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Screening, Production and Industrial Application of Protease Enzyme from Marine Bacteria

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A B S T R A C T

Marine sediment samples were collected from Eastern harbor of Alexandria, Egypt. All bacterial isolates were screened for protease producing microbes. Only one isolate showed maximum proteolytic activity. The isolate was performed and identified as *Bacillus subtilis*. The identification process was carried out according to Bergey's Manual of Determinative Bacteriology. The strain has the ability to tolerate 7%Nacl concentration. The amount of protease produced was expressed in microgram of tyrosine released under standard assay conditions. The total protein content of crude enzyme extracts of *Bacillus subtilis* was quantified which revealed 20.1mg/ml of protein content. The proteolytic bacterium gave an optimum performance was the strain exhibited the enzyme stable at pH7. In the present study *Bacillus subtilis* showed a remarkable activity at 40ºC. Studies pertaining to carbon sources starch were utilized by *Bacillus subtilis* and maximum production was achieved. Among the different nitrogen sources tested yeast extract induced maximum proteolytic activity. The crude enzyme was efficient to remove biological dyes such as crystal violet and saffranin and blood stain by *Bacillus subtilis*.

Introduction

Marine organisms were used to modify products, to improve plants or animals or to develop microorganisms for specific uses (Jha *et al*., 2004). There has been a tremendous interest from researchers to explore marine microorganisms as new source of antibacterial compounds as increasing resistance of pathogen to present antibiotics. Marine microorganisms were proven already to have many beneficial bioactivities such as production of industrial enzymes (Chatellier *et al*., 2000). Bacteria were isolated and cultivated from all possible regions of the earth, on the basis of their habitat, diversity, ecological functions, degree of pathogenicity and biotechnological applications. 70% of the earth's surface is covered by oceans with rich microbial diversity. About 3.6 × 1029 microorganisms were found in marine environments, including subsurface and harbor (Sogin *et al*., 2006). Marine microbes

were now being looked upon as a potential source of various compounds; pharmaceutical, nutritional supplements, agrochemicals, cosmetics and enzymes (Vignesh *et al*., 2011). Proteases were a group of enzymes, whose catalytic function are to, hydrolyse peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $2.9 Billion by 2012. (Jon, 2008).

Proteolytic enzymes were degradative enzymes which catalyze the cleavage of peptide bonds in other proteins. Alkaline proteases, which are referring to proteolytic enzymes which work optimally in alkaline pH are the main enzymes among proteases and constitute 60 to 65% of the global industrial enzyme market (Amoozegara *et al*., 2004). It has wide range of commercial usages in detergents, leather, food and pharmaceutical industries (Deng *et al*., 2010). The genus "Bacillus" is an important source of industrial proteases and were probably the only genera being commercialized for alkaline protease production (Kocher, 2009). Many Bacillus species produce a variety of extracellular and intracellular proteases. Protease constitutes a large and complex group of enzymes that play an important nutritional and regulatory role in nature.

Proteases were (Physiologically) necessary for living organisms, and they are ubiquitous and found in a wide diversity of marine sources. Marine microbial enzymes have unique catalytic properties due to their distinct physiological and metabolic characteristics, efficient nutrient utilization in oligotrophic waters etc. and are also the source of novel biocatalysts like cold adapted enzymes which are economical in terms energy savings (Shanmuga Priya *et al*., 2008).

Materials and Methods

Isolation of Marine Bacteria from Marine Sediments

Marine sediment samples were collected from Eastern harbor of Alexandria, Egypt and screened for protease producing microbes. The collection was performed in plastic containers and transferred to laboratory at room temperature and the process of isolation was initiated immediately.

Screening for Protease Enzyme

The isolated colonies were screened for protease production using skim milk agar medium. All the isolates were streaked on to skim milk agar plates and the plates were incubated for 48 h at 37^0C. The clear zone around the streak of bacteria was evaluated as protease producers.

Characterization of the Effective Protease Producing Isolates

The bacterial isolate with prominent zone of clearance around the streak of bacterial isolate and showing efficient enzyme production was processed for the determination of colony morphology, Gram staining, biochemical tests and enzyme profiles and then identified in accordance with the Bergey's Manual of Determinative Bacteriology.

