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Full Length Research Paper

Alkaline protease production by alkaliphilic marine bacteria isolated from Marsa-Matrouh (Egypt) with special emphasis on *Bacillus cereus* purified protease

Gehan M. Abou-Elela[1]*, Hassan A. H. Ibrahim[1], Sahar W. Hassan[1], Hanan Abd-Elnaby[1] and Nabil M. K. El-Toukhy[2]

[1]National Institute of Oceanography and Fisheries, Microbiology Laboratory Environmental Division, Alanfushy Qayt bay, Alexandria, Egypt.
[2]Protein Research Department, Mubarak City for Scientific Research and Technology Applications, New Borg El-Arab, Alexandria, Egypt.

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Among the eighteen (18) alkaliphilic marine bacterial isolates studied in ten sampling sites in Marsa-Matrouh beaches, the highest alkaliphilic and proteolytic activities were detected in *Bacillus cereus*. Alkaline protease from *B. cereus* was purified by ammonium sulfate precipitation and Sephadex G-200. The molecular mass determined using SDS-PAGE, was nearly 31.0 39 kDa. Some fundamental properties like effects of different temperatures, pH, metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Pb^{3+}, Mn^{2+} and Cd^{2+}) and ethylene diamine tetraacetic acid (EDTA) on protease activity were also studied. Maximum activities were obtained at pH 10, 50°C and only Cu^{2+} ions enhanced the relative enzyme activity up to 112%. The application of alkaline protease for the removal of blood stains from cotton cloth indicates its potential use in detergent formulations. *B. cereus* protease showed excellent stability in the presence of locally available detergents and retained about 60% of its activity with most of them even after 3 h of incubation at temperature of 50°C.

Key words: Protease producing bacteria, detergents, *Bacillus cereus*, purification, enzyme stability and activity.

INTRODUCTION

With increasing emphasis on environmental protection, the use of microbial enzymes particularly from extremephiles has gained considerable attention during the last several years in many industries, including manufacturing of chemicals, textiles; pharmaceuticals, paper, food and agriculture chemicals (Mehta et al., 2006). Alkaliphiles are reported to be a rich source of alkaline active enzymes, for example, amylase, protease, cellulase, xylanase and other enzymes that have numerous applications in many industrial processes due to an interest in their physiological adaptation to high pH (Oskouise et al., 2008).

Proteolytic enzymes are 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., 2007). Moreover, they are used in the food industry in meat tenderization processes, peptide synthesis, infant formula preparations, baking and brewing. They are also used in the detergent industry as additives, in pharmaceutical and medical diagnosis as well as in the textile industry in the process of dehairing and leather processing (Tari et al., 2006; Bhaskar et al., 2007; Dodia et al., 2008).

This study deals with the purification and characterization of alkaline protease from *Bacillus cereus* of Marsa-Matrouh beaches.

*Corresponding author. E-mail: Gege_ta2010@yahoo.com. Tel: 0020128636344.

MATERIALS AND METHODS

Sampling and counting of heterotrophic marine bacteria

Water samples were collected in 500 ml sterile screw-caped bottles as previously described by Austin (1988) from ten different sampling sites (Al-Romulah, Alam-Alroom, Mina-Hashish, Al-Furouz, Romel, Al-Bosut, Cleopatra, Al-Ghram, Al-Obued and Ageebah) that are located in Marsa-Matrouh sea shores (North west of Egypt) at 2 m depth during autumn 2009 with the aid of the National Institute of Oceanography and Fisheries (NIOF) members according to a project sampling process “Evaluation and study of environmental effect on Matrouh beaches”. The samples were brought back to the laboratory and analyzed within 24 h. Heterotrophic bacteria were counted and isolated using the standard plate method. One millimeter of the appropriate dilution was used to surface inoculate sea water agar medium. From each dilution, three plates were inoculated and incubated at 28°C for 48 h.

Isolation of alkaline protease producing alkaliphilic bacteria

Isolation of alkaline protease producing alkaliphilic bacteria was carried out using rich alkaline agar medium containing skimmed milk (modified method of Ventosa et al., 1982). Aliquots (1 ml) of different dilutions of water samples were plated and incubated at 37°C for 72 h. Formation of halo zone around the colonies, resulting from casein hydrolysis was taken as evidence of proteolyses activity. These colonies were isolated and streaked on fresh plates until single uniform colonies were obtained. The most potent isolates which showed the largest halo zones were selected for further studies.

Determination of optimum pH for growth of potent isolates

This experiment was conducted in triplicate in nutrient broth medium, unless otherwise stated. Growth of the selected strains was determined by measuring the optical density at 550 nm with a spectrophotometer (U-1500, Hitachi). Determination of optimum pH for growth was by adjusting the medium pH from 7 to12 with sterilized 1.0 M Na_2CO_3. The isolates optical densities at zero time were adjusted at 0.1.

Characterization of the promising isolate

The characters of the potent organism were studied following the standard microbiological methods as described in Bergy`s Manual of Systematic Microbiology (Holt et al, 1994). Gram reaction, colony morphology, vegetative cell and spore characteristics were observed from 12 h old culture grown on a rotary shaker at 120 rpm, 30°C. The physiological and biochemical characters, included: Indole production, nitrate reduction, urease hydrolysis, citrate utilization, production of oxidase, catalase, sulphide, acetoin (Voges proskauer), tryptophane deaminase, gelatinase, lysine decarbo-xylase, arginine dihydrolase, β-galactosidase and fermentation-oxidation of the following carbon sources (D-glucose, D-mannitol, inositol, D-sorbitol, rhamnose, D-sucrose, D-melibiose, amygdalin and L- arabinose).

