

Research Paper

Extracellular proteases of *Halobacillus blutaparonensis* strain M9, a new moderately halophilic bacterium

Anderson F. Santos^{1,2}, Roberta S. Valle¹, Clarissa A. Pacheco^{1,3}, Vanessa M. Alvarez¹, Lucy Seldin¹, André L.S. Santos^{1,2}

¹Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

²Programa de Pós-Graduação em Bioquímica, Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

³Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

Submitted: May 14, 2012; Approved: April 4, 2013.

Abstract

Halophilic microorganisms are source of potential hydrolytic enzymes to be used in industrial and/or biotechnological processes. In the present study, we have investigated the ability of the moderately halophilic bacterium *Halobacillus blutaparonensis* (strain M9), a novel species described by our group, to release proteolytic enzymes. This bacterial strain abundantly proliferated in Luria-Bertani broth supplemented with 2.5% NaCl as well as secreted proteases to the extracellular environment. The production of proteases occurred in bacterial cells grown under different concentration of salt, ranging from 0.5% to 10% NaCl, in a similar way. The proteases secreted by *H. blutaparonensis* presented the following properties: (i) molecular masses ranging from 30 to 80 kDa, (ii) better hydrolytic activities under neutral-alkaline pH range, (iii) expression modulated according to the culture age, (iv) susceptibility to phenylmethylsulphonyl fluoride, classifying them as serine-type proteases, (v) specific cleavage over the chymotrypsin substrate, and (vi) enzymatic stability in the presence of salt (up to 20% NaCl) and organic solvents (*e.g.*, ether, isooctane and cyclohexane). The proteases described herein are promising for industrial practices due to its haloalkaline properties.

Key words: *Halobacillus blutaparonensis*, halophilic bacterium, serine proteases.

Introduction

Proteases are omnipresent, being found in a wide diversity of sources such as plants, animals and microorganisms. Microbial proteases are the most important industrial enzymes accounting for around 60% of the total enzyme market and they represent approximately 40% of the total enzyme production in the world (Ng and Kenealy, 1986; Rao *et al.*, 1998; Horikoshi, 1999; Gupta *et al.*, 2002). Proteases can be classified as acid, neutral and alkaline, based on their optimal hydrolytic activity pH range. Among those proteases, the alkaline ones are particularly relevant due to their activity and stability in high pH value (Rao *et al.*, 1998; Gupta *et al.*, 1999, 2002a, 2002b) and, consequently,

their wide biotechnological potential for industrial sectors such as laundry detergents, leather tanning, beer, food and pharmaceutical industry (Rao *et al.*, 1998; Schallmeyer *et al.*, 2004; Fujinami and Fujisawa, 2010). Currently, a great number of commercially available alkaline proteases originate from *Bacillus* strains (Rao *et al.*, 1998; Gupta *et al.*, 1999, 2002a, 2002b; Schallmeyer *et al.*, 2004; Fujinami and Fujisawa, 2010).

Over the years, *Bacillus* species as well as *Bacillus*-related genera have emerged as promising extracellular protease producers (Schallmeyer *et al.*, 2004; Fujinami and Fujisawa, 2010). Extracellular proteases from moderately halophilic microorganisms have been thoroughly identified and characterized, including in *Bacillus clausii* I-52 (Joo *et*

al., 2003), *Bacillus subtilis* FP-133 (Setyorini *et al.*, 2006), *Paenibacillus peoriae* NRRL BD-62, *Paenibacillus polymyxa* SCE2 (Alvarez *et al.*, 2006), *Halobacillus* sp. SR5-3 (Namwong *et al.*, 2006), *Halobacterium* sp. SP1(1) (Akolkar *et al.*, 2008), *Virgibacillus* sp. SK37 (Phrommao *et al.*, 2011) and *Geomicrobium* sp. EMB2 (Karan and Khare, 2011).

Our study focused on the identification of enzymes produced by a new bacterium species belonging to the *Halobacillus* genus, which shows the following characteristics: moderately halophilic, Gram-positive, spore-forming, motile, strictly aerobic with rod cells and capability to grow in the absence or in the presence of NaCl. This species was named *Halobacillus blutaparonensis* since it was isolated from the roots of *Blutaparon portulacoides* (Barbosa *et al.*, 2006) that is a coastal perennial rhizomatous herb found on the sand strip parallel to the Restinga of Jurubatiba located in the north of Rio de Janeiro State, Brazil, which is an extremely preserved area comprising a strip of sand of 44 km in length. The plant *B. portulacoides* can endure the salinity stress, high temperatures and tide shift exposure characteristic of the area (Bernardi and Seeliger, 1989). In addition, it is of great medical interest due to the presence of flavonoids, irisone B, sitosterol, vanillic acid, as well as stigmasterol, sitosterol and campesterol steroids (Ferreira and Dias, 2000). Hence, exploring the potential bacteria that are associated to this plant can be a promising source of bioactive compounds. With this task in mind, we have aimed to identify the proteases secreted by the new bacterial species *H. blutaparonensis* strain M9.

