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Protease enzyme: an eco-friendly alternative for leather industry

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Abstract: We report here a novel keratinase from *Bacillus subtilis* that has the potential to replace sodium sulfide in the dehairing process of leather industry. The Protease enzyme produced at laboratory condition has been characterized for its rate of enzyme production, and the environmental influencing factors such as pH and temperature on the activity of the enzyme has also been evaluated. The enzyme produced in pilot scale has been subjected to *in vitro* (spectrophotometrically) as well as *in vivo* assay (on wet goat skin for hair removal). The organism grown in the Dye's synthetic medium at 5.3mg/ml of cell dry weight produced 548 U/ml of protease. The *in vitro* enzyme activity increased with temperature within a range of 25°C to 35°C and found maximum at 45 °C and at pH 11. An index of dehairing comparable to the use of conventional sodium sulfide method was achieved in 7 h of its application on wet goat skin.

Keywords: Protease enzyme, leather industry, *Bacillus sp*, dehairing of skin.

Introduction

Enzymes are vitally important to the existence of life itself. Civilizations have used enzymes for thousands of years without understanding what they were or how they work. However, over the past several generations, science has unlocked the mystery of enzymes and has applied this knowledge to make better use in an ever-growing number of applications. Enzymes play crucial roles in producing the food we eat, the clothes we wear, even in producing fuel for our automobiles. Enzymes are also important in reducing both energy consumption and combating environmental pollution.

Leather processing is one of the important industries closely related to everyday life. Despite making significant contributions to the economy, the leather industry causes severe environmental pollution owing to the use of various chemicals and the release of a variety of detrimental materials. In leather processing, the first step in the beam house is to remove hairs from hides and skins. Unhairing is defined as the removal of hair from hides. Application of lime sulfide – unhairing system, the effluent problems arising from lime are due to its high alkalinity and suspended solids. Besides this sulfide, on the other hand, liberates toxic hydrogen sulfide, a serious hazard for both tannery workers and sewer men. Tanneries are constantly concerned with the obnoxious odor and the pollution caused by the extremely toxic sodium sulfide used in the dehairing process. The conventional dehairing method involves the use of high proportions of lime and sulfide, which contributes to 80-90% of the total pollution load in the leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime (Thanikaivelan *et al.,* 2004). Deaths because of this toxic chemical process have also been reported (Balasubramanian & Pugalenthi., 2000 Gupta *et al.,* 2002).

Worldwide, it is estimated that 315 million bovine leathers are produced per year. Considering the waste treatment cost of $0.30 per m2 of leather produced, more than $1 million is spent per day to treat the waste from tanneries around the world (Ramaswamy, (1996). Enzymatic dehairing in tanneries has been envisaged as an alternative to sulfides (Beynon & Bond, 1989; Altschul *et al.,* 1997). Alkaline proteases can be used which enables the swelling of hair roots, and the subsequent attack of protease on the hair follicle protein allowing easy removal of the hair (Gupta *et al.* 2002). A large proportion of the known alkaline proteases are derived from microorganisms, especially *Bacillus* strains (Wang *et al.,*2006).

Enzymatic unhairing accomplished by proteolytic enzymes is of great commercial importance contributing to more than 40% of the world's commercially produced enzymes. Approximately 50% of the enzymes produced is used for industrial process (Pepper *et al.,* 1963). Further, proteolytic enzymes are more efficient in enzymatic dehairing rather than amylolytic enzymes (Puvankrishanan, 2003). The enzymes cause loosening of the hair, without damaging the fibrous collagen of dermis. The advantages of enzymatic dehairing are as follows: 1) significant reduction or even complete elimination of the use of sodium sulfide, 2) total recovery of hair resulting good quality with good saleable value, and 3) creation of an ecologically conducive atmosphere for the workers.

The chemical composition of fresh hides and skins falls approximately within the following limits: water 60-65%; protein 25-30; fats 5-10% and small amounts of minerals. The chief entity of protein present in skin is collagen. Enzyme treated leather has shown better strength properties and greater surface area. Simplification of pre-tanning process by cutting down one-step *viz* bating. Americans have been practicing the sweating process for unhairing in which the depilation is

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being achieved by the combined actions of the enzymes of autolytic process and those are secreted by the bacterial species (Tailor *et al.,*1987).

