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Application Studies of Alkaline Protease from Marine *Engyodontium album* BTMF S10 in Detergent Industry and in Silver Recovery from Used X-Ray Films

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ABSTRACT

Alkaline protease isolated from marine fungus *Engyodontium album* BTMFS10 was evaluated for its potential use as an additive in detergent formulations in detergent industry. Commercial detergent compatibility studies conducted with the *E. album* protease indicated that in all the detergents tested, the enzyme retained more than 90% of activity even after 3 h of incubation. Comparison of stability of *E. album* protease and other proteases in the presence of commercial detergent (Surf Excel Automatic) at 60 °C showed that *E. album* protease was significantly more active than the commercially available proteases tested. Additionally, wash performance research supported the efficiency of *E. album* protease in removing blood stains from clothing. Esterase activity of the protease indicated by the cleavage of *p*-nitrophenyl derivatives of small chain fatty acids and was confirmed by activity staining. This fungal protease facilitated stripping of the gelatin layer of the used X-ray film towards recovery of silver. The results of the studies testified the potential of marine fungal protease for application as an addition in detergent compositions, and also in silver recovery from used X-ray films.

Key words: *Engyodontium album*, Alkaline protease, Detergent additive, Silver recovery

INTRODUCTION

Enzyme industry is the second largest industry next to pharmaceuticals in the world market. As biocatalysts, they are widely used in several industries including pharmaceuticals, food and feed, detergent, textiles, paper and pulp, leather, bioremediation etc. Further, enzymes are currently the main focus of biotechnological processes and are involved in all biochemical transformations, from the simple fermentation process to the complicated genetic engineering and molecular biology techniques.

Proteases occupy a pivotal position among the different enzymes owing to their wide range of industrial applications. They constitute one of the most significant group of industrial enzymes, accounting for 40-60 percent of all enzyme sales in various industries, such as detergent, food, pharmaceuticals, leather, diagnostics, waste management and silver recovery (Rai and Mukherjee, 2010). Two-third of the proteases produced commercially are of microbial origin. Detergent alkaline proteases that are active and stable in the alkaline pH range have dominated the enzyme market. In fact, proteases with high activity, stability, and resistance to hydrolysis at high temperatures and alkaline ranges are in high demand for bioengineering and biotechnological applications. Thus, there is an escalation in the demand of proteases with specific and novel properties. This scenario has necessitated exploration of newer sources of proteases by investigators. The adoption of enzymes as detergent additives, in the 1960s, sparked their subsequent commercial growth and substantial increase of basic research in this field. Due to alkaline protease's stability and activity in challenging conditions such high temperatures, pH, and the presence of surfactants or oxidising agents, their use in the detergent industry has grown significantly (Joo *et al*., 2003).

Marine microorganisms are unique in many aspects with respect to their physiology and biosynthesis compared to their terrestrial counterparts. By virtue of their wide spectrum of enzymatic activity, they are capable of catalyzing a variety of biological reactions and contribute to the mineralization process and cycling of elements in various environments in the ecosystem (Chandrasekaran, 1997). Microbial protease is one of the major industrial enzymes and a significant focus of intensive research on a global scale. However, proteases from marine microbes, particularly marine fungi remain yet to be harnessed appropriately. In this context we reported earlier the production of a novel extracellular protease from a marine alkaliphilic and salt-tolerant organism, *Engyodontium album* BTMFS10 by solid state fermentation (Chellappan *et al.*, 2006) and the physicochemical characteristics of this enzyme (Chellappan *et al.*, 2011). High productivity in solid state fermentation; activity and stability in alkaline pH, high temperatures, reducing and oxidizing agents, metal ions; and storage stability indicated potential of *E. album* protease enzyme for various industrial applications. In this communication we report, the scope for potential application of this enzyme as an additive in laundry detergent and in silver recovery from used X-ray films.

MATERIALS AND METHODS

Microorganism and preparation of purified protease

Protease enzyme produced by solid state fermentation (SSF) using *Engyodontium album* BTMF S10, isolated from marine sediment of Cochin coast and available as stock culture at Microbial Technology Laboratory, Department of Biotechnology, Cochin University of Science and Technology, India was used in the study. Protease enzyme produced and purified by ion exchange chromatography as described previously (Chellappan *et al.*, 2006) was used in the present study unless otherwise mentioned. Protease activity measurement (Kunitz, 1947) and protein determination (Lowry, 1951) were performed as described earlier (Chellappan *et al*., 2006). Specific activity of the sample was calculated by dividing the enzyme units with the protein content and was expressed as U/mg protein. Residual activity is the percent enzyme activity of the sample with respect to the activity of the control sample.

