bergey’s Manual of Determinative Bacteriology and the isolate was characterized to be a facultative anaerobe, non-motile and appears as mini rods. The isolate M2 was then subjected for 6S rRNA sequencing for further identification of the bacteria.

Amplification of DNA and 16S rRNA gene sequencing of M2 isolate: DNA was extracted from M2 and the homogeneity was checked on 1% agarose gels. For the identification and classification of bacteria at the species level, 16S rRNA analysis has become a dependable tool and is essential to achieve unambiguous identification. 16S rRNA sequence was amplified by using universal primers. A single separated PCR product of 1500 bp was detected on agarose gels (Figure 3). The PCR product was refined to remove impurities. The generated consensus sequence of 16S rRNA was produced using forward and reverse sequence data using aligner software. The obtained sequence was used for BLAST analysis in GenBank, NCBI. The extracted 16S rRNA sequence was deposited in GenBank, NCBI and an accession number MW987795 has been obtained.

Phylogeny: 16S rRNA sequence of the isolate was submitted in BLAST online tool of NCBI Genbank. Phylogeny was carried based on highest identity scores and
the first ten sequences were aligned using multiple alignment software Clustal W and a similarity is shown with *Bacillus paramycoides* (99.93%). The phylogeny was built with 16S rRNA sequence by maximum likelihood process basing on model proposed by Kimura. Distance matrix was created and phylogeny was constructed using MEGA 7 program (Figure 4).

**FAME analysis by GC-MS:** Fatty acid analysis was determined by gas chromatography (GC), which differentiates bacteria based on the physical properties and aids in the determination of fatty acids (FA) in cellular components. The analytes were converted into free fatty acids by base catalysis and esterification to form fatty acid methyl esters (FAMEs) under acidic conditions. FAME analysis showed that the strain M2 contains C16:0 (24.7%), C15:0 iso (9.8%), C17:0 iso (5.2%), C18:0 (4.7%) as high ratio fatty acids (Table 3).

**Assay of alkaline protease:** For the proteolytic activity, the isolate was cultured and supernatant was separated by centrifuging at 10,000 rpm for 20 min. The supernatant was used as enzyme source. Total protein content in the filtrate was determined. Table 4 provides the activity of protease (Units/ml), total protein (mg/ml) and specific activity (Units/mg protein).

Proteases are regarded as one of most important enzymes to be produced commercially and are of immense significance having their applications in food, detergents, pharmaceuticals etc. Several studies have reported the biological production of protease from marine microorganisms. The commercial alkaline proteolytic enzymes produced by alkalophilic bacteria are vital and significant. *Bacilli* strains and *Pseudomonas* sp were reported to be the most potent protease producers.

![Figure 3: Amplification of genomic DNA and 16S rRNA gene of M2 isolate](image)

![Figure 4: Phylogenetic relationship of isolate M2. The phylogeny was built with 16S rRNA sequence by Maximum Likelihood process](image)
The current work interprets the isolation of bacterial strains from marine water sample by serial dilution method and was differentiated by distinct morphological features on nutrient agar medium. The isolates were tested for proteolytic ability on SMA medium. The isolate M2 has been identified as prominent alkaline protease producer based on the appearance of a clear hydrolysis. It has been reported that serial dilution method has been carried for the collected samples and 32 colonies were isolated by following initial screening on skin milk agar media. Numerous species of Bacillus have been reported as producers of alkaline proteases including Bacillus amyloliquefaciens, Bacillus cereus, Bacillus licheniformis, Bacillus mojavensis and Bacillus subtilis. Other than Bacillus sp., serine protease has also been extracted from fermentation of biological municipal solid wastes by Pseudomonas sp. with wide industrial applications.

Optimization of parameters influencing protease production by M2

Effect of pH: Among the physical parameters, pH of the growth medium plays an important role by inducing physiological changes in microbes and their enzyme secretion. The obtained results demonstrated that the isolate M2 multiplied and produced alkaline protease over a pH of (2.0-12.0). At pH 10 production of high enzyme was noted with enzyme activity, 195 ± 0.85 U/ml. A notable decline in the enzyme productivity occurred at both higher and lower pH values (Figure 5).

Effect of temperature: Temperature strongly affects the synthesis of proteases either specifically or non-specifically. The influence of different incubation temperatures on protease production was evaluated. From the results, it was observed that maximum protease production was observed at 40°C with enzyme activity of 198± 0.16 U/ml (Figure 6).

Hence, 40°C was considered as the optimum temperature for enzyme production by the isolate. There is a gradual decrease in protease production at the temperature above 40°C. However, the temperature below or above 40°C resulted in a sharp decrease in protease yield as compared to the optimal temperature.

