Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Characterization and Purification of Alkaline Protease from Novel *Bacillus subtilus* MS1 for Detergent Additives

Manal Selim Mohamed Salim [a], Sahar Saleh Mohamed[a], Sayeda Abdelrazek Abdelhamid[a] and Ahmed Abdelghani Hamed[b]

[a]Microbial Biotechnology Department, National Research Centre, Giza 12622, Egypt
[b]Microbial Biochemistry Department, National Research Centre, Giza 12622, Egypt

Abstract

Proteases are one of the enzymes required that have a significant impact on biotechnology. *Bacillus subtilus* MS1, a bacterial strain obtained from sewage sediment that could produce protease enzyme on skim milk agar. The isolate was identified as *Bacillus subtilus* MS1 with accession number KY621344 based on biochemical tests, microbiological, and 16S rRNA gene sequences. Various cultural conditions were optimized to become the maximum yield of enzyme production of 9.322 U/ml. Based on the optimization results, a significant scale culture was created utilizing glucose as a carbon source and peptone as a nitrogen source at 50°C for 48 h. Purification of the protease was achieved using 60% ammonium sulphate and Sephadex G-100 with a molecular weight of 30 kDa. The protease's activity maximized yields 24.437 U/mg at pH 10 and 50°C, and it remained stable at pH 6.0-11.0 and 30–80°C. In the presence of phenylmethyl sulphonyl fluoride (PMSF) and silver ion (Ag+1) the protease activity was suppressed. The purified protease enzyme occurred a greater stain removal.
Keywords: Bacillus subtilus MS1, protease optimization, protease activity, stain removal.

1. Introduction

Bioactive compounds and extracellular enzymes such as proteases, cellulases, amylases, pectinases, and chitinases were produced by bacterial colonies and fungi. The majority of enzymes employed in biotechnological, and industrial processes nowadays are hydrolytic and they are utilized to degrade a variety of natural compounds [1]. Extracellular protease and amylase account for 60% of the global industrial enzyme industry, with a wide range of applications in leather, detergents, food, and pharmaceutical industries [2]. Proteases were the main class of industrial enzymes that account for over 60% of the global enzyme market and 40% of global enzyme sales. Proteases were made up of a group of enzymes that include (peptidases, proteinases, and amidases) and are not only a single enzyme [3]. Proteinases hydrolyzed entire protein molecules, releasing amino acids and peptones in the process. Peptidases hydrolyzed peptones and release amino acids, whereas amidases accelerate amino acid hydrolysis and release ammonia [4, 5]. Proteases were classed as acidic with pH 2.0-6.0, neutral with pH 6.0-8.0, or alkaline with pH 8.0-13.0 enzymes based on their pH [3, 6]. Catalytic activity, substrate selectivity, temperature, pH, stability profiles, and active site specificity are all characteristics of distinct types of proteases [7]. Proteases could be found in a wide species including plants, microbes, and animals. Nonetheless, plant and animal-derived proteases are unable to fulfill rising industrial needs prompting a surge in interest in microbial proteases [8-10]. Alkaline proteases (APases) made up more than half of all microbial proteases produced worldwide, with specific applications [11, 12]. Because microbial APases had a shorter doubling time than plants and animals, they could meet market demand for a variety of industrial processes [1]. Several microbes included *Vibrio* spp. [13], *Bacillus* sp. [11, 14], *Pseudoalteromonas* sp. [15], *Streptomyces* sp. [16], and *Haloarchaea* [17] might be of great interest as potential APases producers. As a result, in this investigation, an attempt was undertaken to screen protease-producing bacterial isolates in sewage sediment from the industrial region

_____

of West Alexandria for potential detergent applications.

2. Experimental:

All used chemicals in this study were purchased from the Sigma Chemical company

2.1 Source of samples

Sewage sediment of an industrial region in West Alexandria and transferred to the laboratory in an icebox maintained at 4ºC for further study. **2.2 Isolation of bacteria**

Bacteria were isolated for protease enzyme using a serial dilution method described by [18]. Samples were inoculated on skim milk agar plates containing g/l: peptone 1, NaCl 5, and skim milk 100 at pH 10, then incubated at $37 \pm 2°C$ for two days [19]. Bacterial isolates were primarily purified on nutrient agar medium and routinely maintained at $4°C$ on culture purity was determined from colony morphology.

2.3 Screening for best strain produced protease

The isolates were screened for best strain-produced protease by plate assay using protease specific medium containing (g/l): K_2HPO_4 2, glucose 1, peptone 5, gelatin 15, and agar 20. The clear zone diameters were measured after 24 h of incubation at 28°C by flooding the plates with mercuric chloride solution, this method was referred to as the gelatin clear zone method [20].