Commercial detergent compatibility of enzyme

The most common detergents on the Indian market were used to test the stability of the enzyme in the presence of commercial detergents. The commercial detergents used in the study are presented in **Table 1**. They included Ujala washing powder (Jyothi Laboratories Limited, Mumbai, India), Surf Multi Action with Kids Stain Formula (Hindustan Unilever Limited, Mumbai, India), Surf Excel (Hindustan Unilever Limited, Mumbai, India), Surf Excel Automatic (Hindustan Unilever Limited, Mumbai, India), Ariel Compact (Procter and Gamble, Mumbai, India), Henko Stain Champion (Henkel India Limited), Henko Power Pearls (Henkel India Limited), Tide (Procter and Gamble, Mumbai, India), Rin Shakti (Hindustan Unilever Limited, Mumbai, India), Sunlight Extrabright with colourlock (Hindustan Unilever Limited, Mumbai, India), Wheel (Hindustan Unilever Limited, Mumbai, India), Mr. White (Henkel India Limited), and Speed (Well-made Manufacturing Corporation, Philippines)each at a concentration of 7 mg/ml (w/v); Godrej dish wash liquid (Godrej Consumer Products, Mumbai, India) and Harpic Power toilet cleaner(Reckitt Benckiser India, Limited)each at a concentration of 1% (v/v).

Table 1: Commercial detergents used in the studies: Concentration and pH of different detergent after preparation as solutions

Detergents	Concentration	pH
Ujala washing powder	7 mg/ml (w/v)	9.79
Surf Multiaction	7 mg/ml (w/v)	9.55
Surf Excel	7 mg/ml (w/v)	9.70
Surf Excel Automatic	7 mg/ml (w/v)	9.98
Ariel Compact	7 mg/ml (w/v)	9.92
Henko Stain Champion	7 mg/ml (w/v)	10.12
Henko Power Pearls	7 mg/ml (w/v)	9.73
Tide	7 mg/ml (w/v)	9.89
Rin Shakti	7 mg/ml (w/v)	9.93
Sunlight Extra Bright with Color Lock	7 mg/ml (w/v)	10.00
Wheel	7 mg/ml (w/v)	10.03
Mr.White	7 mg/ml (w/v)	9.90
Speed	7 mg/ml (w/v)	10.02
Godrej dish wash liquid	1% (v/v)	6.72
Harpic toilet cleaner	7 mg/ml (w/v)	2.13

All the detergent powders and solutions were first boiled for 10 min towards heat inactivation of inherent enzymes, if any, already present. Then to 50ml of heat inactivated solution 2 ml of the purified test enzyme sample (285 U/ml) was added and incubated for 3 h at room temperature (RT 28±2 °C). Samples were drawn at intervals of 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h and the residual activities were determined. The enzyme activity of the sample without addition of any detergent was considered as control and enzyme activity in the control was taken as 100% for all computation purposes.

Comparison of performance of E. *album* protease with different commercial proteases in the presence of detergent at 60 °C

A comparative evaluation on the performance of *E. album* protease and commercially available proteases was performed by determining the residual enzyme activity after incubation with

commercial detergent Surf Excel Automatic (7 mg/ml) for 3 h at 60 °C. The commercial proteases used in this study are presented in **Table 2**. They included subtilisin (EC.3.4.21.14, Product No. P5380, Sigma-Aldrich), esperase (EC.3.4.21.62, Novozyme), pronase E (EC.3.4.24.31, Product No. P6911, Sigma-Aldrich), proteinase K (EC.3.4.21.64, Product No. P2308, Sigma-Aldrich), and that obtained from *Bacillus* sp. (Product No. P3111, Sigma-Aldrich), *Bacillus licheniformis* (Product No. P4860, Sigma-Aldrich), *B. amyloliquefaciens* (Product No. P1236, Sigma-Aldrich) and *Aspergillus oryzae* (Product No. P6110, Sigma-Aldrich). Initially, enzymes already present in the detergent solution (taken as 50 ml aliquots) were heat inactivated by boiling for 10 min and then the test enzymes, appropriately diluted, were added and incubated. Samples were drawn at intervals of 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, and 3 h and residual activities were determined. The enzyme activity without any detergent was considered as control and taken as 100% for comparison studies.

Table 2: Commercially available proteases used in the study.

S. No.	Enzyme
1	Subtilisin (EC.3.4.21.14, Product No. P5380, Sigma-Aldrich)
2	Esperase (EC.3.4.21.62, Novozyme)
3	Pronase E (EC.3.4.24.31, Product No. P6911, Sigma-Aldrich)
4	Proteinase K (EC.3.4.21.64, Product No. P2308, Sigma-Aldrich)
5	From *Bacillus* sp. (Product No. P3111, Sigma-Aldrich)
6	From *Bacillus licheniformis* (Product No. P4860, Sigma-Aldrich
7	From *B. amyloliquefaciens* (Product No. P1236, Sigma-Aldrich)
8	From *Aspergillus oryzae* (Product No. P6110, Sigma-Aldrich)

Wash performance studies

Wash performance analysis of purified protease (40-90% ammonium sulfate precipitated enzyme with activity of 480 U/ml) was studied on white cotton cloth pieces (5 x 5 cm) stained with human blood (25 µl). The stained cloth pieces were taken in separate flasks and subjected to the following wash treatment studies.

