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Partial Purification and Characterization of Protease Enzyme from *Bacillus subtilis* and *Bacillus cereus*

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Abstract

The aim of this experimental study was to isolate and partially purify protease enzyme from *Bacillus cereus* and *Bacillus subtilis*. Protease enzyme is obtained by inducing spore genesis of bacteria from *Bacillus* species in suitable nutrient plates. The partial purification was realized by applying, respectively, ammonium sulfate precipitation, dialysis, and DEAE-cellulose ion-exchange chromatography to the supernatant that was produced later. Optimum pH, optimum temperature, pH stability, and temperature stability were determined, as well as the effects of pH, temperature, substrate concentration, reaction time, and inhibitors and activators on enzyme activity. In addition, the molecular mass of the obtained enzyme was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The specific activity of partially purified enzyme from *B. subtilis* was determined to be 84 U/mg. The final enzyme preparation was eight-fold more pure than the crude homogenate. The molecular mass of the partially purified enzyme was found to be 45 kDa by using SDS-PAGE. The protease enzyme that was partially purified from *B. cereus* was purified 1.2-fold after ammonium sulfate precipitation. The molecular mass of the partially purified enzyme was determined to be 37 kDa by using SDS-PAGE.

Index Entries: Enzyme; protease; *Bacillus subtilis*; *Bacillus cereus*; purification; isolation.

Introduction

Proteases are one of the most important industrial enzymes, accounting for nearly 60% of total worldwide sales of enzyme. Of these, alkaline proteases are employed primarily as cleansing additives. Among various proteases, bacterial proteases are most significant, compared with animal and fungal proteases. Proteases constitute one of the most important groups

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of industrial enzymes, being extensively used in the food, detergent, and other industries. Ideally, proteases used in a detergent formulation should have a high level of activity over a broad range of pH values and temperatures *(1,2)*.

Bacillus subtilis is well known for its use in the production of industrially important enzymes such as amylase, and proteases. The bacteria of genus *Bacillus* are active producers of extracellular proteases, and the characteristics of enzyme production by *Bacillus* species have been well studied. The production of protease depends on the availability of both nitrogen and carbon sources in the medium; either an excess or a low level of nitrogen may cause an inhibition of the biosynthesis of protease by *Bacillus* species. The production of protease may also be inhibited by the level of carbon source *(2)*.

Bacillus cereus may cause postoperative infections and inflammations occurring in the lung, eye, cerebral leptomeninges, endocardium, and connective tissue. *B. cereus* produces an emetic or diarrhea syndrome induced by emetic toxin and enterotoxin, respectively. Proteases, among other toxins, are produced during growth. These may contribute to the pathogenicity of *B. cereus* in nongastrointestinal disease *(3,4)*.

In the present study, we attemped to isolate bacteria from soil and optimize it for protease production by a promising strain. Different bacterial strains were isolated from soil and screened for their ability to produce protease, and two potential producers were examined and identified as *B. subtilis* and *B. cereus.*

Materials and Methods

Chemicals

The compounds obtained from Sigma (Dorset, England) were Bacto Tryptone, Bacto Soytone, Bacto Agar, peptone, beef extract, ammonium sulfate, manganese chloride, calcium chloride, sodium carbonate, and sodium bicarbonate. Manganese sulfate, acetic acid, tricholoroacetic acid (TCA), sodium acetate, boric acid, sodium hydroxide, casein, ammonium sulfate, HCL, sodium chloride, ethyl alcohol, EDTA, Tris, sodium dodecyl sulfate, ammonium persulfate, glycerol, and tin sulfate were purchased from Merck, (Darmstadt, Germany). Acrylamide, bisacrylamide, and glysine were obtained from Bio-Rad (Marnes la Qoquette, France). DEAE Cellulose anion exchanger from Sigma (Dorset, England) was used for column chromatography.