2.4 Production of Protease

The culture-positive for alkaline protease was grown under optimal conditions for the enzyme production in 100 ml of Horikoshi I medium [21] containing (g/l): glucose 5, peptone 7.5, sodium chloride 5, magnesium sulfate 5, and ferrous sulfate 0.1 with the pH of 10.0. The medium was incubated for 48h in a shaker incubator (120 rpm) at 37°C. The fermented broth was filtered and the filtrate was centrifuged at 5000 rpm for 5 minutes to extract the crude extracellular protease. The culture filtrate was used for further assay procedures.

2.5 Protease assay

Protease activity was determined by modifying Anson's method [22]. The reaction mixture containing 2 ml of 1% (w/v) casein solution in 0.05 M glycine-NaOH buffer (pH 10) and 1 ml of enzyme solution were incubated at 40°C for 20 min. The reaction was then stopped by adding 3 ml of 10% (w/v) trichloroacetic acid. After that, the entire mixture was centrifuged at 5000 rpm for 20 min at 4°C and absorbance of the liberated tyrosine was measured at 280 nm against blank. One unit of protease was defined as the amount of enzyme that released1 micromole of tyrosine per minute under the assay conditions [22].

2.6 Identification of the highest protease activity

The promising strain which produces the highest protease activity was identified based on morphological, physiological, and biochemical characteristics [23] combined with 16S rRNA gene sequencing using universal primers 27F5'(AGAGTTTGA CCTGGCTCAG)3' and 1492R 5'(GGTTACCTTGTTACGACTT)3' [24]. Data were submitted to the Gene Bank database. The DNA sequence was compared with the Gene Bank database in the NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST program [25].

2.7 Optimization of the production medium

The promising stain was subjected to different culture physicochemical and nutritional conditions to derive the optimum conditions for maximum protease was evaluated.

2.7.1 Effect of physicochemical parameters

Subsequently, the medium component studied included the effect of different incubation times (1, 2, 3, 4, and 5 days), different pH (6, 7, 8, 9, 10, and 11), and different temperatures (20, 30, 40, 50, 60 and 70°C). All the experiments were carried out in a 500-ml Erlenmeyer flask containing 100 ml of basal medium in triplicate.

2.7.2 Effect of nutritional parameters

Glucose in the production medium was substituted with other carbon sources, including 1% (w/v) (maltose, fructose, sucrose, lactose, glycerol, and starch) the various carbon sources were autoclaved separately and added to the medium on an equal carbon basis, and different nitrogen sources were investigated by substituting the peptone in the production medium (Horikoshi-I), with different sources of organic and inorganic nitrogen sources (0.5%, w/v) the organic nitrogen sources included yeast extract, casein, skim milk, and beef extract; while inorganic nitrogen sources included ammonium nitrate, ammonium sulfate, sodium nitrate, and urea.

2.8 Determination of protein content

Protein content was measured according to Bradford, [26] using bovine serum albumin as the standard.

2.9 Purification of protease

Cell-free supernatant was precipitated from the supernatant with ammonium sulfate (0–80%) saturation according to (Dixon, 1953). Protease active fraction was pooled, centrifuged (5,000 rpm, 30 min) and the precipitate was dissolved in a minimal amount of 0.05 M Tris-HCl buffer pH 8.0 and dialyzed overnight at 4°C against the same buffer. The Sephadex column was equilibrated with 0.05 M Tris-HCl buffer pH 8.0. The dialyzed enzyme sample was loaded onto a Sephadex G-100 column (2.6 × 75 cm) and then eluted with the same buffer. Fractions each of 5 ml were collected at a flow rate of 30 ml/h. The fractions showing absorbance at 280 nm were analyzed for protease activities and the active fractions were pooled and dialyzed against the same buffer. The protein content was measured and protease activity was assayed as described before.

2.10 Characterization and properties of the purified protease

2.10.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of the purified protease was determined by SDS-PAGE according to the method of Laemmli, [27]. The strength of the gel was 12% (w/v) and the protein bands were stained with Coomassie brilliant blue R 250. Medium range (29-205 kDa). Molecular weight markers were used to determine the molecular mass of purified protease. The markers were: carbonicanhydrase (29 kDa), albumin (egg) (45 kDa), bovine serum albumin (66 kDa), phosphorylase (97 kDa), β-galactosidase (116 kDa), and myosin (205 kDa).

2.10.2 Effect of temperature on the activity and stability of protease

The effect of reaction temperature on protease activity was determined by incubating the reaction mixture at different temperatures ranging from 20 to 80°C in 0.05 M glycine- NaOH buffer. The thermal stability of the purified enzyme was determined by pre-incubating the enzyme solution for up to1 h at various temperatures (from 20°C to 80°C) in the absence of substrate. At different times (10–60 min), aliquots were removed and cooled and the residual activity was measured by the standard assay method as previously mentioned.