1. 100 ml distilled water + stained cloth piece
2. 100 ml distilled water + 1ml enzyme solution + stained cloth piece
3. 100 ml heat inactivated detergent (7 mg/ml) + stained cloth piece
4. 100 ml heat inactivated detergent (7 mg/ml) + 1ml enzyme solution + stained cloth piece

The cloth pieces were removed from the water bath shaker after 30 minutes of incubation at 50 °C, rinsed under running water, dried, and visually inspected to determine whether the stains had been effectively removed.

Casein hydrolysis

To study the effectiveness of *E. album* protease towards protein hydrolysate production, 0.2 ml of purified enzyme (285 U/ml) was added to 1 ml of casein (2%) and incubated for 30 min at 40 °C. After 30 min, 2.5 ml of 0.44 M TCA (trichloroacetic acid) was added to coagulate the undigested protein and casein hydrolysis was noticed by visual observation.

Decomposition of gelatin layer of X-ray film

Potential of the enzyme to hydrolyze the gelatin layer of the X-ray film for the recovery of silver was investigated by incubating 2 g of X-ray film (Kodak) in enzyme solution (enzyme purified by 40-90% ammonium sulfate precipitation with activity of 330 U/ml) (Chellappan *et al*., 2006). Following sets were prepared in flasks and studied.

1. 19 ml carbonate-bicarbonate buffer (pH 10) + 1 ml enzyme solution + 2g X-ray film
2. 20 ml carbonate-bicarbonate buffer (pH 10) + 2 g X-ray film
3. 19 ml distilled water + 1 ml enzyme solution + 2 g X-ray film
4. 20 ml distilled water + 2 g X-ray film

The flasks were incubated on a rotary shaker with 120 rpm at room temperature. After being incubated for 90 minutes, the X-ray film was removed, cleaned with tap water, dried, and examined visually. Protein stripped to the supernatant by the action of the enzyme was estimated by the method of Lowry *et al.* (1951).

Esterase activity of the protease enzyme

Esterase activity of purified protease enzyme was determined by measuring the release of *p*-nitrophenol (*p*NP) from different *p*NP derivatives of fatty acids according to modified method of Prim *et al*. (2003) in microtitre plate. *p*NP Acetate (Sigma-Aldrich), *p*NP Butyrate (Sigma-Aldrich), *p*NP Caprylate (Fluka), *p*NP Laurate (Fluka) and

*p*NP Palmitate (Sigma-Aldrich) were used as substrates. An aliquot of 230 µl of buffered substrate [prepared by drop wise addition of 1 ml of solution A (0.15% stock solution of each substrate in Isopropanol i.e. substrates were dissolved in isopropanol and sonicated for 6 minutes in a continuous mode for proper emulsification) to 9 ml of continuously stirred solution of B (50 mM Tris buffer (pH 8.0) containing 0.1% gum arabic and 0.4% Triton X-100] was incubated at 40 °C for 10 min in the ELISA plate reader (Bio-Rad). After adding 20 µl of suitably diluted enzyme solution to the pre-incubated buffered substrate, it was incubated at 40 °C for 30 min. Following incubation, the absorbance at 415 nm was measured and compared to appropriate blanks to determine the amount of released pNP. One unit of activity was defined as the amount of enzyme that released 1 µmol of *p*NP per minute under the assay conditions described.

Activity staining for detection of esterase activity

Esterase activity was further confirmed by activity staining which releases the fluorescent 4-methylumbelliferone (MUF) from 4-methylumbelliferyl butyrate (Prim *et al.,* 2003).For activity staining, SDS-PAGE (Laemmlli, 1970) was conducted using the purified protease and after the run, the gel was soaked for 30 min in 2.5% TritonX-100 at RT, briefly washed in 50 mM Tris buffer (pH 8.0), and immersed in a solution of 100 µM methylumbelliferyl butyrate (diluted with 50 mM Tris buffer from a stock of 25 mM solution in Methyl cellosolve) and incubated for a short period at RT. Under UV light, activity bands (blue colour) were seen. Gel was stained with Coomassie Brilliant Blue R-250 after zymogram analysis to reveal protein bands.

