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Original Research Article

Detergent Compatibility and De-hairing Ability of Protease Produced by *Bacillus subtilis* Strain PS03 Isolated from Tannery Effluent

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Abstract

The present study was aimed to investigate the de-hairing ability of protease produced by bacteria isolated from tannery effluent. The proteolytic bacteria were isolated from the tannery effluent collected near Chennai, TN, India and identified as *Bacillus subtilis* PS03 based on morphological and 16S rRNA analysis. The *B. subtilis* PS03 showed the logarithmic phase after 8 to 24 h and the stable growth phase being till 40 h. The enzyme retained its activity during elevated temperature and pH which elucidated its adaptability to industrial conditions. The protease produced by *B. subtilis* PS03 was found to be biocompatible with leading detergent brands. The enzyme showed promising de-hairing activity when treated with cattle hide for 12 h which can make them to be an alternate to chemical de-hairing techniques. Considering the promising activities, the isolate *B. subtilis* PS03 may be used as a potential candidate for different biotechnological applications.

Keywords

Bacillus subtilis
De-hairing
Detergent compatibility
Protease

Introduction

Proteases are found to be industrially important enzymes. They are produced by various types of microorganism such as, bacteria, fungi and actinomycetes (Gessesse, 1997). The potential of proteases has been revealed in various industries, viz., textile industry, in de-inking, laundry detergents, etc. Most commercial proteases are from fungal origin. Bacteria are considered as potential protease producers in recent days due to their increased growth rate, stability and ability to produce multi-enzyme complexes (Yang et al., 2000). Among various bacteria, *Bacillus* species are reported to produce industrially important proteolytic enzymes. Several *Bacillus* species have been reported to be involved in protease production which include, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, etc. (Johnvesly and Naik, 2001; Santhi, 2014).

The largest application of proteases was found in laundry detergents and tannery industries. In detergent formulations, the enzyme helps in removing protein stains from clothing. In order to be a detergent additive, an enzyme should be be stable in the presence of detergent ingredients such as bleaching agents, surfactants, fabric softeners, etc. (Vijayaraghavan et al., 2014).

De-hairing is one of the most important operations performed in leather processing unit where the hair is removed using conventional chemical methods which lead to various pollution problems by directly affecting the ecosystem. So, it is essential to replace the conventional method by other biological methods such as enzyme assisted de-hairing, an eco-friendly technique which reduces nearly 40% of BOD and 50% of COD in leather processing industry (Senthilvelan et al., 2012). Against this background, the present investigation was aimed to isolate a potential proteolytic bacterium from the tannery effluent. Further, the enzyme production was optimized and their de-hairing property was assessed for the purposes of exploiting its potential in the detergent and leather industry.

Materials and methods

Isolation of proteolytic bacteria

Effluent samples were collected in sterile plastic bags from tannery industry located near Chennai, TN, India. The effluent samples were brought to the laboratory and processed immediately. One milliliter of the sample was diluted with 100 ml of distilled water and homogenized to make the effluent stock solution. The stock solution was gradient diluted and the samples were spread on the surface of a nutrient agar plate containing 1% casein and incubated at 37°C for 24 h (Suganthi et al., 2013).

After incubation, the plates were observed for the appearance of proteolytic zones around the bacterial colonies and the strains showing clearance zone were isolated for further studies. The selected strains were separately inoculated in nutrient broth medium containing 1% casein and incubated at 37°C for 24 h under shaken condition. After incubation, the fermentation extracts of the bacterial strains were subjected to protease enzyme assay for the determination of protease. The bacterial strain possessing high protease activity was chosen and best strain was used in further experiments.

Molecular identification of proteolytic bacteria

The selected proteolytic bacterial strains were morphologically characterized by Gram staining and endospore staining. The identification of the bacterial strains was done by 16S rRNA sequence analysis (Suganthi et al., 2013). The 16S rRNA sequence was amplified from the genomic DNA isolated from the selected proteolytic bacteria by PCR (Biorad Thermocycler) using universal primer pair 27F/1492R. The purified PCR product was sequenced by Chromous, India. The 16S rRNA gene sequence homology analysis was carried out using Basic Local Alignment Search Tool (BLAST) program hosted by National Centre for Biotechnological Information (NCBI).

