Original Article Open Access

A novel low molecular weight extracellular protease from a moderately halophilic bacterium *Salinivibrio* sp. strain MS-7: production and biochemical properties

Mahnaz Shahbazi[1], Hamid Reza Karbalaei-Heidari[1, 2, *]

1) Molecular Biotechnology Lab, Department of Biology, Faculty of Sciences, Shiraz University, Shiraz 71454, Iran
2) Institute of Biotechnology, Shiraz University, Shiraz, Iran

A B S T R A C T

Kinetics of bacterial growth and protease production were monitored on a novel isolated moderately halophilic bacterium, *Salinivibrio* sp. strain MS-7, and maximum growth and protease activity was achieved after 48 hours at 30°C and 180 rpm. To determine the effect of various carbon sources on protease production, glucose, lactose, sucrose and maltose were investigated and maximum production of the enzyme was obtained in a basal medium (pH 8.0) containing maltose as a carbon source (494 U/ml). The protease was isolated from a stationary phase culture, purified 3.6-fold with 56% yield by a simple procedure and characterized biochemically. The enzyme revealed a monomeric structure with a relative molecular mass of 21 KDa by running on SDS-PAGE. Maximum caseinolytic activity of the enzyme was observed at 50°C, pH 8.0 and 0–0.5 M NaCl with a high tolerance to salt concentrations of up to 3 M. The effect of various metal ions and inhibitors on caseinolytic activity of the purified protease revealed that it probably belongs to the subclass of serine metalloproteases. These findings suggest that the protease secreted by *Salinivibrio* sp. strain MS-7 can be introduced as a candidate for biotechnological applications based on its haloalkaline properties.

Key words: *Salinivibrio* sp. strain MS-7; Biochemical properties; Low molecular weight protease; Moderately halophilic bacterium; Production optimization

INTRODUCTION

It is well evident that microbial proteases constitute one of the most important groups of industrial enzymes, as they account for at least a quarter of the total global enzyme production [1]. Microbial proteases are classified into various groups, depending on whether they are active under acidic, neutral or alkaline conditions, and the characteristics of their active sites, i.e., metallo, aspartic, cysteine, and serine-type [2]. Besides their physiological importance, they

* Address for correspondence: Molecular Biotechnology Lab, Department of Biology, Faculty of Sciences, Shiraz University, Shiraz 71454, Iran; P. O. Tel: +98 711 2280916; Fax: + 98 711 2280926;
E-mail: karbalaei@shirazu.ac.ir

ISSN 2322-181X

constitute a class of enzymes with broad applications in industrial fields. They are used in detergents, proteins, breweries, photography, leather, meat and dairy products and waste treatment industries [3].

Many extreme halophiles have been isolated and investigated for possible biotechnological applications, including the production of compatible solutes, enhanced oil recovery and the degradation of industrial pollutants in saline habitats [4-6]. Moreover, considerable attention has been drawn to enzymes of moderately halophilic microorganisms and their biotechnological potentials [7]. This class of halophilic enzymes, while performing identical enzymatic functions as their non-halophilic or extreme halophilic counterparts, exhibits the capability to be active in either presence or absence of salt [8].

The genus *Salinivibrio* includes moderately halophilic bacteria belonging to the family *Vibrionaceae* within the *Gammaproteobacteria* and, is represented so far by four species: *Salinivibrio costicola*, with three subspecies: *S. costicola* subsp. *costicola* [9], *S. costicola* subsp. *vallismortis* [10], *S. costicola* subsp. *alcaliphilus* [11], and *Salinivibrio proteolyticus* [12], *Salinivibrio siamensis* [13], and the newly published name *Salinivibrio sharmensis* [14].

In recent years, a number of studies have been conducted to characterize extracellular proteases from *Salinivibrio* genus. Lama *et al*. [15] reported a 38 KDa serine metalloprotease from *Salinivibrio* sp. strain AG18 with a synergetic increasing effect of calcium ion and NaCl on its activity. Moreover, we previously reported a newly isolated haloalkaliphilic strain AF-2004 belonging to the genus *Salinivibrio*. The strain AF-2004 (*Salinivibrio proteolyticus*) was isolated from Bakhtegan salt lake in Iran, and exhibited two proteinases (38 and 29 KDa) based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) activity staining. The 38-KDa purified protease showed its optimal activity at 65 ◦C, pH 8.5 with a high tolerance to salt concentrations of up to 4 M and was classified as a zinc-metalloprotease [16]. Another extracellular protease with MW of 29 KDa from *Salinivibrio proteolyticus* revealed good stability in the presence of organic solvents and was classified as a serine metalloprotease [17].