RESULTS AND DISCUSSION

Commercial detergent compatibility of the enzyme

Serine proteases made by *Bacillus* strains are the detergent proteases now in use on the market. Whereas fungal alkaline proteases are advantageous, since they enable simple downstream processing and cell-free enzyme is easily obtained. Marine fungal enzymes are lesser known for their industrial applications and particularly for use as an additive in detergent formulations in detergent industry although enzymes of terrestrial sources are widely used as additives. In this context in the present study protease isolated from marine fungus *E. album* was evaluated for its potential application as an additive in detergent industry. The enzyme was reported earlier to have a pH of 10.0-11.0 as the optimum for maximal activity and is stable over a broad range of pH from 5.0 to 12.0. Further the enzyme was also recorded to be stable, even after 24 h, over a wide range of temperatures from 30 °C to 60 °C (Chellappan *et al.,* 2011). The enzyme's compatibility with commercial detergents was assessed by incubating *E. album* protease in various detergent solutions for a period of 3 h and measuring the residual enzyme activity every 30 min. Result depicted in **Figure 1**, indicated that all of the investigated detergents maintained more than 90% of the enzyme activity even after 3 h at RT, except with Harpic toilet cleaner. In fact, the protease was observed to be 100% compatible with several commercial detergent powders including Surf Excel, Surf Excel Automatic, Ariel, Rin, Wheel, Speed, and Godrej Dish Wash; and no reduction in enzyme activity was recorded with these detergent solutions compared to control. This result can be substantiated with the pH value of each detergent solutions **(Table 1)**, where all the detergents, (except Harpic), had an alkaline or near neutral pH that could have positively supported stability of the enzyme. Loss of enzyme activity in "Harpic" could be attributed to its highly acidity pH (2.13) and strong bleach. Interestingly, it was also noted that the enzyme incubated in "Godrej dish wash" having a pH of 6.72 also retained 100% of its activity even after 3 h of incubation. The pH has an impact on amino acid ionization, which determines the primary and secondary structure of the enzyme and so regulates its activity (Dutta and Dutta, 2005).

Incorporation of enzymes into detergent formulations has been known to have numerous practical problems. Proteases are prone to autolytic destruction, oxidation, and denaturation; these processes are frequently accelerated by the surfactants, bleaches, and water softening builders that are required in laundry detergent formulations. Proteases also facilitate the lytic breakdown of additional detergent enzymes that may be included in formulations. (Crutzen and Douglass, 1999).All currently used detergent-compatible enzymes are typically alkaline, thermostable, and have a high pH (9.0-12.0) and temperature (50 to 70 °C) optima, since the pH and the washing conditions are in that range. The majority of alkaline proteases that are now used in industry come from *Bacillus* species (Gupta *et al.,* 2002).

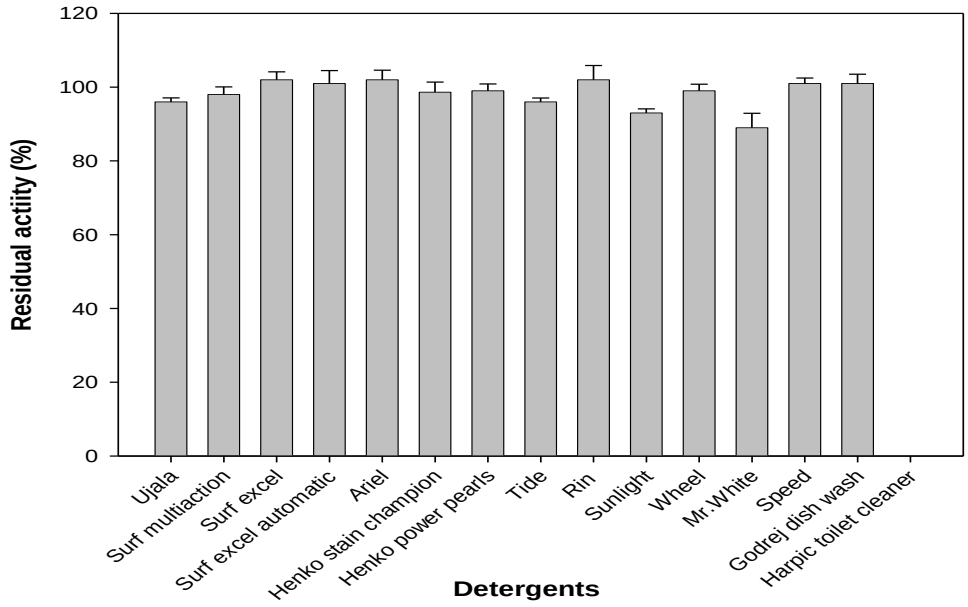

Figure 1: Compatibility of *E. album* protease with commercial detergents.