Molecular identification

The identification was carried out at Mubarak City for Scientific Research and technology applications, Arid Land Institute, Molecular Plant Pathology Department, New Borg El Arab City, 21934, Alexandria, Egypt.

Production of alkaline protease

Protease production was carried out in medium containing (g/l) casein 2 and peptone 5; pH was adjusted to 10 and prepared with sea water (modified method of Ramakrishna et al., 2010). The culture incubated overnight at 180 rpm, 37°C and then, sub-cultured into a 500 ml Erlenmeyer flask containing 200 ml of the same medium and incubated at 37°C for 48 h. Cells and insoluble materials were removed by centrifugation at 9000 ' g for 10 min at 4°C and the cell-free supernatant was filtered through 0.45 µm pore-size membrane filter and was used as the source of the crude alkaline protease enzyme.

Determination of protease enzyme activity

The protease enzyme activity was measured according to the method described by Yamaguchi et al. (1982).

Determination of protein concentration

The determination of the protease concentration generally depended on spectrophoto-metric methods according to Bradford (1976).

To discover the protein content of the purified protease, absorption at 280 nm was used.

Desalting and concentrating of purified protein

The purified protease was desalted and concentrated using the ultra filtration tubes at a speed 3,000 rpm for 30 min at 4°C in centricon-10 (Amicon, USA) ultrafiltration concentrators (membrane cut off 10 kDa).

Purification using liquid chromatography

Purification of the protease enzyme was performed through ÄKTA fast protein liquid chromatography (FPLC) by Amersham Pharmacia Biotech. The HiPrep 16/10 FF-DEAE-Sepharose CL 6B column as an anion-exchange liquid chromatography technique was used for separation and purification of the protease were the running buffer was 20 mM glycine buffer (pH 10.0) and the elution buffer was 1.0 M NaCl in 20 mM glycine (pH 10.0). The flow rate was 0.50 ml/min with elution rate of 0 to 100% and the fraction size was 6.0 ml and the sample volume was 5 ml.

The HiPrep 16/60 Sephacryl S-200 high resolution column as a gel filtration liquid chromatography technique was used for the separation and purification of the protease that pooled from the anion-exchange separation. The running buffer used was 20 mM phosphate buffer (pH 8.0) with 200 mM sodium chloride and the flow rate was1.0 ml/min with collecting fraction size of 5.0 ml.

Polyacrylamide gel electrophoresis (PAGE)

The denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE) has been used according to the method of Laemmli (1970), on SCIE-PLAS TV100 YK-EBSYS TV-Modular electroblotting system with 10% polyacrylamide concentration. This is for detecting the protease enzyme homogeneity and estimation of its molecular mass using ferments protein molecular weight marker.

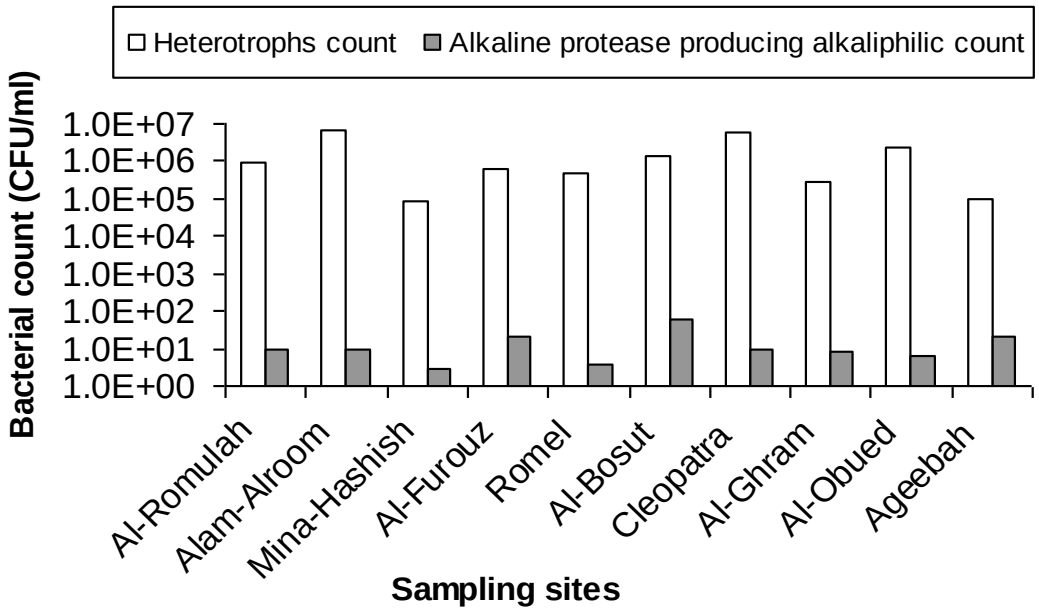

Figure 1. Heterotrophs and alkaline protease producing alkaliphilic bacterial count

Characterization of protease

Effect of temperature on the stability of the purified alkaline protease

The effect of temperature on alkaline protease stability was determined by incubating the reaction mixture (pH 10) for 20 min at different temperature ranging from 30 to 80°C .The activity of the protease was then, measured as standard protease assay (Abu Sayem et al, 2006).

