Lime and Sulphide-Free Dehairing of Animal Skin Using Collagenase-Free Alkaline Protease from *Vibrio metschnikovii* NG155

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Abstract The objective of this work was to isolate a microorganism producing alkaline protease that can be used as an ecofriendly alternative to chemicals in dehairing process of leather manufacture. Alkaline protease producing bacterium *Vibrio metschnikovii* NG155 was isolated from soil samples of leather industry. The protease was highly effective in dehairing of goat skin, completely eliminating the use of lime and sulfide. Histological studies of the skin after dehairing showed that the enzyme did not damage the collagen layer and brought good fiber opening. Absence of collagenase activity was confirmed by reacting pure collagen with the enzyme and analyzing it on SDS PAGE, which showed no degradation of collagen. The enzyme was stable in a wide range of pH (7–11) and temperature (10–50 °C), which makes it suitable for industrial application.

Keywords Alkaline protease · *Vibrio metschnikovii* · Dehairing · Leather industry

Introduction

Awareness of environmental problems has increased considerably during recent years. Processing industries cause adverse effects on environment, leather industry being one

such unit. Despite making significant contributions to the economy, the leather industry causes severe environmental pollution because of the use of hazardous chemicals. In leather manufacture, dehairing is an essential step, in which the hair along with epidermis, non-collagenous proteins and other cementing substances are removed from the skin [1]. The conventional dehairing method involves the use of high proportions of lime and sulfide. This process contributes 80–90 % of the total pollution caused by leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime [2]. Nowadays, chemical processes are being replaced by enzyme based processes in number of industries [3–6]. Accordingly, as an alternative to chemical dehairing, enzyme-based dehairing processes using proteases are being developed. Although there are numerous studies on the use of enzymes for dehairing [7, 8], its commercial application in the leather manufacturing is not much prevalent. Tanners are hesitant to use enzymes because of certain disadvantages like stability in industrial conditions such as wide range of pH and temperature, duration of consistent performance and cost of production. Besides, many proteases can remove hair but they usually cause the degradation of collagen at the same time, which gives unacceptable physical properties to the finished leather, thus reducing the leather quality [9]. Therefore, till now, no protease product has been widely accepted for dehairing at industrial level. Consequently, the search is on to isolate/develop novel dehairing enzymes, which have properties suitable for industrial application and enzyme activity is specifically directed towards the epidermis, thereby minimizing the damage to collagen. This work describes isolation of an environmental isolate, *V. metschnikovii* NG155 which produces collagenase-free alkaline protease having potential application in the dehairing process of leather manufacture.

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Materials and Methods

Isolation and Protease Production

Soil and water samples were collected from leather and meat industry and dispersed into sterile saline. For the isolation of alkalophiles, appropriate dilutions of the samples were plated on Horikoshi medium (pH 10) (1 % dextrose, 0.5 % yeast extract, 0.5 % peptone, 0.02 % MgSO_4 and 0.1 % KH_2PO_4) and incubated for 24 h at 37 °C. Protease activity was observed by visualization of clear zone of hydrolysis around the colonies on flooding the plates with 1 N HCl. For protease production 20 ml of Horikoshi medium (pH 10) was inoculated with 1 % inoculum of overnight grown cells. Cell free supernatant was recovered after 24 h incubation at 37 °C by centrifugation ($7,826 \times g$) and used as source of protease enzyme.

Protease Activity

Protease activity was determined by casienolytic method [10]. One unit of protease activity was defined as the amount of enzyme that releases 1 μmol tyrosine per minute under the assay conditions.

Analysis of Collagenase Activity

To determine the collagenase activity of the enzyme, Calf skin collagen (1 mg ml^{-1}) was incubated with 5 U ml^{-1} of NG155 crude enzyme for 24 h at 28 °C. As a positive control, collagen was incubated with collagenase from *Clostridium histolyticum*. Samples were collected at 0 and 24 h of incubation and analyzed on SDS PAGE [11].

Dehairing and Histological Studies

For the dehairing experiment, fresh goat skin was washed and cut into $4 \times 4 \text{ cm}$ pieces and soaked in water for overnight. Enzymatic dehairing was performed by dip method [12] where, pieces of skin were treated with 10 U ml^{-1} enzyme at 37 °C for 18 h. Control experiment was set up using heat inactivated enzyme. Conventional dehairing was performed by paste method [13] in which a paste was made with 10 % w/w lime, 2 % w/w sodium sulphide and 10 % water, and applied on the flesh side of skin. At the end of the process, the skin pieces were gently scraped with fingers to remove loose hairs. The efficiency of dehairing was estimated by depilated area seen at the end of the treatment. The effect of enzymatic dehairing on skin collagen and fiber opening was determined by histological analysis of skin after fixing with formalin and staining with hematoxylin and eosin [14]. The quality of hair removed after depilation was studied by light microscope.

Identification

The selected isolate was examined for morphological, physiological and biochemical characteristics. Furthermore, the isolate was identified by 16S rDNA sequencing. Genomic DNA was isolated and by using consensus primers (forward primer: 5'-AGAGTRTGATCMTYGCTWAC-3' and reverse primer 5'CGYTAMCTTWTACGRCT-3') about 1.5 kb of 16S rDNA fragment was amplified using *Taq* DNA polymerase. Genomic DNA was isolated and amplified using *Taq* DNA polymerase. PCR Conditions were as follows: Initial denaturation at 96 °C for 1 min (25 cycles), denaturation at 96 °C for 10 s, hybridization at 50 °C for 5 s and Elongation at 60 °C for 4 min. The amplified product was sequenced and sequences homologous to isolate were obtained using BLAST. Sequences with high query coverage and homology were selected for phylogenetic analysis. Multiple sequence alignment was done using multiple sequence alignment with hierarchical clustering (MultAlin) version 5 and phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.

