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PRODUCTION OF ALKALINE PROTEASE BY *BACILLUS SUBTILIS* AND ITS APPLICATION AS A DEPILATING AGENT IN LEATHER PROCESSING

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Abstract

The work describes the production of alkaline protease in a lab scale fermentor from *Bacillus subtilis* IH-72. The production of alkaline protease was enhanced by optimization of cultural conditions in a stirred fermentor. The alkaline protease so produced was applied to the goat skin for the removal of hair. The skin pieces were treated in three different ways; singly with the enzyme, in combination with the lime sulphide and singly with lime sulphide. The best results with the skin processing were obtained, when skin was treated with crude enzyme in combination with 7 % lime sulphide. The quality of pelt (color, grain, stretch, scud etc) and physical properties of the finally prepared leather (tensile strength, tear strength, bursting strength etc) were also improved with the use of proteolytic enzymes produced by *Bacillus subtilis* IH-72.

Introduction

Proteases possess some characteristics of biotechnological interest due to which these have become the most important industrial enzymes (Barindra, *et. al.*, 2006). Almost all proteases are heat resistant (Mehler, 1957), vary widely in their specific activities, optimum pH, pH stability range (2-13), heat sensitivity, substrate specificity, active site, catalytic mechanism and stability profiles (Raymonde & Othmer, 1941; Ward, 1985). On the basis of their acid-base behavior, proteases have been classified in to three categories i.e., acid, neutral and alkaline proteases. The acid proteases are those which have pH optima in the range of 2.0-5.0 and are mainly fungal in origin (Haq & Mukhtar, 2007). Proteases having pH optima in the range of 7.0 or around are called neutral proteases. They are mainly of plant origin however; some bacteria and fungi also produce neutral proteases. While proteases that have pH optima in the range of 8.0-11.0 are grouped under the category of alkaline proteases. Some of the important alkaline proteases are solanain, hurain and proteolytic enzymes of *Bacillus* and *Streptomyces* species (Hameed *et al.*, 1996; Lee *et al.*, 2002; Tang *et al*., 2004).

Alkaline proteases are envisaged to have extensive applications in leather industry. In a tannery, a rawhide is subjected to a series of chemical treatments prior to tanning and finally converted to finished leather. Proteases may play a vital role in these treatments by replacing these hazardous chemicals especially involved in soaking, dehairing and bating (Puvankrishnan & Dhar, 1986). Increased usage of enzymes for dehairing and bating not only prevents pollution problems, but also is effective in saving energy.

In general, proteases play a vital role in leather processing starting from soaking of hides to finished products (Sachidanandham *et al.*, 1999; Mukhtar & Haq, 2007). In advanced tanneries, soaking is usually performed with combination of proteolytic enzymes that are optimally active in the alkaline or neutral pH (Ward, 1985; Christner,

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1995; Godfrey, 1996). The conventional method for depilation has now been clearly recognized to be environmentally objectionable accounting for the discharge of 100 % of sulfide and 80 % of the suspended solids in the tannery effluent (Peper & Wyatt, 1989).

The use of enzymes for unhairing skin and hides has been widely discussed since the beginning of this century and many microbial sources and methods of application have been suggested. The enzymes catalyze the breakdown of the protein keratin in the hair and allow the hair to be easily removed. The pH at which these enzymes are active ranges from acidic, in which pepsin and papain can be used, to neutral and alkaline for enzymes such as from *Bacillus subtilis*. The advantage of using proteases for dehairing of skins are the reduction of the sulfide contents in the effluent, recovery of the hair/wool which is of good quality, an increased yield of leather area, easy handling of the pelts by workmen, simplification of the pretreatment, the elimination of the bate in the deliming stage and finally the production of a good quality pelts/leather (Dhar, 1974; Mitra & Chakraverty, 1998). However, there are some disadvantages such as the enzymatic process is more expensive than the conventional chemical process; it requires careful control and lakes swelling of the collagen during the enzymatic dehairing (Malathi & Dhar, 1987).

Materials and Methods

Microorganism: The bacterial strain of *Bacillus subtilis* IH-72 was isolated from a soil sample collected from the tannery area. One gram soil was suspended in 10 ml of sterilized saline and was subjected to serial dilution. 0.5 ml from an appropriate dilution was speeded on a test plate containing nutrient broth-casein-agar medium. The strain was isolated on the basis of formation of a clear zone of casein hydrolysis on the test plate and was transferred to nutrient agar slant for growth and maintenance.