2.10.3 Effect of pH on the activity and stability of protease

The optimum pH for the purified protease activity was measured at different pH values (6-12) by using a 1% (w/v) solution of casein as a substrate dissolved in different buffers determined (0.05 M): phosphate (pH 6-7) Tris–HCl (pH 8-9) and glycine-NaOH (pH 10-12). After incubating each reaction at 37ºC for 20 min., enzymatic activity was detected. The pH stability of the enzyme was determined by pre-incubating the enzyme solution at different pH values ranging from 6.0 to 11.0 for 2 h. at 4ºC. The residual activities were then measured according to the standard assay procedure.

2.10.4 Effect of inhibitors and metal ions on protease activity

Effects on protease activity of various inhibitors (each at 5mM) such as phenylmethyl sulphonyl fluoride (PMSF), cysteine inhibitors p-chloromercuric benzoate (pCMB), ethylene diamine tetraacetic acid (EDTA), and metal ions (Ca^{+2}, Zn^{+2}, Mg^{+2}, Co^{+2}, and Ag^{+1}), each at 5 mM concentration on protease activity were studied. The purified enzyme was pre-incubated with the above inhibitors and metal ions for 30 min at 40°C. Then the remaining activity was measured routinely.

2.11 Mode of a protease enzyme on the stains

The application of protease enzyme as a detergent additive was studied on white cotton cloth pieces (5×5 cm) stained with human blood and chocolate as per [28]. The cloths were incubated with 2 ml of the purified protease at 50ºC for 20 min and 40 min. After incubation time, each piece was rinsed with water for 2 min and then dried. The same procedure was done for the control except for incubation with the enzyme solution.

3. Results and Discussion

3.1 Isolation and screening of protease producing microbes

Microbial proteases are one of the enzymes required that had a significant impact on biotechnology. The potentiality of bacterial-producing isolates was determined by measuring the clear zone produced around the colony. The potential bacterial isolates 4 were selected as the most potential protease producer isolate from a total of 10 morphologically distinct bacterial colonies isolated from the sediment of sewage. Colorimetric evaluation [29], Zymographic tools [30], and incorporation of different substrates in nutrient agar plates, such as casein [31], and skim milk [32] were all described as methods for estimating proteolytic activity. In this study, *Bacillus* sp. strain was the most protease producer isolate so it was selected among 4 potential bacterial isolates (Figure 1). Proteolytic activity was evaluated utilizing skim milk agar and recorded as the diameter of a clear zone in mm, which was 17 mm in this study.
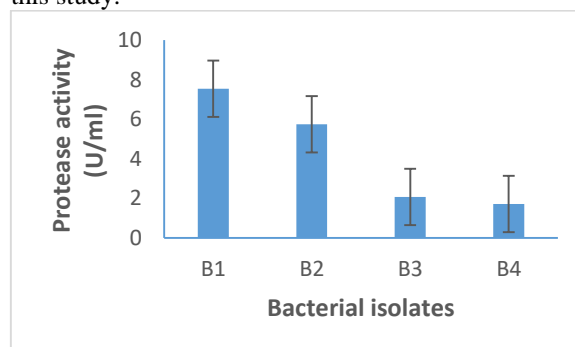

Fig. 1 Protease activity of selected bacterial isolates

3.2 Identification of isolate by 16S rRNA gene sequencing

Biochemical tests of the selected isolate indicated that was *Bacillus* sp. The results were Gram-positive, rod shape, motile, and catalase-positive while, the Indole, oxidase, and nitrate reduction were negative. To prove and further confirm the isolate's species identification, a molecular approach was applied. The 16S rDNA partial sequence was evaluated and compared to Gene Bank databases. *Bacillus subtilus* MS1 with accession number KY621344 similarity 99% to *Bacillus subtilus* was identified. The neighbor-joining technique was used to create phylogenetic relationships based on several *Bacillus* species (Figure 2). The neighbor-joining

technique was used to create phylogenetic relationships based on several *Bacillus* species Similar to [31], who obtained 52 bacteria from sand and water samples, and *Bacillus cereus* being the best isolate.
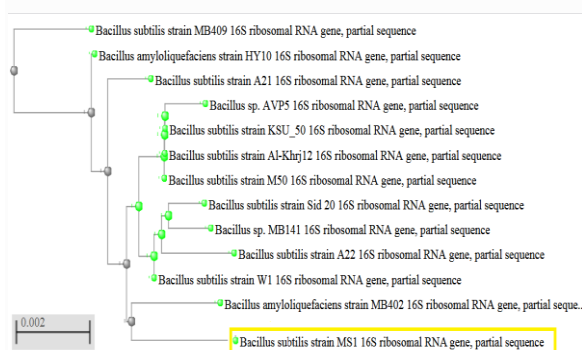

Fig 2. Phylogenetic tree of the *Bacillus subtilus* MS1 partial 16S rRNA sequence in comparison to closely similar sequences in GenBank databases.

