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Alkaline protease from *Bacillus cereus* VITSN04: Potential application as a dehairing agent

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The objective of this work is to use protease enzyme as an ecofriendly alternative to chemicals in dehairing. An alkaline protease producing bacterium was isolated from protein-rich soil sample. The bacterium was identified as *Bacillus cereus* VITSN04 by 16S rRNA gene sequencing method. Growth characteristics and protease activity were studied in yeast, malt, beef, nutrient broth and soybean casein digest media and the enzyme secretion was found to correspond with growth. Maximum protease production was obtained in soybean casein digest medium at 16 h with the activity of 200.1 ± 0.68 U/ml and a correlation coefficient of 0.965 between growth and enzyme production. The crude enzyme was found to have maximum activity at 30°C and pH 8.0. The protease was purified by ammonium sulphate precipitation, Sephadex G-50 and G-100 gel filtration chromatography. The purified protease was homogeneous on non-denaturing PAGE and its molecular weight was estimated to be 32 kDa. The purified protease was of the serine type as it was inhibited by phenylmethylsulphonyl fluoride. The crude enzyme preparation was found to be effective in dehairing goat skins in leather processing.

[**Key words:** *Bacillus cereus* VITSN04; Alkaline protease; Dehairing; Goat skin; Leather]

Proteases from microbial source are the most important hydrolytic enzymes, which not only play important roles in cellular metabolic processes but have also gained importance in industrial sectors (1). A research report in 2007 on world enzymes revealed that enzymes were expected to grow at the rate of 7.6% every year to about US$ 6 billion by 2011 in the world market (2). The literature survey showed that alkaline proteases from *Bacillus* sp. are the best commercial sources available to date (3). Although, proteolytic enzymes are produced and are in continuous usage, they are not sufficient enough to meet the growing demands in the world market for different applications. Leather industry is one of the industries looking up to enzymes to reduce the impact of tanning processes on the environment (4).

Leather industry faces challenges all over the world on account of pollution related problems, though it has been contributing significantly to economic development and employment and export potentials in many developing countries. Apart from the close monitoring from the pollution control authorities, increased awareness among the consumers of leather products is also adding to the pressure on the industry to adopt cleaner processing methods. Though the end-of-treatment methods have been given priority so far for complying with the pollution control norms, containing pollution in-process by adoption of cleaner processing methods is considered essential for finding a lasting solution to the pollution problems in the recent times (5).

Leather processing involves four distinct set of unit operations, *viz.*, pretanning or beam house, tanning, posttanning or wet finishing and finishing operations. On analysis of the pollution loads generated in different unit operations, it becomes obvious that the pretanning operations contribute the most to the total dissolved solids and chemical and biochemical oxygen demand in the composite waste liquor (6). Liming is an important pretanning operation in the leather making and it is said that the leather is made or marred in this operation (7). The primary objective of this operation is to remove hair and flesh and open up the fibre structure suitably to get the desired properties in the final leather. Conventional liming process involves the use of lime and sodium sulphide which results in the destruction of the hair and other proteinous materials resulting in high pollution load in terms of chemical oxygen demand (COD) and biochemical oxygen demand (BOD). Moreover, sulphide is toxic and high amount of lime used creates huge solid waste (8). Hence, the researchers in the area have been actively involved in the development of enzyme based dehairing; defleshing and fibre opening processes to replace the conventional process (9–11). The enzyme dehairing involves the use of proteases to cleave the cementing substances holding the hair to the skin so that the hair can be removed without destruction. This is in stark contrast to the chemical dehairing process in which the di-sulphide linkage in the cysteine residues in keratin is cleaved leading to the destruction and partial solubilization of the hair (12). Though, many proteases have been screened for their dehairing efficacy, the search for enzymes to give satisfactory dehairing without the use of chemicals is still active and the present work is one such effort.

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In the present study, an attempt has been made to screen the microorganisms from the soil sample collected near the protein-rich dumping site for the isolation of protease producing bacterium. The growth of the organism, activity and purification of extracellular proteolytic enzyme from the isolated bacterium *Bacillus cereus* VITSN04 has been studied and the application of the enzyme in dehairing of goat skin investigated.

MATERIALS AND METHODS

Chemicals Pepstatin A, iodoactetate, PMSF, 1,10-phenanthroline, and Sephadex G-100, G-50 were purchased from Sigma-Aldrich, India. Soybean casein digest broth and other media were purchased from Himedia, India. All other chemicals used were of analytical grade.

Screening, isolation and characterization Alkaline soil sample was collected near protein-rich place, milk vending shop at Vellore, India. 1 g of soil was suspended in 10 ml of sterile saline water and serially diluted and plated on skim milk medium and incubated at 37°C overnight. Microorganisms showing clear zone of hydrolysis around their colonies were picked up, sub-cultured and maintained in glycerol stock at −20°C. Morphological and Gram characteristics of the colonies were studied. Molecular characterization was further done by the 16S rRNA gene sequencing method.