**Table 3**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Fatty acid</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C18:0</td>
<td>4.7</td>
</tr>
<tr>
<td>2</td>
<td>C17:1 iso ω10c</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>C17:0 iso</td>
<td>5.2</td>
</tr>
<tr>
<td>5</td>
<td>C17:0 anteiso</td>
<td>1.2</td>
</tr>
<tr>
<td>6</td>
<td>C16:0 iso</td>
<td>2.7</td>
</tr>
<tr>
<td>7</td>
<td>C16:0</td>
<td>24.7</td>
</tr>
<tr>
<td>8</td>
<td>C15:0 iso</td>
<td>9.8</td>
</tr>
<tr>
<td>9</td>
<td>C14:0 iso</td>
<td>0.9</td>
</tr>
<tr>
<td>10</td>
<td>C14:0</td>
<td>4.2</td>
</tr>
<tr>
<td>11</td>
<td>C13:0 iso</td>
<td>4.3</td>
</tr>
<tr>
<td>12</td>
<td>C12:0 iso</td>
<td>1.1</td>
</tr>
<tr>
<td>13</td>
<td>C12:0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

**Figure 5:** Effect of pH on protease production by M2 isolate
The rate of microbial enzyme production is highly dependent on different culture environmental parameters like temperature, pH, duration, agitation speed, carbons, inoculum size and nitrogen sources and is required for ensuring the productivity and activity of microbial enzyme at industrial scale. The isolate M2 produced alkaline protease at a pH range of 2.0-12.0 and high enzyme was produced at pH 10. Maximum protease production at pH 9.0 has been reported for Bacillus subtilis. At higher and lower pH, enzyme production was inhibited as it strikingly affects the microbial growth. Alkaline protease isolated from Bacillus cereus TKU006 exhibited optimum pH at 9 with its high stability to the surfactants.

It was observed that the maximum protease production was observed at 40°C and hence considered as the optimum temperature for protease production by the isolate. Reports relating to alkaline protease production by different Bacillus species have shown a pH range of activity within 9-12 and temperatures between 40º to 70ºC.

Many researchers investigated and reported the optimum temperature and pH for protease production by different Bacillus species such as 70ºC and 9 pH for Bacillus licheniformis A109 and 35ºC and 9.5 pH for Bacillus cereus FT13. The results in the present study were reasonably reproducible and showed a related range of enzyme activity within 9-12 and temperatures between 40º to 70ºC.

Effect of incubation period: Production of protease was determined at different incubation periods and the isolate showed maximum protease production for incubation period of 72 h with protease activity of 198±1.4U/ml (Figure 7).

Effect of carbon source: In the present study, an experiment was designed to investigate the effect of different carbon sources on protease production by the isolate. Here, molasses was better utilized and high yield of protease was noted (195.0 ± 0.80 U/ml) by the isolate M2 (Figure 8).

Effect of Nitrogen source: Different organic and inorganic nitrogen supplements were tested for better protease production. Maximum production of protease was reported with peptone (192 ± 0.80 U/ml) (Figure 9). This shows the versatility of the M2 in utilizing a wide range of nitrogenous metabolites.

Bacillus sp. produced high amount of protease when medium was enriched with wheat bran and peptone. Highest alkaline protease yield at 6 % glucose by B. licheniformis has been reported. Likewise, strain NPST-AK15 utilized fructose as an exclusive carbon supplements for better enzyme production. Inorganic nitrogen supplements were identified to be good nitrogen sources for both growth as well as for the production of protease in some microorganisms.

Table 4
Assay of alkaline protease of the isolate M2

<table>
<thead>
<tr>
<th>S. N.</th>
<th>Isolate</th>
<th>Protease activity (U/ml)</th>
<th>Total protein (mg/ml)</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>M2</td>
<td>195 ± 0.85</td>
<td>15.7</td>
<td>12.42</td>
</tr>
</tbody>
</table>

The production of protease occurs at the end of exponential phase.

Decreased enzyme production due to extended incubation time may be due to autodigestion of proteases and proteolytic attack by other proteases. Based on this finding, incubation time of 72 h was used for production of enzyme throughout the study. Several researchers have reported a broad incubation period ranging from 36 h to 96h for the maximum yield of protease enzyme by Bacillus strains. Maximum enzyme production is at 72 h for the isolated strain of Bacillus SNR01.

Figure 6: Effect of temperature on protease production by M2 isolate
Figure 7: Effect of incubation time on protease production by M2 isolate

Figure 8: Effect of different carbon sources on protease production by M2 isolate


Figure 9: Effect of inorganic and organic nitrogenous supplements

It has been studied and reported that beef extract is better nitrogen source for *Bacillus subtilis* and yeast extract as sole nitrogen source for PD-4 strain for highest enzyme production.

