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ORIGINAL PAPER

Production, optimization and purification of a novel extracellular protease from the moderately halophilic bacterium *Halobacillus karajensis*

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Abstract The production of a protease was investigated under conditions of high salinity by the moderately halophilic bacterium *Halobacillus karajensis* strain MA-2 in a basal medium containing peptone, beef extract, maltose and NaCl when the culture reached the stationary growth phase. Effect of various temperatures, initial pH, salt and different nutrient sources on protease production revealed that the maximum secretion occurred at 34°C, pH 8.0–8.5, and in the presence of gelatin. Replacement of NaCl by various concentrations of sodium nitrate in the basal medium also increased the protease production. The secreted protease was purified 24-fold with 68% recovery by a simple approach including a combination of acetone precipitation and Q-Sepharose ion exchange chromatography. The enzyme revealed a monomeric structure with a relative molecular mass of 36 kDa by running on SDS-PAGE. Maximum caseinolytic activity of the enzyme was observed at 50°C, pH 9.0 and 0.5 M NaCl, although at higher salinities (up to 3 M) activity still remained. The maximum enzyme activity was obtained at a broad pH range of 8.0–10.0, with 55 and 50% activity remaining at pH 6 and 11, respectively. Moreover, the enzyme activity was strongly inhibited by phenylmethylsulfonyl fluoride (PMSF), Pefabloc SC and EDTA; indicating that it probably belongs to the subclass of serine metalloproteases. These findings suggest that the protease secreted by *Halobacillus karajensis* has a potential for biotechnological applications from its haloalkaline properties point of view.

Keywords Haloalkaline protease · *Halobacillus karajensis* · Moderately halophilic bacteria · Protease production · Serine metalloproteases

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Introduction

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively. This group of enzymes represents one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sales in the world [1]. They have numerous applications in the industrial production of different items including detergents, foods, pharmaceutical, leather, diagnostics, waste management and silver recovery [2]. Despite the fact that many different proteases have been purified and characterized so far, and some of them have been used in biotechnological and industrial applications, the present known proteases are not sufficient to meet most of the industrial demands [3]. Industrial processes are carried out under many harsh conditions, which cannot always be adjusted to the optimal values required for the activity and stability of the available enzymes. Therefore, it would be of great importance to have available enzymes showing optimal activities at extreme values of pH, temperature, and different concentrations of salts.

Recently, considerable interest has been drawn on enzymes of moderately halophilic microorganisms and their biotechnological potentials [4]. This class of halophilic enzymes, while performing identical enzymatic functions as their non-halophilic or extreme halophilic counterparts, exhibit the capability to be active in either presence or absence of salt. Therefore, they have a new potential to be used in some industrial processes where the concentration of salt solutions used would vary during time [5]. Protease production and purification have been shown in some moderate halophiles such as *Pseudoalteromonas* sp. strain CP76 [6], an aerobic haloalkaliphilic strain belonging to the genus *Salinivibrio* [7], *Halobacillus* sp. SR5-3 [8], *Salinivibrio* sp. strain AF-2004 [3] and a haloalkaliphilic bacterium sp. AH-6 [9].

In this investigation, we report the production, optimization and effects of different parameters on bacterial growth and protease secretion. Also, the purification of a novel extracellular protease which was produced by a new moderate halophile bacterium, *Halobacillus karajensis* was performed and characteristics of the purified enzyme were compared with those of previously reported enzymes.

Methods

Bacterial strain and culture conditions

The organism used in this study was *Halobacillus karajensis* strain MA-2 (DSM 14948), which was isolated from saline soil (5% w/v total salt) in Iran and cultivated aerobically at 34°C in nutrient broth containing 10% (w/v) NaCl. Details on morphological, cultural, physiological and biochemical properties of the isolate MA-2 have been reported elsewhere [10]. A basal medium of the following composition ($g\ l^{-1}$) was used for the production of protease in 500 ml Erlenmeyer flasks containing 100 ml of the broth medium: peptone (Difco) 10; beef extract (Difco) 10; maltose (Merck) 5; NaCl 81; $MgCl_2$ 7; $MgSO_4$ 9.6; $CaCl_2$ 0.36; KCl 2; $NaHCO_3$ 2; NaBr 0.026 and pH 8.0. To determine the effects of various salts and carbon sources on protease production; either sodium chloride or maltose were replaced in the basal medium. Luria-Bertani broth (LB), tryptic soy broth (TSB), nutrient broth (NB), gelatin (1%) + yeast extract, nutrient gelatin broth and skim milk broth were also used for enzyme production. The kinetics of growth and the enzyme production were measured at different time intervals. Bacterial growth, along with enzyme activity, was measured by spectrophotometric method (Shimadzu model UV-160A). The data presented here are the average of triplicate measurements.