A great deal of work has been reported in the past, since 1960 employing enzymes for unhairing. However, the use of enzymes in leather manufacturing process particularly for unhairing has not been accepted by the industry to the desired level. This is mainly because: a) enzymes are not effective enough to eliminate the sulfide completely, b) there is an apprehension that the enzymes assisted process needs stringent process control, and c) the cost of enzymes is not encouraging. Hence, the present work has focused on screening for proteolytic enzyme from a suitable microorganism, which is economically viable and effective enough to eliminate the sulfide completely.

Materials and methods

Isolation and identification of protease producing organisms

One gram of soil sample was collected from 0.15 cm layer near the mutton stall at Peravurani and dissolved in 100ml of distilled water, marked as 10^{-2} dilution. The soil suspensions were serially diluted up to 10^{-7}. An aliquot of 0.1ml was drawn from 10^{-5} and 10^{-6} dilutions and streaked into sterile petriplates. The plates were incubated at 28-37°C for 24 hours. Identification was done using standard methods based on culture morphology, staining, motility and biochemical characteristics.

Screening of protease activity

The identified bacterial isolates were plated on the skim milk agar plates and incubated at 37°C for 24 hours until a clear zone of skim hydrolysis give an indication of protease producing organism. Depending on the zone of clearance and the growth of organism, *Bacillus* species were selected for further studies.

Inoculums preparation

Primary inoculum: The bacterial culture obtained from soil sample was inoculated in a 100ml of yeast extract glucose broth (Glucose- 0.3g; yeast extract - 0.3g; Peptone- 0.3g; sodium chloride-0.5g; water to make 100ml; pH- 7). The culture was then incubated in a rotary shaker (ORBIT Shaker Incubator, Neo lab) for 72 hours at 37°C.

Secondary inoculum: A 50ml medium contains: Bacto peptone or tryptone- 0.25g; sodium chloride- 0.25g; 0.25ml of inoculum was added after strization and incubated for 72 hours at 37°C.

Production media: Production media (500ml) contains: Groundnut cake powder- 7.5g; horsegram flour- 2.5g; Glucose- 2.5g; potassium phosphate - 0.5g; micronutrient solution- 0.5g; pH is adjusted to pH 7.

Preparation of micronutrient: Ferrous sulfate- 10mg; copper sulfate- 5mg; sodium molybdate- 5mg; manganus chloride -5mg; magnesium sulfate- 10mg; boric acid- 5mg; zinc sulfate- 5mg; Make up to 10 ml with distilled water.

Protease activity at various environmental conditions

The parameters such as pH, temperature and substrate concentration were altered in the assay procedure one at a time. The conditions that favored maximum activity were taken to be optimum conditions. The standard conditions used were pH – 11.0 and temperatures – 45°C.

pH: In the substrate and the culture filtrate mixture, the pH, was altered from 3.5 to 8.0. Which is achieved by preparing the substrate using different buffers with different pH. For pH 3.5 to 5.5 acetate buffer (0.2M), and for pH 6.0 to 8.0 Phosphate buffer (0.2m) was used. Starch (1%) was prepared using the buffer 0.5ml and added to 0.5ml of culture filtrate for the assay. A graph was plotted taking pH values at X – axis against enzymes on Y-axis. The optimum pH for enzyme activity was extrapolated from the graph.

Temperature: The incubation temperature ranged from 30°C to 80°C and a graph was plotted taking temperature range in X – axis and enzymes activity on Y – axis.

Activity of enzyme: Protease reacts with the casein liberating tyrosine and the liberated tyrosine in alkaline conditions causes the reduction of Phospho molybdate and Phospho- tungstate with Folin – ciocalteau reagent to give blue colour. The colour developed is measured at 620 nm. The absorbance was the parameter for the estimation of tyrosine produced.