Even after 1 h of incubation at 40 °C and 50 °C, it was observed that protease made from *B. cereus* maintained more than 80% of its activity in all commercial detergents tested (Banik and Prakash, 2004)**.** Serine alkaline protease from *Bacillus sp*. SSR1 showed nearly 70-80% of activity in most of the detergents tested at 40 °C (Singh *et al*., 2001). Further, protease enzymes were reported to retain increased activity in commercial detergents following the addition of additives $CaCl_2$ and glycine (Banerjee *et al*., 1999, Bhosale *et al*., 1995, Singh *et al*., 2001) and alkaline protease produced by the marine *Streptomyces fungicidicus*MML1614 was observed to show excellent stability and compatibility with standard detergents at 60 °C in the presence of $CaCl_2$ and glycine as stabilizers. Present study indicated that *E. album* protease has more desirable characteristics for use as detergent additive compared to others, since it maintains more than 90% activity for a longer period of time while using laundry detergents and without the addition of any additives.

Comparison of performance of *E. album* protease with different commercial proteases in the presence of detergent at 60 °C

Stability of *E. album* protease and other proteases in the presence of commercial detergent at higher temperature was studied by incubating various proteases in Surf Excel Automatic (7 mg/ml) for 3 h at 60 °C and determining the residual activity at intervals of every 30 min. From the results presented in **Figure 2** for the specific activity determined after 30 minutes of incubation showed that *E. album* protease retained the maximal specific activity. It was observed that commercially known protease-Esperase, retained less specific activity compared to marine *E. album* protease and the protease subtilisin Carlsberg was totally inactive after 30 minutes of incubation at 60 °C. Further it was also noted that in the presence of commercial detergents, except in the case of Esperase, Pronase E and *B. licheniformis* proteases, all the other enzymes were totally inactive after 30 minutes of incubation. The results clearly indicated that marine *E. album* protease has desirable properties for use as additive at par with the other available commercial proteases when incubated with the detergent Surf Excel at high temperature.

Wash performance studies

In laundry detergents, alkaline proteases have a specialized catalytic function in the hydrolysis of protein stains like blood, milk, human perspiration, etc. The protease is now used more frequently as a detergent ingredient since it can clean effectively in non-phosphate, environmentally friendly detergents (Mei and Jiang, 2005; Gouda, 2006). An earlier investigation into the substrate preferences of *E. album* protease revealed that casein and haemoglobin were the two substrates for which the enzyme had the highest affinity (Chellappan *et al*., 2011). Hence, in the present study the wash performance of the protease was assessed in terms of their ability to remove blood stain from white cotton cloth. Visual inspection of the soiled textile pieces after washing proved the *E. album* protease's efficacy in removing stains (**Figure 3**). The results also indicated that both the detergent and the protease enzyme can work together to remove blood stains effectively. It has been observed that *Spilosoma obliqua* protease is effective at removing blood stains from cotton fabric both with and without the use of detergents (Anwar and Saleemuddin, 1997).

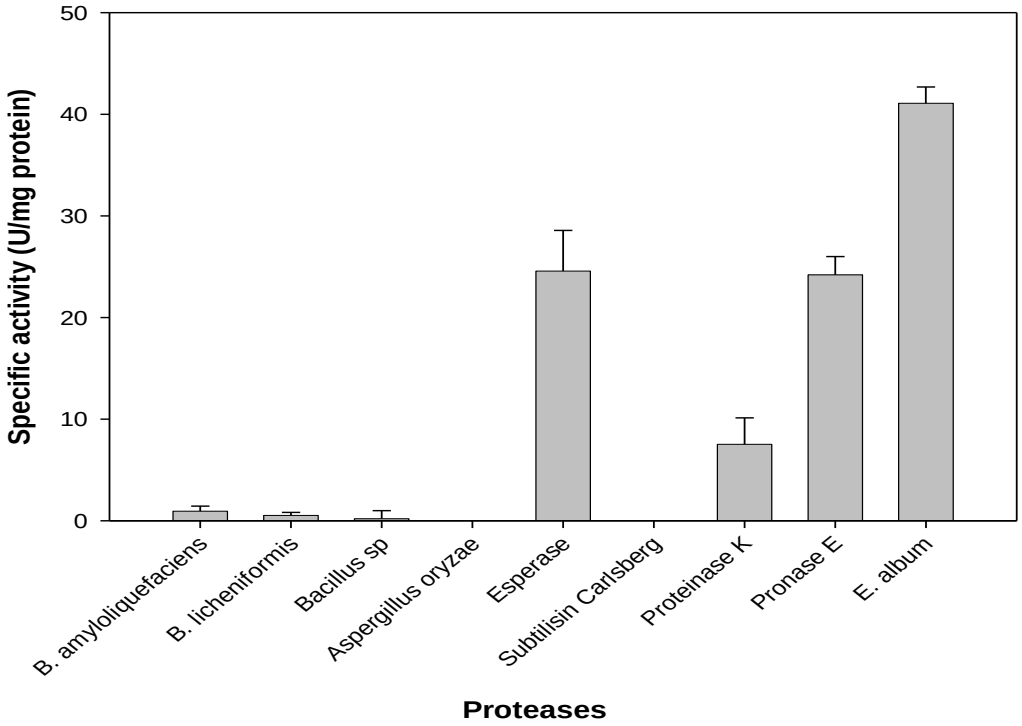

Figure 2: Comparison of *E. album* protease activity with different commercial proteases in the presence of detergent at 60 ºC.

