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Industrial Applications of Alkaline Protease with Novel Properties from *Bacillus Cereus* Strain S8

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Abstract

Proteases are the enzymes which catalyze the hydrolysis of peptide bonds in proteins and they are widely used industrial enzymes. The present paper reports the applications of alkaline protease from Bacillus cereus strain S8 in different industrial sectors. The protease was optimally active at pH 10. 0 and temperature 70^{0}C. This enzyme has been used as an alternative for conventional chemical treatments for dehairing of goat skins, chitin recovery from shrimp waste, silk degumming agent and as well as in detergent formulations. Thus, this protease is ecofriendly by reducing the pollution load by replacing conventional chemical methods. The deproteinization activity of Bacillus cereus strain S8 protease was better than many proteases reported due to its thermostability and its activity at alkaline pH. The percentage of protein removal from natural shrimp waste was 87% after 3 h of incubation at 70°C. The protease is effective as silk degumming agent as it promotes sericin hydrolysis with 19% weight loss of treated silk threads. Silk degumming is a very essential step for the removal of sericin protein from silk fibers for giving it shine and softness. The protease is also effective and profitable in dehairing of goat skins by promoting fine leather without affecting its quality as evidenced by Scanning electron micrograph (SEM) micrographs. Due to its unique properties it also compatible with various detergent formulations in industrial sector. These results suggest that the alkaline protease from Bacillus cereus strain S8 is significant in the industry from the prospects of its ability to withstand harsh conditions in various industrial applications.

Keywords: *Bacillus cereus strain S8, Alkaline protease, SEM, Industrial applications.*

1. Introduction

Proteases are the most important class of enzymes, which occupy a pivotal status with respect to their commercial applications (Mohisha *et al*., 2018). They are the most important hydrolytic enzymes from microbes and have been extensively studied with the advent of enzymology (Haki and Rakshit, 2003). The significant study of proteolytic enzymes is not only due to their recognition as important enzymes in cellular metabolic processes but have also gained considerable attention in the industrial community.

Bacillus strains are preferred because of their ability to produce enzymes extracellularly in a short time (Maurer, 2004), in order to produce alkaline protease in a cost-effective manner. Proteases with high activity at different pH values and at high temperatures have novel application potential in pharma, diagnostic, detergent, tannery, amino acid production, contact-lens cleaning agents, effluent treatment, enzymatic debridement, supporting the natural healing process in the skin ulcerations. In addition, their functional and thermal stability of protein chemistry and protein engineering are the most important parameters to be investigated to understand their utility in different sectors.

Derivatives of chitin or chitin itself has great potential by possessing versatile biological activities with excellent biocompatibility and complete biodegradability. Shrimps and crabs are the principal sources of chitin. Conventional chitin recovery methods involve harsh chemical treatments, an alternative approach is eco-friendly processes by the use of proteolytic enzymes (Manni *et al*., 2010). Similarly, leather processing industry is one of the worst offenders of the environment, due to pre-tanning

operations involve quite toxic chemicals. Proteolytic enzymes are more efficient in enzymatic dehairing than amylolytic enzymes (George *et al.,* 1995). Microbial proteases, especially from *Bacillus* sp. are widely used in leather processing. Natural silk (22-25%) is composed of sericin protein that gives harsh and stiff texture to the fibre, but diminishes the shine and whiteness of silk that affects the dyeing process. Enzymatic removal of sericin is an environment friendly process of silk degumming (Arami *et al*., 2007). In finishing of textiles enzymatic treatment has been a focus of interest in recent days for fabric softness and good performance by cheapen the manufacturing process. Today, enzymes have become an integral part of silk finishing process.

Due to these significant roles of proteases in industrial sector, it is desirable to search for new proteases with novel properties. In this investigation, efficacy of the thermostable alkaline protease produced from *Bacillus cereus* strain S8 under optimal conditions was used for application studies in various industries.