Enzyme production

For enzyme production studies, protease production medium containing (g/L): galactose 10g, casein 5g, peptone 5g, KH_2PO_4 2g, Na_2CO_3 10g and $MgSO_47H_2O$ 2g, (Pant et al., 2015) was used. The pH of the medium was adjusted to 7.2 and the medium was autoclaved. A 250 ml Erlenmeyer flask containing 100 ml of sterile production medium was inoculated with 1 mL of overnight bacterial suspension and was incubated in an orbital shaker for 72 h. The culture medium was withdrawn at regular time intervals, centrifuged and the cell free supernatant was subjected to proteolytic assay and bacterial growth study.

Protease assay

The proteolytic enzyme activity of the cell free supernatant was assayed using casein as substrate (Pant et al., 2015). The reaction mixture containing 1 mL of 1% (w/v) substrate solution (Casein, pH-7.5) prepared in 50 mM potassium phosphate buffer and 1 ml of appropriate concentration of enzyme solution, was incubated at 37°C. After 10 min of incubation time, the reaction was terminated by adding 2 ml of 10% trichloroacetic acid reagent and incubated for 30 min. The resulting sample was centrifuged for 15 min at 10000 rpm and to the 3 mL of supernatant, 500 mM sodium carbonate solution was added and absorbance was measured at 280 nm. One unit (U) of protease activity towards the casein was defined as 1 μmol of tyrosine equivalent released per minute under the defined assay conditions.

Partial purification of enzymes

Ammonium sulfate precipitation was carried out by adding 60% saturated ammonium sulfate to the cell free supernatant to obtain partially purified protease with constant stirring at room temperature. The crude enzyme was collected by centrifuging the obtained precipitate at 10000 rpm for 20 min. The obtained crude enzyme

precipitate from ammonium sulphate precipitation was dissolved in 10 mM sodium phosphate buffer, and dialyzed overnight using the same buffer with three buffer changes at 8 h intervals. The dialyzed, partially purified, protease enzyme was concentrated by lyophilization and stored at -20°C.

Effect of the temperature and pH in the activity and stability of protease

The effect of temperature on the protease activity was determined by performing the standard assay procedure at varying temperatures. The thermal stability of protease produced by *Bacillus subtilis* PS03 was assayed by determining the enzyme activity after incubation of the enzymes at 30-90°C for 1 h in a constant-temperature using water bath. Similarly, the effect of pH on the activity and stability of the protease enzyme was assayed by determining the enzyme activity after incubation of the enzymes at different pH (6-11) for 1 h. The residual protease activity and stability was estimated quantitatively using spectrophotometer.

Detergent compatibility of protease with commercial detergents

The detergent brands used were Surf®, Power®, Rin®, Ariel® and Tide®. The selected detergent powders were diluted in distilled water at defined concentration (7 mg/mL) and boiled at 100°C for 15 min to deactivate the native enzymes that could be part of the detergents. To the detergent solutions, the partially purified protease enzyme at definite concentration (20 mg/mL) was added and incubated at 37°C for 1 h. After incubation, aliquots were recovered at different time intervals, and the residual protease activity was measured and compared with the control without detergent (Ladeira et al., 2015).

Enzymatic de-hairing

The cattle hide (unbleached skin of cattle) of 5 × 5 cm size was used for the study. Two pieces of cattle hides were selected, one served as control which was incubated in the flask containing distilled water. The other piece was incubated in the flask containing the partially purified protease enzyme solution. The cattle hides were incubated for 24 h at 50°C and the de-hairing extent was assessed by the hair removal, trail with fingers (Mohsen et al., 2013).

Results and discussion

Using casein as the substrate in selective media for preliminary isolation of proteolytic bacteria is a widely accepted method for screening of proteolytic bacteria. However, repeated screening is necessary for the quantitative estimation of the proteolytic activity as the plate-screening methods are not quantitative and also because of the poor correlation between protease activity and the size of the zone of clearance (Okafor and Anosike, 2012). Many proteolytic bacteria have been screened from different sources, such as the bovine rumen, organic waste, soil, etc. Most of the protease producers belong to the species of *Bacillus, Clostridium*, etc. (Kembhavi and Kulkarni, 1993).