Effect of pH on the stability of the purified alkaline protease

The effect of pH value on alkaline protease from *B. cereus* was determined by assaying the enzyme activity at 50°C at different pH values ranging from 6.0 to 12.0, using the following buffer systems: phosphate (pH 6 to 7), tris-HCl (pH 8 to 9) and glycine -NaOH (pH 10 to 12). The concentration of each buffer was 0.05 M (Abu Sayem et al., 2006).

Effect of metal ions on the activity of the purified alkaline protease

The effect of metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Pb^{3+}, Mn^{2+} and Cd^{2+}) and ethylene diamine tetraacetic acid (EDTA) on protease activity was investigated to further characterize the enzyme. The alkaline protease was pre-incubated with the earlier mentioned chemicals for 30 min at 50°C; afterwards the residual activity (%) was measured by standard protease assay. The final concentration of each ion was 5 mM at the time of pre-incubation (Joshi et al., 2007).

Protease applications

Application of alkaline protease in removing the blood stains

The application of alkaline protease in removing the blood stains was observed according to the method of Najafi and Deobagkar, (2005) after slight modification. A clean piece of pure white cotton cloth was soaked in animal blood for 15 min and then, allowed to dry at 80°C for 5 min in hot air oven. The dried cloth was cut into equal sizes (4 × 4 cm^2) and incubated with crude enzyme at 50°C for different incubation periods (10, 20, 30, 40 and 50 min). After a given incubation, the cloth was rinsed with tap water for 2 min without scrubbing and then, dried in open air. The same procedure was done with the control without the enzyme exposure.

Compatibility with detergents

The compatibility of protease with local laundry detergents was studied in the presence of 10 mM $CaCl_2$ and 1.0 M glycine. Detergents used were Ariel (Procter and Gamble, Egypt); Persil (Henkel PDC, Egypt); Bonex (Procter and Gamble, Egypt); Sunlight (Unilever, Egypt). The detergents were diluted in distilled water (0.7% w/v) and incubated with protease for different periods at 50°C and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100% (Adinarayana et al., 2003).

RESULTS

Heterotrophs and alkaline protease producing alkaliphilic bacterial counts

As indicated in Figure 1, the highest total viable counts were detected in Alam-Alroom (7×10^6 CFU/ml) and Kilo-Batra (6×10^6 CFU/ml). The lowest counts were detected in Mina-Hashish and Ageebah (9×10^4 and 10^5 CFU/ml, respectively). The counts of alkaline protease producing alkaliphilic group were very low as indicated by Figure 1. The highest count was detected in Al-Bosut (60 CFU/ml).

Table 1. Proteolyses zones of the different isolates on milk agar.

Isolate code	Site of isolation	Colony diameter (mm)	Proteolyses zone diameter (mm)
S_1	Alam-Alroom	3.0	8.0
S_2	Alam-Alroom	2.0	4.0
S_3	Alam-Alroom	2.0	4.0
S_4	Alam-Alroom	2.0	6.0
S_5	Alam-Alroom	3.0	6.0
S_6	Mina-Hashish	2.0	20.0
S_7	Romel	2.0	5.0
S_8	Al-Bosut	2.0	14.0
S_9	Al-Bosut	3.0	20.0
S_{10}	Al-Bosut	3.0	6.0
S_{11}	Al-Bosut	1.0	3.0
S_{12}	Al-Ghram	6.0	10.0
S_{13}	Al-Ghram	4.0	9.0
S_{14}	Al-Ghram	2.0	4.0
S_{15}	Al-Ghram	4.0	6.0
S_{16}	Al-Ghram	2.0	16.0
S_{17}	Ageebah	1.0	10.0
S_{18}	Al-Obued	3.0	6.0

Al-Furouz and Ageeba achieved the same count (20 CFU/ml). Al-Romulah, Alam-Alroom and Cleopatra counts were (10 CFU/ml). Lower numbers were detected in El-Gharam, El-Obued, Romel and Mena-Hashish.

Detection of the proteolytic zones on milk agar

Eighteen (18) isolates were isolated on milk agar, with wide range of proteolytic ability. Alam-Alroom (5 isolates), Al-Bosut (4 isolates), Al-Ghram (5 isolates), while one isolate from each of the following sites (Mina-Hashish, Romel, Ageebah and Al-Obued). The highest proteolysis zones as shown in Table 1 were produced by S_6, S_8, S_9, S_{16} and S_{17}.

Growth of the promising strains at different pH values

Isolates with codes (S_6, S_8, S_9, S_{16} and S_{17}) were subjected to growth at alkaline nutrient broths with different pH values. All strains favored the alkaline conditions. All strains gave their maximum growth as indicated with their optical densities at pH range from 8 to 9, while strain number S_6 gave its maximum growth at pH range from 9 to 10 as shown in Table 2, so that, it was chosen for further studies.

The morphological and biochemical characteristics of the most promising isolate

Morphological studies for promising isolates with code no. S_6 revealed that, its colony color was white with convex elevation and irregular margin. The growing cells were Gram-positive aerobes. The isolate was positive for enzyme tests of β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, catalase, oxidase and gelatinase. No sulphide or indole production but acetoin production (Voges Proskauer) was detected. Citrate was not utilized. Urease was not produced. D-glucose was utilized as carbon source, while the following carbon sources: D-mannitol, inositol, D-sorbitol, D-rhamnose, D-sucrose, D-melibiose and L-arabinose were not utilized, (Table 3).