Protease from *Pseudomonas aeruginosa* PD-100 was also reported to efficiently remove blood stains from cotton cloth without the addition of detergent (Najafi *et al*., 2005).Recently proteases from fungi *Graphium putredinis* and *Trichoderma harzianum* were reported to have potential to be used as additives in commercial detergents to improve wash performance (Savitha *et al*., 2011).

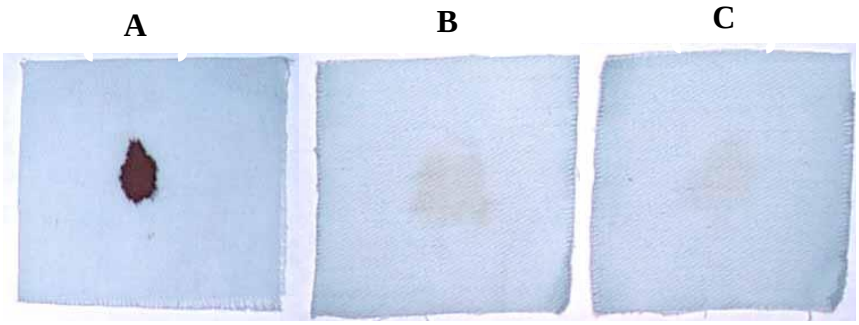

Figure 3 : Wash performance studies using marine *E. album* protease. A, Cotton cloth stained with blood (before washing); B, blood stained cloth washed in commercial detergent; C, blood stained cloth washed with commercial detergent+protease.

Casein hydrolysis

Protein hydrolysis is the primary function of proteases, and this ability has been used to generate protein hydrolysates and peptides of high nutritional value. Alkaline proteases are employed in the generation of hydrolysates with clearly characterized peptide profiles from a variety of natural protein substrates. The commercial protein hydrolysates are made from casein (Miprodan®; Arla Food Ingredients Group, Denmark), whey (Lacprodan®; Arla Food Ingredients Group, Denmark) and soy protein (Proup®; Novo Nordisk, Bagsvaerd, Denmark). The hydrolysates of casein, whey protein, and soy protein are frequently included in formulations of hypoallergenic baby food (Clemente, 2000). Alkaline protease was used by Fujimaki *et al*. (1970) for production of soy protein hydrolysates and by Perea *et al*. (1993) for the production of whey protein hydrolysates. In the present study the results documented in **Figure 4** clearly demonstrated the ability of marine *E. album* protease to hydrolyse casein, which strongly testify the merits of this enzyme for its possible use in protein hydrolysate production and also in the removal of milk protein stains.

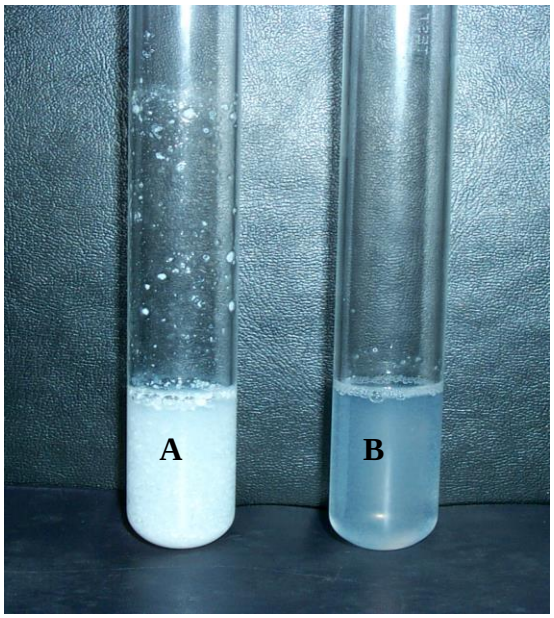

Figure 4: Hydrolysis of casein by *E. album* protease. A, casein alone ; B, casein treated with protease.