In view of the remarkable industrial and commercial value of halophilic proteases, the search for new microbial sources for these enzymes is of continuous interest and for this reason, in this report we describe the production and characterization of a new low molecular weight proteolytic enzyme from *Salinivibrio* sp. strain MS-7, isolated from Maharlou salt lake, which is a part of our attempt to identify novel proteases from the salt lakes of Fars province, Iran.

MATERIALS AND METHODS

Isolation of bacterial strain: The microorganism used for the present investigation was isolated from Maharlou salt lake (a hypersaline lake) in Iran. Among the many strains which were streaked onto the skim milk agar plates containing 5% (w/v) skim milk, 2% agar and 10% NaCl, the best producer with a good halo around the colony was selected as a strain MS-7. The clear zones around the colonies indicated the hydrolysis of casein by extracellular protease.

Growth conditions and media compositions: The bacterial strain MS-7 was aerobically cultured at 30 ^{o}C and 180 rpm in a nutrient broth containing 10% NaCl and 25% tap water. For the production of protease a basal medium containing (g/l): peptone 10; yeast extract 5; maltose 5; NaCl 81; $MgSO_4$ 9.6; $MgCl_2$ 7; $CaCl_2$ 0.36; $NaHCO_3$ 2 and pH 8.0 was used.

Growth kinetics and protease production: For evaluation of bacterial growth and protease production, different carbon sources and various pH's of the above mentioned medium were tested. Luria-Bertani broth (LB) and tryptic soy broth (TSB) were also used for enzyme production. Growth kinetics and enzyme production were measured at different time intervals. Bacterial growth, along with caseinolytic activity was monitored by spectrophotometric method (Shimadzu model UV-120). The data presents the triplicate measurement averages.

Identification of bacterial isolate: The isolated strain was identified by morphological and biochemical tests. The shape and size of cells, gram reaction, anaerobic growth, spore and pigment production, motility, oxidase, catalase, urease activities, citrate utilization, indole and H_2S production, nitrate reduction, hydrolysis of starch and casein, Voges-Proskauer and methyl red tests were carried out as recommended by Smibert and Krieg [18]. The ability to oxidase or ferment a variety of substrates was assessed as recommended by Ventosa et al [19]. The effect of different pH, temperature and salt concentrations on bacterial growth were determined by using the aerobic growth medium.

DNA amplification, sequencing and phylogenetic analysis: Strain MS-7 was also identified by the analysis of 16S rRNA gene sequence. Genomic DNA was extracted by QIAamp DNA mini kit (Qiagen, Germany) and 16S rDNA gene sequences were amplified using the universal primers HRK1 (5'-ACTCCTACGGGAGGCAGCAG-3') and HRK2 (5'-TGACGGGCGGTGTG TACAAG-3'). The amplification was done by initial denaturation at 95 ^{o}C for 4 min followed by 35 cycles of 93 ^{o}C for 1 min, 58 ^{o}C for 45 sec, and 72 ^{o}C for 1.5 min with a final extension step of 10 min at 72 ^{o}C. The amplified DNA was analyzed by 1% agarose gel electrophoresis, and the fragment was purified using the Roche agarose gel DNA extraction kit (Germany). The purified PCR product was sequenced in both directions using an automated sequencer by SeqLab Company, Germany. Database searches were conducted with the BLAST programs available at the National Center for Biotechnology Information (Bethesda, USA). For phylogenetic tree construction, the sequence was compared with 16S rRNA gene sequences in GenBank database using the alignment tool of the MEGA software package. A phylogram was constructed with MEGA 4.0 software programs [20] using neighbor-joining procedure.