Molecular identification

DNA of the promising isolate was extracted and the extracted 16S rRNA gene was amplified, the produced amplicons was analyzed using agarose gel electrophoresis. The GenBank accession number for the 16S rDNA sequences GU201872.1, the isolate was identified as *B. cereus* with similarity percentage 98%, its classification under (Division: Firmicutes). Figure 2 represents the phylogenetic relationships among representative

Table 2. Optical density (O.D.) of the promising isolates at different pH after 24 h

Isolate code	Optical density at different pH					
	7	**8**	**9**	**10**	**11**	**12**
S6	0.268	1.122	1.376	1.156	0.962	0.660
S8	0.247	1.109	1.235	0.832	0.826	0.485
S9	0.330	1.003	1.189	0.808	0.639	0.559
S16	0.280	0.975	0.985	0.904	0.528	0.422
S17	0.304	1.152	0.924	0.628	0.544	0.524

Table 3. Characteristics of the most promising isolate.

Characteristic	Observation
Morphological characters	
Colony color	White
Colony margin	Irregular
Colony elevation	Convex
Colony configuration	Lobate
Gram reaction	+
Presence of spores	+
Physiological characters	
β-galactosidase production	+
Arginine dihydrolase	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Citrate utilization	-
H_2S production	-
Urease production	-
Tryptophane deaminase	+
Indole production	-
Acetoin production	+
Gelatinase production	+
Utilization of	
D-glucose	+
D-mannitol	-
Inositol	-
D-sorbitol	-
D-rhamnose	-
D-sucrose	-
D-melibiose	-
Amygdalin	+
L-arabinose	-
Oxidase production	+
Catalase production	+
Nitrate reduction	-

experimental strain and the most closely related *Bacillus* species.

Enzyme purification

B. cereus was grown on alkaline nutrient broth. Total protein content and total protease activity were measured in the culture filtrate and found to be 3.955 mg and 1549.25 U, respectively, with a specific activity of 391.72 U/mg and after dialyzing and concentrating of the culture filtrate using the centricon-10 system, the specific activity was increased to 465.76 U/mg. After separation process

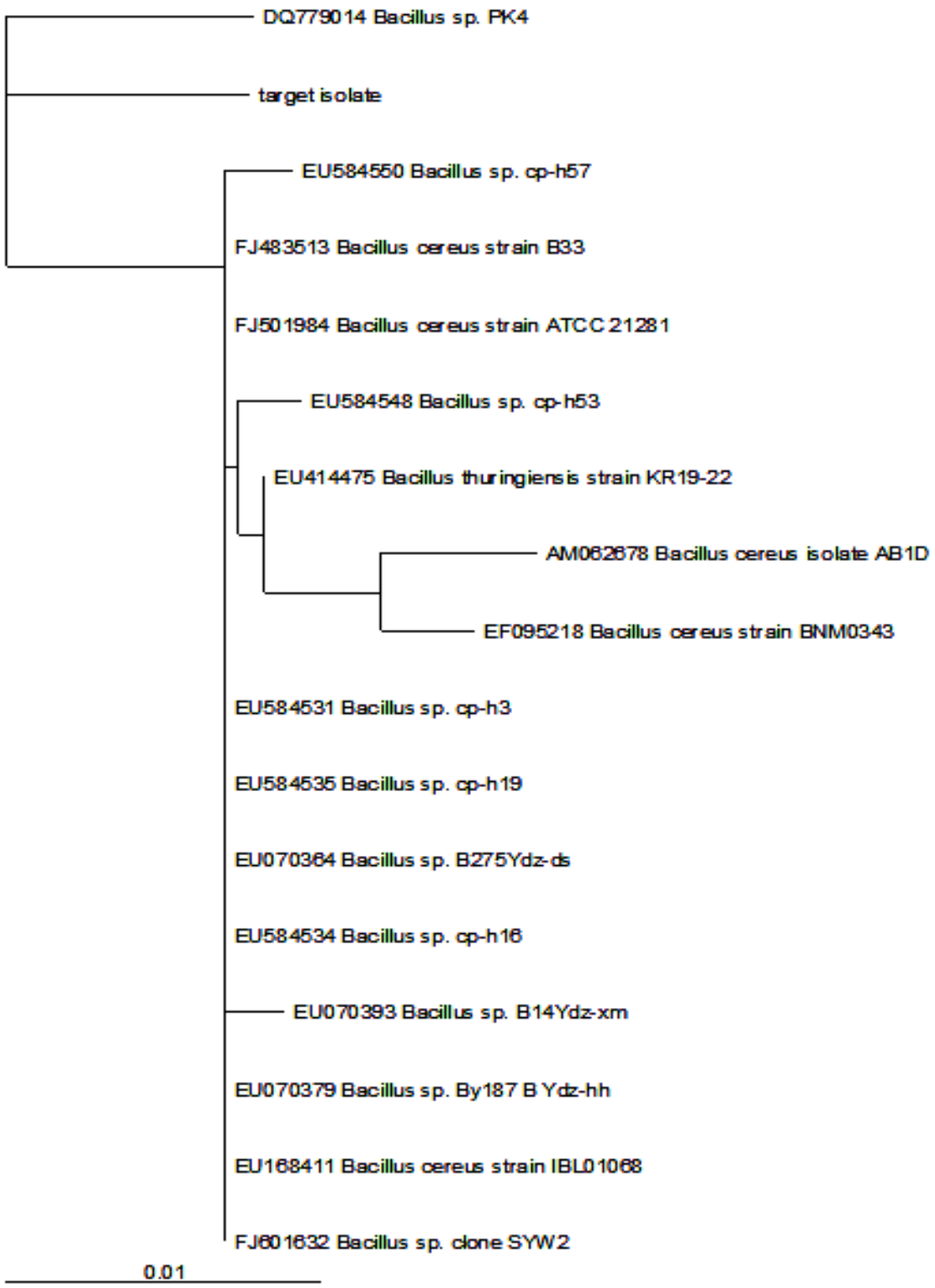

Figure 2. Phylogenetic relationships among representative experimental strain and the most closely related *Bacillus* species. The dendogram was generated using tree view program.