Molecular taxonomy, sequencing and phylogenetic analysis DNA was isolated from the biomass harvested at 48 h. The isolated DNA was then amplified by polymerase chain reaction using a Medoxmix kit. The primer sequences were chosen from the conserved regions previously reported for the bacterial 16S rDNA (13). Partial sequencing was done with the primers 5C27 (5′-AGAGTTTGATCCTG-3) and RC1492 (5′-TACGGCTACCTTGTTACGACTT-3). The conditions for PCR and the methodology for sequencing were followed as per procedure (14). The 16S rRNA partial gene sequence similarities were studied using the National Centre for Biotechnology Information-BLAST search.

Growth and enzyme activity profile The kinetics of the growth was studied in the nutrient broth for a period of 48 h at 37°C and 120 rpm on a rotary shaker. The generation time (g) was calculated by measuring viable cell count at every 4 h interval during growth by a serial dilution plating technique. Growth of the organism and enzyme production was carried out in five different media: (i) malt medium containing 35 g of malt, 10 g of peptone and 5 g of NaCl in 1 l of distilled water (ii) beef medium containing 35 g of beef extract, 10 g of peptone and 5 g of NaCl in 1 l of distilled water (iii) yeast medium containing 35 g of yeast extract, 10 g of peptone and 5 g of NaCl in 1 l of distilled water (iv) nutrient broth and (v) soybean casein digest broth. Each sterile 50 ml of production medium was inoculated with 1 ml of inoculum with the cell density being 3.1×10^7 cells/ml (1 OD_{600}) and incubated at 37°C at 120 rpm. Growth was measured in terms of absorbance at 600 nm using a UV Visible spectrophotometer (Shimadzu). Extracellular supernatants were checked for the activity of enzyme by the caseinolytic method at regular time intervals up to 48 h.

Assay of protease activity The proteolytic activity was determined by the caseinolytic method (15) using casein as a substrate with slight modification. The overnight culture broth was centrifuged at 8000 rpm for 10 min and the supernatant served as the crude enzyme source. The cell-free supernatant (0.5 ml) was mixed with 2 ml of 2% casein dissolved in 0.2 M phosphate buffer (pH 8.0) and was incubated at 37°C for 30 min. The reaction was stopped by the addition of 2.5 ml of 1.2 M trichloroacetic acid. A test blank (control) sample was also prepared by adding TCA prior to the enzyme addition. Test and test blank solutions were then filtered through a Whatman No. 1 filter paper. For 2.5 ml of the filtrate 5 ml of 0.4 M Na_2CO_3 and 0.5 ml of 0.5 N Folin Ciocalteu reagent were added and mixed thoroughly. The absorbance was measured at 660 nm by the UV Visible spectrophotometer. One unit of protease activity is defined as the amount of enzyme which liberated 1 μmol of tyrosine per min at 37°C.

Purification of alkaline protease The cell-free supernatant was collected from the 48 h old culture by centrifugation at 8000 rpm for 10 min at 4°C. The supernatant was subjected to ammonium sulphate fractionation according to the chart of Gomori (16) at 4°C. The active fraction between 30 and 80% saturation was separated by centrifugation at 10,000 rpm for 15 min at 4°C and the pellets were individually dissolved in 0.05 M phosphate buffer, pH 8.0 and dialyzed against the same buffer overnight. The dialyzed samples were lyophilized. The lyophilized sample was subjected to a gel filtration on a Sephadex G-50 column equilibrated and eluted with 0.05 M of phosphate buffer. The fractions were collected at the flow rate of 1 ml/min and the protein content was measured at 280 nm. The enzyme activity in each fraction was measured and the fractions showing activities were pooled.

The pooled fractions were lyophilized and loaded on to the Sephadex G-200 column equilibrated and eluted with 0.05 M of phosphate buffer. The fractions were collected at the flow rate of 0.05 ml/min. Protein content (Abs 280 nm) and protease activity were measured. The fractions showing high enzyme activity were pooled and lyophilized for further analysis.

Electrophoretic techniques The crude and purified (lyophilized) samples were subjected to native-polyacrylamide gel electrophoresis (Native-PAGE) according to the method of Ornstein (17) and Davis (18) to determine the homogeneity and molecular weight of the enzyme.

In zymogram, gelatin (10 mg/ml) as a copolymerized substrate was added in the resolving part of native-polyacrylamide gel electrophoresis (19). After the run, the gel was incubated at 37°C for 1 h in 0.05 M phosphate buffer, pH 8.0 and was stained with Coomassie Brilliant Blue R-250.