Inoculum preparation: Vegetative inoculum was used during the present studies. Fifty milliliter of pre-culture medium consisting of 0.8 % nutrient broth (Peptone, 0.3%; yeast extract, 0.4%; pH 7.5) was transferred to the individual cotton plugged 250 ml Erlenmeyer flask and sterilized in an autoclave for 15 min at 15 lb/inch^2 (121ºC). After cooling at room temperature, the flasks were inoculated aseptically with a loopful of bacteria from 48 hrs old slant. The flasks were then rotated at 200 rpm in the rotary shaking incubator (Sanyo GallenKamp UK) at 37°C for 24 hrs.

Fermentation experiments: The fermentation experiments for the production of extracellular alkaline protease by *Bacillus subtilis* IH-72 were carried out in a laboratory scale 7.5 L batch bioreactor (Bioflow 110 Fermentor/Bioreactor, New Brunswick Scientific, USA) with a working volume of 5.0 L. The bioreactor was equipped with monitors, which were used to measure and control foam, temperature, pH, stirring rate and dissolved oxygen. The vessel of the bioreactor was equipped with a four-blade turbine. A peristaltic pump was used to control the foam by automatic addition of an antifoam silicon agent. For tests with automatic pH control, a system operating with an ingold sterilizable electrode and automatic addition of oxalic acid/KOH solutions through peristaltic pump was used. Controls were performed at different levels of different parameters. The vegetative bacterial inoculum was transferred to the basal medium at a level of 2 % (v/v) based on total working volume of the fermentation medium. The batch

was carried out at an incubation temperature of 37°C, agitation speed of 200 rpm and aeration rate of 1.0 vvm.

Application of protease for dehairing: The crude preparation of alkaline protease from *Bacillus subtilis* IH-72 was applied to the pieces of raw hide to evaluate the depilating (unhairing) efficiency of the enzyme. The depilating experiments were carried out at the Institute of Leather Technology, G.T. Road, Gujranwala. Goat skin was selected for the studies which were washed with water to remove salts and other debris. It was cut into standard sized pieces measuring 10x5 cm dimension which were latter treated with the enzyme. The skin pieces were treated as with either crude enzyme preparation only (Treatment # 1); 2: 7 % Sodium sulphide, lime and crude enzyme preparation (Treatment #) or 3: 14 % Sodium sulphide and lime (Treatment # as a control).

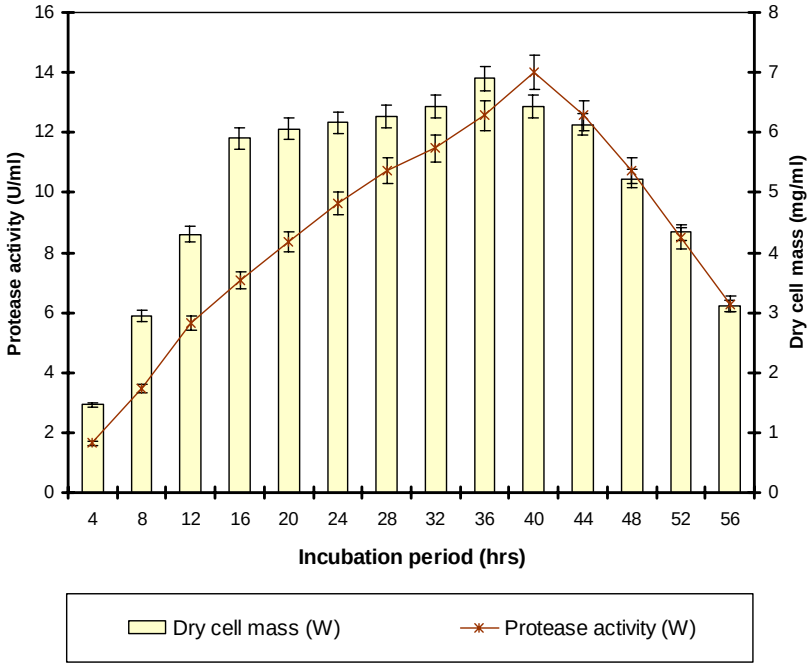
The skin pieces after above treatments were examined for depilation time, depilation extent, pelt color and scud to evaluate the process of depilation with different depilating agents. The pelt so produced was further processed by conventional method to be converted into leather. The finally prepared pieces of leather were examined for different physical tests such as tensile strength, tear strength, bursting strength and percentage elongation at break. The quality of the finally prepared leather was also assessed by examining the features such as color, grain, stretchability, scud removal and general appearance of the body. All the experiments were carried out in triplicate on both the parallel and perpendicular pieces of the skin.