Enzyme purification: Purification was the next step in this study, performed at 4 ^{o}C. Cell free supernatant of strain MS-7 was collected by centrifugation at 9000 g for 10 min after 48 h of cultivation. Cold acetone was slowly added to the supernatant up to 40% saturation and after throwing away the precipitate, further acetone was added to the solution up to 80% saturation with slow stirring and left for 30 min. The precipitate obtained after centrifugation at 12000 g for 20 min, was suspended in a minimal volume of 20 mM Tris-HCl, pH 8.0 containing 50 mM NaCl and 0.5 mM $CaCl_2$ (buffer A) and dialysed against the same buffer for 24 h with two buffer changes. The enzyme preparation was loaded on a DEAD-Cellulose column (2.5×10 cm), previously equilibrated with buffer A. Washing until the optical density of effluent at 280 nm almost reached zero, the column was then eluted with a linear gradient (0.05-0.6 M) of NaCl in the same buffer at a flow rate of 0.5 ml/min. Protein content and protease activity in the fractions were then determined. Active fractions were pooled and concentrated using ultrafiltration (Centricon, Americon, USA) and stored as aliquots at -20 ^{o}C for further use.

Protease activity assay: The proteolytic activity was assayed by the previously described method [16] using casein as a substrate at a concentration of 0.5% in a 20 mM Tris-HCl buffer,

pH 8.0. 475 µl of the substrate solution was pre-incubated at 50 ^{o}C for 3 min. The reaction was initiated by the addition of 25 µl purified enzyme, at 50 ^{o}C for 10 min. The reaction was terminated by the addition of 500 µl trichloroacetic acid (10% (w/v)). The mixture was allowed to stand at room temperature for 20 min and then centrifuged at 14000 g for 10 min to remove the precipitate. The absorbance of the supernatant was determined against a blank at 280 nm. One unit (U) of activity was defined as the amount of enzyme required to release 1 µg of Tyr/min under experimental conditions.

Total protein determination: Protein content of the enzyme solution was measured by Bradford's method [21], using Bovine Serum Albumin (BSA) as a standard, and estimated by observing the absorbance at 280 nm during different stages of purification.

SDS-Polyacrylamide gel electrophoresis: SDS-PAGE was carried out according to Laemmli's method [22] on a 5% stacking and a 12% resolving gel for control of the purity and estimation of enzyme molecular weight. Electrophoretic migration of the protease was compared with that of standard molecular weight markers (Vivantis; PR0602). The molecular weight of the enzyme was extrapolated from the plot of log molecular weight versus electrophoretic mobility of molecular weight marker.

Biochemical characterization pH and temperature optima: The optimum pH of the purified protease against casein 0.5% (w/v) as a substrate was determined by estimating the protease activity over a pH range of 5 to 12. The following buffer systems were used: 50 mM sodium acetate (pH 4-5.5), 25 mM sodium phosphate (pH 5.0-7.5), 50 mM Tris-HCl (pH 8.0-10.0), and 50 mM glycine-NaOH (pH 9.5-12). Similarly, the effect of temperature on caseinolytic activity was studied to determine the temperature optimum of the enzyme. Catalytic activity was measured by conducting the assay under different temperature ranges of 20 to 80 ^{o}C at pH 8.0 for 10 min.

Determination of K_m and V_{max}: The initial reaction rates of the purified enzyme were obtained using casein as a substrate at different concentrations ranging from 0.25 to 5 mg/ml at 50 ^{o}C and 20 mM Tris-HCl (pH 8.0). K_m and V_{max} values of the pure enzyme were calculated from Lineweaver-Burk plot.

Influence of metal ions and inhibitors on protease activity: The effect of some ions (K^+, Ba^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+}, Ni^{2+}, Fe^{2+}) on enzyme activity were investigated by measuring the casein hydrolysis activity of the purified enzyme after incubating it with ions (in chloride salts) at 37 ^{o}C for 30 min under standard assay conditions. To determine protease type, the effect of inhibitors on the protease activity was examined after the protease had been pre-incubated with the inhibitor for 30 min at 37 ◦C, and the residual activity was determined by standard assay method. The concentrations of metal ions and inhibitors (in the pre-incubation mixture) are listed in Table II. The enzyme activity without metal ions and inhibitors was taken as control and considered to be 100%.

RESULTS AND DISCUSSION

Characterization of the best protease producer and phylogenic analysis: Several bacterial strains with the capability to secrete protease were isolated from Maharlou salt lake in Iran. Among these, one strain exhibited prominent clear zones around the colonies on skim-milk agar plates indicating that it secretes significant amounts of protease (Figure 1).