2. Materials And Methods

Production of crude alkaline protease

Production of crude alkaline protease from *Bacillus cereus* strain S8 (MTCC NO 11901) was carried out by statistically optimized media supplemented with molasses, 1% (w/v); potassium nitrate, 0.75% (w/v); salt solution, 5% (v/v) – {$MgSO_4.7H_2O$, 0.5% (w/v); KH_2PO_4, 0.5% (w/v)}; $FeSO_4.7H_2O$, 0.01% (w/v) and $CaCO_3$, 0.5% formulated by (Lakshmi and Hemalatha, 2016).The protease is optically active at pH 10.0 and temperature 70ºC with great stability towards pH and temperature with casein as a specific substrate. The enzyme is a serine protease of trypsin type as it is completely inhibited by PMSF and TLCK. The enzyme exhibits a great stability towards organic solvents, oxidizing and bleaching agents and it is negatively influenced by Li^{2+} and Co^{2+} metal ions (Lakshmi *et al*., 2018).

Shrimp waste deproteinization

Shrimp waste (50%, w/v) was minced and cooked at 100°C for 20 min to inactivate endogenous enzymes. Cooked sample was homogenized for 2 min in a Moulinex® blender. Adjust the pH of the mixture to 10.0 and then shrimp waste proteins were digested with crude protease (208 ± 0.89 U/ml). The reaction was stopped after 3 h incubation at 75°C by heating the solution for 20 min at 100°C. Then, the shrimp waste protein hydrolysate was centrifuged for 20 min at 5000g to separate soluble and insoluble fractions. Wash the solid phase with distilled water and dried for 1 h at 60°C. Deproteinization (DP) was expressed as percentage and computed by the following equation as described by Rao *et al*., (2000).

$$\% \text{ DP} = \frac{[(PO \times O) - (PR \times R)] \times 100}{PO \times O}$$

Where PO and PR are protein concentrations (%) before and after hydrolysis; while, O and R represent the mass (g) of original sample and hydrolyzed residue in dry weight basis, respectively.

Silk degumming method

Raw silk threads were dried at 80°C to constant weight and then treated with protease of *Bacillus cereus* strain S8 at 70°C in Glycine-NaOH buffer (pH 10.0) for 1 h. Wash the treated fibres with water and dried at 80°C. Weight difference between treated and untreated silk threads was measured. Structures of treated and untreated fibers were observed under scanning electron microscope.

Weight loss determination

Fabric weight loss was recorded as dried sample weight loss. The drying conditions were 80°C in an air-circulated oven for 1 h. The samples were weighed, after cooling in a desiccator. The following equation was used to calculate the weight loss (wt %):

$$\text{wt \%} = \left(\frac{W1-W2}{W1}\right) x100$$

Where, W1 and W2 are the weights of the fabric before and after treatment, respectively (Kalantzi *et al*., 2008).

Dehairing studies

Dehairing of common salt preserved goat skins procured from the local market were cut into two pieces. One piece was chosen for dehairing using lime-sulphide to serve as control in the study and the other was taken for enzyme application trials. The skins were soaked with three changes of water till they are free from dirt, dung, blood and other contaminating materials and also free from sodium chloride as checked by silver nitrate solution. After soaking, the skins were piled to drain water for about an hour

before the application. Control was applied on the flesh side uniformly with a paste of lime (10%) and 3% sodium sulphide. Other piece was dipped in 50 ml of Glycine-NaOH buffer (pH 10.0) supplemented with protease (206 ± 0.19 U/ml). After application, they left overnight. Next day (after about 18 h), the skins were dehaired manually on a wooden beam using a knife in tune with the commercial practice in the leather industry and the dehairing efficacy was assessed according to the depilated area of the skin at the end of the process and the quality of the dehaired skin was estimated by the naked eye after treatment.

SEM studies

Samples measuring 5×2 mm were cut from an identical location on control and enzyme treated leathers and mounted both vertically and horizontally on aluminum stubs and sputter coated with gold (JEOL, JSM-6610LV). The micrographs of the surface view recorded.