Protease purification

For protease purification, at the first step, pre-chilled acetone was gradually added to the supernatant of the culture medium up to 80% concentration with gentle stirring and left for 1 h. The precipitate formed by 80% saturation of acetone was collected by centrifugation at 12,000*g* for 20 min, dissolved in a minimum amount of 20 mM Tris–HCl, pH 8.5 containing 50 mM NaCl and 0.5 mM $CaCl_2$ and dialyzed against the same buffer for 24 h. The enzyme preparation was loaded on a Q-Sepharose HP column (1.6 × 20 cm), which had been equilibrated with the same buffer. The column was washed with equilibration buffer until no absorbance at 280 nm was detectable. The bound proteins were eluted by applying a linear gradient of 0.05–1 M NaCl at a flow rate of 1 ml/min. Active fractions (5 ml) were pooled and concentrated by ultrafiltration (Centricon, Amicon, USA) and used as the purified enzyme for further characterization. All the purification steps were performed at 4°C.

Protease activity assay

The proteolytic activity of the enzyme, with casein as the substrate, was determined by the modified method of Kunitz [11]. Enzyme solution with suitable concentration (50 µl) was added to 450 µl of substrate solution (0.5% casein in 20 mM Tris–HCl buffer, pH 8.5) and the mixture was incubated at 50°C. The reaction was stopped by adding 500 µl of 10% trichloroacetic acid (TCA) and kept at room temperature. After 15 min, the supernatant was separated by centrifugation at 14,000*g* for 10 min and absorbance at 280 nm was determined. The blanks were prepared in which 500 µl of TCA was added before enzyme addition. In some cases (effect of inhibitors) for enzyme activity measurement, to 500 µl of the TCA precipitated supernatant, 1.5 ml of Na_2CO_3 solution and 0.5 ml of 1 N Folin-Ciocalteu reagent were added and mixed thoroughly. The color developed after 20 min of incubation at 37°C was measured at 660 nm. All assays were done in triplicate. One unit of protease activity was defined as the amount of the enzyme yielding the equivalent of 1 µg of tyrosine per minute under the assay conditions (pH 8.5, 50°C). The specific activity is expressed in the units of enzyme activity per milligram of protein.

Protein determination

Protein concentration was measured by the method of Bradford [12] using BSA as the standard. During chromatographic purification steps, protein concentration was estimated by observing the absorbance at 280 nm.

Polyacrylamide gel electrophoresis and zymograms

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [13]. After heating the samples at 80°C for 4 min, molecular mass of the protease was determined in a 12% cross-linked polyacrylamide gel containing 0.1% sodium dodecyl sulfate at 4°C. A ready to use molecular marker (Fermentase; SM#0661) was used as a standard. Following running the gel, the proteins were stained in a solution containing 30% methanol, 10% acetic acid, and 0.2% Coomassie Brilliant Blue R-250. Gelatin zymography was performed in polyacrylamide slab gels containing SDS and gelatin (0.1%) as co-polymerized substrate, as described by Heussen and Dowdle [14] with some modifications. After electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 1 h at 25°C to remove SDS and were incubated under optimal assay conditions (20 mM Tris–HCl buffer, pH 8.5 and 50°C) for 30 min to perform the proteolytic activity. Finally, the gels were stained in a solution of 0.5% (w/v) amido black 10B. The activity band was observed as a clear colorless area depleted of gelatin in the gel against the blue background when destained in water–methanol–acetic acid (60:30:10) solution.

Influence of temperature, pH and salt on protease activity

To determine the optimum temperature for purified enzyme, the activity values of protease were measured at various temperatures from 20 to 80°C. The effect of pH on protease activity was studied by incubating the reaction mixture at pH values ranging from 5.0 to 12.0, in the following buffer systems 0.1 M sodium phosphate (pH 5.0–7.5); 0.1 M Tris–HCl (pH 8.0–10.0); 0.1 M glycine–NaOH (pH 9.5–12.0). Also, the effect of NaCl concentration on protease activity was determined in the presence of 0–4 M NaCl in enzyme reaction mixture.