using the DEAE anion-exchange technique, an estimation of the total protein content and the protease activity in the pooled fractions were found to be 1.085 mg and 1284.3 U, respectively. Continuing purification using gel-filtration technique results in a good purification fold of the enzyme as shown in Table 4. The purification-fold

Table 4. Purification profile of alkaline protease.

Step	Total volume (ml)	Total protease activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture filtrate	200	1549.25	3.955	391.7193426	100	1
After concentrating	50	1465.75	3.147	465.7610423	94.61	1.19
After DEAE column	30	1284.3	1.085	1183.686636	82.90	3.02
After gel-filtration	20	1005.7	0.275	3657.090909	64.92	9.34

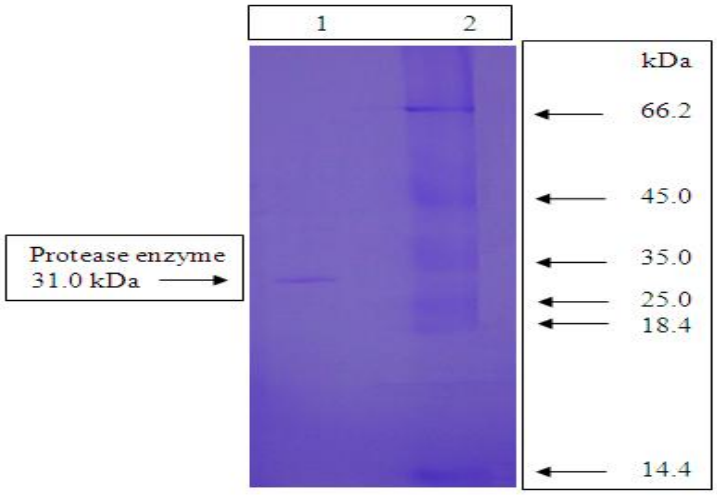

Figure 3.SDS-gel polyacrylamide electrophoresis (SDS-PAGE) showing the purified enzyme.

rise to 9.34 fold and this showed that such separation technique was an excellent method for protease purification. SDS-PAGE with Coomassie-brilliant blue staining was conducted to determine the molecular weight of the protease enzyme. By comparing our protease molecular weight with the protein marker, an estimation molecular weight was found to be 31.0 kDa as shown in Figure 3.

Characterization of purified protease

Effect of different temperature on protease stability

As shown in Figure 4, the enzyme was stable at a temperature range from 50 to 70°C. Maximum stability detected at 50°C. The residual activity sharply decreased at 80°C.

Effect of different pH on protease stability

The effect of pH on the stability of protease was determined. The enzyme was found stable over a broad range of pH (8 to 11). Maximum activity was determined at pH 10 and the enzyme lost more than 50% of its activity at pH 8 and 12 as shown in Figure 5.

Effect of various metals ions on protease activity

Most of the metal ions tested had an inhibitory effect on enzyme activity. Only (Cu^{2+}) ions had a stimulatory effect and increased the activity by 12% as shown in Figure 6. EDTA also lowered the activity of the protease to 64%.

Protease applications

Removal of blood stain

The blood stain was removed from a white cotton cloth after incubating the cloth in enzyme broth for different time intervals from 10 to 50 min. Figure 7 showed the gradual removal of blood stain by increasing the contact time with the enzyme broth.

Compatibility of protease with detergents

B. cereus protease showed excellent stability in the

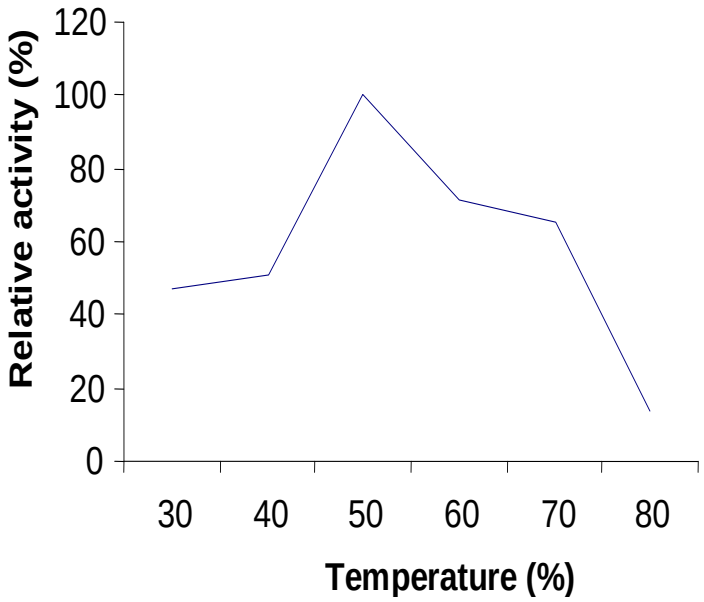

Figure 4. Effect of different temperatures on protease stability.