Destaining studies and detergent additive

Application of alkaline protease as a detergent additive was studied on white cotton cloth pieces (4x4 cm) stained with human blood and spicy food material. Effect of five different commercial detergents (Surf excel, Arial, Tide, Rin and wheel) on the enzyme activity was performed under optimal conditions at 5 mg/ml concentration. Based on the result obtained, detergent (Enzyme retained maximum activity) was selected for further study. The stained cloth pieces were taken in separate flasks. The following sets were prepared.

Set-I

(a) Control: stained cloth (human blood)
(b) Stained cloth (human blood) + distilled water (100 ml)
(c) Stained cloth (human blood) + distilled water (100 ml) + 1 ml detergent (5 mg/ml)
(d) Stained cloth (human blood) + distilled water (100 ml) + 1 ml enzyme (186 U/ml)
(e) Stained cloth (human blood) + distilled water (100ml) + 1 ml detergent (5 mg/ml) + 1 ml enzyme solution (suitably diluted).

Set-II

(a) Control: stained cloth (spicy food)
(b) Stained cloth (spicy food) + distilled water (100 ml)
(c) Stained cloth (spicy food) + distilled water (100 ml) + 1 ml detergent (5 mg/ml)
(d) Stained cloth (spicy food) + distilled water (100 ml) + 1 ml enzyme (186 U/ml)
(e) Stained cloth (spicy food) + distilled water (100ml) + 1 ml detergent (5 mg/ml) + 1 ml enzyme solution (suitably diluted).

The above flasks were incubated at 70°C for 15 min. Thereafter, the cloth pieces were taken out, rinsed with water and dried for visual examination. Untreated cloth pieces stained with blood and spicy food material were used as control.

3. Results and Discussion

Shrimp waste de-proteinization

The bioconversion of shrimp waste is attracting biotechnological interest since it might represent an alternative method of waste management method that could be coupled with the production of valuable products. Thus, these waste materials can be converted into economically useful products. The crude protease from *Bacillus cereus* strain S8 was tested for the deproteinization of shrimp waste in order to extract chitin.

Removing proteins from shrimp shell waste constitute a crucial step in the extraction procedure for obtaining chitin. Few studies on the use of proteolytic enzymes for the deproteinization of shrimp wastes have been reported. Many reports have demonstrated the application of proteolytic microorganisms for the deproteinization of marine crustacean wastes to produce chitin (Manni *et al*., 2010). The deproteinization activity of *Bacillus cereus* strain S8 protease was better than many proteases reported in many previous studies. The percentage of protein removal from natural shrimp waste was 87% after 3 h of incubation at 70°C. This deproteinization rate was in agreement with those reported for other microbial proteases for shrimp waste deproteinization. Bustos and Healy (1994), compared the effects of microbial and enzymatic deproteinization. A maximum deproteinization value of 82% was achieved with *Pseudomonas maltophilia* after 6 days of incubation, but no more than 64% deproteinization was achieved by using microbial protease under the same condition. The explanation why 100% deproteinization could not be further increased might be because proteins in the inner layer of squid pen were protected, by the outer layer chitin, from the attack of proteases and thus no further proteolysis

could occur (Wang *et al*., 2006). A maximum of 88% of deproteinization was achieved with a ratio of E/S = 20 U/mg protein by *B. cereus* SV1 proteolytic preparation (Manni *et al*., 2010).

Rim Nasri *et al*., (2011), reported that alkaline proteases from *Z. ophiocephalus*, *R. clavata*, and *S. scrofa* were applied for the deproteinization of shrimp waste to produce chitin and protein hydrolysate. The efficient shrimp waste deproteinization (80%) was shown by *S. scrofa* crude extract and deproteinization with *Z. ophiocephalus* and *R. clavata* crude enzymes was 76%. According to Rayda siala *et al*., (2015), 83% deproteinization rate was observed with the protease produced by *A. arilaitensis* Re117.