Effect of inhibitors on proteolytic activity

The effect of inhibitors on the protease activity was examined after the protease had been pre-incubated with inhibitor for 30 min at 37°C, and the residual activity was determined by the standard assay method. The level of inhibition was expressed as percent activity remaining compared to a control without inhibitor.

Results and discussion

Protease production and effects of different parameters

Halobacillus karajensis strain MA-2 grew in various carbon sources and produced protease. However, the amount of the secreted enzyme varied in the presence of different carbon sources (Table 1). The highest protease production in basal medium occurred in the presence of lactose (35.6 units/O.D.$_{600}$), but in the case of glucose and sucrose in spite of good cell growth, the enzyme production was repressed. A catabolic repression mechanism for extracellular enzyme production is assumed as it has been reported for several proteases [6, 9, 15, 16]; however, enhancement of protease activity caused by lactose is not a common behavior.

Of the six nutrient sources investigated, the maximum protease secretion was observed in nutrient gelatin broth (47.8 units/O.D.$_{600}$). The comparative production of enzyme in presence of TSB, NB, LB, skim milk and nutrient gelatin broth revealed that while growth was similar in all cases, enzyme production varied extensively. The presence of gelatin had significant positive effect on the yield of protease activity with respect to nutrient broth medium. This activity increase in nutrient gelatin broth may be due to induction of the protease production by gelatin.

Kinetics of bacterial growth and protease production were investigated in the basal medium. The lag phase of bacterial growth was short (~2 h) and after 16 h the bacterial growth reached the stationary phase (Fig. 1). No extracellular proteolytic activity was shown during the early- and mid-exponential growth phase. Like other halophilic microorganisms, this strain showed proteolytic activity at the post-exponential phase of growth and reached to a plateau during the stationary phase. However, maximum protease production was detected in mid-stationary phase, while in another moderate halophile, *Pseudoalteromonas*

Table 1 The effect of various nutrient sources on protease production by *Halobacillus karajensis* strain MA-2

Nutrient sources	Cell growth (O.D.$_{600}$)	Activity (units/ml)	Protease production (units/O.D.$_{600}$)
Glucose	1.3	0	0
Maltose	1.6	50.2 ± 2.0	31.3
Sucrose	1.4	0	0
Lactose	1.6	57 ± 2.0	35.6
Skim milk	1.2	42.4 ± 2.0	35.3
TSB	1.6	38.7 ± 2.0	24.2
Leuria Bertani broth	1.6	48.9 ± 2.0	30.6
Nutrient broth	1.9	52.2 ± 2.0	27.5
Nutrient gel. broth	2.0	95.7 ± 2.0	47.8
Gel. + yeast extract	1.8	50.7 ± 2.0	28.2

The culture was grown for production of protease as described in "Methods". Maltose in the medium was replaced by other nutrient sources as listed. Sugars were added in 0.5, 1, and 2% to the basal medium. pH of the medium was adjusted to 8.0. Values are the average of three independent experiments ±SD

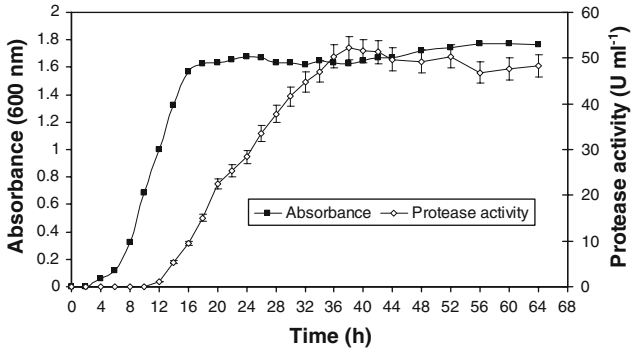

Fig. 1 Kinetics of growth and protease production in *Halobacillus karajensis* sp. strain MA-2 in the basal medium. Results represent the means of three experiments, and *bars* indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols

sp. strain CP76, maximum enzyme production was shown at the end of the exponential growth phase [6] and in a halo-alkaliphilic *Bacillus*, optimum proteolytic activity was shown in the early stationary phase [16].