Assay of protease: The method of McDonald and Chen (1965) was used for the assay of protease. Casein (1% solution in 0.1 M Phosphate buffer of pH 8.0) was incubated with one ml of enzyme sample at 30°C for one hour. The reaction was arrested by the addition of five ml of 5% trichloroacetic acid (TCA) solution. The mixture was centrifuged at 5000 rpm for 10 min. and one ml of supernatant was mixed with five ml of alkaline reagent. To this mixture one ml of 1N NaOH was added to make the contents of the tube alkaline. After 10 min., 0.5 ml of Folin and Ciocalteau reagent was added to the test tubes and mixed. The blue color produced was measured with UV spectrophotometer (CECIL, CE 7200, Cambridge, England) at 700 nm after 30 min. One unit of protease activity is defined as the amount of enzyme required to produce an increase of 0.1 in optical density at 700 nm under the defined conditions.

Results and Discussion

Production of alkaline protease: The rate of fermentation of alkaline protease by *Bacillus subtilis* IH-72 was investigated in a stirred bioreactor (Fig 1). The samples of fermented broth were withdrawn aseptically from the fermentor after regular intervals of 4 hrs up to 56 hrs of fermentation and were assayed for biomass and enzyme production. It was found that the growth of the microorganism and enzyme production was gradually increased as the fermentation process progressed and reached maximum after 40 hrs of incubation. The maximum growth and enzyme production was found 13.80±0.53 mg/ml and 7.0±0.36 U/ml. A rapid decline in the enzyme production was observed when the fermentation was extended beyond the optimum incubation period. It was also found that there was very little production of the enzyme during lag and log phase of the bacterial growth.

Figure 1: Production of alkaline protease by *Bacillus subtilis* IH-72 in fermentor*



Each value is an average of three parallel replicates. Y error bars indicate the standard error among the replicates. *Initial pH 8.5, incubation temperature 37°C, agitation 200 rpm, aeration rate 1.0 vvm, working volume 72 %, inoculum size, 3 % (v/v).

Table 1: Evaluation of the pelt after treatment with alkaline protease from *Bacillus subtilis* IH-72

Experiment	Time for Depilation (hrs)	Scud	Pelt color	Extent of Bating (%)
Treatment # 1	07	Little	White	50
Treatment # 2	04	No	White	75
Treatment # 3	1.5	Too much	Blackish	Nil

Table 2: Physical analysis of the finally prepared leather treated with alkaline protease from *Bacillus subtilis* IH-72

Experiment	Cut of leather	Tensile strength (N/mm^2)	Tear strength (N/mm^2)	Bursting strength (Kg/cm)	Elongation at break (%)
Treatment # 1	Parallel	21.2	66.8	476	26
	Perpendicular	29.8	73.5	483	21
Treatment # 2	Parallel	14.7	57.5	460	20
	Perpendicular	19.8	68.0	462	36
Treatment # 3	Parallel	12.2	56.0	366	25
	Perpendicular	17.4	62.2	368	30

Table 3: Evaluation of quality of the finally prepared leather treated with alkaline protease from *Bacillus subtilis* IH-72

Experiment	Color	Grain	Stretch	Scud	Appearance
Treatment # 1	Grey	Smooth	Good	No	Normal
Treatment # 2	Grey	Smooth	Good	No	Normal
Treatment # 3	Grey-blue	Rough	Fair	Too much	Normal

The production of alkaline protease by bacteria is directly related with the incubation period/growth phase of the microorganism. *Bacillus* species are known to produce maximum amount of proteases in the stationery phase or post exponential phase of their growth (Schaeffer, 1969; Sharipova *et al.*, 2000). The stationery phase of bacterial growth was started at about 28 hrs and lasted till 48 hrs of incubation in the fermentor. This correlation was attributable to an increased need for turnover of cell proteins at the slower growth rate (Mandelstam, 1958). Some other bacilli synthesize proteases during the exponential growth phase, but it mainly depends on the composition of the medium and some other factors (Chaloupka, 1969).

