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Research Article

PRODUCTION OF ALKALINE PROTEASE BY PD4 STRAIN AND ITS APPLICATION IN LEATHER PROCESS

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ABSTRACT

The demand for alkaline proteases in industries is increasing worldwide. The present research describes the production of alkaline protease in a lab scale fermentor from PD4 strain. The production of alkaline protease was enhanced by optimization of cultural condition in a stirred bed reactor (SBR). The alkaline protease so produced was applied to the goat skin for the removal of hair. Dehairing process was considered to be one of the major steps in tannery industries. The chemicals used in this work were replaced by alkaline protease enzymes due to their potentiality for removing the hairs 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. Treatment with enzyme preparation and lime sulphide showed complete depilation of the hide after 24 h of treatment with the resulting pelt of white color having no scud.

Keywords: *Bacillus subtilis,* alkaline protease, submerged fermentation, dehairing activity.

INTRODUCTION

Proteases, one among the three largest groups of industrial enzymes, accounts for about 60% of the total worldwide sale of enzymes from biological sources since they possess almost all characteristics desired for their biotechnological applications (Adinarayana*et al.,* 2003). Production of alkaline protease was carried out generally using submerged fermentation. It is advantageous than other methods due to its consistent enzyme production with defined medium, better process conditions and improved downstream processing (Prakasham*et al*., 2006). Microbial proteases producing industries are always in search of new and cheaper methods to enhance the protease production as well as to decrease the market price of this enzyme (Mukherjee *et al*., 2008). The use of cost effective growth medium for the production of alkaline proteases from an alkalophilic microorganisms is especially important, because these enzyme account for approximately 25% of the world wide enzyme consumption(Gessesse, 1997).

The growth and enzyme production of the organism are strongly influenced by medium components like carbon and nitrogen sources. Besides the nutritional factors the cultural parameters is the primary task in a biological process. So, the media components and cultural conditions are need be optimized (Das and Prasad,2010). Alkaline proteases of microbial origin posses considerable industrial potential due to their biochemical diversity and wide applications in detergents, leather processing, silver recovery, medical purposes, food

processing, feeds, and chemical industries, as well as tannery waste treatment (Vijay *et al.,* 2010).
Dehairing of raw hide is one of the most important steps which define removal of hair, fat and other unnecessary things from raw hide. The most convenient way to achieve perfect dehairing is use of enzyme and specifically protease which dissolve the hair protein without affecting structure of skin. In this process generally alkaline and neutral proteases have been used extensively since last two decades (Madhavi*et al*., 2011) Alkaline proteases with elastolytic and keratinolytic activity are used for dehairing and bating process to obtain a desired grain, softness and tightness of leather in a short time. Protease showing keratinolytic activity from *B. subtilis* has been studied as a potential for replacing sodium sulfide in the dehairing process of leather industry, and also showed the use of protease from *Thermoactinomyces*sp. RM4 for dehairing goat hides. Alkaline proteases with keratinolytic activity have been reported for remarkable dehairing properties (Giongo*et al*., 2007). Improved hide unhairing and liming methods resulted in reducing decomposition of products and sulfide ion pollution by treating it with proteolytic enzyme solution (Padmapriya*et al*., 2012).The present investigation was aimed to enhance the alkaline protease production and its application in leather process.

MATERIALS AND METHODS

Collection of samples

Three different solid samples (leather, food industrial waste and slaughter house waste) were collected in sterile container according to microbiological procedures and shifted to the laboratory for further analysis.

Screening of protease producers

The collected samples were serially diluted and streaked on skin milk agar plates. The plates were incubated for 48h at 37°C and protease producers were selected by observation of zone of hydrolysis around the colonies (Genkal*et al.,* 2006).