Silk degumming agent

Sericin was successfully removed from the silk fibre by the treatment with protease of *Bacillus cereus* strain S8. After the removal of unwanted sericin layer, the silk fibre appeared smoother in SEM micrographs (Fig.1). The treatment with alkaline protease results in the complete and uniform removal of sericin.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the released sericin gave bands of higher molecular weight (180 KDa, 137 KDa) and 71, 51, 10 KDa respectively (Fig. 2).

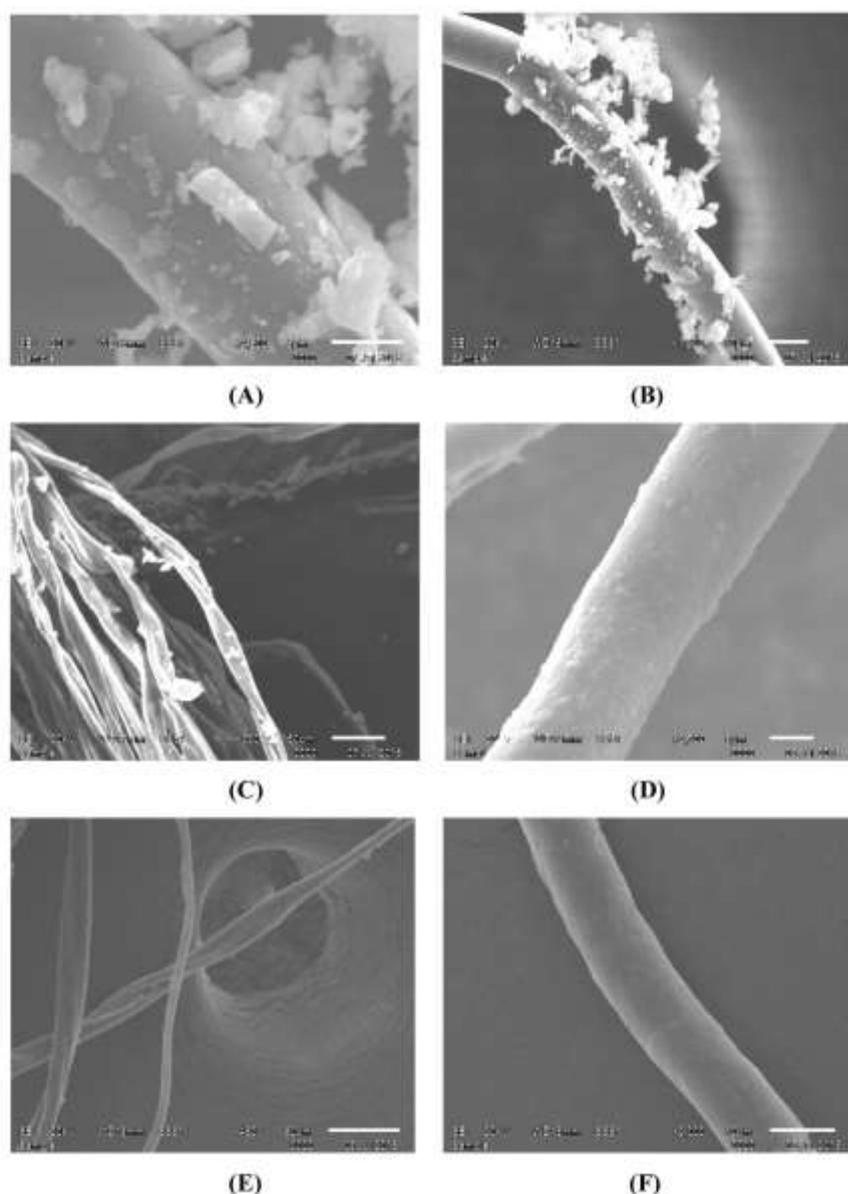

Fig.1. Scanning electron micrographs (SEM) of untreated silk fibre (a-c) and protease treated silk fibre (d-f) at different resolutions.