Decomposition of gelatin layer of X-ray film

The gelatin layer of X-ray or photographic waste films contains 1.5-2.0% silver by weight, making them a valuable source of silver for a number of uses. Traditionally, silver is extracted from discarded X-ray films by burning them however this practice pollutes the environment. In addition to aiding in the extraction of silver from gelatin by enzymatic hydrolysis, polyester film base can also be recycled and used again. Hence in the present study hydrolytic activity of the marine fungal protease on the gelatin layer of the X-ray film was evaluated by incubating the X-ray film in enzyme solution. Protein content of the supernatant estimated after incubation clearly evidenced the ability of the enzyme to degrade the protein layer of the X-ray film. From the results depicted in **Figure 5A**, it was inferred that the X-ray film incubated in enzyme solution with pH 10.0 yielded higher protein content (13.6 mg/ml) compared to the X-ray film in enzyme solution diluted in distilled water (11.9 mg/ml). In the control (without enzyme), protein content of the supernatant was very less (1.46 mg/ml). The result clearly indicated that the enzyme was able to degrade the protein layer of the X-ray film even in distilled water. The capacity of the enzyme to break down the gelatin layer of the X-ray film was demonstrated by visual examination of the film (**Figure 5B**). The alkaline proteases from *Bacillus* sp. B21-2 (Ishikawa *et al.*, 1993), *Bacillus* sp. B18' (Fujiwara *et al.*, 1991), *B. subtilis* (Fujiwara *et al.*, 1981), and *B. coagulans* PB-77 (Gajju *et al.*, 1996) were reported to be effective at breaking down the gelatinous layer on used X-ray films such that the silver could be retrieved. Results of the present study demonstrated the potential of the marine fungal enzyme for application towards the recovery of silver and recycling and reuse of the films.

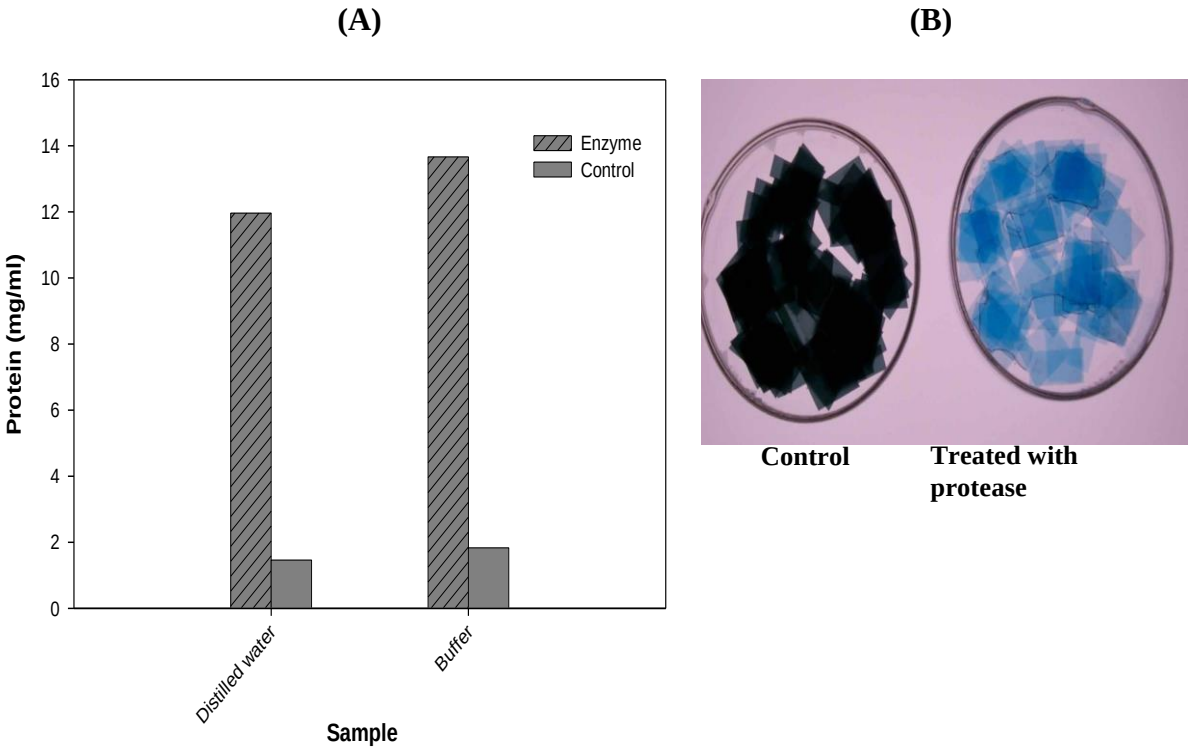

Figure 5: Degradation of gelatin layer of X-ray film for the recovery of silver. A, Protein content of supernatant estimated after the treatment of X-ray film with *E. album* protease ; B, X-ray film before and after treatment with *E. album* protease.