Quantitative assay of protease activity

The total protein contents of the samples were determined according to the method described by Lowry's method using Bovine Serum Albumin (BSA) as standard. Enzyme activity was determined using culture supernatant collected by centrifuging culture broth at 10, 000 rpm for 15min. Protease activity was measured by standard assay procedure proposed by Akcan and Uyar, 2011. About 0.5ml of 0.5% casein and 1.25ml of tris buffer (pH-8.0 to 14.0) was added into 0.2ml of each of the culture supernatant separately. Mixture was incubated for 30 min at 37^{0}C. About 3ml of trichloroacetic acid was added and incubated at 40^{0}C for 10 min to form precipitate. The mixture was centrifuged at 10,000rpm for 15min and 0.5ml of supernatant was collected. Reagent containing sodium carbonate, copper sulphate, sodium potassium tartarate was mixed with 1ml of Folin-phenol reagent. The mixture was incubated at dark for 30 minutes to form blue colour. The absorbance was read at 660 nm to determine the optical density of each sample. The obtained OD was extrapolated in the standard graph. The standard curve was obtained for series of known concentrations of bovine serum albumin. From the graph, the amount of protein liberated due to the action of enzyme protease in the supernatant was determined. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg/ml tyrosine under the experimental conditions.
Enzyme activity = OD value X amount of protein released (μg)/ concentration of substrate X
time of incubation X weight of the sample

Inoculum preparation (Olajuyigbe and Ajela, 2005)

The production media was prepared and transferred aseptically to the sterile SBR. Inoculation of the production media was done after three different stages of inoculum development. About 9 ml of 1% of casein broth was inoculated with 1ml (10% inoculum size) of PD_4 culture as initial inoculum. After 24 h incubation the turbidity of the culture was measured and the whole 10ml was transferred to the second stage of inoculum development. The 10ml inoculum volume was transferred to 90ml of 1% casein broth and incubated for 48h. After measuring the optical density of the culture, the whole 100ml was transferred to 900ml of production media.

Fermentation process

The fermentation process for the production of extracellular alkaline protease by *Bacillus subtilis*were carried out in a laboratory scale 3 L batch bioreactor with a working volume of 1 L . After inoculation, fermentation was carried out at 37°C at 200 rpm for 48–72 h. At the end of each fermentation period, the culture broth was centrifuged at 10,000 rpm for 15 min to remove

the cellular debris and the clear supernatant was used for enzyme preparation

Application of protease for dehairing (Madhavi*et al*., 2011)

The crude preparation of alkaline protease was applied to the pieces of raw hide to evaluate the depilating (unhairing) efficiency of the enzyme. 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 10x5cm dimension which were latter treated with the enzyme. The skin pieces were treated with prepared enzyme, 2:7 % Sodium sulphide, lime and enzyme, 3:14 % Sodium sulphide and lime (control). All the treated hides were incubated for 24 hours at 37ºC and the depilating efficiency was compared.

RESULTS AND DISCUSSION

Alkaline proteases has considerable industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, feeds and chemical industries, as well as tannery waste treatment . At present, the largest part of the hydrolytic enzyme market is occupied by the alkali proteases. Extreme environments are important sources for isolation of microorganisms for novel industries and enzymes production. Hence, in this present study the protease producing bacteria were isolated from tannery, food industry; and slaughter house industrial effluent discharge site. The zone of hydrolysis was due to protease enzyme produced by the isolates on Skim milk agar media. Narendraet al., (2012) reported that about 25 organisms were recovered from different fields near to Ravulapalem village, East Godavari district, Andhra Pradesh

Bulk production of *Bacillus subtilis* (PD4) was carried out under controlled cultural conditions in a stirred bed reactor. The selected optimized conditions favourable for the growth of organism and production of protease were made during the fermentation process. At the end of each fermentation period, the culture broth was harvested to remove the cellular debris and the clear supernatant was used for enzyme analysis.