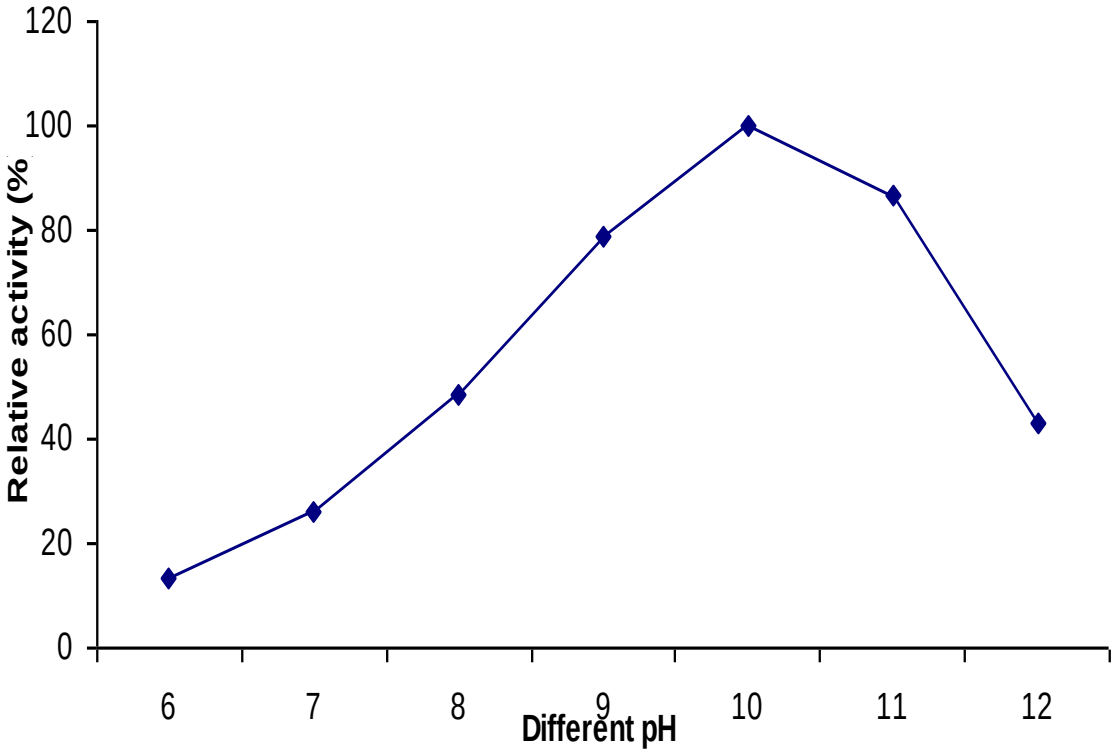

Figure 5. Effect of different pH on protease stability. Buffer used were phosphate buffer (pH 6 to 7), Tris-HCl buffer (pH 8 to 9) and glycine-NaOH buffer (pH 9.5-10). The concentration of each buffer was 0.05 M.

presence of locally available detergents (Ariel, Persil, Bonex and Sunlight) at 50°C in the presence of $CaCl_2$ and glycine as stabilizers as shown in Table 5. The studied protease showed the highest stability with Persil followed by Bonex, then, Sunlight. After 1 h of incubation, the activity ranged from 82 to 95% with respect to Ariel