The weight of the treated silk was reduced from 1.0 to 0.81 g (19% weight loss) as a result of sericin hydrolysis by protease. Silk degumming is a very essential step for the removal of sericin protein from silk fibres for giving it shine and softness. The use of crude proteases for this purpose is cheaper and

eco-friendly. Arami *et al*., (2007) has reported 22.43% loss in the weight of Persian silk on treatment with alcalase, savinase, and mixtures of these enzymes in different ratios. Swati and Satyanarayana (2013), reported that treatment of silk fibres with recombinant alkaline serine protease from a novel bacterium *Bacillus lehensis* achieved 12% weight loss.

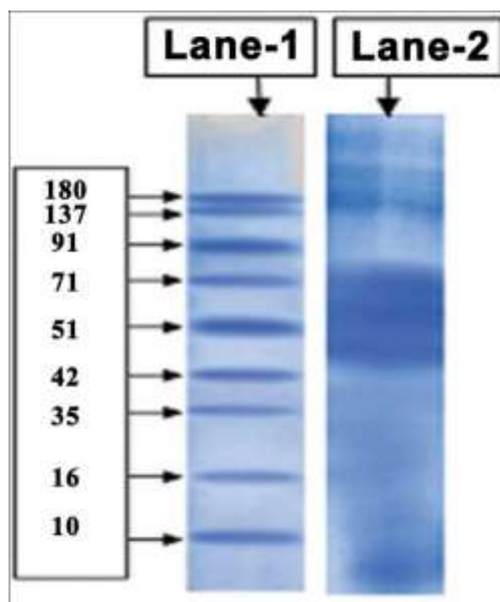

Fig.2. SDS-PAGE profile of the standard recombinant molecular weight markers (Lane-1) and released sericin (Lane-2).

De-hairing of goat skin

Enzymatic dehairing process has been gaining importance as an alternative chemical methodology in present day scenario as this process is significant in reduction of toxicity in addition to improvement of leather quality. There is not much published literature available concerning enzymatic dehairing process. In the leather industry, alkaline conditions are used to facilitate the bulge of hair followed by the subsequent attack of protease, which allows for easy hair removal (Gupta *et al*., 2002). Therefore, efforts have been directed towards developing novel alkaline proteases for dehairing. Some microorganisms producing extracellular enzyme with dehairing activity have been described; *Streptomyces* sp. isolated from soil, degraded human hair, chicken feather, silk, wool, and unhaired goat skin (Mukhopadhyay and Chandra, 1993). In addition, the potential of using strains of *B. subtilis* and *B. amyloliquefaciens* for dehairing purposes has been examined (Nashy *et al*., 2005). Incubation of the crude enzyme preparation with goat skin for dehairing showed that after incubation for 18 h at pH 10.0 and at 37°C, hair was removed very easily from skin. The dehairing function during leather processing is generally carried out at a relatively high pH values ranging from 8.0 to 10.0 (Dayanandan *et al*., 2003). Thus, the protease from *Bacillus cereus* strain S8 could be used for dehairing during the leather process since it displayed high activity at pH 10.0. Similar results were obtained with *Aspergillus tamari* alkaline protease on goat skin after 18-24 h incubation at pH 9.0-11.0 and at 30-37°C (Dayanandan *et al*., 2003). Alkaline proteases with high keratinolytic activity from *B. pumilus*, were also found to dehair bovine hair (kumar *et al*., 2008), cow hides (Wang *et al*., 2007) and goat skins (Huang *et al*., 2003). The obtained results from the study indicate that the crude enzyme preparation could also be used in leather processing.

The dehairing efficacy of the enzyme was assessed in comparison with that of the chemical dehairing system. In the case of the enzyme dehairing, the hair was removed with the epidermis whereas in the case of the chemical method, short hairs were intact in the neck region. The enzyme treated skins were cleaner, whiter compared to the chemically treated skins. The enzyme treated skins lacked in terms of plumping compared to control skins which looked plumpier due to osmotic swelling brought about by lime and sulphide but this can be adjusted in the subsequent processes for the enzyme treated skins (Fig.3).