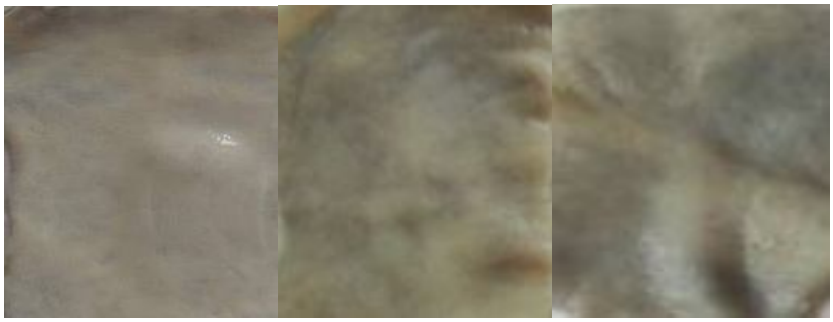
Dehairing activity

The comparative analysis of dehairing ability of purified alkaline protease with other chemically used dehairing agents. After 24 h of treatment with pure enzyme, pelt was completely turned to white in colour whereas chemical treatment of resulted in partial depilation (Table-1, Plate-1and2)

Table 1: Evaluation of the pelt after treatment with alkaline protease

S.No.	Treatment	Sample	Time for depilation (h)	Scud	Pelt colour
1.	Sodium sulphide and lime	Goat skin	24	Too much	black
2.	Sodium sulphide and lime and partially purified enzyme		24	No	white
3.	Pure enzyme		24	little	Pure white

Plate 1: Evaluation of the pelt before dehairing



(A): Treatment-1 (B): Treatment-2 (C): Treatment-3

Plate-2:Evaluation of the pelt after dehairing

Varela *et al.,* (1997) reported that alkaline protease speed up the process of dehairing, because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair in goat hide. The most convenient way to achieve perfect dehairing is use of enzyme and specifically alkaline protease which dissolve the hair protein without affecting its structure. A class of protease that is alkaline proteases has been sued most significantly in last few decades. Yu (2000)reported that alkaline protease based leather dehairing has been considered an environmentally friendly alternative to the conventional chemical process. With this main objective, in the present study alkaline protease harvested as crude enzyme extract from PD4 was used to determine the dehairing process. So the extracted enzyme can be used for tannery industrial applications.

Treatment-1 (Treatment with crude enzyme preparation)

The animal skin pieces were treated with crude enzyme preparation for 24h at 37°C with a shaking speed of 50rpm was recorded. Complete depilation (unhairing) was achieved after 36h of enzyme treatment. In Plate-2(A), the pelt had a little scud and was white in color was noticed. 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. Pelt was of good quality showing white color, smooth surface, good stretching, and no scud and had a normal appearance.

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 24 hrs at 37°C with a shaking speed of 50 rpm. The results of this treatment were even better than the treatment 1 with respect to almost all the parameters. In table 10 the complete depilation of the hide was achieved after 24 hours of treatment with the resulting pelt of white color having no scud. In Plate-2(B), pelt was of bluish grey color, smooth grain surface, good stretch ability, no scud and normal body appearance was presented.

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 24 hours at 37°C with a shaking speed of 50 rpm which is a conventional method of depilation. In table 10 the results of this treatment showed that the depilation was completed in 24 hours. In Plate-2(C), the pelt having much scud and blackish color, rough surface, fair stretch ability, too much scud and a normal appearance was presented. Similar works were also performed by different researchers recently. The researcher's spectral data were compared with the present findings, in order to prove supportive.

Mukhtar and Ulhaq (2008) proved that advantage of using alkaline proteases for dehairing of skin was the reduction of the sulfide contents in the effluent, recovery of the hair/wool which was 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. They reported that 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.

Mane *et al*., (2013) investigated alkaline protease for dehairing skin. The researchers found that 50% reduction of sulphide was observed in the waste water, as well as 40% reduction in the suspended solids level. The researchers concluded that the reduction in sulphide helps for reduction of odors in the final sulphate waste water content. Kamini*et al*., (2005) revealed that enzymatic dehairing made fabric more lengthy, stronger wool, which requires minimum washing afterwards. Hair gets removed along with epidermal layer and this makes the process of hair-loosening easier. The enzymatic dehairing process is a more environmentally friendly process, and studies of its kinetics will help to improve the process.

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