Stock tyrosine was prepared by dissolving 50 mg of tyrosine in 1N HCl and made up to 100ml using distilled water. 0.5ml of concentrated casein 2% was taken in 2 test tubes labeled test and control (t1 and c1). To this 1ml of citrate phosphate buffer was added. The tubes were incubated at 37°C for 5minutes. Then 2ml of enzyme, (whose activity is to be estimated), was added to the tube labeled test. Again the tubes were incubated at 37°C for 30 minutes. After incubation, 2ml of 10% TCA was added

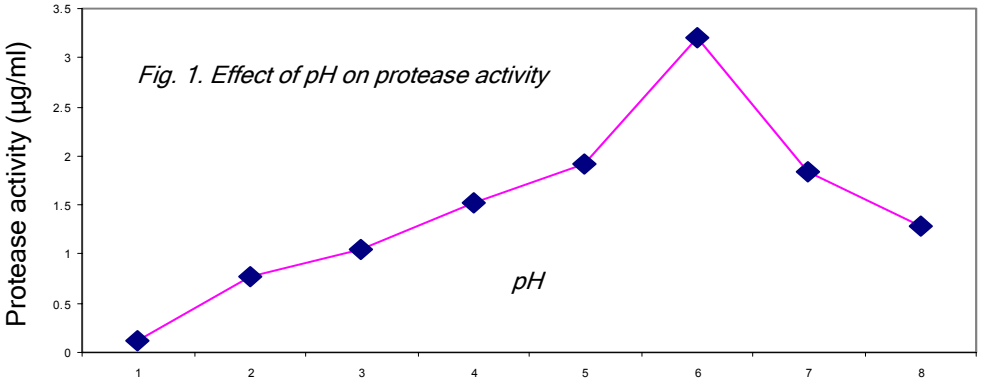

Fig. 1. Effect of pH on protease activity

Indian Journal of Science and Technology Vol.2 No. 12 (Dec. 2009) ISSN: 0974- 6846

to both the control and the test tubes and then 2ml of enzyme was added to the control tube. The tubes were centrifuged and supernatant was taken for the assay.

Standard tyrosine of 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml was taken in five test tubes labelled S1 to S5. Then 0.5ml of supernatant was taken in 2 tubes, labeled U1 and U2. The volumes in the tubes were made up to 2.4 ml with distilled water. Sodium hydroxide with a concentration of 0.5N of volume 2.0 ml was added to all the tubes followed by the addition of 0.6ml of Folin ciocalteau reagent. The tubes were incubated at room temperature for 10 to 20 minutes. Absorbance was measured at 620 nm using red filter. Taking the concentration along the X- axis and optical density along the Y-axis a standard graph was prepared.

Enzymes recovery

Fermented broth was filtered through sterile 0.22 µm hydrophilic durapore members filters, filtrate used as enzyme source. Enzyme suspension is taken in Erlenmeyer flask containing 0.5M $Cacl_2$ in 10% (v/v). The setup is allowed in a condition undisturbed (5h) for settling of suspension. Decant the supernatant from the flask and the solids were used. Again the remaining supernatant is removed by squeezing and sodium chloride is added to the solid sediments and allowed it to dry in a shallow pan for 6hrs.

Application procedure

The dehairing efficacy of the enzymes is being assessed by applying the same on the Goatskins. Wet salted Goat skins numbering four were selected and cut along the backbone. The right half was used for dehairing using sodium sulfide whiles the left half using the enzyme in varying concentration (Toyoda & Futami, 1956).

Soaking 1: Four right halves and four left halves viz R1 to R4 and L1 to L4) water 350% - the skins were washed.

Soaking II : Water 350% - the skins were soaked for 4hours

Soaking III : Water 350% - the skins were soaked for overnight.

Liming (control)

The four right halves were pasted with the following composition and piled for six hours: Water 20%; Lime 7%; Sodium sulfide 2.5%. The skins were dehaired and relimed.

Liming (experiment): The four left halves (L1 to L4) were applied with the lime – enzyme paste as follows: Water 20%; Lime 7%.