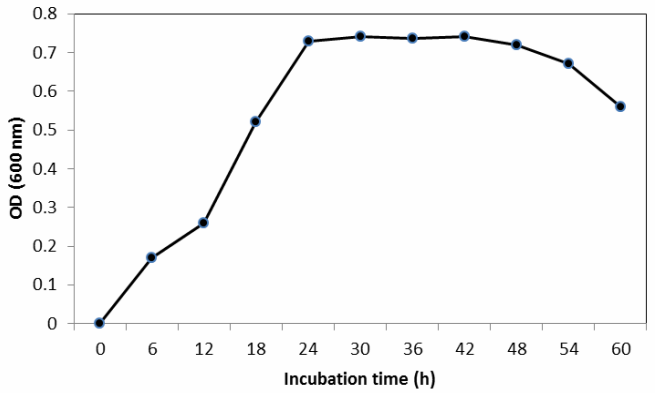
In the present investigation, bacterial strains isolated from the tannery effluent were screened for the protease producing ability. From the tannery effluent, 7 morphologically different bacteria were isolated among which three strains were found to be high protease producers (PS01, PS03, and PS06). The three selected strains were further subjected for quantitative assay using protease production medium. Further, the enzyme activity of the three bacterial strains was quantitatively assayed and the strain PS03 which showed the highest enzyme activity was selected for further research.

Morphological examination of the bacterial strain PS03 showed rough surfaced colonies and microscopic examination confirmed the strain to be rod shaped, Gram-positive and endospore producing bacteria. From the morphological and microscopic observations, it was found that the bacteria belong to *Bacillus* species. Further identification of the bacterial strain PS03 was done by 16S rRNA sequence analysis. The 16S rRNA sequence of the proteolytic bacterial strain PS03 was found to be 1371 bp. BLAST homology analysis revealed the degree of sequence similarity of the strain PS03 to its closely related species. The strain PS03 is related to *Bacillus subtilis* strain NA26 (GenBank Accession No.: JN585723), with a maximum identity of 99%. Thus, the 16S rRNA molecular characterization methods showed that the selected proteolytic bacterium PS03 was a *Bacillus subtilis* strain. The 16S rRNA of the strain PS03 was submitted to GenBank and was acquired with the Accession No. KT160016.

Bacillus subtilis strain PS03 reached its logarithmic phase during 6 - 20 h after the inoculation, beyond which reached a plateau which extended for about 24 h

(Fig. 1). Based on the growth characteristics of the strain PS03, the optimum protease production period for *Bacillus subtilis* strain PS03 was found between 20 - 44 h of incubation. The bacterial enzyme production was found maximum during 24th h of incubation and hence used as optimum time for further production experiments.

Fig. 1: Growth curve of *Bacillus subtilis* PS03.



The strain PS03 grew gradually and the exponential phase was found to occur during the incubation time from 6 to 20 h. Maximum protease activity (4.16 U/mL) was observed after 24 h incubation, beyond which, the protease production was gradually decreased. The protease producing ability of PS03 was found to be consistent with its predicted growth characteristics. The fermentation time of PS03 for the maximum protease yield is shorter than that of the *Bacillus* strains isolated by Kumar and Parrack (2003) and Prakasham et al. (2006). The protease production decreased after 24 h of incubation which might be due to the excessive consumption of nutrients in the medium (Johnvesly and Naik, 2001).

The cell free supernatant from cultures of *Bacillus subtilis* PS03 was used as crude enzyme solution and was subjected to partial purification by ammonium sulfate precipitation followed by dialysis. This dialyzed fraction was lyophilized and stored at -4°C till further analysis. A pH range from 6.0 to 11.0 was used to study the effect of pH on protease enzyme activity. Protease showed optimum activity at pH 8.0. The protease produced by *Bacillus subtilis* PS03 revealed the maximum stability at a pH range between 7.0 and 9.0, where it retained more than 70% of its maximum activities, after incubation at room temperature within this pH range (Fig. 2). The protease from *Bacillus subtilis* PS03 was significantly active in the temperature range tested. Protease activity increased with the increase in temperature from 30°C to 70°C and a reduction in enzyme activities was observed beyond 70°C. The optimum temperature of the protease was also observed at 70°C and remained 100% stable up to 60°C (Fig. 3).

Fig. 2: Effect of pH on enzyme activity and stability.