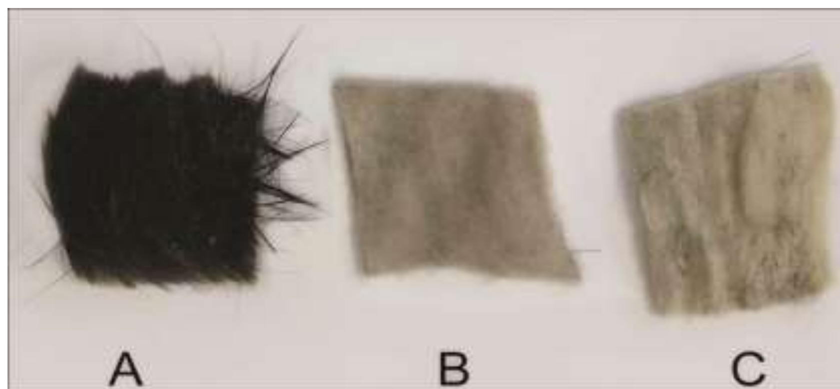

Fig.3. (A). Control (Untreated skin), (B). Enzymatically treated skin, (C). Chemically treated skin.

Scanning electron microscope (SEM) pictures (Fig.4 a-d) depicting the surface characteristics of the leather indicate that there is no damage occurred due to the enzyme treatment and enzyme treatment has brought about some fibre opening at the macro level but the fibre bundles are not split up due to insufficient time of exposure during enzyme treatment. The results from the skin application trials indicate that the enzyme can be profitably employed in place of polluting lime-sulphide method for reducing pollution load in effluent considerably without affecting the quality of the leather produced and indicating its potential application in leather industry for economizing the process. These results were highly significant than the observations made with *B. licheniformis* RP1 (Haddar *et al*., 2011) and *B. circulans* (Subba Rao *et al*., 2008).