**Effect of NaCl:** The influence of NaCl on enzyme production by isolate M2 showed optimum in the production medium containing 1% NaCl after 72 h of incubation with protease activity of 196 ± 0.22 U/ml (Figure 10). The isolate M2 exhibited growth tolerance up to 5% NaCl but there is a downfall in the protease production when salt concentration increased above 1% NaCl. The greater ability of M2 to grow over a range of NaCl concentration indicates the halotolerant nature of strain.

**Effect of metal ions:** Among the metal ions tested, Ca²⁺ and Ba²⁺ had a stimulatory effect on the isolate M2 for the maximum protease production (Table 5). On the other hand, rest of the metal ions had a negative effect on the protease production.

In the current study, influence of NaCl on enzyme production by isolate M2 showed optimum in the production medium containing 1% NaCl after 72 h of incubation. Studies on the effect of salinity on the growth of halotolerant bacteria have shown changes in the polar lipid composition of the cell membranes and a decrease of growth rate causing reduced enzyme production. Ca²⁺ and Ba²⁺ had a stimulatory effect on the isolate M2 for the maximum protease production whereas rest of the metal ions had a negative effect on the protease production. There are huge number of metals playing a major role in production of protease such as calcium, magnesium, zinc, iron, copper, cobalt, manganese, potassium, sodium etc.

Kohlmann et al. reported that *Neisseria flavescence* utilized ZnCl₂ metal ion for the maximum production of protease as activator but CuSO₄ inhibited the production. Metal ions like Ca²⁺, Mg²⁺ and Mn²⁺ increased and stabilized the protease activity of the enzyme and have been reported to increase the thermal stability of *Bacillus* alkaline proteases. Studies reported metal ions apparently protecting the enzymes against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperatures.

**Dehairing efficiency of M2 alkaline protease:** The dehairing ability of the M2 alkaline protease was evaluated.
in comparison with that of chemical method. Treatment with crude alkaline protease (195 U/ml in Glycine-NaOH buffer, pH 10.0) exhibited noticeable unhairing property after 20 hrs of incubation. Enzyme treated skin hairs may be well removed by plucking with forceps whereas in chemical method, hair loosening was not observed as they are intact and therefore it is difficult to remove hairs on the skin. When compared to the chemically treated skins, enzyme cured skins were cleaner and whiter. The enzyme treated skins did not exhibit collapsing in comparison to control skins as they were plumpier due to osmotic swelling by lime and sulphide but this can be overcome with enzyme treated skins (Figure 11).

Dehairing ability of the protease was then visualized by Scanning Electron Microscopy on the sections of treated skin (Figure 12) which illustrate the surface features. SEM pictures of skin indicate that no damage has occurred with enzyme treatment and this has brought some fibre openings at the macro level.

Hamza et al\textsuperscript{22} reported that at 50ºC and alkaline pH, \textit{Bacillus} sp. AMUa38 exhibited maximal activity of protease production indicating high suitability of enzyme in leather industries. The partially purified alkaline protease from \textit{Bacillus subtilis} AKAL7 isolated from poultry wastes mixed soil substantially dehaired cow skin completely after 12 h of incubation\textsuperscript{3}. A novel alkaline protease from alkaliphilic \textit{Idiomarina} sp. C9-1 has also been reported for its potential application in enzymatic dehairing of cattle hide and skins of goat, pig and rabbit in 8–12 h without causing significant damage to hairs and grain surface\textsuperscript{50}.

It has been reported that the use of protease with maximum enzyme activity at high pH can enter into the skin with more ease\textsuperscript{34}. Protease of \textit{Bacillus megaterium} RRM2 was reported to have dehairing ability on goat skin after 12 h of incubation\textsuperscript{11}. An extracellular protease from \textit{Bacillus} sp. served for unhairing the pelts of goat\textsuperscript{4}. Alkaline protease from \textit{Aspergillus tamarii} has also been reported for dehairing goat skins and it dehaired efficiently with 2% enzyme concentration at 32-37°C, pH 9.0-11.0 with an incubation period of 18 to 24 h\textsuperscript{5}. Dehairing application trials with goat skin supports the suitability of alkaline protease in the place of chemical method for reducing pollution load in effluent significantly without altering the quality of the leather.

\textbf{Conclusion}

In the present study, isolate M2 was identified as potent alkaline protease producer based on the appearance of clear hydrolysis on skim milk agar plate. The molecular and phylogenetic analysis of 16S rRNA gene sequence revealed the isolate M2 showing maximum similarity (99.93\%) to \textit{Bacillus paramycoides}. The optimum physical and nutritional parameters for maximal production of enzyme for the isolate M2 were studied.
The results related to dehairing efficacy of the M2 crude alkaline protease were found to be promising in the removal of hair from the goat skin. The results in the current study support that the alkaline protease of M2 isolate could have promising future and is suitable for leather processing industries.

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