3.3 Optimization of protease production

To enhance enzyme production and make the process less cost-effective, process parameters should be optimized. Despite the reality that different *Bacillus* species have similar growth patterns and enzyme profiles, the optimal environment condition for each strain varies. *Bacillus cereus* HP RZ17 and *Paenibacillus xylanilyticus* HP RZ19 produced the most protease throughout two days at 30°C and an alkaline pH (Jadhav et al., 2020). In the current study, the *Bacillus subtilis* MS1 strain was treated with several growth conditions in determining the most effective culture conditions for protease production. The conclusions of the incubation period investigation revealed the highest growth rate and protease activity were observed 7.482 U/ml at was observed at incubation period after 2 days (Figure 3), and the maximum bacterial growth and protease production were noted at 7.576 U/ml at pH 10.0 (Figure 4), Protease was increased by increasing the incubation temperature and the maximum yield was found 9.322 U/ml at 50°C (Figure 5) and then an increase of temperature decreased protease synthesis, different carbon sources (maltose, sucrose, fructose, lactose, starch, and glycerol) at a concentration of 1% the maximum protease activity was found in case of glucose as a carbon source that 9.298 U/ml (Figure 6), and the various organic and inorganic nitrogen sources effect of the bacterial growth and protease synthesis from *Bacillus subtilus* MS1was indicated in Figure (7) peptone gave the best enzyme productivity 9.33 U/ml. Kanmani et al [31] investigated that the *Bacillus* generally produces extracellular protease during the late proliferative growth, according to an experiment to investigate protease production at pH 9-13.
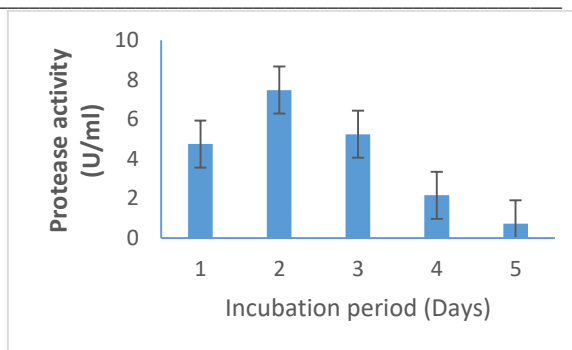

Fig.3 The effect of the incubation period on the production of alkaline protease
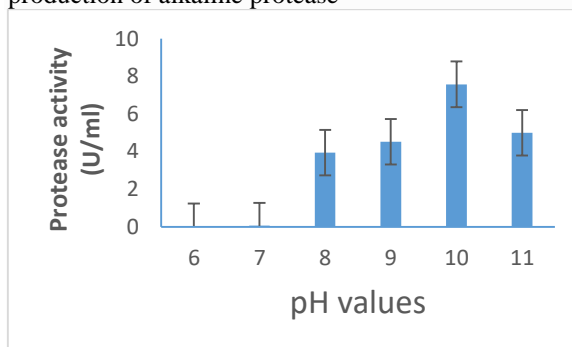

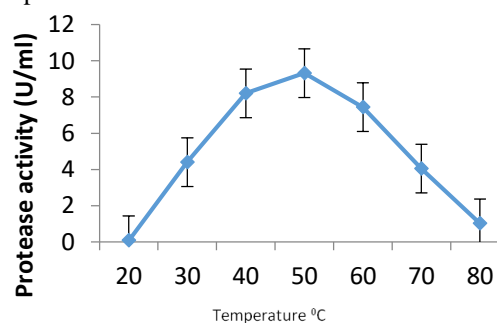**Fig.4** The effect of the pH on the production of alkaline protease

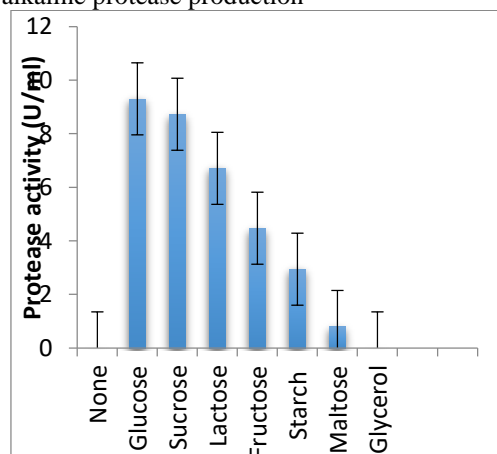**Fig. 5**. The effect of the temperature on the production of alkaline protease production

Fig. 6. The effect of the different carbon sources on alkaline protease production
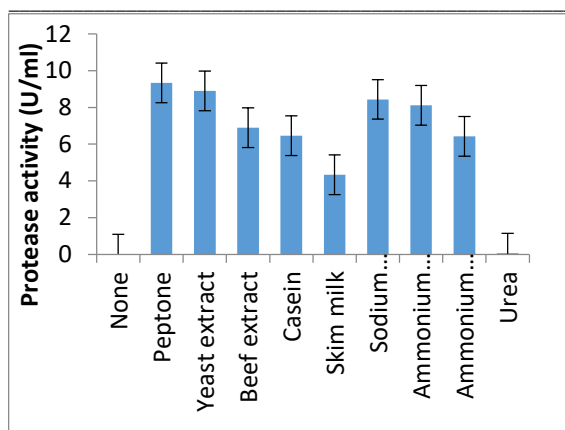
Fig.7 The effect of different nitrogen sources on alkaline protease production