Esterase activity of *E. album* protease

Esterase activity of protease enzyme was determined using different *p*-nitrophenyl derivatives and the data obtained is depicted in **Figure 6A**. Results indicated that the enzyme has a higher affinity towards the short chain fatty acid derivatives like *p*-nitrophenyl butyrate (four carbon containing fatty acid) followed by *p*-nitrophenyl caprylate (eight carbon containing fatty acid). The enzyme was not able to cleave *p*-nitrophenyl laurate and *p*-nitro- phenyl palmitate, which are the ideal substrates for lipase. This observation clearly endorsed the esterase activity of the *E. album* protease. Aqualysin I (a thermophilic alkaline serine protease) was shown to have esterase activity (Matsuzawa *et al*., 1988), while the metalloprotease from *B. thuringiensis* did not show esterase activity (Li and Yousten, 1988). The esterase activity of *E. album* protease was further confirmed by activity staining which released the fluorescent 4-methylumbelliferone (MUF) that appeared as a blue band in the gel (**Figure 6B**). The MUF-butyrate approach has a much higher sensitivity range than conventional zymographic systems, which enables it to detect 1.5 X 10^{-7} units of Pancrealipase® in less than 15 min. The short amount of time needed for activity detection on gels significantly aids in preventing protein diffusion, enabling the most precise estimation of the molecular weight of the protein. This zymographic method has the significant benefit that, following activity detection, the same gels may next be stained with a standard dye to calculate the molecular mass of the active proteins. (Prim *et al*., 2003).In the presence of the majority of the studied organic solvents, such as DMSO, isopropanol, acetonitrile, ethanol, petroleum ether, acetone, and ethyl ether, *E. album* protease maintained a significant amount of activity (Chellappan *et al*., 2011). Organic solvent stability and esterase activity of this enzyme suggest scope for this serine protease to be used in various esterification reactions.

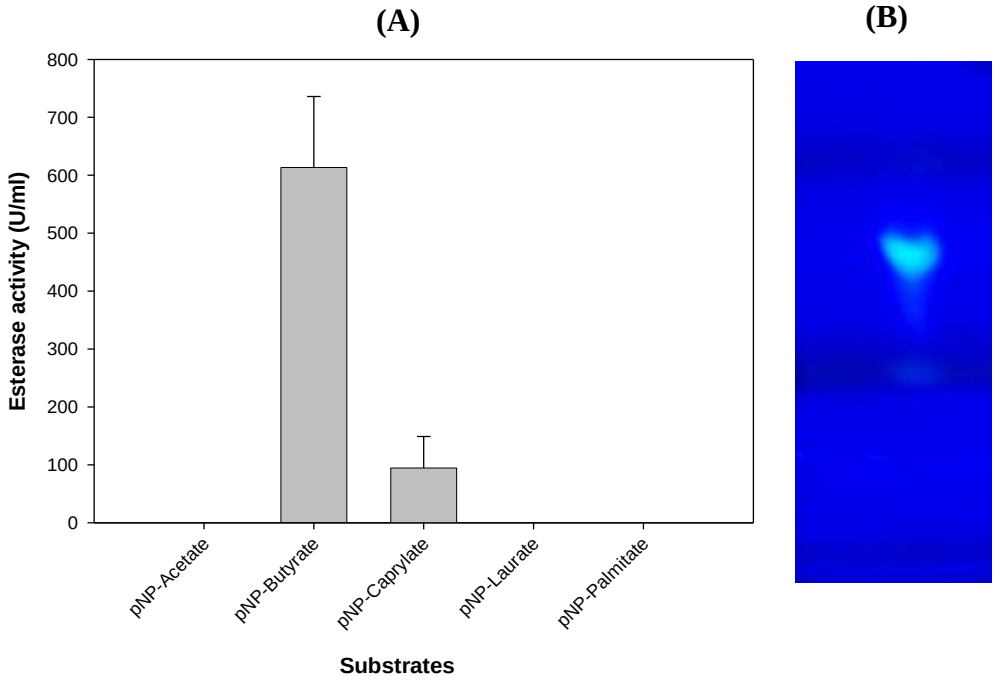

Figure 6: A, Esterase activity of *E. album* protease using different p-NP substrates; B, Activity staining for esterase showing fluorescent band.

CONCLUSION

Activity and stability demonstrated by the marine fungal *E. album* protease in the presence of standard commercial detergents, ability to remove stains, esterase activity and the ability to hydrolyse the gelatin layer of X-ray film for the recovery of silver indicate definite scope for application of this enzyme as an additive in the detergent formulation and for recycling of photographic films. To the best of our knowledge, this is the first report on detergent compatibility of alkaline protease produced by any marine fungus. Further results obtained from the present study add strength to the hypothesis that marine microbes could return many industrial enzymes of potential application. There is ample scope for further investigation on the biochemical and biophysical studies of the marine fungal enzyme, which could lead to development of this enzyme as an ideal biocatalyst for detergent industry besides enriching new scientific knowledge on marine fungal enzymes.

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