Isolation, Screening, and Identification of Protease-Producing Strains

Different bacterial strains were isolated from the waste-discharge area of the leather industry and were screened for their ability to produce protease. Samples collected from different leather factories in Turkey were analyzed for their content of microorganisms, especially for the genus

Bacillus. Two hundred microliters of each sample was spread onto different nutrient agar plates containing 5 g/L of peptone, 3 g/L of meat extract, and 15 g/L of agar (pH adjusted to 7.0) and cultured at 37°C for 24 h. Some colonies were detected. Each of these colonies then transferred to new agar plates and grown under the same conditions. First, the purity of the strains was checked. Second, by Gram staining test, the strains were examined under a microscope to determine whether they had the shape of a spore-forming rod and also whether they were Gram positive. Colonies that had the appropriate characteristics were further grown on agar plates to ensure adequate amounts of bacteria.

Specific identification of bacterial strains was done with an API 50 CH test kit (Biomérieux, Boulogne France), which is a standardized system of 50 biochemical tests for the study of carbohydrate metabolism of microorganisms. The API 50 CH strip consists of 50 microtubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids). The fermentation tests were inoculated with a suspension of microorganisms prepared with the suitable API CHB/E medium containing 2 g/L of ammonium sulfate, 0.5 g/L of yeast extract, 1 g/L of tryptone, 3.22 g/L of disodium phosphate, 0.12 g/L of monopotassium phosphate, 0.18 g/L of phenol red, and 10 mL of trace elements. This medium served to rehydrate the substrates. During incubation, fermentation was revealed by a color change in the tube, caused by the anaerobic production of acid and detected by the pH indicator present in the medium.

All the bacteria from the culture plate were picked up with an inoculation loop. Then, in a sterile solution of NaCl (0.85%), the bacteria were suspended to prepare a heavy suspension (S). To another tube of 5 mL of NaCl (0.85%) solution, drops were added from the suspension (S) to have a turbidity equivalent to 2 McFarland (approx 600×10^6 bacteria/mL). The number of drops was recorded as (n) number. The medium used to inoculate the strips (10 mL of API 50 CHB/E medium) was then inoculated by adding twice the number of drops ($2n$) from the suspension (S). Each tube in the test strip was inoculated with the bacteria-medium suspension and incubated at 37°C for 48 h.

Strips were read after 24 and 48 h of incubation. A positive test corresponded to an acidification revealed by the phenol red indicator contained in the medium changing to yellow. A positive test could become negative at the second reading, because of the production of ammonia from peptone, which neutralizes the acids. Such tests were recorded as positive. The results recorded as "+" or "–" were entered in the identification software. Two different bacterial strains were identified as *B. cereus* and *B. subtilis*.

To determine the strain that produced the most proteases, an API ZYM enzymatic activity research system was used. Using the same inoculation principle as for the API 50 CH strip, the strips containing 20 different sub-

strates were inoculated with a dense suspension of organisms to rehydrate the enzymatic substrates. The base of these strips was made of nonwoven fibers containing synthetic substrates. This allowed enzymatic reaction to take place even if the substrates were insoluble. The metabolic end products produced during the incubation period were detected through colored reactions revealed by the addition of reagents. Experiments were then carried out using the strain that this test determined was able to produce the most proteases.

Production of Enzyme

The cultivation of isolates was carried out in 1-L Erlenmeyer flasks containing 200 mL of nutrient broth (5 g of peptone, 3 g of meat extract, 10 mg of $MnSO_4 \cdot H_2O$ for 1 L of medium) as liquid medium. The isolates were cultivated for 20–22 h at 37°C with agitation of 200–250 rpm.