3.4 Protease purification

The purification of protease by ammonium sulfate then Sephadex G-100 the precipitation of protein was 4.65 and 1.024 mg, and the specific activity for the enzyme was 67.558 and 237.773 U/mg respectively (Table 1). At the end of the purification yields 24.437 U/mg by using the Sephadex G-100 from the crude extract (Figure 8). The proteins in fractions 4-10 were collected and concentrated by ammonium sulphate precipitation for further purification by gel filtration chromatography, according to the quantity of protease activity from *Limosilactobacillus fermentum* R6 [33]. Also, Kanmani et al [31] used ammonium sulphate saturation to partially purify and lyophilize an enzyme from *Bacillus cereus*. The 80 percent saturation gave a maximum saturation of 0.9 g, while Rao et al. [34] used an ethanol precipitation protocol to partially purify and concentrate three major proteins with protease activity from the crude extract.

3.5 Optimization of the purified protease activity

The purified extracellular protease from *Bacillus subtilus* MS1 in the temperature range of 30-80°C and exhibited the maximum level of initial velocities at a temperature of 50°C the activity was 12.181 U/ml (Figure 9) and the thermal stability examined at range 30-100°C for 10-60 minutes Figure (10) indicated that the activity of the enzyme after 50°C start decrease till 100°C and the stable range 30-80°C. Figure (11) showed that expressed protease activity over a broad pH range (6-11) and exhibited a maximum level of initial velocities at pH 10 the activity was 12.177 U/ml and for the pH stability at range (8-11) showed in Table (2). Effects on protease activity of various inhibitors phenylmethyl sulphonyl fluoride, cysteine inhibitors p-chloromercuric benzoate, ethylenediamine tetra acetic acid and metal ions of calcium (Ca^{+2}), zinc (Zn^{+2}), magnesium (Mg^{+2}), cobalt (Co^{+2}), and silver (Ag^{+1}) indicated in table 3 the highest relative activity 140% for Ca^{+2} and could be inhibited by PMSF and Ag^{+1}. Sun et al. [33] study the protease produced by *Lactobacillus fermentum* R6 had the highest activity at pH 6, 40°C and protease activity

could be inhibited by ethylenediamine tetra acetic acid disodium salt (EDTA). The purity of protease was assured by SDS-PAGE where the molecular weight of the enzyme was 30 kDa. Beg and Gupta, [35] stated that the molecular mass of protease from *B. mojavensis* was 30 kDa. Mostly, the molecular weight of alkaline proteases from different *Bacillus* species extended between 17-40 kDa [36-41], with little exceptions of high molecular weight, up to 90 kDa from *Bacillus* subtilis [42]. Temperature 40°C, pH 9.0, 35 ppt salinity, and 3% casein as the ideal substrate were used to optimize the protease enzyme from *Bacillus cereus* [31].

Table. 1. Summary of the purification of protease

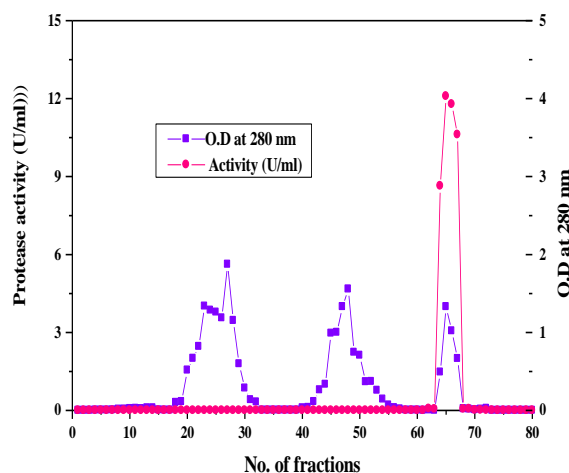| Purification steps	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Purification (fold)	Recovery (%)
Crude	9332	959	9.730	1	100
$(NH_4)_2SO_4$ (60%)	314.145	4.65	67.558	6.943	3.366
Sephadex G-100	243.48	1.024	237.773	24.437	2.609