Identification and Characterization of Isolated Organisms

The protease producing marine bacterium was identified based on morphological and biochemical characterization.

Production of Protease Enzyme

For enzyme production media consisting of

Casein – 2.0%, Dextroses– 1.0%, Peptone - 1.0%, KH2PO4 - 2.0%, NaCl2 – 0.2%, CaCl2 – 0.002% at pH 7.0 was used. Inoculums (OD 660 nm) were developed by growing the isolate in nutrient broth for 24 h. For production of enzyme, 1.0% inoculums was added to 50 ml production medium in 250 ml conical flasks and then incubated at 37℃ for 3 – 4 days. Sample withdrawn at specific time intervals were centrifuged at 5,000 rpm for 20 min and the supernatant has been used as enzyme source for assay.

Quantitative Estimation of Protein

The quantitative estimation of protein was determined by the method Lowry's (Lowry *et al*., 1951) using bovine serum albumin as the standard.

Determination of Molecular Weight of Protein in SDS - PAGE

The molecular weight of the crude enzyme was determined by sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli *et al*., 1970).

Protease Enzyme Assay

Proteolytic activity was carried out according to casein – pholine method culture media was centrifuged at 7200 rpm for 10 min and supernatant was used as enzyme source. However, 1% Casein (in 0.1 M phosphate buffer and pH 7.0) was used as substrate. 1 ml each of enzyme and substrate was incubated at 50℃ for 60 min. The reaction was terminated by adding 3 ml of Trichloroacetic acid (TCA).

One unit of protease activity was defined as the increase of 0.1 unit optical density at 1 h incubation period. It was then centrifuged at 5000 rpm for 15 min. From this, 0.5 M of supernatant was taken, to this 2.5 ml of 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of folin phenol reagent and the absorbance was read at 660 nm using spectrophotometer. The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity was expressed in microgram of tyrosine released by 1 ml of enzyme in 30 minutes at 30℃ on tyrosine equivalent.

Optimization Parameters for Protease Enzyme Production

Effect of Temperature on Enzyme Activity

The influence of different temperature on proteolytic activity of the crude enzyme was determined by holding the reaction mixture at various temperatures (30ºC, 40ºC, 50ºC, 60ºC, and 70℃) for 30 min during standard enzymes assay.

Effect of pH on Enzyme Activity

To optimize the pH of enzyme activity, 1 ml of enzyme solution was reacted with 5 ml of casein solution (1% (w/v)) in various pH values (4, 5, 6, 7, 8, 9, and 10) at 37℃ for 30 min and the enzyme activity was assayed.

Effect of Nitrogen Sources on Enzyme Production

To optimize the nitrogen source for enzyme production, five different nitrogen source (1%) (Urea, Gelatin, Casein, Ammonium chloride and Yeast extract) were added to nutrient broth and the organism was inoculated and incubated for 48 h at 37℃. The enzyme activity was assay in the culture supernatant.

Effect of Carbon Sources on Enzyme Production

To find the optimum carbon source for enzyme production, five carbon source (1%) (Xylose, Glucose, Starch, Lactose and Sucrose) were supplemented to nutrient broth the organism was inoculated and incubated for 48 h at 37^0C and the enzyme activity was assayed in the culture supernatant.

Application of Protease Enzyme: Wash Performance Test

Stability of the protease in commercial detergents was tested by incubating measured quantity of the enzyme (500µl) with the solutions of the different commercial detergent concentration of 7 mg/ml (to simulate washing conditions) for 1h. The detergents tested were Ariel, Tide and Persil, which are widely used in Egypt. Suitable aliquots were drawn at different time intervals (at15, 30 and 60 min), for 1h, and their activity was measured by standard assay procedure and compared with the control (incubated under similar conditions, without any detergent) and the relative activity was expressed in % taking the value given by control as100%. Application of protease as a detergent additive was studied on white cotton cloth pieces (4X5cm) stained with crystal violet (0.1%), saffranin (0.1%) and blood stain.

The stained cloth pieces were taken in separate flasks. The following sets were prepared:

a. Flask with distilled water (100 ml) + 1ml stained cloth (crystal violet (0.1%) saffranin (0.1%) and blood).

b. Flask with distilled water (100ml) +1ml detergent (7 mg/ml).

c. Flask with distilled water (100ml) + 1ml detergent (7 mg/ml) + 2ml enzyme solution.