Partial Purification of Protease

After incubation, the culture broth was centrifuged at 4°C and 20,000*g* for 20 min. The supernatants were collected. The enzyme in the cell-free supernatant was precipitated between 40 and 70% saturation of ammonium sulfate. They were left for 30 min after the addition of salt, and the resulting precipitates were collected by centrifuging at 25,000*g* for 30 min. The precipitates were then dissolved in a minimal volume of 50 m*M* Tris-HCl, 5m*M* $CaCl_2$ (pH 7.5). These solutions were dialyzed overnight against 500 mL of the same buffer at 4°C. These solutions were applied to DEAE-cellulose ion-exchange chromatography. The supernatant that was obtained after ammonium sulfate saturation, dialysis, and centrifugation was loaded onto a DEAE-cellulose ion-exchange chromatography column (2 × 60 cm) that had been preequilibrated with Borax-NaOH buffer. The protein was eluted with the same buffer using a fraction size of 5 mL at a rate of 1/6 mL/min. Fractions containing the majority of the protease activity were pooled for activity assay. The activity of protease enzyme at the end of each step was measured by a spectropho- tometric method. The molecular mass of the obtained enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protease Assay

For measuring protease activity, a diluted enzyme solution (0.5 mL) was mixed with 2.5 mL of 0.6% casein solution (in Tris-HCl buffer, pH 7.2) and incubated for 10 min at 30°C. The reaction was terminated by adding 2.5 mL of TCA to the solution. The reaction mixture was filtered through Whatman no. 1 filter paper. The amount of tyrosine in the solution was measured by reading the absorbance at 275 nm. One unit of activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine in

1 min at 30°C. A tyrosine standard was prepared by dissolving different amounts of tyrosine in TCA solution.

Determination of Protein

Protein concentration was determined with 2,2´-bichinolyl-4,4´-dicarbonic acid, Na_2-salt at 570 nm *(16)* in a Bio-Rad Model 3550 UV Microplate Reader (Hercules, CA). Bovine serum albumin (BSA) was used as a standard.

Stability of Protease and Activity Profiles

Optimum pH, pH stability, optimum temperature, and temperature stability, as well as the effects of substrate concentration, time, and activators and inhibitors were determined for partially purified protease enzyme by using casein solution as substrate.

Effect of pH on Enzyme Activity and Stability

The optimum pH was determined at 30°C with casein solution prepared in Britton-Robinson (B-R) buffer ranging from pH 4.8 to 9.1.

pH stability was also determined at the same pH range in the same B-R buffer systems. Five hundred microliters of enzyme solution was incubated with 150 µL of B-R buffer for 20 h at 30°C. Then it was diluted with 0.05 *M* Tris-HCl buffer (pH 7.5) to 1 mL. For the reference value, 50 µL of enyzme was left at +4°C for 20 h in 150 µL of 0.05 *M* Tris-HCl buffer (pH 7.5) and then diluted with the same buffer to 1 mL.

Effect of Temperature on Enzyme Activity and Stability

Optimum temperature was determined by measuring the partial purified protease activity in a temperature range using the activity assay procedure at related temperature.

Temperature stability was determined by measuring the residual activity. An enzyme solution (50 µL) was incubated in 950 µL of 0.05 *M* Tris-HCl buffer (pH 7.5) for 30 min at different temperatures ranging from 30 to 70°C. Then the samples were put on ice and the relative activities were assayed at standard assay conditions.

The activity of partially purified enzyme was assayed at the same temperature range for 10 min. A 0.6% casein solution was preincubated at respective temperatures for 5 min before the enzymatic assay.

Effect of Substrate Concentration and Time on Enzyme Activity

Substrate concentration is an important factor in determining the degree of enzyme reaction. Casein substrate solution was prepared at concentrations of 1.2, 0.6, 0.3, 0.15, and 0.075%. Activity was assayed for every substrate concentration at 10, 30, 50, and 70 min.

Effect of Activator and Inhibitor on Enzyme Activity

The effects of some divalent cations on the activity of protease enzyme were also analyzed. Mn^{+2}, Fe^{+2}, and Ca^{+2} were used in the study.