Fig 8. Elution profile of protease by Sephadex G-100 column (2.6 × 75 cm) with a flow rate of 30 ml/h
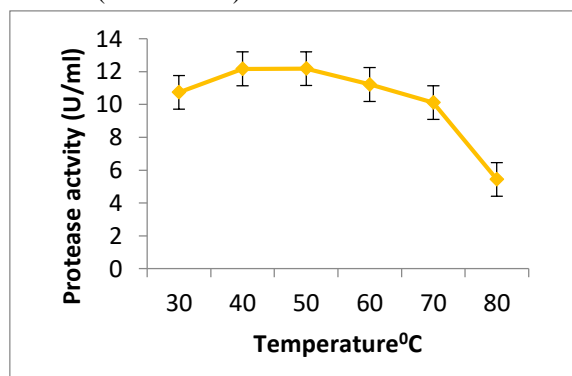

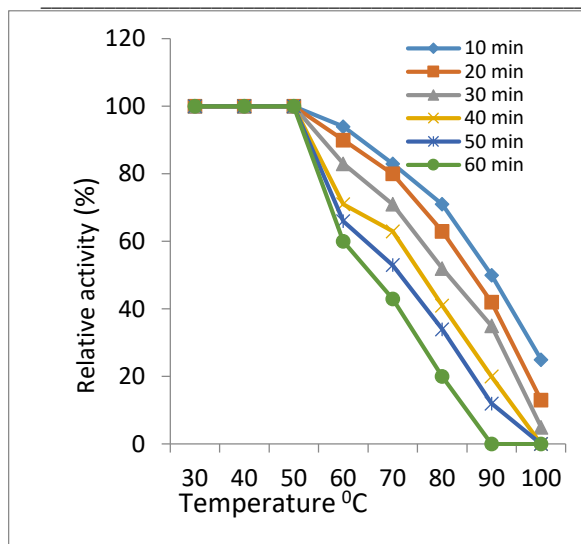**Fig.9** Effect of temperature on the activity of purified protease
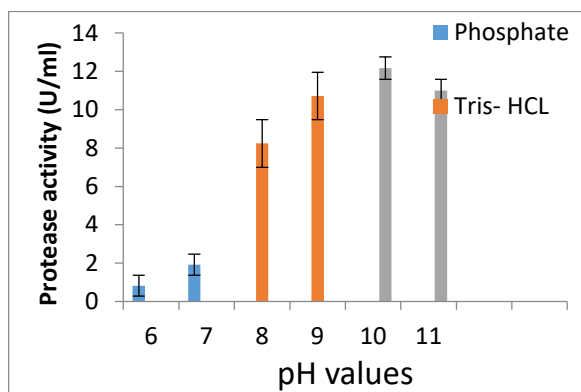
Fig.10 Thermal stability of the purified protease

Fig. 11 Effect of pH on the activity of purified protease

Table 2. Effect of pH on the Stability of purified protease

pH	Residual activity (%)
7	10
8	78.63
9	90.88
10	100
11	95.96

Table 3. Effect of inhibitors and metal ions on protease activity

Inhibitors/ Activator (5mM)	Relative Activity (%)
Control	100
PMSF	0
*P*CMB	60
EDTA	50
Ca^{2+} (CaCl2)	140
Mg^{2+} (MgCl2)	105
Zn^{2+} (ZnCl2)	80
Co^{2+} (CoCl2)	60
Ag^{+} (AgNO$_3$)	0

3.6 Mode of a protease enzyme on the stains

The enzyme preparation was supplemented as detergent significantly improves the cleaning within 20, and 40 minutes after different incubation times, each piece was rinsed with water for 2 min and then dried Figure 12 showed the effect of the enzyme to remove both blood and chocolate so, we can have used the purified enzyme. The usage of protease from *Bacillus subtilus* MS1 as a detergent addition was investigated to see if it improved the washing performance of detergent stained with human blood and chocolate similar to that study Kanmani et al [31], purified enzyme from *Bacillus cereus* was used in the stain removal of egg yolk, blood, and chocolate stains, and Nadeem et al. [43] agreed that *Bacillus licheniformis* N-2 had a high capability of removing blood stains, indicating its potential in the detergent industry.
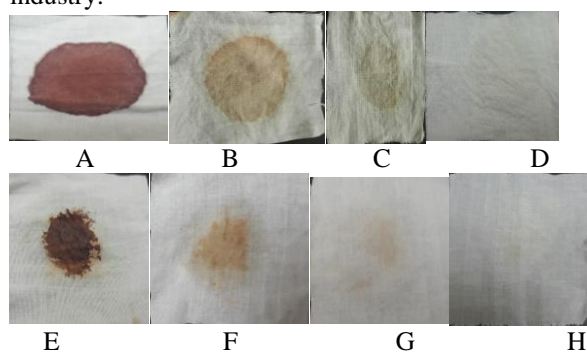

Figure 12. A. Bloodstain cloth, B. Bloodstain cloth washed with water only (control), C. Bloodstain cloth washed with protease for 20 min., D. Bloodstain cloth with protease for 40 min. E. Chocolate stain cloth, F. Chocolate stain cloth washed with water only (control), G. Chocolate stain cloth washed with protease for 20 min. H. Chocolate stain cloth with protease for 40 min.