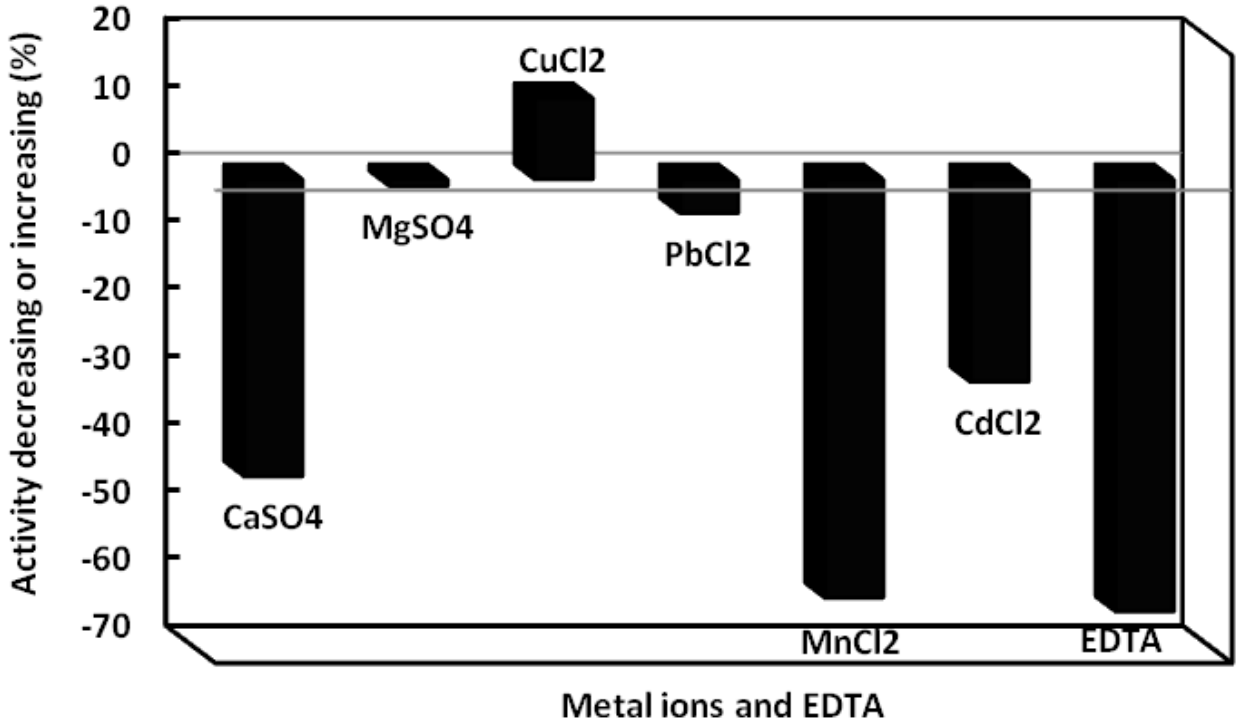

Figure 6. Effect of some metal ions and EDTA on enzyme activity.

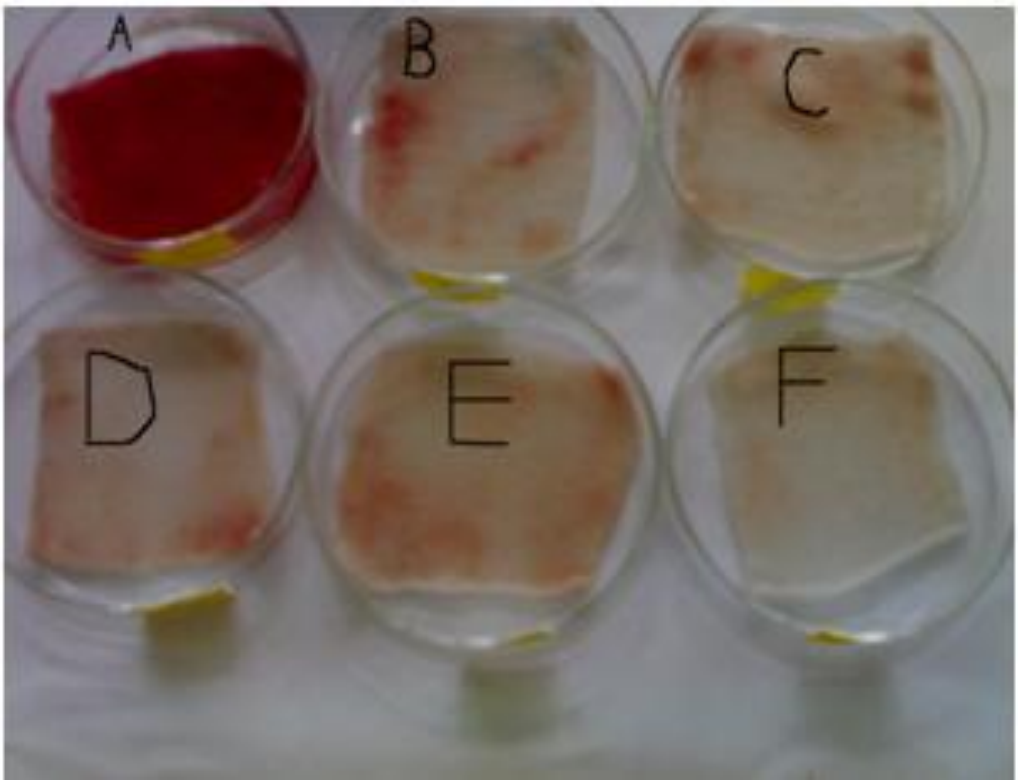

Figure 7. Removal of blood stains from cotton cloth pieces (4 × 4 cm^2) by alkaline protease of *B. cereus*. The blood-stained cotton cloth pieces were incubated with Alkaline protease broth at 50°C for different time intervals. A, control (without treatment); B,10 min;
C, 20 min; D, 30 min; E, 40 min; F, 50 min.

Table 5. Compatibility of alkaline protease with commercial detergents at 50°C.

Time (min)	Relative residual alkaline protease activity (%)				
	Control	Ariel	Persil	Bonex	Sunlight
0.0	100	100	100	100	100
30	98	85	97	97	88
60	96	82	95	94	83
90	90	79	90	86	79
120	86	69	88	80	75
150	82	64	77	72	69
180	78	58	64	60	58

and Persil. The enzyme retained about 60% of its activity with most of the commercially used detergents even after 3 h of incubation at 50°C after the supplementation of $CaCl_2$ and glycine.

DISCUSSION

Extremophiles, the microbes dwelling in unusual habitats, can potentially serve in a verity of industrial applications. As a result of adaptation to extreme environments, extremophiles have evolved unique properties, which can be of biotechnological and commercial significance (Martins et al., 2001). Extremophiles include halophiles, acidophiles, thermophiles and haloalkaliphiles (Dodia et al., 2006). Alam-Alroom achieved the highest heterotrophic count (7×10^6 CFU/ml) this may be due to the high values of total organic carbon (1.62%) and total organic matter (2.95 mg/g) in the sediments of this area (Project report of National institute Of Oceanography and Fisheries (NIOF) Evaluation and study of environmental effect on Matrouh Beaches). The counts of alkaline protease producing alkaliphilic bacteria in Marsa-Matrouh were very low with respect to the total heterotrophs counts. Up to our knowledge, the detection of this group in Egyptian marine environment is not well studied. The first trial was done by Ibrahim et al. (2007) who studied the isolation and identification of alkaline protease producing alkaliphilic bacteria from an Egyptian Soda Lake.