The effect of different salts at various concentrations on growth of the strain and protease production is shown in Table 2. No growth of strain was detected in the medium lacking any salt and maximum production was exhibited when $NaNO_3$ (1.5 M) was added (55.8 units/O.D.$_{600}$). The isolate was capable of producing protease in the presence of NaCl, Na_2SO_4, KCl, $NaNO_3$ and $NaCH_3COOH$ in the following order: $NaNO_3 > Na_2SO_4 > NaCH_3COOH > NaCl > KCl$. The isolate was not able to growth in the medium containing sodium citrate. The salinity of the growth medium strongly influenced the protease production by this strain. The results clearly indicate the halophilic nature of strain MA-2 which the salt appears to be a prerequisite for growth and enzyme production. There is a good correlation between the optimal salt concentration for growth and protease production; similar behaviors were reported for other moderately halophilic bacteria with the capability of producing extracellular proteases [3, 7]. However, maximum production of the enzyme in the presence of $NaNO_3$ is unique for this strain and has not been reported so far [3, 6, 7, 9, 17].

Also the effects of initial pH of the medium and incubation temperature on protease production in the basal medium were investigated. Protease secretion was observed at a broad pH range from 5.0 to 10.0 and temperature range of 20–40°C with maximum production at pH 8.0–8.5 and 30–35°C (data not shown).

Purification and properties of the protease

The extracellular protease produced by *Halobacillus karajnesis* strain MA-2 was purified in two steps by 80% acetone precipitation followed by anion exchange chromatography on Q-Sepharose resin. The recovered active fraction from 80% acetone precipitated of culture broth was completely adsorbed on the Q-Sepharose matrix and no activity was detected in washing step. The bound protease was eluted with 0.4 M NaCl (in 20 mM Tris–HCl buffer, pH 8.5) by using a linear gradient of NaCl concentration between 0.05 and 1 M (data not shown). The results of the protease purification are summarized in Table 3. The protease was purified approximately 24-fold and about 68% of the total activity units was recovered. The specific activity of the purified enzyme was 1,523 units/mg. To our knowledge, the yield of the purification of this protease is unique amongst other purification reports. Proteases from other moderately halophilic bacteria have been purified by various combinations of chromatographic procedures. Namwong et al. [8] purified a serine protease by combination

Table 2 The effect of various salts on protease production by *Halobacillus karajensis* strain MA-2

Salt concentration (M)	Cell growth (O.D.$_{600}$)	Activity (units/ml)	Protease production (units/O.D.$_{600}$)
None	–	–	–
Sodium chloride			
0.5	1.8	51.3	28.5
1.0	1.8	47.1	26.2
1.5	1.6	43.2	27
2	1.3	36.2	27.8
Sodium nitrate			
0.5	1.7	80.3	47.2
1.0	1.5	81.2	54.1
1.5	1.5	83.7	55.8
2.0	1.5	78.5	52.3
Sodium sulphate			
0.5	1.7	58	34.1
1.0	0.4	6.8	17
1.5	0.1	0.7	7
2.0	0.08	0	0
Sodium acetate			
0.5	1.9	49.7	26.2
1.0	1.9	55.7	29.3
1.5	1.7	42.7	25.1
2.0	1.4	27.3	19.5
Sodium citrate			
0.5, 1.0, 1.5, 2.0	0	0	0
Potassium chloride			
0.5	1.6	47.7	29.8
1.0	1.6	47.1	29.4
1.5	1.6	41.3	25.8
2.0	1.5	38.8	25.9

Sodium chloride in the medium replaced by the salts listed. Values are averages of three independent experiments

Table 3 Purification of the serine metalloprotease from *Halobacillus karajensis* strain MA-2

Purification steps	Total activity (units)	Total protein (mg)	Sp. act. (units/mg)	Purification (fold)	Yield (%)
Culture supernatant	5,241	83.2	63	1.0	100
80% acetone	4,365	41.2	106	1.7	83
Q-Sepharose column	3,561	2.34	1,522	24	68

of ammonium sulfate precipitation and bacitracin–Sepharose affinity chromatography to 270-fold and 39% yield. Lama et al. [7] obtained 2.97% yield and 86.6-fold purification of a protease produced by *Salinivibrio* sp. strain 18AG by ammonium sulfate precipitation, Q-Sepharose, and Superdex 200 gel filtration chromatography. By employing Q-Sepharose anion-exchange and gel filtration chromatography, 2.9% yield and 64.8-fold purification was reported in case of *Pseudoalteromonas* sp. strain CP76 by Sánchez-Porro et al. [6]. Overall, it seems that using acetone as a solvent to precipitate proteins at high saturation is a potential method to get rid out other proteinaceous component from protease mixture. This supposes a method which can be used to purify protease solutions from a huge amount of other proteinaceous material at industrial scale.