4. **Conclusion**

The parameters and characteristics of *Bacillus subtilus* MS1 protease, which was isolated from sewage sediment in West Alexandria was useful for potential applications on detergent additives to remove the stains.

5. **Conflicts of interest**

The authors declare that they have no competing interests.

6. **Formatting of funding sources**

Not applicable

7. **Acknowledgments**

Not applicable

8. **References**

1. Barzkar, N. (2020). Marine microbial alkaline protease: An efficient and essential tool for various industrial applications. International Journal of Biological Macromolecules *161*, 1216-1229.

2. Majethiya, V., and Gohel, S. (2020). Isolation and Screening of Extracellular Enzymes Producing Actinobacteria Associated With Sea Weed. Available at SSRN 3560095.

3. Naveed, M., Nadeem, F., Mehmood, T., Bilal, M., Anwar, Z., and Amjad, F. (2021). Protease—a versatile and ecofriendly biocatalyst with multi-industrial applications: an updated review. Catalysis Letters *151*, 307-323.

4. Maitig, A.M.A., Alhoot, M.A., and Tiwari, K. (2018). Isolation and screening of extracellular protease enzyme from fungal isolates of soil. Journal of Pure and Applied Microbiology *12*, 2059-2068.

5. Munawar, T.M., Aruna, K., and Swamy, A. (2014). Production, purification and characterization of alkaline protease from agro industrial wastes by using Aspergillus terreus (AB661667) under solid state fermentation. International Journal of Advanced Research in Engineering and Applied Sciences *3*, 12-23.

6. Souza, P.M.d., Bittencourt, M.L.d.A., Caprara, C.C., Freitas, M.d., Almeida, R.P.C.d., Silveira, D., Fonseca, Y.M., Ferreira Filho, E.X., Pessoa Junior, A., and Magalhães, P.O. (2015). A biotechnology perspective of fungal proteases. Brazilian Journal of Microbiology *46*, 337-346.

7. Hariharan, A., Rajadurai, U.M., and Palanivel, I. (2019). Isolation, Purification and Mass Production of Protease from Bacillus Subtilis. Available at SSRN 3370124.

8. Kumar, S., Sharma, N.S., Saharan, M.R., and Singh, R. (2005). Extracellular acid protease from Rhizopus oryzae: purification and characterization. Process Biochemistry *40*, 1701-1705.

9. Wang, H., Liu, J., Chen, Q., Kong, B., and Sun, F. (2021). Biochemical properties of extracellular protease from Staphylococcus epidermidis isolated from Harbin dry sausages and its hydrolysis of meat protein. Food Bioscience, 101130.

10. Rifaat, H.M., El-Said, O.H., Hassanein, S.M., and Selim, M.S. (2007). Protease activity of some mesophilic streptomycetes isolated from Egyptian habitats.

11. Hakim, A., Bhuiyan, F.R., Iqbal, A., Emon, T.H., Ahmed, J., and Azad, A.K. (2018). Production and partial characterization of dehairing alkaline protease from Bacillus subtilis AKAL7 and Exiguobacterium indicum AKAL11 by using organic municipal solid wastes. Heliyon *4*, e00646.

12. Rifaat, H.M., Hassanein, S.M., El-Said, O.H., Saleh, S.A., and Selim, M.S. (2005). Purification and characterisation of extracellular neutral protease from Streptomyces microflavus. Arab. J. Biotechnol *9*, 51-60575.

13. Subhashini, P., Annamalai, N., Saravanakumar, A., and Balasubramanian, T. (2012). Thermostable alkaline protease from newly isolated Vibrio sp.: extraction, purification and characterisation. Biologia *67*, 629-635.

14. Jadhav, H., Sonawane, M., Khairnar, M., and Sayyed, R. (2020). Production of alkaline protease by rhizospheric Bacillus cereus HP_RZ17 and Paenibacillus xylanilyticus HP_RZ19. Environmental Sustainability *3*, 5-13.

15. Wu, S., Liu, G., Zhang, D., Li, C., and Sun, C. (2015). Purification and biochemical characterization of an alkaline protease from marine bacteria Pseudoalteromonas sp. 129-1. Journal of basic microbiology *55*, 1427-1434.

16. Ramesh, S., Rajesh, M., and Mathivanan, N. (2009). Characterization of a thermostable alkaline protease produced by marine Streptomyces fungicidicus MML1614. Bioprocess and biosystems engineering *32*, 791-800.

17. Hou, J., Yin, X.-M., Li, Y., Han, D., Lü, B., Zhang, J.-Y., and Cui, H.-L. (2021). Biochemical characterization of a low salt-adapted extracellular protease from the extremely halophilic archaeon Halococcus salifodinae. International Journal of Biological Macromolecules *176*, 253-259.

18. Sjödahl, J., Emmer, Å., Vincent, J., and Roeraade, J. (2002). Characterization of proteinases from Antarctic krill (Euphausia superba). Protein expression and purification *26*, 153-161.