Figure 1: Skim milk agar plate of *Salinivibrio* sp. strain MS-7 indicating casein hydrolysis

Morphological, physiological and biochemical analysis showed that the strain MS-7 was Gram negative, short curved-rod shaped, 1.71 µm, without spore and pigment. No growth was observed above 50 ^{o}C. The bacterium grew within a pH range of 5.0-9.0 with an optimum of 8.0. This strain grew well at various concentrations of NaCl ranging from 5 to 20% (w/v) with an optimum growth observed at 10% (w/v) NaCl. No growth was observed without NaCl, therefore, strain MS-7 was identified as a moderately halophilic bacterium. The isolated bacterium was positive for oxidase and citrate utilization, casein hydrolysis and Voges-Proskauer test and negative for H_2S and indole production, starch hydrolysis and methyl red test. Acid was produced aerobically from glucose, lactose, maltose, sucrose and anaerobically from glucose, maltose, and sucrose but not from lactose. 1067 bp 16S rRNA gene fragment was sequenced and submitted to NCBI GenBank (accession no. HQ832893.1). According to morphological and physiological characteristics and comparative sequence analysis of the 16S rRNA gene of strain MS-7 and other 16S rRNA available in the GenBank database, it was tentatively named as "*Salinivibrio* sp. strain MS-7". The phylogenetic tree (Figure 2), constructed by the neighbor-joining method, indicated that isolate MS-7 was part of the cluster within the genus *Salinivibrio*. Among the described sub species, the closest relative of isolate MS-7 was *Salinivibrio* sp. N9-1 and *Salinivibrio costicola* (X95530.1). Although strain MS-7 was closely related to the genus *Salinivibrio* species, characteristics such as nitrate and nitrite reduction, temperature range and NaCl concentration for growth and Voges-Proskauer and methyl red were different from other species of *Salinivibrio*.

Effect of cultural conditions on protease production by strain MS-7: The kinetic of bacterial growth and protease production was assessed during 72 h of cultivation in the basal medium. After about 4 h for the lag phase and 24 h for the exponential phase, bacterial growth reached the stationary phase (Figure 3).

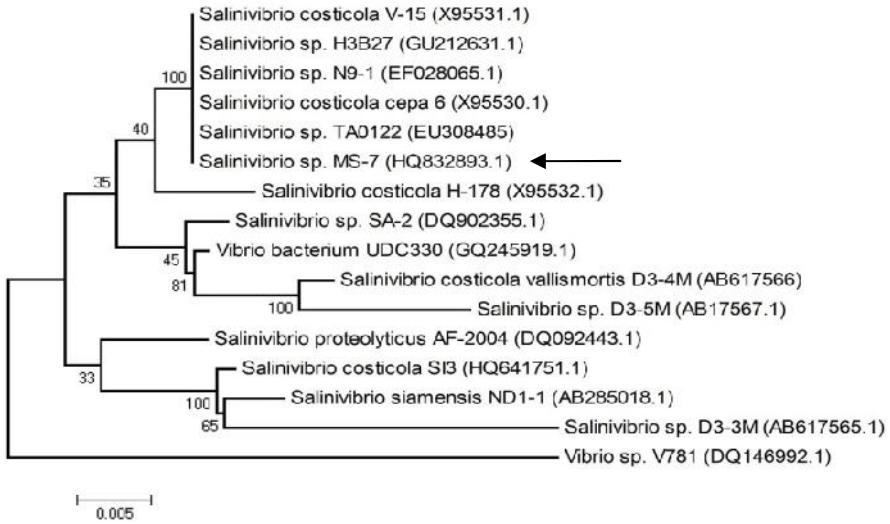

Figure 2: Phylogenetic tree showing the taxonomic position of isolate *Salinivibrio* sp. strain MS-7 and relationships between strain MS-7, species of the genus *Salinivibrio* and related taxa based on 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method

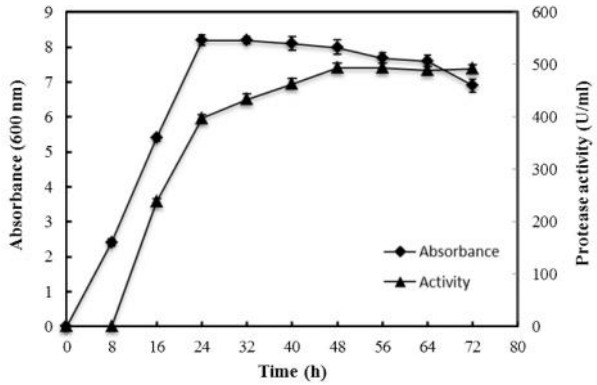

Figure 3: Kinetics of growth and protease production in *Salinivibrio* sp. strain MS-7in the basal medium. Results represent the means of three experiments. The errors were smaller than symbols.