$MnCl_2$, $FeCl_2$, and $CaCl_2$ solutions at 10^{-3} *M* were prepared, and 100 µL of activator solution and casein substrate solution was completed 2.5 mL. The solution was stored in a water bath at 30°C for 5 min, 0.5 mL of enzyme solution was added, and the reaction was stopped after 1 min by using 2.5 mL of TCA solution.

The effects of EDTA and Cu^{+2} were investigated. For this purpose, 10^{-3} *M* $CuCl_2$ and EDTA solutions were prepared, and 100 µL of inhibitor solution and casein substrate solution completed 2.5 mL. The solution was stored in a water bath at 30°C for 5 min, 0.5 mL of enzyme solution was added, and the reaction was stopped after 1 min by using 2.5 mL of TCA solution.

Determination of Molecular Mass of Partially Purified Enzyme

The molecular mass of partially purified protease enzyme from *B. subtilis* and *B. cereus* bacteria was analyzed by SDS-PAGE. These proteins standards (β-galactosidase: 116 kDa; BSA: 66.2 kDa; ovalbumin: 45 kDa; lactate dehydrogenase: 35 kDa; endonuclease Bsp 981: 25 kDa; β-lactoglobulin: 18.4 kDa; lysosyme: 14.4 kDa) were used as protein markers in the method.

Results and Discussion

Purification of Protease

Protease enzymes from two different *Bacillus* species, *B. subtilis* and *B. cereus*, were partially purified with 70% ammonium sulfate precipitation followed by dialysis. Only *B. subtilis* protease was loaded onto a DEAE-cellulose ion-exchange column. Their specific activities and degrees of purification are given in Tables 1 and 2.

Characterization of Protease

Optimum temperature and pH values of *B. subtilis* protease and *B. cereus* protease and their thermal stabilities were determined. The optimum temperature for *B. subtilis* protease and *B. cereus* protease was found to be 60°C, as shown in Fig. 1. This optimum temperature is higher than for other recorded proteases, which have optimum temperatures of 40 *(2)*, 45 *(5)*, and 50°C *(6)*. The thermal stability studies suggested that enzyme from *B. subtilis* protease was stable between 50 and 60°C and from *B. cereus* protease was stable between 50 and 70°C, as shown in Fig. 2.

The optimum pH of protease enzyme from *B. subtilis* protease was estimated to be 8.4 and from *B. cereus* protease, 7.4, as shown in Fig. 3. The pH stability of protease enzyme from *B. subtilis* protease and *B. cereus* protease was determined to be 3.5–6.5 and 4.0–6.0, respectively. After a pH of approx 7.5, a slow loss of activity was followed by a slight decrease, as shown in Fig. 4. On the other hand, the activity was more stable under acidic conditions such as pH 6.0 when compared with the other protease,

Table 1
Partial Purification of Protease Enzyme from *B. subtilis*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	258	2838	11	100	1
DEAE-cellulose ion-exchange column[a]	2.4	201.6	84	7.1	8

[a]After saturation to 40–70% with $(NH_4)_2\ SO_4$.

Table 2
Partial Purification of Protease Enzyme from *B. cereus*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	239	2868	12	100	1
Saturation to 40–70% with $(NH_4)_2SO_4$	150	2100	14	73.2	1.2