19. Uyar, F., Porsuk, I., Kizil, G., and Yilmaz, E.I. (2011). Optimal conditions for production of extracellular protease from newly isolated Bacillus cereus strain CA15. EurAsian Journal of BioSciences *5*, 1-9.

20. Ali, O.A.G. (1992). Formation of proteases by Aspergillus fumigatus and Pencillium sp. King Saud University–Science *4*, 127-136.

21. Sundararajan, S., Kannan, C.N., and Chittibabu, S. (2011). Alkaline protease from Bacillus cereus VITSN04: potential application as a dehairing agent. Journal of bioscience and bioengineering *111*, 128-133.

22. Yang, S., and Huang, C. (1994). Protease production by amylolytic fungi in solid state fermentation. JOURNAL-CHINESE AGRICULTURAL CHEMICAL SOCIETY *32*, 589-589.

23. Sneath, P.H. (1986). Endospore-forming Gram-positive rods and cocci. Bergey's manual of systematic bacteriology *2*, 1104-1207.

24. Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. Journal of bacteriology *173*, 697-703.

25. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5:

molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution *28*, 2731-2739.

26. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry *72*, 248-254.

27. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. nature *227*, 680-685.

28. Adinarayana, K., Ellaiah, P., and Prasad, D.S. (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-11. Aaps Pharmscitech *4*, 440-448.

29. Rinderknecht, H., Geokas, M., Silverman, P., and Haverback, B. (1968). A new ultrasensitive method for the determination of proteolytic activity. Clinica chimica acta *21*, 197-203.

30. Lantz, M.S., and Ciborowski, P. (1994). [45] Zymographic techniques for detection and characterization of microbial proteases. Methods in enzymology *235*, 563-594.

31. Kanmani, R., Dhivya, S., Jayalakshmi, S., and Vijayabaskar, P. (2011). Studies on detergent additives of protease enzyme from an estuarine bacterium Bacillus cereus. Int. Res. J. Biotechnol *2*, 157-163.

32. Ahearn, D., Meyers, S., and Nichols, R. (1968). Extracellular proteinases of yeasts and yeastlike fungi. Applied Microbiology *16*, 1370-1374.

33. Sun, F., Hu, Y., Yin, X., Kong, B., and Qin, L. (2020). Production, purification and biochemical characterization of the microbial protease produced by Lactobacillus fermentum R6 isolated from Harbin dry sausages. Process Biochemistry *89*, 37-45.

34. Rao, M.B., Tanksale, A.M., Ghatge, M.S., and Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. Microbiology and molecular biology reviews *62*, 597-635.

35. Beg, Q.K., and Gupta, R. (2003). Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from Bacillus mojavensis. Enzyme and Microbial Technology *32*, 294-304.

36. Tang, X.-M., Lakay, F., Shen, W., Shao, W.-L., Fang, H.-Y., Prior, B., Wang, Z.-X., and Zhuge, J. (2004). Purification and characterisation of an alkaline protease used in tannery industry from Bacillus licheniformis. Biotechnology letters *26*, 1421-1424.

37. Kim, W., and Kim, S. (2005). Purification and characterization of Bacillus subtilis JM-3 protease from anchovy sauce. Journal of food biochemistry *29*, 591-610.

38. Guangrong, H., Tiejing, Y., Po, H., and Jiaxing, J. (2006). Purification and characterization of a protease from thermophilic Bacillus strain HS08. African Journal of Biotechnology *5*.

39. Yossan, S., Reungsang, A., and Yasuda, M. (2006). Purification and characterization of alkaline protease from Bacillus megaterium isolated from Thai fish sauce fermentation process. Science Asia *32*, 377-383.

40. Haddar, A., Agrebi, R., Bougatef, A., Hmidet, N., Sellami-Kamoun, A., and Nasri, M. (2009). Two detergent stable alkaline serine-proteases from Bacillus mojavensis A21: purification, characterization and potential application as a laundry detergent additive. Bioresource Technology *100*, 3366-3373.

41. Padmapriya, B., Rajeswari, T., Nandita, R., and Raj, F. (2012). Production and purification of alkaline serine protease from marine Bacillus species and its application in detergent industry. Eur J Appl Sci *4*, 21-26.

42. Kato, T., Yamagata, Y., Arai, T., and Ichishima, E. (1992). Purification of a new extracellular 90–kDa serine proteinase with isoelectric point of 3.9 from Bacillus subtilis (natto) and elucidation of its distinct mode of action. Bioscience, biotechnology, and biochemistry *56*, 1166-1168.

43. Nadeem, M., Qazi, J.I., Baig, S., and Syed, Q.-u.-A. (2008). Effect of medium composition on commercially important alkaline protease production by Bacillus licheniformis N-2. Food Technology and Biotechnology *46*, 388-394.