Results and Discussion

Isolation and Screening of Protease Producing Microorganisms

In the present study, different marine sediment samples were collected from Eastern harbor of Alexandria, Egypt. The samples were serially diluted, spread plated and incubated at 37^0 C for 48 h. About four dominant morphologically distinct colonies were selected and pure cultured by repeated streaking on the nutrient agar plates. The four isolated bacterial strains were screened for protease producing ability on skim milk agar. The zone formation around the streak of bacterial growth was identified as the positive protease producers which may be due to hydrolysis of casein. Among the four isolates screened, only one isolate showed a proteolytic activity with a zone of clearance around the streak of the isolate (Figure 1). Therefore, this efficient protease producing strain was selected for further experimental studies and biochemical tests.

Identification of the Efficient Marine Protease Producing Strain

Morphological (Table1) and biochemical characterization were performed in accordance was Bergey's manual of determinative bacteriology methods. It was identified as *Bacillus subtilis*.

Salt Tolerance of the Isolate

The salt tolerance of the isolate was studied at various NaCl concentrations such as 3% NaCl, 4%NaCl, 5%NaCl, 6%NaCl, and 7% NaCl. The results were shown in (Figure 2). It was found that the tested strain has the ability to tolerate up to 7% NaCl concentration.

Protease Enzyme Assay

Crude enzyme extract was studied for protein degrading activity and the amount of amino acid produced was measured by using Folin phenol reagent and the absorbance was read at 660 nm using colorimeter. The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions. Based on the tyrosine released the protease activity was expressed in microgram of tyrosine released by 1ml of enzyme in 30 min at 30˚C on tyrosine equivalent.

Determination of Total Protein Content

The total protein content of crude enzyme extracts from *Bacillus subtilis* was quantified by Lowry *et al*., method with BSA standard. It was found out that *Bacillus subtilis* revealed 20.1mg/ml.

Determination of Molecular Weight by SDS - PAGE

The molecular weight of purified enzyme was determined by SDS - PAGE. The crude protease enzyme was concentrated by centrifugation and approximately 90-100% of proteolytic activity was observed. Determination of molecular weight by SDS-PAGE for the separated fractions of the protease enzyme exhibited a predominant peak (band) of 28 kDa which indicate that protease have high activity and this lyse within the molecular weight range of standard marker between 14-160kDa protein marker (Figure3).

Effect of Temperature on Protease Enzyme Activity

Temperature also played an important role in activating and inactivation of enzymes. In the present study, the effect of temperature on protease production was studied with various temperatures ranging from 30 - 70℃. The protease activity is relatively stable in the temperature range 40℃ for *Bacillus subtilis* (0.232 U/ml).).The enzyme activity was found to be decreased above and below those temperatures for both strains. The present investigation showed that *Bacillus subtilis* produced maximum protease at 40ºC. The obtained results were noted in (Figure 4).

Effect of pH on Protease Enzyme Activity

pH played an important role in the enzyme production. In the present study the pH optima of protease activity was studied at different pH ranging from 4 to 10. The highest protease production was observed in pH 7 for *Bacillus subtilis* with 0.174 U/ml. Below and above that pH the enzyme activity was found to be decreased. The obtained results were presented in (Figure 5).

Effect of Nitrogen Sources On Protease Activity

In the present investigation, urea, gelatin, casein, ammonium chloride and yeast extract were added in the medium for determining protease activity. Among the different nitrogen sources tested maximum protease production was observed at yeast extract with 0.213 U/ml for *Bacillus subtilis*. The addition of other nitrogen sources caused a drastic reduction in enzyme activity. These results were noted in (Figure 6).

Effect of Carbon Sources On Protease Activity

The effect of different carbon sources viz. Xylose, Glucose, Starch, Lactose and Sucrose on enzyme activity was studied.

Maximum protease production was observed in *Bacillus subtilis* when starch (0.270 U/ml) was supplemented as carbon source. The least enzyme activity was observed in Lactose (0.157 U/ml). The results were presented in (Figure 7).