Protease production initiated at the early exponential phase and reached its maximum level (494 U/ml) in the mid-stationary growth phase (48 h). Many other reported bacteria normally reached maximal protease secretion in the stationary phase [23,24]. Moreover, maximal protease production from *Pseudoalteromonas* sp. strain CP76, a moderately halophilic bacterium, has been reported at the end of the exponential growth phase [25] and in the halophilic *Bacillus* sp. maximum protease activity was observed in the early stationary phase [26]. Also, various carbon sources in the basal medium (other than maltose) were studied at a concentration of 0.5% (w/v). The results revealed that maltose (494 U/ml) and lactose (474 U/ml) were the most suitable substrates and glucose (357 U/ml) and sucrose (313 U/ml) caused a significant reduction in protease production (Table 1).

Table 1. Effect of different carbon sources and complex media on the growth and protease production in *Salinivibrio* sp. strain MS-7.

Nutrient Sources	Cell growth (OD. 600)	Activity (U/ml)	Protease production (U/OD. 600)[a]
Basal medium +			
Maltose	9.6±0.1	494±2.5	51.5±0.7
Lactose	8.8±0.07	474±2.3	53.9±0.6
Glucose	7.5±0.07	357±2.3	47.6±0.6
Sucrose	6.7±0.05	313±2.2	46.7±0.5
NB	8.2±0.07	371±2.3	45.2±0.6
TSB	7.1±0.1	333±2.5	46.9±0.7
LB	2.4±0.1	31±2.5	12.9±0.9

[a] The concentration of NaCl in all media was 10%. After 48 h incubation at 30 °C and 180 rpm the growth and protease activity were evaluated. Mean ± SD values for three independent experiments are shown

The data are different from those for strain AF-2004 protease secretion, in which the presence of maltose in a basal medium decreased protease production drastically. However, a catabolic repression mechanism was suggested in the case of glucose and sucrose. When comparing the three complex media (TSB, NB, LB), the maximum secretion of the protease (371 U/ml) and the bacterial growth observed in the NB medium, the minimum was found to be the LB medium (31 U/ml). Different carbon sources have been reported to be favorable substrates for protease production by various bacteria. Maltose and lactose were also suitable C sources for protease production by the moderately halophilic bacterium *Halobacillus Karajenesis* [27]. But our findings are different from those of Ningthoujam et al [28] who have introduced sucrose as the best carbon source of moderately halophilic alkalithermotolerant indigenous Actinomycetes.

Purification of extracellular protease and biochemical properties: The purification of an extracellular protease from the culture supernatant was achieved using a combination of 40-80% acetone precipitation and anion exchange chromatography. The protease was purified about 3.6-fold from the initial culture broth with a specific activity of 177 U/mg on casein and a final yield of 56% (data not shown). Judged by SDS-PAGE analysis, the protease purified from Salinivibrio sp. strain MS-7 is a single polypeptide chain of about 21 KDa (Figure 4).

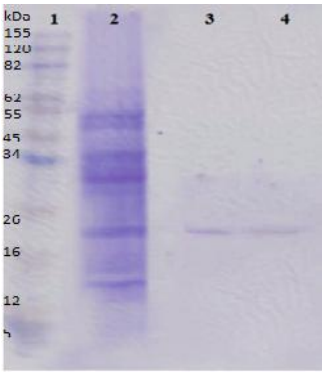

Figure 4: SDS-PAGE pattern of various purification steps of MS-7 extracellular protease. Lanes: 1, molecular markers; 2, Supernatant of culture medium. 3, acetone precipitated (40–80%); 4, DEAD-Cellulose column purified protease. Further details are described in Materials and Methods.

The molecular mass of purified proteases from various bacterial strains is commonly in the range of 30-50 KDa. For example, the molecular mass of an extracellular thermostable protease from *Bacillus* strain HUTBS71 was estimated to be 49 kDa [29]. Although some low molecular weight extracellular proteases have been previously reported from bacteria [30,1], to the best of our knowledge, this is the first report of a low molecular weight extracellular haloalkaline protease in moderately halophilic bacteria. In addition, the K_m and V_{max} values of MS-7 purified protease, derived from Lineweaver-Burk plot, are 1.14 mg/ml and 7.24 U, respectively, using casein as a substrate. The temperature profile is shown in (Figure 5A).