SDS-PAGE of the crude and purified enzyme preparations is shown in Fig. 2a. The purified enzyme showed a single band corresponding to an apparent molecular mass of 36 kDa. This value is slightly smaller than that reported for some moderately halophilic proteases [3, 6, 7]. However, in comparison with other alkaline proteases from haloalkaliphilic *Bacillus* [16] and *Salinivibrio* sp. strain AF-2004 [18], this protease has higher molecular mass. Zymogram activity staining also revealed one clear zone of proteolytic activity against the blue background for both crude and purified samples at corresponding positions in SDS-PAGE (Fig. 2b).

Protease activity and the effects of different parameters

The characterization of the purified protease revealed that the enzyme exhibited optimum temperature for maximum protease activity at 50°C and pH 9.0. The protease undergoes thermal activation above 30°C with maximum activity between 45 and 55°C, followed by thermal inactivation above 60°C. The effect of temperature on protease activity is shown in Fig. 3a. The isolate showed 20 and 50% activity reductions at temperatures of 60 and 65°C, respectively. Khalil Beg and Gupta [19] reported a similar temperature optimum (50°C) for a protease produced by *Bacillus mojavensis*.

The pH values altered the protease activity at 50°C. The protease showed activity over a broad pH range (5.0–12.0) for casein hydrolysis and the optimum pH for the enzyme activity was pH 9.0 (Fig. 3b). The enzyme showed 40% activity reduction at pH 6.0 and 11.0. Biochemical properties of the enzyme such as temperature and pH profile revealed a moderate thermoactive and alkaliphilic character (maximum activity at 50°C and pH 9.0). These characteristics are roughly similar to alkaline proteases from *Salinivibrio* [3, 7] and the protease CP1 from *Pseudoalteromonas* sp. strain CP76 [6].

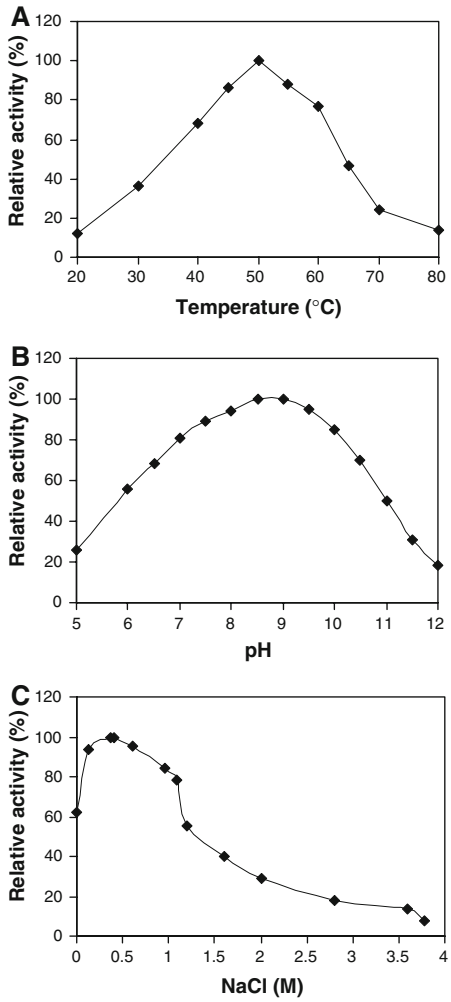

Fig. 2 Effect of temperature (pH 9.0) (**a**), pH (at 50°C) (**b**), and NaCl (**c**) on the caseinolytic activity of the serine metalloprotease of *Halobacillus karajensis*. The relative activity was defined as the percentage of activity detected with respect to the maximum protease activity. See "Methods" for further details

Enzyme activity was also determined at various NaCl concentrations (0–3.5 M) at 50°C and pH 9.0 (Fig. 3c). The highest activity was shown at 0.2–1 M NaCl. The enzyme