Results

Selection of the Isolate on the Basis of Dehairing Ability and Absence of Collagenase

Twenty isolates showing protease production on HK-Casein plates (pH 10) were screened by liquid assay, six isolates showing enzyme activity more than 10 U ml^{-1} and stability in wider range of pH (7–11) and temperature (10–50 °C) were selected. Out of these six, NG155 was selected for further studies as it could efficiently remove hair from animal skin (Fig. 1a). The enzymatically dehaired skin was of good quality showing clean hair pore and clear grain structure whereas chemical treated skin became black due to the use of sulphide and residual hair were also visible in the hair pores. Moreover, the hair recovered after dehairing with protease from NG155, was intact with hair root and was of good quality, which can be a used as saleable byproduct (Fig. 1b). The histological studies of enzyme treated skin showed absence of epidermis, hair follicle, hair shaft and other glandular structures while the collagen layer retained the same morphological structure as the control with fiber opening. Contrarily in the chemical treated skin section, hair follicle with residual hair and other glandular structures were visible (Fig. 1c). The absence of collagenase activity in the crude enzyme from isolate NG155 was further confirmed by treating pure collagen with the crude enzyme and analyzing the products on SDS-PAGE. Even after 24 h of incubation, no

Fig. 1 **a** Dehairing of goat skin: (1) enzyme treated; (2) chemical treated; (3) control skin; **b** enzymatically removed hair showing intact hair with hair root; **c** histological studies of animal skin after dehairing; (1) control (2) chemical treated skin (3) enzyme treated skin (Hematoxylin & Eosin, $\times 175$)

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the products resulting from the treatment of bovine collagen (1 mg ml^{-1}) with enzyme. [Lane1, collagen; lane 2, collagen treated with collagenase from *Clostridium histolyticum* after 24 h; lane 3 and 4, collagen treated with NG155 supernatant after 0 and 24 h respectively]

degradation of collagen by the enzyme was seen (Fig. 2). The production of enzyme from isolate NG 155 was optimized using statistical methods to 200 U ml^{-1} (from the initial 15 U ml^{-1}) using cheap nutritional sources, making the production economically viable. (Data not shown).

Identification

According to the biochemical and physiological characteristics isolate NG155 was found similar to that of genus *Vibrio*. The 16S rDNA gene sequence (GenBank Accession

No. JN837684) analysis revealed that the organism phylogenetically belonged to genus *Vibrio* and was closely related to the type strain *V. metschnikovii* (99 % similarity) (Online Resource 1). The strain has been deposited in the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India (MTCC No. 11407).

Discussion

In the conventional dehairing process of leather manufacture, animal skins are subjected to drastic chemical treatments using lime and sodium sulfide which produces large amounts of hazardous wastes. Currently, as an alternative to chemical dehairing, enzyme based dehairing processes, using proteases to avoid the use of lime and sulfide are being developed because of their environmental benefits. There are numerous studies on the use of enzyme for dehairing but, its commercial application in the leather industry is limited. The important factors for an enzyme to be used as a dehairing agent depend on an efficient dehairing capability and inactivity on collagen [9]. This study describes the production and application of extracellular protease from an environmental isolate *V. metschnikovii* NG155. Some of the other *Vibrio* strains like *V. metschnikovii* RH530, *V. metschnikovii* DL33-51 and *V. metschnikovii* J1 have been known to produce proteases [15–17], with stability in range of pH 8–11 and 30–50 °C, have application in detergents but their application in dehairing has not been explored and have lower enzyme yield when compared to *V. metschnikovii*

NG155. The protease in this study could efficiently remove hair from animal skin with good fiber opening while maintaining the morphological structure of collagen layer, which makes it applicable in dehairing step of leather manufacturing. The inactivity of protease on skin collagen is one of the prerequisite for its application in dehairing. Some proteases with dehairing ability have been reported but they damage the collagen of the grain layer to a certain extent, which can impart unfavorable properties to finished leather [18, 19]. Moreover, as leather manufacturing is a water intensive process and pH and temperature of water varies depending on source, region and season, for the application of an enzyme in leather industry, it should be active and stable in wide range of pH and temperature. Compared to some other similar studies [12, 20], the protease from *V. metschnikovii* NG155 is stable in broad range of pH (7–11) and temperature (10–50 °C) which makes it highly suitable for leather industry. In addition, under the optimized conditions, the high yield of enzyme (200 U ml⁻¹) makes the process more economical. The application of the enzyme at industrial level is being explored in our laboratory.

Conclusion

To conclude, the newly isolated *V. metschnikovii* NG155 produces alkaline protease having good activity in wide range of pH and temperature. The enzyme has good dehairing ability and with the histological studies and analysis on SDS PAGE, it is clear that the enzyme does not damage the collagen layer. All these properties make it highly suitable for dehairing of animal skin in actual industrial conditions and remark the biotechnological potential of this enzyme to be used for dehairing process in leather industry.

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