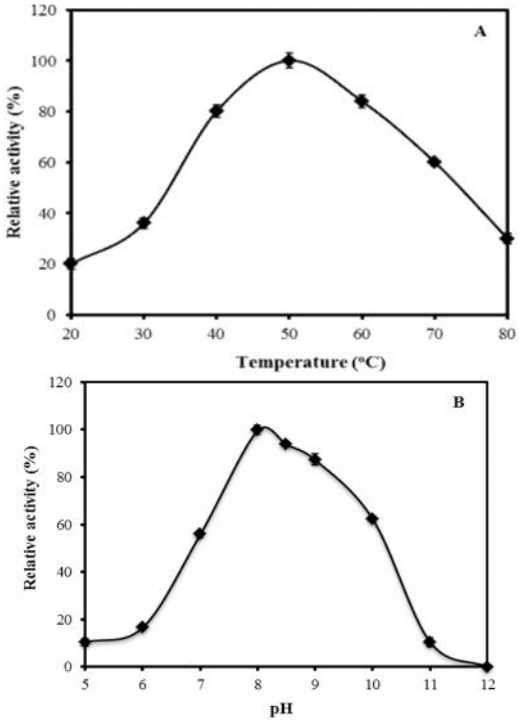

Figure 5: Effect of temperature (pH 8.0) (A), and pH (at 50 ◦C) (B) on the caseinolytic activity of purified protease. The relative activity was defined as the percentage of activity detected with respect to the maximum protease activity. See Materials and Methods for further details.

The activity increased rapidly above 30 ^{o}C and maximum protease activity was found to be 50 ^{o}C, followed by a thermal inactivation above 60 ^{o}C and retaining about 60% activity at 70 ^{o}C. The temperature profile pattern of the purified enzyme is almost similar to other halophilic reported proteases. Similar temperature profiles have been reported for other halophilic proteases previously characterized [27, 15]. The protease was active in the pH range of 5-11, with an optimum at pH 8.0. The enzyme retained 56% and 62.5% activity at pH 7.0 and 10.0, respectively (Figure 5B).

Similar optimum pH of 8.0 for a moderately halo-alkalithermotolerant *Bacillus Subtilis* strain SH1 protease has also been reported [31]. Furthermore, the enzyme was very stable in the presence of high concentrations of NaCl (1 to 3 M) after incubation for 60 min and the enzyme's full activity remained in 3 M NaCl (data not shown). Moreover, the MS-7 protease had optimal activity even in the absence of NaCl, which is a desirable characteristic of the enzyme for application in wide biotechnological processes. According to the above mentioned the protease from strain MS-7 is introduced as a haloalkalophile enzyme with a moderate thermal stability.

Influence of different metal ions and inhibitors on proteolytic activity: The influence of various cations and inhibitors on the caseinolytic activity of the purified protease was studied, the results summarized in Table 2.

Table 2. Effect of various metal ions and inhibitors on strain MS-7 protease activity

Ions or Inhibitors	Concentration (mM)	Residual activity (%)[a]
$MgCl_2$	2	117±2.2
$CaCl_2$	2	131±1.8
$BaCl_2$	2	158±2.1
$FeCl_2$	2	112±3.0
KCl	2	100±4.5
$HgCl_2$	2	79±3.5
$ZnCl_2$	0.5	85±2.2
$NiCl_2$	2	34±3.5
PMSF	1	0.0
EDTA-Na2	1	26±2.4
EDTA-Na2	10	4.9±3.5
1,10-Phenanthroline	1	118±2.5

[a] The enzyme was incubated with different inhibitors at 37 °C for 30 min.
The residual protease activity was measured as described in Materials and Methods. Mean ± SD values for three independent experiments are shown.