Enzymes suspension X% (X varies from 0.6, 1.2, 2.4 and 4.8 respectively on L1, L2, L3, and L4). The skins were piled and the dehairing efficacy was assessed. X presents the case of dehairing of the enzyme-applied skins with respect to time. The pH of the paste was 11.0

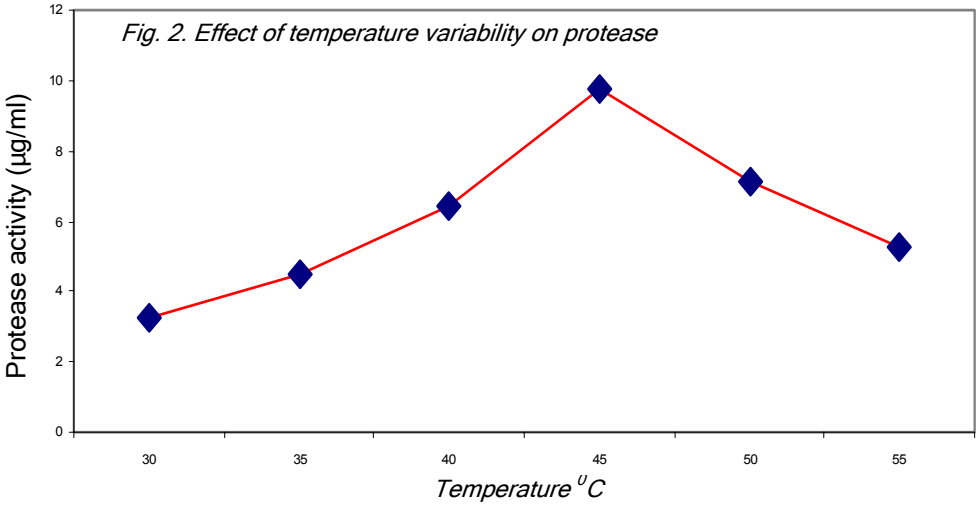

Fig. 2. Effect of temperature variability on protease

Results and discussion

Protease enzyme produced from *Bacillus* species at laboratory condition has been characterized and the protease assay is being carried out. Further, the effect of temperature and pH on the optimum activity of the enzymes requires 11pH at 45°C.

Coolbear *et al.* (1992) has stated the enzyme yields have remained relatively low since little work has been done on strain selection, growth optimization and enzyme yields. The *Bacillus* species produces a large variety of extra cellular enzymes of which protease is of significant industrial importance. In order to obtain protease on commercial scale the *Bacillus* species is being given priority in our investigation.

Bacillus species grown in Dye's synthetic medium (Dye's 1967) for two days is being used for the production of protease enzyme. The test organism grew well in the medium by producing 5.3mg/ml of cell dry weight producing 548 U/ml.

The effect of various parameters on protease activity of *Bacillus* species has been studied. The activity at pH 6.0 was low, but increased to a maximum at pH 11.0 but slowly decreasing to pH 11.0.(Green, 1961, 1961a; Gupta *et al.*, 2002) have demonstrated the pH optimum for *Bacillus* alkaline protease varies but generally optimum range is from 8-11 (Fig.1).

The enzyme activity increased with temperature within a range of 25°C to 35°C in the present observation as reported by Everett and Cordon (1956) and Joo *et al.* (2002). However, the maximum enzyme activity could be achieved at 45°C as shown in Fig. 2.

Indian Journal of Science and Technology Vol.2 No. 12 (Dec. 2009) ISSN: 0974- 6846

Application studies

The enzyme was then in a pilot scale following the same procedure and the enzyme was applied on skins for assessing the unhairing efficacy. It could be seen from the results that even with the minimum amount of enzyme fairly good degree of hair loosening could be achieved (Table 1).

The potential use of protease enzymes in leather processing eliminates the pollution causing chemicals such as sodium, lime and solvents. Future might witness ecolabelled leather products emerging as niche products by the use of protease enzyme technology and the experience gained by the Indian leather industry in this area might greatly help to emerge as a global leader. Our investigation of a novel keratinase from *Bacillus subtilis* has the potential to replace sodium sulfide in the dehairing process.

Table 1. Enzymatic de-hairing on wet goat-skin (index 1 to 10)

Experiment	After 3h	After 4h	After 5h	After 6h	After 7h
L1	3	4	5	6	7
L2	5	6	7	7	8
L3	5	6	7	8	9
L4	6	7	8	9	10

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