Effect of pH on the activity and stability of the enzyme The optimum pH of the enzyme was studied over a pH range of 3–10. Egg albumin (0.1%) and casein (2%) were used as substrates in acidic and alkaline pH respectively. Citrate buffer (pH 3–5), Tris–HCl buffer (pH 6–8), and Glycine–NaOH buffer (pH 9–10) were the buffers used to determine the enzyme activity. The pH of the reaction mixture was adjusted with the above mentioned buffers at 0.2 M concentration and the enzyme activity was measured by assay method. The stability of the enzyme was studied by pre-incubating the enzyme for 30 and 60 min at 37°C with each buffer. The assay was then carried out at pH values ranging from 3 to 10 using buffered substrate with pre-incubated enzyme to determine the stability of enzyme.

Effect of temperature on the activity and stability of the enzyme The optimum temperature of the enzyme was studied by incubating the reaction mixture over the temperature range of 20–80°C and measured the enzyme activity by casein assay method. The thermal stability of the enzyme was studied by pre-incubating the enzyme at different temperatures ranging from 20 to 80°C for 30 and 60 min at a constant pH of 8.0. The casein assay was then carried out to determine thermal stability of the enzyme.

Effect of inhibitors on the enzyme activity Inhibition studies were carried out to determine the specific class of protease using PMSF (phenylmethylsulphonyl fluoride): serine protease inhibitor, EDTA (ethylenediaminetetraacetic acid) and 1,10-phenanthroline: metallo protease inhibitor, iodoacetate: cysteine protease inhibitor, pepstatin A: aspartate protease inhibitor. The concentration of each inhibitor is 2 and 5 mM. Detergents (0.5%, 1% and 2%) such as sodium dodecyl sulphate (SDS), Tween 80, Triton-X-100 and denaturant DTT (dithiothreitol 2 and 5 mM) were also used for inhibition studies. To analyze the effect of inhibitors, purified enzyme was pre-incubated with and without each inhibitor (control) for 1 h at 37°C. The caseinolytic activity of the enzyme was then assayed.

Crude enzyme preparation for leather processing The cell-free supernatant was subjected to 50% (w/v) saturation with ammonium sulphate. The precipitated proteins were centrifuged at 10,000 rpm for 15 min at 4°C. The resultant pellet was dissolved in 0.05 M phosphate buffer (pH 8.0) and dialyzed against the same buffer. Dialyzed samples were then subjected to lyophilization. The lyophilized enzyme was used for dehairing in leather processing.

Dehairing of goat skin Common salt preserved goat skins procured from the local market were cut into two halves along the backbone. The left half was chosen for dehairing using lime–sulphide to serve as control in the study and the right half was taken for enzyme application trials. The skins were soaked with three changes of water (300% on the weight of the skins) till they are free from dirt, dung, blood and other contaminating materials and also free from sodium chloride as checked by silver nitrate solution. After soaking, the skins were piled to drain water for about an hour before the application. The soaked weight of the skins was noted separately for the left and right halves. The weight of the chemicals/enzymes used in the trials was based on this weight. The left half was applied on the flesh side uniformly with a paste of lime (10%) and 3% sodium sulphide of 60% purity and 10% water. The right half was applied on the flesh side with 2% enzyme and 10% water. After application, the left and right halves were piled separately with flesh and covered with a gunny cloth and left overnight. Next day (after about 18 h), the skins were dehaired manually on a wooden beam using a knife in tune with the commercial practice in the leather industry and the dehairing efficacy of the enzyme assessed by an experienced leather technologist subjectively in comparison with the control process based on lime–sulphide system.

Histological studies Histological examination was carried out on the limed pelt after defleshing. Samples from identical locations were cut from both control and enzyme treated pelts and fixed in 10% formalin for 48 h. The formalin fixed samples were dehydrated in an aqueous alcohol series (50 to 100%) and then cleared in xylene. The samples were finally embedded in paraffin wax and 10 μm sections were cut on a microtome and the sections were stained with hematoxylin and counter stained with eosin.

Measurements of pollution load The sectional waste liquors were quantitatively collected at the end of the reliming process for control and experiment. It was then analyzed for pollution parameters, *viz*., COD and BOD to assess the effect of enzyme treatment on pollution load generated in the liming process by following the standard analytical procedure (20). The dehaired skins were taken for further processing using a standard process recipe and converted to shoe upper leathers.

Physical test and visual assessment of the leathers The enzyme treated leathers were visually assessed for quality and tested for strength characteristics as per standard procedures in comparison with lime–sulphide treated leathers. The samples for physical testing were cut from the official butt portion of the leathers and conditioned at 20 ± 2°C and 65 ± 4% RH for 48 h. The tensile strength and tear strength were determined as per standard IUP methods 6 (21) and 8 (22) respectively. The crust leathers were assessed for quality by an experienced leather technologist.

SEM studies Samples measuring 5×2 mm were cut from an identical location on control and enzyme treated leathers and mounted both vertically and horizontally on aluminum stubs and sputter coated with gold (Edwards E-306). The micrographs of the surface and cross sectional view at 200× were recorded on a FEI-Quanta 200 microscope at an accelerating voltage of 12 kV.

Statistical analysis Arithmetic means, standard deviations and ANOVA were employed on the data for protease activity at different time intervals, pH, temperature,