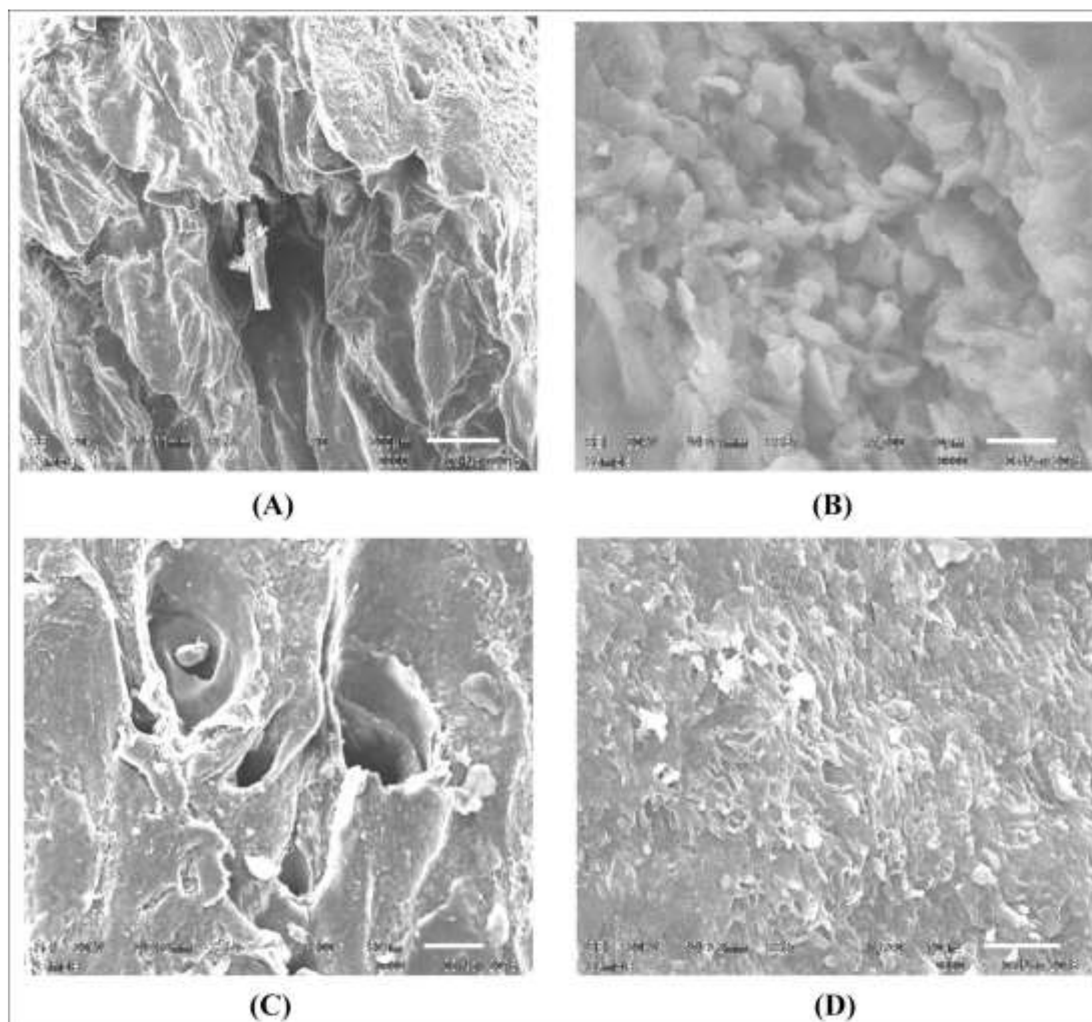

Fig.4. Scanning electron micrographs-surface view: (A) and (B) control skin, (C) and (D) Enzyme treated skin showing no damage to the skin when treated with enzyme.

Protease from *B. cereus* strain AT was found to be effective in leather processing (Zambare *et al*., 2010). To evaluate the effect of alkaline proteases on goat hide, it was incubated with a crude protease sample for 18 h (pH 9.0) at room temperature. The enzyme produced by *B. cereus* strain AT revealed its activity

on goat hides and removed fine hairs. The alkaline protease from this organism effectively dehaired the goat hide within 18 h of incubation.

Washing tests with protease preparation (destaining technique)

From the previous studies it was observed that enzyme was highly active and stable at high pH (12.0) and temperature (70°C) and exhibits stability towards both ionic, non-ionic surfactants like SDS, Tritox-100, Tween-80 etc. These are the interesting and essential characteristic features of the enzyme for its use in detergent formulation (Banik *et al*., 2004) and these optimal parameters of alkaline protease S8 were most efficient than cited in the previous literature. Studies on five different commercially available detergents revealed that the enzyme was highly stable in Tide detergent retaining 94% (Table.1) of its original activity. Hence further destaining studies were continued by using Tide detergent (5mg/ml).

Table .1. Effect of commercial detergents on protease activity.

S. No	Name of the Detergent	Concentration (mg/ml)	Residual activity (%)
1	None	5	100
2	Surf excels	5	91
3	Arial	5	79
4	Tide	5	94
5	Rin	5	88
6	Wheel	5	86

The destaining studies gave good results on the cotton cloths stained with human blood and spicy food materials. The slight removal of stain could be seen with the stained cloths with the detergent (5mg/ml) and enzyme (196 ± 0.71 U/ml) used alone with 15 min of incubation, but the complete removal of stains was observed when 1 ml of crude enzyme was used with detergent as an additive (Fig.5.0 A-B). Similar studies were also carried by Adinarayana *et al.,* (2003), using different isolates of *Bacillus* sp. under submerged cultivation.