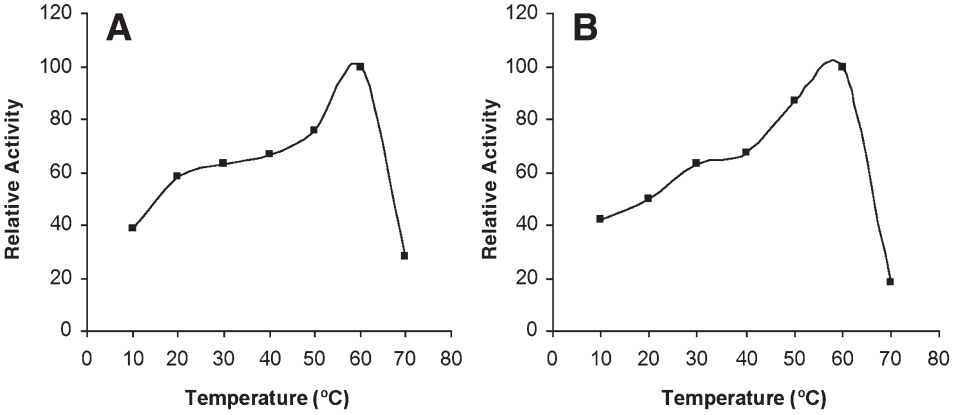

Fig. 1. Effect of temperature on activity of partially purified protease from **(A)** *B. subtilis* and **(B)** *B. cereus*.

but there was a decrease in the activity of *B. cereus* protease under alkaline conditions such as pH 8.0.

Substrate concentration is an important factor in determining the degree of enzyme reaction. Our results for *B. subtilis* protease are given in Table 3 and for *B. cereus* protease in Table 4. Generally, casein is used as the substrate for protease studies reported in the literature *(9,10)*. In our study,

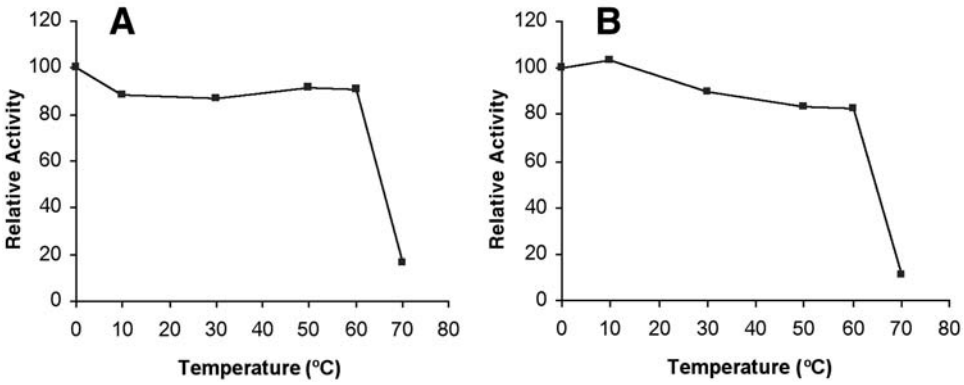

Fig. 2. Thermostability of partially purified protease from **(A)** *B. subtilis* and **(B)** *B. cereus*.

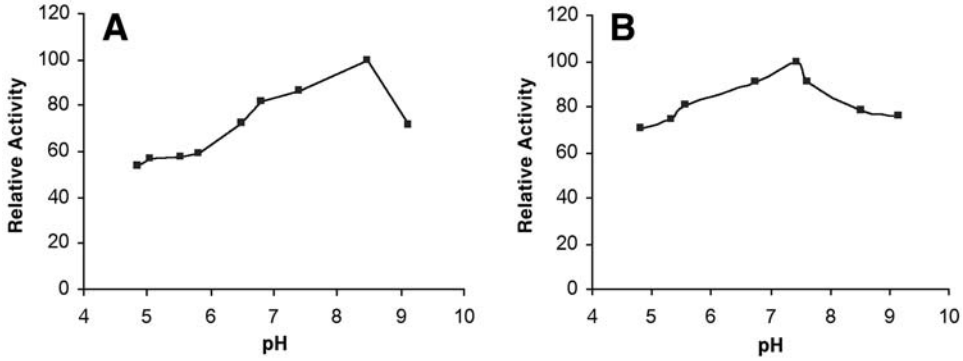

Fig. 3. Effect of pH on activity of partially purified protease from **(A)** *B. subtilis* and **(B)** *B. cereus*.