Application of the Protease Enzymes in Stain Removal

In order to find out the dye removal efficacy of the dyes and strain, a white cotton cloth (4 x 5 cm) was stained with crystal violet (0.1%), saffranin (0.1%) and dried in hot air oven for 2 h. Then the stained cloth was incubated at 50^{o}C in enzyme broth for different time intervals. The enzyme was able to remove crystal violet (0.1%), saffranin (0.1%) from the test fabric cloth after 2 hours at 50^{o}C. These results were noted in (Figure). The enzyme was also efficient to remove blood stain within 30 minutes. It was seen that protease produced by *Bacillus subtilis* had high capability of removing the dye and blood stain from fabric cloth, which indicated its potential in detergent industries. The obtained results were noted in (Figure 8)

From the present study it is revealed that the protease enzyme produced by *Bacillus subtilis* showed a positive result in the removal of dye and blood stain from the cloth. Therefore this enzyme was used in the industrial applications in pilot scale.

Proteases from microbial sources are preferred than the enzymes obtained from plant and animal sources since they possess almost all characteristics desired for their biotechnological applications. Proteolytic bacteria are wide spread in nature and are able to grow under various growth conditions, such as different temperatures, pH and ionic strength (Sanchez-Porro *et al*., 2003). The bacteria that exist in the marine samples represent a very important and diversified enzymatic potential. But sufficient information is not much available on their role in industrial and hence the present study was carried to isolate and characterize the potent protease positive bacteria from the marine sediment sample of Eastern Alexandria Harbor (Egypt).It has been reported that the production of extracellular proteases by different microorganisms can be strongly influenced by the culture conditions. So, it becomes necessary to understand the natural proteases and their catalytic potentiality under different conditions. (Johnvely *et al*., 2002). In this experiment, one bacterial isolate from the four bacterial isolates was able to produce proteolytic enzymes by formation of clear zone on skim milk around the peak of bacterial as indication of good growth with the ability of protease production (Boominadhan *et al*., 2009). Determination of molecular weight by SDS-PAGE for the separated fractions of the protease enzyme exhibited a predominant peak (band) of 28 kDa which indicate that protease have high activity and this lyse within the molecular weight range of standard marker between 14-160kDa protein marker. Our results are in total agreement with the work of which emphasized that protease fractions of appeared as a single band just below 28 kDa isolated from B.species. It is concluded that B. subtilis capable of producing proteolytic enzyme giving optimum performance and serves to be an ideal potential candidate for industrial applications. The enzyme was considerably stable at pH 7 for the strain, but below and above that pH the enzyme stability gradually decreased. Similar results were reported by (Abu Sayem Alam *et al*., 2006). The present observations disagreed with the results of in which they reported that Bacillus sp was highly active and stable at pH 9-10 (Abou Elalla *et al*., 2009) reported that optimum

protease activity and stability was recorded at pH 9-9.5. Temperature plays an important role in inactivation and activation of enzymes. Each enzyme has an optimum temperature for maximum enzyme activity. In the present study, *Bacillus subtilis* showed maximum activity at 40oC. Our results coincide with the work of in which he demonstrated that at temperatures of 60 and 70oC, the enzyme lost its activity rapidly whereas the optimum temperature were found to be 40oC.

Table.1 Morphological and Physiological Characterization of the Efficient Protease Producing Isolate

Tests	*Bacillus subtilis*
Colony morphology	On nutrient agar, it produced creamy white, irregular undulate colonies.
Morphological characteristics. a) Gram staining b) Spore staining	Positive rod Endospore forming

Fig.1 Screening of marine bacterial isolates for proteolytic activity in skim milk agar

Fig.2 The tested strain has the ability to tolerate 6% NaCl concentration. Protein profile of the crude protease of *Bacillus subtilis* in SDS-PAGE