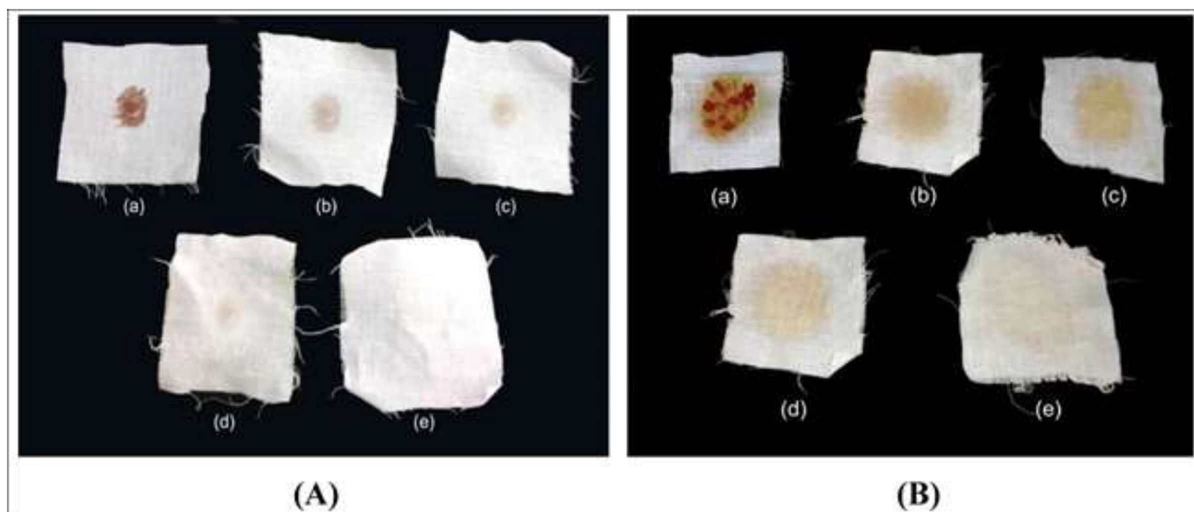

Fig.5. Destaining activity of stained cloth: (A). Destaining activity of blood-stained cloth. (B). Destaining activity of spicy food-stained cloth. *(a-control, b-with water, c-detergent alone, d-protease alone, e-both protease and detergent (tide).*

These results were highly significant than other observation of *B. licheniformis* MP1 (Jellouli *et al*., 2011). MP1 crude protease effectively removed the blood stain from white cloth with 1 h incubation along with 7mg/ml of Axion laundry detergent. Another researcher (Jayashree *et al.,* 2012) reported the thermostable alkaline protease from alkaliphilic strain of *Bacillus pumilus* MCAS 8 which removed stain from fabric after 10 min of incubation at room temperature. Saminathan and Sriman Narayanan (2015) experimentally revealed that alkaline metalloprotease from *Bacillus subtilis* completely removed the blood stain within 30 min with solid detergent (surf excel). The results clearly indicate that preparation of enzyme as a detergent additive significantly enhanced destaining property from cotton cloth and these properties clearly indicated its possible way of use in detergent formulation. The present investigation clearly indicates that the alkaline protease of *Bacillus cereus* strain S8 is a robust enzyme with considerable industrial potential in Shrimp waste deproteinization, in leather industry as dehairing agent as well as in detergent formulation. This study indicates the scopes for utilization of this enzyme in industry after pilot plant scale up studies were conducted.

4. Conclusion

The present investigation clearly indicates that the alkaline protease of Bacillus cereus strain S8 is a robust enzyme with its unique properties like thermostability, halotolerancy, compatibility with various organic solvents, detergents etc., due to these unique features, it overcomes the current difficulties of industrial sector in the area of deproteinization industries, detergents industries, silk processing industries, leather industries etc. This study concludes better scope in enzyme industry in a cost effective and economical way in terms of its commercial scale up process and applications to meet the current scenario of industries.

Future scope

The future scope of industrial applications of alkaline protease with novel properties from Bacillus cereus strain S8 can be promising and diverse. Alkaline proteases have a wide range of applications in various industries, and the discovery of a protease with unique properties can open up new possibilities and also research and development in enzyme engineering and biotechnology may uncover new uses for proteases with unique properties. The successful commercialization of such enzymes may depend on factors like cost-effectiveness, stability, and regulatory compliance. Furthermore, sustainable and environmentally friendly solutions are increasingly valued in various industries, making the discovery of enzymes like alkaline proteases from Bacillus cereus strain S8.

Conflict of Interest: None

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