Of all the ions tested, Ba^{2+} and Ca^{2+} ions were particularly effective in activating the enzyme, causing 58% and 31% stimulation, respectively. Mg^{2+} ions showed fewer positive effects at the same concentration. Fe^{2+} was observed to have 12% stimulatory effect, which has been rarely reported so far (Setyorini et al., 2006). On the contrary, a highly inhibitory effect on the MS-7 protease activity was observed with Ni^{2+} (34% residual activity). Hg^{2+} and Zn^{2+} had some negative impact. K^{+} showed no influence on the enzyme activity. Likewise, Deng et al [32] observed stimulatory effects of Ba^{2+}, Ca^{2+} and Mg^{2+} on the purified protease of *Bacillus* sp. B001. Jellouli et al. [33] reported stimulatory effect of Ca^{2+} and Mg^{2+}, but showed the inhibitory effect of Ba^{2+} ions on alkaline protease from *Bacillus licheniformis* MP1. These results suggest that the presence of divalent cations has an important role in the stability of the protease structure and consequently influences the catalysis of the purified enzyme. On the other hand, Hg^{2+} and Zn^{2+} ions had negative impacts (see Table 2). Ni^{+2} ions also showed 79% inhibitory effects on protease activity. Inhibitory effects of Ni^{2+}, Hg^{+2} and Zn^{+2} ions have been reported by many researchers [16,15, 34]. Proteolytic enzyme types were assorted according to their inhibitory functions. PMSF, a common serine protease inhibitor, inactivated the purified protease completely at a concentration of 1 mM. Also, in the presence of EDTA, a metal ion chelator, the protease lost 74% and 95% of its activity at 1 mM and 10 mM, respectively. Whereas 1,10 phenanthroline not only had no inhibitory effect, but stimulated proteolytic activity of the enzyme as well. On the basis of this inhibition profile, the extracellular protease from *Salinivibrio* sp. strain MS-7 could belong to the family of serine metalloproteases. The positive effect of 1,10 phenanthroline (18% simulation at 10 mM concentration) showed no dependency of the enzyme to Zn^{2+} ions. In addition, the chelate of trace amounts from the assay mixture exhibited an enhanced enzyme catalytic activity, an interesting result which has not been reported so far.

Serious attention has been recently given to isolate halophilic enzymes due to novel applications in industrial fields. MS-7 protease, as a newly identified protease in the *Salinivibrio*

genus, revealed a lower molecular mass in comparison to other previously reported halophile-origin enzymes and probably has different functional behaviors. Considering its stability in the presence of high concentrations of salt and even without salt, together with its haloalkalophilic features, potential applications in the treatment of proteinaceous waste solutions with or without salt contents may also be found.

Acknowledgments

Financial support for this work was provided by the Research Council, Shiraz University.

REFERENCE

1. Mala M, Srividya S. Partial purification and properties of a laundry detergent compatible alkaline protease from a newly isolated *Bacillus* species Y. Indian J Microbiol 2010;50: 309-317.
2. Gupta R, Beg QK, Lorenz P. Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 2002;59:15-32.
3. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 1998;62:597-635.
4. Hough DW, Danson MJ. Extremozymes. Curr Opin Chem Biol 1999;3:39-46.
5. Margesin R, Schinner F. Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 2001;5:73-83.
6. Van Den Burg B. Extremophiles as a source for novel enzymes. Curr Opin Microbiol 2003;6:213-218.
7. Mellado E, Sánchez-Porro C, Martín S, Ventosa A. Extracellular hydrolytic enzymes produced by moderately halophilic bacteria, Halophilic microorganisms. Springer, Berlin, 2004, pp. 285–295.
8. Sanchez-Porro C, Martin S, Mellado E, Ventosa A. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. J Appl Microbiol 2003;94:295-300.
9. Mellado E, Moore ER, Nieto JJ, Ventosa A. Analysis of 16S rRNA gene sequences of Vibrio costicola strains: description of *Salinivibrio costicola* gen. nov., comb. nov. Int J Syst Bacteriol 1996;46:817-821.
10. Huang CY, Garcia JL, Patel BK, Cayol JL, Baresi L, Mah RA. *Salinivibrio costicola subsp. vallismortis* subsp. nov., a halotolerant facultative anaerobe from Death Valley, and emended description of *Salinivibrio costicola*. Int J Syst Evol Microbiol 2000;50:615-622.
11. Romano I, Gambacorta A, Lama L, Nicolaus B, Giordano A. *Salinivibrio costicola* subsp. *alcaliphilus* subsp. nov., a haloalkaliphilic aerobe from Campania Region (Italy). Syst Appl Microbiol 2005;28:34-42.
12. Amoozegar MA, Schumann P, Hajighasemi M, Fatemi AZ, Karbalaei-Heidari HR. *Salinivibrio proteolyticus* sp. nov., a moderately halophilic and proteolytic species from a hypersaline lake in Iran. Int J Syst Evol Microbiol 2008;58:1159-1163.