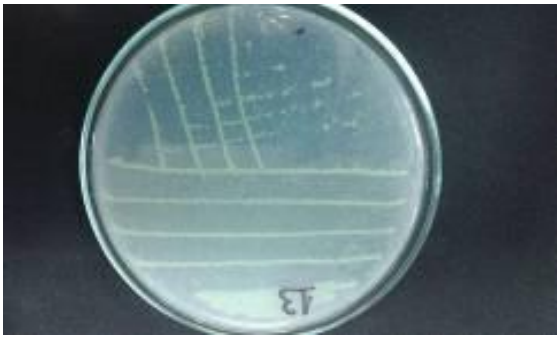


Fig.3 Protein profile of the crude protease of *Bacillus subtilis* in SDS-PAGE
Lane1: Marker protein and Lane 2&3: *Bacillus subtilis* crude protease (28 kDa)

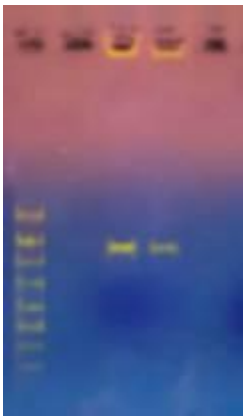


Fig.4 Effect of pH on Protease Enzyme Activity

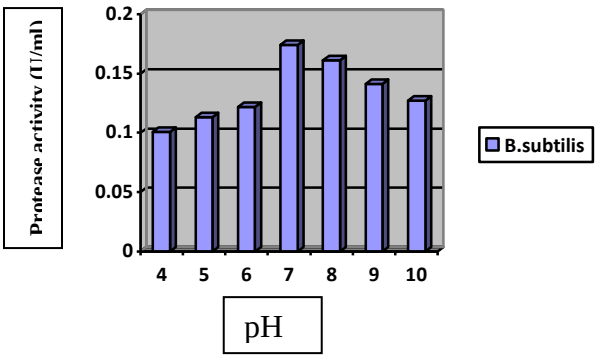


Fig.5 Effect of temperature on Protease Enzyme Activity

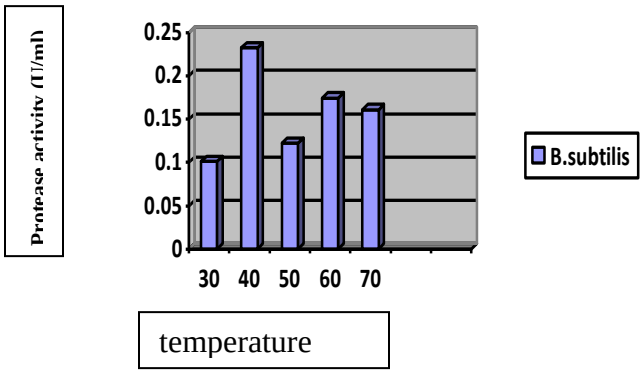


Fig.6 Effect of Carbon Source on Protease Enzyme Activity

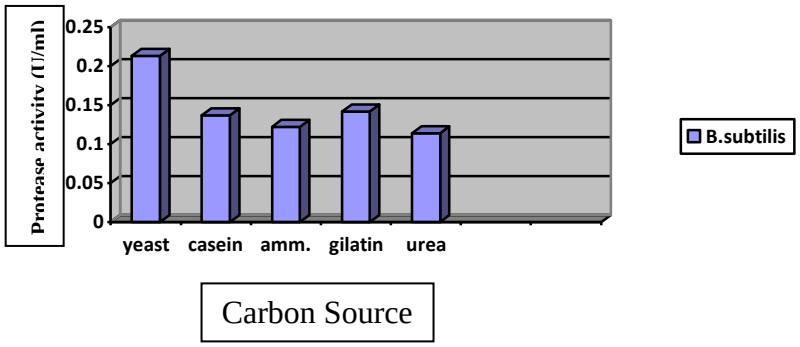


Fig.7 Effect of Nitrogen Source on Protease Enzyme Activity

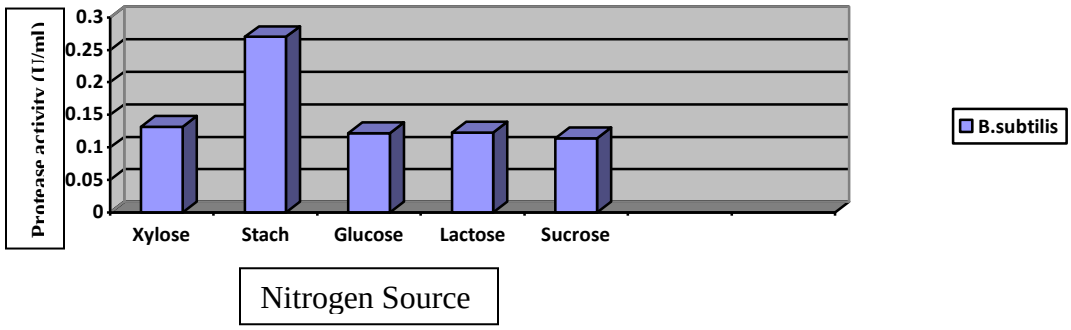
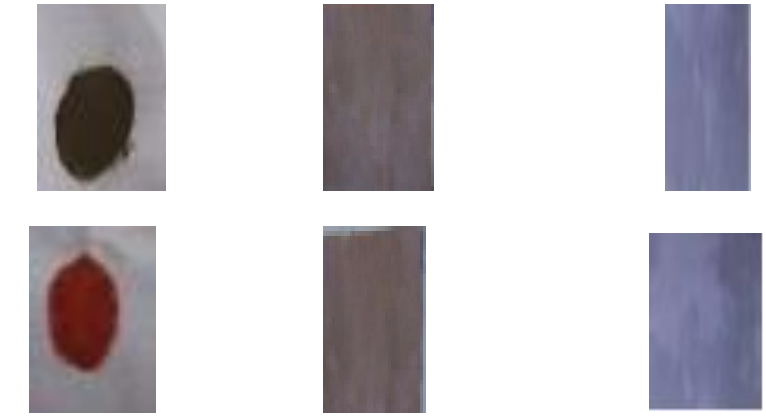


Fig.8 Effect of *Bacillus subtilis* protease on crystal violet dye and blood stain Removal at 500C



The results of the present study agreed with the mentioned results of (Nehra *et al*., 2002). The addition of carbon sources in the medium influence the production of protease enzyme. Maximum production was observed in starch for B.subtilis. It is worthy to note that except starch, the addition of other carbon sources in the production medium led to the remarkable decreases in protease production. These results are in accordance with the finding of (Srinivas Naik *et al*., 2013). The nitrogen sources are the secondary energy sources for microorganisms which play an important role in the growth of the organisms and enzyme production. In the presence study, the extra addition of yeast extract to the medium induced the proteolytic activity. The other sources showed no remarkable increase or depressive effective on protease production. Similar work was carried out by (Feroz Khan, 2013).