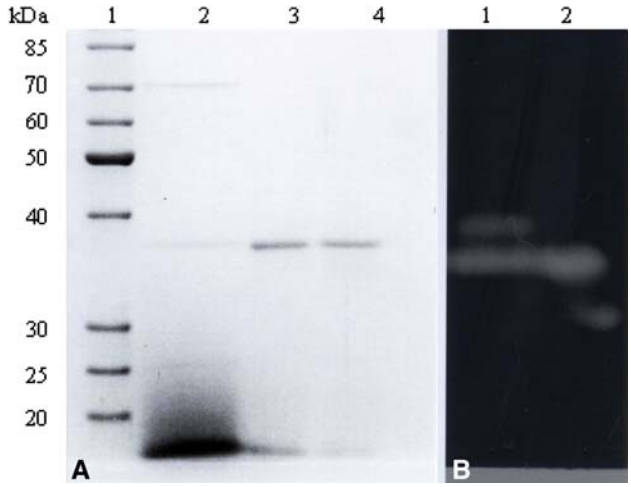

Fig. 3 **a** SDS-PAGE analysis of various purification steps of protease produced by *Halobacillus karajensis*. *Lane 1* molecular markers, *lane 2* culture media, *lane 3* Acetone precipitated (80%), *lane 4* Q-Sepharose column; **b** gelatin zymography of the purified protease in SDS-PAGE as described in "Methods"; *lane 1* Acetone precipitated (80%), *lane 2* purified protease after Q-Sepharose column

Table 4 Effect of different inhibitors on purified protease activity

Inhibitors	Final concentration	Residual activity (%)
Antipain	4 μM	90
Bestatin	130 μM	98
Chymostatin	100 μM	100
E-64	28 μM	99
Leupeptin	10 μM	90
Pepstatin	1 μM	100
	16.6 μM	97
Pefabloc SC	4 mM	4
PMSF	3.3 mM	0
Aprotinin (BPTI)	0.3 μM	98
	5 μM	93
Phosphoramidon	0.6 mM	83
EDTA-Na_2	1 mM	22
1,10-phenanthroline	10 mM	96

The enzyme was incubated with different inhibitors at 37°C for 30 min. The residual protease activity was measured as described in "Methods". The values shown are the percentages of the activity without inhibitors

retained 20 and 49% of its initial activity in 3 and 0 M NaCl, respectively. In contrast to proteases from the *Salinivibrio* sp. strain AF-2004 [3, 18] and similar with metalloprotease CP1 from *Pseudoalteromonas* sp. strain CP76 [6]; this enzyme needs salinity conditions to show maximum activity (0.2 M NaCl).

The effect of chelating agents and group-specific reagents on purified protease activity is summarized in Table 4. The complete inhibition of proteolytic activity was shown by serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and Pefabloc SC at concentrations of 4 and 3.3 mM, respectively. These results indicate that there is an active hydroxyl group in the active site microenvironment of the enzyme. No significant inhibition was detected with Bestatin, Chymostatin, E-64, Leupeptin or Pepstatin. Enzyme activity was also inhibited to 80% by 1 mM ethylene diamine tetraacetic acid (EDTA), while 1, 10-phenanthroline (a zinc-specific chelator) had no effect. Inhibition of the enzyme by EDTA suggested that this protease is a metalloprotease and no effect of O-Phe revealed that Zn^{2+} ions do not have important role in its catalytic function. Taken together, these data suggest that probably this protease belongs to the subclass of serine metalloproteases. Similar behavior was shown by a serine metalloprotease from *Burkholderia pseudomallei* [20] and also other metalloproteases that have been characterized from moderately halophilic bacteria [3, 6, 7].

Conclusions

Although halophilic microorganisms have increasing interest in recent years, most studies have been performed in extreme halophiles. However, moderately halophilic bacteria represent an excellent model of adaptation to frequent changes in extracellular osmolality and constitute an interesting group of microorganisms from a biotechnological point of view. The ability of the moderately halophilic bacteria to grow and produce enzymes over a very wide range of salinities make them very attractive for research and for the isolation of novel enzymes with unusual properties. The efficient purification of this protease by acetone precipitation and ion exchange chromatography suggests that acetone can be used as a good solvent to purify protease mixtures. Characterization of the purified protease and comparison with proteases from other moderately halophilic bacteria also reveals a high potential for this haloalkaliphilic protease in industrial applications.

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