13. Chamroensaksri N, Tanasupawat S, Akaracharanya A, Visessanguan W, Kudo T, Itoh T. *Salinivibrio siamensis* sp. nov., from fermented fish (pla-ra) in Thailand. Int J Syst Evol Microbiol 2009;59:880-885.
14. Romano I, Orlando P, Gambacorta A, Nicolaus B, Dipasquale L, Pascual J, Giordano A, Lama L. *Salinivibrio sharmensis* sp. nov., a novel haloalkaliphilic bacterium from a saline lake in Ras Mohammed Park (Egypt). Extremophiles 2011;15:213-220.
15. Lama L, Romano I, Calandrelli V, Nicolaus B, Gambacorta A. Purification and characterization of a protease produced by an aerobic haloalkaliphilic species belonging to the *Salinivibrio* genus. Res Microbiol 2005;156:478-484.
16. Karbalaei-Heidari HR, Ziaee A-A, Schaller J, Amoozegar MA. Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium, *Salinivibrio* sp. strain AF-2004. Enzyme Microb Technol 2007;40: 266-272.
17. .Karbalaei-Heidari HR, Ziaee AA, Amoozegar MA. Purification and biochemical characterization of a protease secreted by the *Salinivibrio* sp. strain AF-2004 and its behavior in organic solvents. Extremophiles 2007;11:237-243.
18. Smibert RM, Krieg NR. 1994. Phenotypic characterization, p. 615. In P. Gerhardt, R. Murray, W. Wood, and N. Krieg (ed.), Methods for general and molecular bacteriology. ASM Press, Washington, DC.
19. Ventosa A, Quesada E, Rodrı´guez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. J Gen Microbiol 128, 1959–1968.
20. Tamura K, Dudley. J, Neim M. MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007;24:1596–1599.
21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.
22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.
23. Chu WH. Optimization of extracellular alkaline protease production from species of Bacillus. J Ind Microbiol Biotechnol 2007;34:241-245.
24. Joo HS, Kumar CG, Park GC, Paik SR, Chang CS. Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J Appl Microbiol 2003;95:267-272.
25. Sanchez-Porro C, Mellado E, Bertoldo C, Antranikian G, Ventosa A. Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76. Extremophiles 2003;7:221-228.
26. Patel R, Dodia M, Singh SP. Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization. Process Biochem 2005;40: 3569-3575.
27. Karbalaei-Heidari HR, Amoozegar MA, Hajighasemi M, Ziaee AA, Ventosa A. Production, optimization and purification of a novel extracellular protease from the moderately halophilic bacterium *Halobacillus karajensis*. J Ind Microbiol Biotechnol 2009;36:21-27.
28. Ningthoujam DS, Kshetri P, Sanasam S, Nimaichand S. Screening, identification of best producers and optimization of extracellular proteases from moderately halophilic alkalithermotolerant indigenous actinomycetes. World Appl Sci J 2009;7:907-916.

29. Akel H, Al-Quadan F, Yousef TK. Characterization of a purified thermostable protease from hyperthermophilic *Bacillus* strain HUTBS71. Eur J Sci Res 2009;31:280-288.
30. Dutta JR, Dutta PK, Baberjee R. Kinetic study of a low molecular weight protease from newly isolated *Pseudomonas* sp. using artificial neural network. Indian J Biotechnol 2005;4:127-133.
31. Ningthoujam DS, Kshetri P. A thermostable alkaline protease from a moderately halo-alkalithermotolerant *Bacillus Subtilis* strain SH1. Aus J Basic and Appl Sci 2010;4:5126-5134.
32. Deng A, Wu J, Zhang Y, Zhang G, Wen T. Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. Bioresour Technol 2010; 101:7111-7117.
33. Jellouli K, Ghorbel-Bellaaj O, Ayed HB, Manni L, Agrebi R, Nasri M. Alkaline-protease from *Bacillus licheniformis* MP1: Purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization. Process Biochem 2011;46: 1248-1256.
34. Setyorini E, Takenaka S, Murakami S, Aoki K. Purification and characterization of two novel halotolerant extracellular proteases from *Bacillus subtilis* strain FP-133. Biosci Biotechnol Biochem 2006;70:433-440.