In laundry detergent, the use of enzymes such as proteases in very common about 50% of liquid detergent, 25% of powder detergent had almost all powder bleach additives now contain enzymes to help in breaking down of stains that are otherwise hard to remove with conventional surfactants alone. They work as scissors to cut off the stain physand piece by piece from the surface of the fabrics (Panagal Mani *et al*., 2012). The efficiency of B.subtilis protease was tested for removing the blood stain and crystal violet (0.1%), saffranin (0.1%) dye from test fabrics at 50°C with enzymes and 1% V/V of 15mg/ml detergents (Tide). The enzyme was efficient to remove blood stain within 30minutes. The enzyme was also able to remove dye within 2h and also showed computability with the detergents the protease enzymes were also used as an additive detergent to check the contribution of the enzymes in improving the washing performance of the detergents. The supplementation of the enzyme preparation in Tide could significant improving & cleansing performance towards proteinase stain (blood stain & dye).

In conclusion, proteases are important enzymes obtained from marine microorganisms which synthesis bioactive compounds. The bacterial strain isolated from marine environment where identified as *Bacillus subtilis*. Screening was performed and maximum proteolytic activity was revealed. *Bacillus subtilis* has tolerated up to 7% NaCl concentration. Whereas optimization of pH revealed that *Bacillus subtilis* exhibited to increase protease production at pH7. Different carbon sources tested starch was found suitable for protease. Similarly supplementation of nitrogen sources was found favorable for *Bacillus subtilis* utilized yeast extract as nitrogen source. Molecular analysis of the crude enzyme showed prominent band with the molecular weight of 28Kda. The role of protease enzyme in wash performance using detergent was found to hydrolysed large protein molecules associated with hard dyes and stain. Hence it is imperative to use the crude protease enzyme for stain removal.

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