The alkaline conditions were prevailing in the sea water of the study area; Abou Elalla and Shalaby (2009) reported that, the total alkalinity as $CaCO_3$ in EL-Gharam beach (Western side of Alexandria) was 146 mg/ml, so that the detection of alkaliphiles was reasonable.

Formation of clear zone on skim milk around the isolated colonies was considered as indication of good growth with the ability of alkaline protease production extracellular, therefore, the largest zones producing strains were selected for further studies, in spite of Coolbear et al., (1991) who established that, there is no necessarily good correlation between zones of clearing around colonies on milk-agar plates and levels of proteinase activity. All isolates in this study grew well in the broth medium at pH range of 8 to 9 indicating that, these isolates are alkaliphilic. In this study, strain (S_6) from Mina-Hashish achieved the largest proteolytic zone (20 mm) with respect to the colony diameter (2 mm) and its optimum growth estimated at higher alkaline range (9 to 10), therefore, this strain was chosen for further studies.

Microbial proteases are produced from high yielding strains including species of *Bacillus* sp., *Alcaligenes faecalis, Pseudomonas fluorescens* and *Aeromonas hydrophilia* grown under submerged culture conditions. Among these, *Bacillus* sp. is the most important group of bacteria that are involved in the enzyme industry and this bacterium is also known to produce proteolytic enzymes quite effectively (Boominadhan et al., 2009). Asokan and Jayanth (2010), Das and Prasad, (2010), Kumar and Vats (2010) and Ramakrishna et al. (2010) studied the production of alkaline protease from different *Bacillus* species.

The most promised strain in this study is identified as *B. cereus* according to morphological and physiological tests and 16S rDNA sequence analysis. *Bacillus* species are widely distributed in soil and other natural environments characterized with wide range of different physiological conditions. Most of the commercially important alkaline proteases are derived from *Bacillus* species. In fact, these bacteria are known for their abilities to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures (Pastor et al., 2001). In another study concerned with the production of alkaline protease, it was observed that 27 bacteria out of 40 isolates from soil samples belonged to the genus *Bacillus* (Belma et al., 2002).

The enzyme purity was confirmed by SDS-PAGE which demonstrated a single band indicating a homogeneous preparation. The final purification step presented 9.34-fold enzyme purification with a specific activity of 3657.09 U/mg protein. These results indicated the effectiveness of the purification method.

Temperature also plays an important role in activation and inactivation of enzymes. Each enzyme has an optimum temperature for maximum enzyme activity. Abu

Sayem et al. (2006) demonstrated that, at temperatures of 60 and 70°C, the enzyme lost its activity rapidly with the optimum at 40°C. For *Bacillus licheniformis* S-40, it has been reported to be 50°C (Sen and Saytyanarayana, 1993). The results of the present study agreed with the mentioned results but disagreed with the results of Ferrero et al. (1996) on *B*. *licheniformis* MIR-29, in which case the enzyme retained 90% stability at 70°C.

The protease was considerably stable when exposed to pH range (9 to 11) with maximum stability at pH 10, but at lower pH, the stability gradually decreased, this indicates that the enzyme was alkaline in nature. Similar results were also reported for *Bacillus* sp. in which the enzyme was highly active and stable at pH 9 to 10 (Johnvesly and Naik, 2001). Dodia et al. (2006) reported that, optimum protease activity and stability was recorded at pH 9 to 9.5 for S_5 strain. Protease from B*. licheniformis* MZK03 remained stable over a wide pH range (6 to 11) and maximum activity was found at pH 8.5 (Abu Sayem et al., 2006). The enzyme studied by Malathi and Dhar (1987) and Manachini and Parini (1988) had a pH optimum range of 9.0 to 9.5.

The various metal ions tested revealed that, Cu^{2+} ions only increased the relative enzyme activity up to 12% and the other ions (Cd^{2+}, Mn^{2+}, Pb^{2+}, Mg^{2+} and Ca^{2+}) in addition to EDTA exerted a negative effect on the enzyme. On the other hand, Siriporn et al. (2006) reported that, Mn^{2+}, Ca^{2+} and Mg^{2+} ions have been described to increase the relative protease activity produced by *Bacillus megatarium* isolated from Thai fish sauce.

Abu Sayem et al. (2006) reported that, enzyme activity was accelerated by the addition of Mg^{2+}, Ca^{2+} and Mn^{2+}, whereas, it was inhibited by Hg^{2+}. EDTA reduced the protease activity of *B. cereus* MTCC 6840 to 52% (Joshi et al., 2007). Very few workers have reported such unusual inhibition of bacterial protease by EDTA (Kim et al., 2005). *B. cereus* protease enzyme retained about 60% of its activity with most of the commercially used detergents even after 3 h incubation at 50°C and in this respect, this study agreed with many studies. Madan et al. (2002) studied the compatibility of alkaline protease from *Bacillus polymyxa* which retained 20 to 84.5% of its activity in various detergents while Devi et al. (2008) reported that, alkaline protease from *Aspergillus niger* retained more than 50% activity after 60 min incubation at 40°C in the presence of detergents such as Tide, Surf, Wheel and Henko indicating its suitability for application in detergent industry.Blood removing study revealed that, *B. cereus* protease is a promising additive for detergent industry and that well established by many authors. Nadeem et al. (2008) studied the high capacity of blood stain removal by *B. licheniformis* N-2.

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