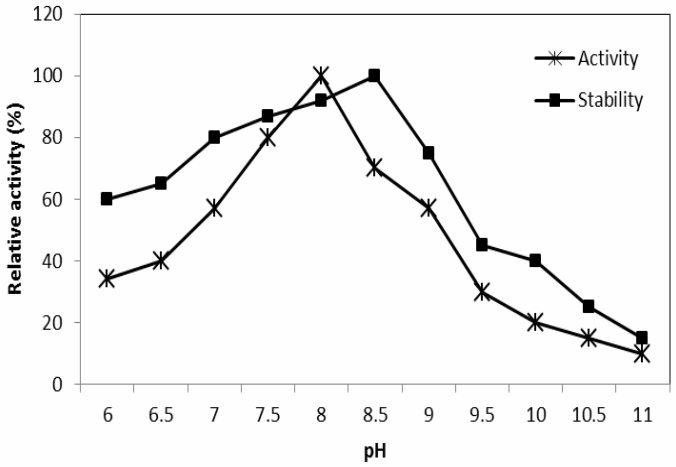


Fig. 3: Effect of temperature on enzyme activity and stability.

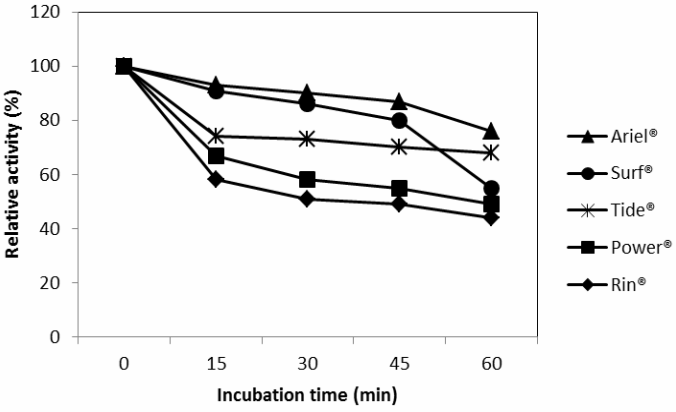
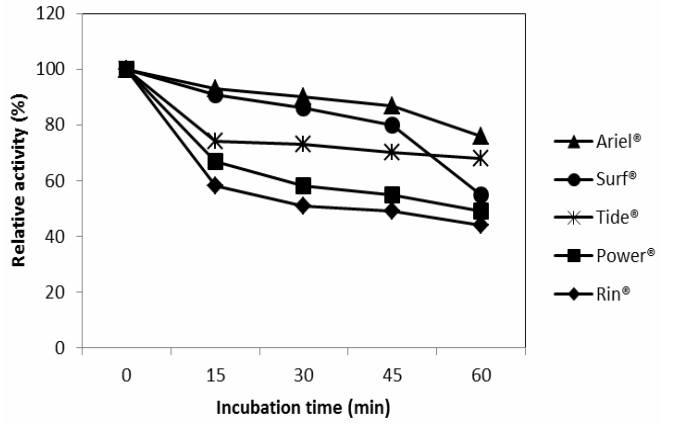


Fig. 4: Biocompatibility of protease from *Bacillus subtilis* PS03 with commercial detergents.



The detergent compatibility testing of the protease produced by *Bacillus subtilis* PS03 revealed that the enzyme retained its activity (>70%) after incubation with the detergent brands Ariel®, Surf® and Tide® (Fig. 4). The relative activity of the enzyme falls below 50% after incubation with the detergent brands Power® and Rin® which might be due to the increased amount of surfactants present in the selected brands (Ladeira et al., 2015). From the results obtained, the protease produced by *Bacillus subtilis* PS03 was found to be compatible with the leading detergent brands, Ariel®, Surf® and Tide®.

To evaluate the potential application of protease produced by *Bacillus subtilis* PS03 in leather industries, its de-hairing potential was evaluated against cattle hides. Under optimal conditions, complete de-hairing was achieved by the enzyme after 12 h of incubation. The results obtained were found to be on par with the available reports. While it took 24 h for de-hairing of cow hide using chemicals (Vijayaraghavan et al., 2014), protease from *Aspergillus flavus* took 20 h to de-hair (Malathi and Chakraborty, 1991) and keratinases from *B. subtilis* required 9 h (Macedo et al., 2005). Thus, protease produced by *Bacillus subtilis* PS03 has a potential to substitute toxic de-hairing chemicals in leather industries and will assist in the production of quality leather products.

Conclusion

In the present investigation, the protease produced by *Bacillus subtilis* PS03 was found to be having applications in detergent and leather industries. The biocompatibility of the enzyme with the leading detergent brands showed that the enzyme can be used as a detergent additive for effective stain removal. De-hairing process using the protease from *B. subtilis* PS03 was found to be an alternative to the chemical methodology. In the current scenario, the enzymatic de-hairing process significantly reduces the toxicity and also in addition it will improve the quality of the leather products.

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