Treatment # 1 (Treatment with crude enzyme preparation): In the first experiment, skin pieces were treated with crude enzyme preparation for 10 hrs at 37 °C with a shaking speed of 50 rpm. Complete depilation (unhairing) was achieved after 07 hrs of treatment. The pelt had a little scud and was white in color (Table 1). However, no scud was observed after 10 hrs of treatment with the enzyme preparation. A significant finding of this treatment was that the hair was removed from the root level rather than broken off at the skin surface as in the case with lime sulphide treatment. Physical analysis of the finally prepared leather showed that the tensile strength, tear strength, bursting strength and percentage elongation at break of parallel piece of leather were 21.2 N/mm^2, 66.8 N/mm^2, 476 Kg/mm and 26 % respectively while in case of perpendicular piece of leather these values were 29.8 N/mm^2, 73.5 N/mm^2, 483 Kg/mm and 21 %, respectively (Table 2). As for as the quality of finally prepared leather is concerned, it was of good quality showing grey color, smooth surface, good stretching, no scud and having a normal appearance (Table 3).

Treatment # 2 (Treatment with enzyme preparation and lime sulphide): In the second treatment, skin pieces were dipped in a solution containing enzyme preparation, 7 % sodium sulphide and lime. The skin pieces were treated with the said combination for 10 hrs at 37°C with a shaking speed of 50 rpm. The results of this treatment were even better than the first treatment with respect to almost all the parameters. The complete depilation was achieved after 4 hrs of treatment with the resulting pelt of white color having no scud (Table 1). Physical testing of the finally prepared leather revealed that the parallel pieces of leather showed the tensile strength of 14.7 N/mm^2, tear strength of 57.5 N/mm^2, bursting strength of 460 Kg/mm and percentage elongation at break of 20 %. On the other hand, the perpendicular pieces showed the tensile strength of 19.8 N/mm^2, tear strength of 68.0 N/mm^2, bursting strength of 462 Kg/mm and percentage elongation at break of 36 % (Table 2). The finally prepared leather was evaluated for its quality and was found to have bluish grey color, smooth grain surface, good stretchability, no scud and normal body appearance (Table 3).

Treatment # 3 (Treatment with lime sulphide; conventional depilation): In this treatment, the skin pieces were dipped in a solution containing 14 % sodium sulphide and lime for 6 hrs at 37°C with a shaking speed of 50 rpm which is a conventional method of depilation used in the tanneries of Pakistan. The results of this treatment showed that the depilation was completed in 90 min with the pelt having much scud and blackish color (Table 1).

The results of the physical tests showed that the parallel and perpendicular pieces of finally prepared leather showed tensile strength of 12.2 and 17.4 N/mm^2, tear strength of 56.0 and 62.2 N/mm^2, bursting strength of 366 and 368 Kg/mm and percentage elongation at break of 25 and 30 % respectively (Table 2). The gross evaluation of the finally prepared leather revealed that it had grayish blue color, rough surface, fair stretch ability, too much scud and a normal appearance (Table 3).

Leather processing is one of the most important industrial activities in Pakistan. Depilation of hides is an important and unavoidable step involved in the processing of leather in the tanneries. The conventional lime sulphide method employed for this purpose creates pollution hazards (Chandrasekaran *et al*., 1985). Hence an attempt to minimize the use of lime sulphide in the tanneries and to develop an environment-friendly method of depilation was made in present studies. Complete depilation of the skin was achieved after 4 hrs of treatment and the resulting pelt was of white color with no scud. Mitra & Chakraverty, (1998) have found that optimum time for depilation of goat skin using proteolytic enzymes of microbes was 6 hrs, which is more than present studies.

As for as physical evaluation of leather is concerned, the pelts obtained after treatment with a mixture of enzyme and lime sulphide showed better physical properties as compared to the pelts obtained after conventional treatment. Physical evaluation data showed that all the values of physical parameters were also better than reported by other workers (Malathi & Chakraborty, 1991;). The results were also compared with the commercially used proteases such as Milenzyme® 8X and Novo Unhairing Enzyme®, which brought about complete dehairing after about 6 hrs contact at 37°C having a pH of 8-9. So our results with the enzyme produced were even better than the commercially available enzymes.

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