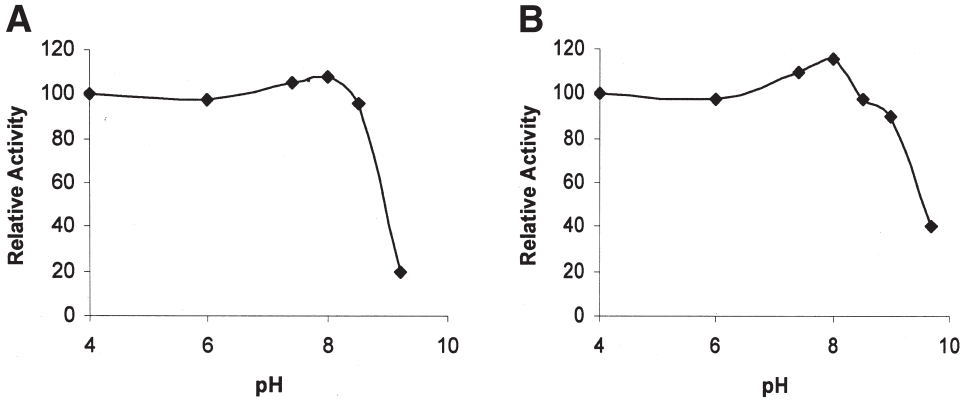

Fig. 4. pH stability of partially purified protease from **(A)** *B. subtilis* and **(B)** *B. cereus*.

Table 3
Effect of Substrate Concentration and Time on Activity of Partially Purified Protease from *B. subtilis*[a]

	Substrate concentration (%)				
Time (min)	1.2	0.6	0.3	0.15	0.075
10	61.8	62.6	65.3	56.5	81.2
30	70.3	62.7	70.8	68.4	61.5
50	31.4	83.4	85.8	83.3	32.8
70	95.8	98.9	104.9	97.9	85.8

[a]Activity values are in units/milliliter.

Table 4
Effect of Substrate Concentration and Time on Activity of Partially Purified Protease from *B. cereus*[a]

	Substrate concentration (%)				
Time (min)	1.2	0.6	0.3	0.15	0.075
10	40.4	37.5	44.8	39.8	47.1
30	45.4	35.3	38.6	46.9	36.6
50	26.2	58.4	54.4	54.4	26.2
70	73.0	69.4	63.9	58.6	50.9

[a]Activity values are in units/milliliter.

B. subtilis protease activity increased until 70 min for all concentrations studied. After this period, the activity was stable but *B. cereus* protease activity increased with an increment in both reaction time and substrate concentration. After 50 min, all substrate concentrations showed the same characteristics. This led us to believe that a concentration of 0.6% casein solution could be used in further studies.

The molecular mass of the enzyme obtained as a result of SDS-PAGE was found to be approx 37 kDa for *B. subtilis* protease and 45 kDa for BCP bacteria species, as shown in Fig. 5. Comparison of the molecular masses of both enzymes reveals that they are different from that of a protease examined by another group of researchers: a mol mass of 66 vs a mol mass of 45 for our study.

The presence of Mn^{+2} ion at 100 µL increased the protease activity for both bacteria species, as shown for *B. subtilis* protease in Table 5 and BCP in Table 6. EDTA at 100 µL produced a partial inhibitory effect for protease enzyme from *B. subtilis* bacteria, and $CuCl_2$ (100 µL) was able to decrease the velocity of the reaction very strongly for protease enzyme from *B. cereus* bacteria, as shown for *B. subtilis* protease in Table 7 and BCP in Table 8. Several researchers *(7–15)* examined the proteolytic activities of *B. subtilis*

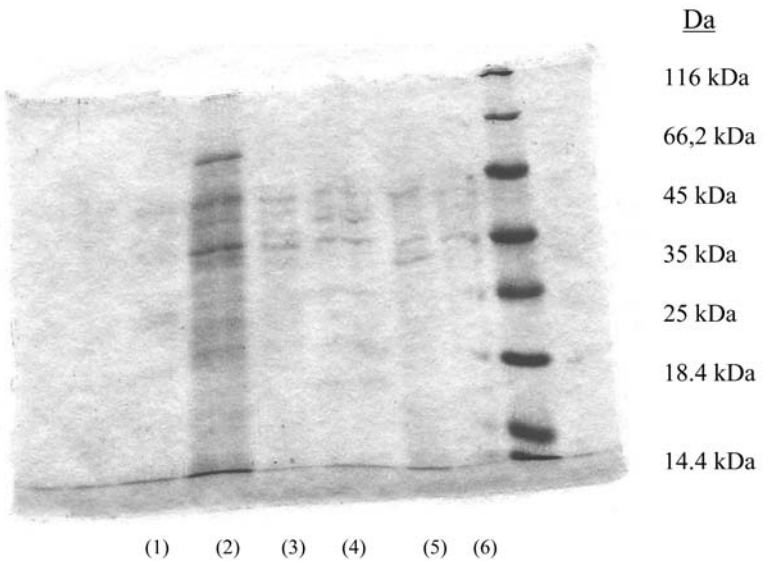

Fig. 5. Molecular mass of partially purified protease from *B. subtilis and B. cereus.* Lane 1, ammonium sulfate precipitation (*B. subtilis*); lane 3, ammonium sulfate precipitation (*B. cereus)*; lane 5, supernatant (*B. cereus*).

Table 5
Effect of Activator on Activity of Partially Purified Protease from *B. subtilis*

Substrate concentration (%)	Relative activity			
	$MnCl_2$ 10^{-3} (mol/L)	$FeCl_2$ 10^{-3} (mol/L)	$CaCl_2$ 10^{-3} (mol/L)	Absence of activator
0.6	119.5	101.6	105	100
0.3	117.4	95.1	106.9	100
0.15	118.1	99.9	95.6	100
0.075	130.7	118.3	100.0	100

Table 6
Effect of Activator on Activity of Partially Purified Protease from *B. cereus*

Substrate concentration (%)	Relative activity			
	$MnCl_2$ 10^{-3} (mol/L)	$FeCl_2$ 10^{-3} (mol/L)	$CaCl_2$ 10^{-3} (mol/L)	Absence of activator
0.6	124.6	124.7	105.1	100
0.3	131.2	105.9	116.4	100
0.15	123.8	116.3	106.3	100
0.075	117.1	108.8	107.9	100

Table 7
Effect of Inhibitor on Activity of Partially Purified Protease from *B. subtilis*

Substrate concentration (%)	Relative activity		
	$CuCl_2$ 10^{-3} (mol/L)	EDTA 10^{-3} (mol/L)	Absence of inhibitor
0.6	81.4	75.9	100
0.3	106.2	71.8	100
0.15	99.0	95.7	100
0.075	90.6	91.8	100

Table 8
Effect of Inhibitor on Activity of Partially Purified Protease from *B. cereus*

Substrate concentration (%)	Relative activity		
	$CuCl_2$ 10^{-3} (mol/l)	EDTA 10^{-3} (mol/l)	Absent of Inhibitor
0.6	96.4	103.3	100
0.3	78.0	92.9	100
0.15	95.0	113.7	100
0.075	99.2	96.4	100

and *B. cereus.* Some studied the protease obtained from the inside of the microorganisms cell. The others examined the typical extracellular proteolytic enzymes or proteases from spores. By comparing the two proteases (BSP and BCP) examined by the other researchers with the enzymes isolated in our study, it can be concluded that BCP is a metalloenzyme, as suggested by inhibition with EDTA *(8–15)*.

Conclusion

A high optimum pH is a feature of alkaline proteases *(8)*. In our study, proteases produced by two different *Bacillus* strains showed maximum activity at high temperatures. *B. subtilis* protease and BCP showed a good thermal stability. Good sporulation could also be obtained when the temperature was room temperature. These thermostable proteases are suitable for inclusion in detergent compositions and have potential uses in other biotechnological applications that would require higher working temperatures. Additional work is necessary in order to enable the full characterization (amino acid analysis, column chromatography for BCP) of the examined proteases.

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