Materials and Methods

Microorganism and culture conditions

The strain M9 of *Halobacillus blutaparonensis*, originally described by Barbosa *et al.* (2006), was cultivated in Luria-Bertani (LB) broth that contains in its formulation 0.5% NaCl. Cells were incubated without agitation at 32 °C for 48 h. Working cultures were kept on LB agar plates at 4 °C, while long-term storage was followed in LB slants supplemented with 1% CaCO₃ (Seldin *et al.*, 1983).

Influence of salt on the bacterial growth

Bacterial cells were incubated in LB broth supplemented with different concentrations of NaCl (0.5%, 2.5%, 5.0% and 10.0%) at 32 °C up to 96 h. In order to evaluate the growth kinetics, an aliquot of each system was recovered every 24 h, diluted (10⁻³ to 10⁻⁶) and then plated onto LB solid medium containing the respective NaCl concentration. Afterwards, all the plates were incubated for 48 h to count the colony-forming units (CFU).

Influence of salt on the production of proteolytic enzymes

Determination of protease production was primarily performed by using LB agar plates (containing 0.5%, 2.5%,

5.0% and 10.0% NaCl) supplemented with 0.4% gelatin (Sigma-Aldrich Chemical Co., USA). Briefly, ten microliters of the bacterial suspension were placed in the center of agar plates (9-cm diameter Petri dishes) and then incubated at 32 °C for 24, 48, 72 and 96 h. The gelatin degradation was revealed by flooding the plates with a solution containing 15% (w/v) mercuric chloride in 20% HCl. After 10 min, a clear transparent zone indicated hydrolysis of the gelatin by extracellular proteolytic activity, whereas the rest of the plate became opaque because of the coagulation of gelatin by HgCl₂ (Frazier, 1926). The colony diameter (a) and the diameter of colony plus the clear zone (b) were measured by a digital paquimeter. The enzymatic index (EI) was expressed as follows: EI = b/a. The EI value of three different samples was measured to obtain the average value.

Cell-free culture supernatants

H. blutaparonensis cultures (200 mL) were centrifuged (15,000 g / 20 min / 4 °C) and the supernatants were filtered through a 0.22-µm membrane (Millipore). The cell-free culture supernatants were concentrated 50-fold using a 10,000 molecular weight cut-off Amicon micropartition system (Stirred Cell Model 8200) (Alvarez *et al.*, 2006). Protein concentration was determined by the method described by Lowry *et al.* (1951), using bovine serum albumin (BSA) as standard.

Zymography

Proteases were assayed and partial characterized by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 0.1% co-polymerized gelatin as substrate (Heussen and Dowdle, 1980). Gels were loaded with 50 µg of protein per slot. After electrophoresis, at a constant current of 120 V at 4 °C, SDS was removed by incubation with 10 volumes of 2.5% Triton X-100 for 1 h at room temperature under constant agitation. In order to promote the proteolysis, the gels were incubated for 48 h at 37 °C in the following buffer systems: 20 mM sodium phosphate buffer (pH 5.0 and 7.0) and 20 mM glycine-NaOH (pH 9.0) in the absence or in the presence of proteolytic inhibitors (Sigma) (10 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM 1,10-phenanthroline, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 µM *trans*-epoxysuccinyl L-leucylamido-(4-guanidino) butane (E-64) and 10 µM pepstatin A). The gels were stained for 2 h with 0.2% Coomassie brilliant blue R-250 in methanol:acetic acid:water (50:10:40) and destained overnight in a solution containing methanol:acetic acid:water (5:10:85), to intensify the digestion halos. The molecular masses of the proteases were calculated by comparison with the mobility of low molecular mass standards. The gels were dried, scanned and digitally processed (Alvarez *et al.*, 2006). The quantification of proteolytic halos was performed by densito-

metrical analyzes using the public domain Image J software.

Cleavage of chromogenic peptide substrates

The crude supernatant fluid of *H. blutaparionensis* was tested to cleave the following chromogenic substrates (Sigma): *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (elastase substrate), *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (chymotrypsin substrate) and *N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide (trypsin substrate). The reactions were started by the addition of each substrate at 1 mM to the supernatant fluid (50 µg) diluted in 20 mM glycine-NaOH, pH 9.0. The reaction mixtures were incubated at 37 °C up to 120 min and the rate of substrate hydrolyses (the formation of *p*-nitroaniline) were determined by Thermomax Molecular Device microplate reader at 405 nm. A control for each chromogenic substrate, where the substrate was added just after the reactions were stopped, was used as blank. One unit of proteolytic activity was defined as the amount of enzyme that caused an increase of 0.001 in absorbance unit, under standard assay conditions.

Effects of salt and organic solvents on protease activity

The stability of the crude proteases was also investigated in the presence of various organic solvents (at 25% v/v) and salt concentrations (up to 20% NaCl). The reaction mixtures and the rate of substrate hydrolyses were determined as described above.

Statistical analysis

The experiments were performed in triplicate, in three independent experimental sets. The data were analyzed statistically by means of Student's *t*-test using EPI-INFO 6.04 (Database and Statistics Program for Public Health) computer software. *P* values of 0.05 or less were considered statistically significant.

Results and Discussion

Figure 1 shows the growth curves of *H. blutaparionensis* when cultivated at 32 °C for a period of 1 to 4 days in LB broth supplemented with different salt concentrations. This bacterial species attained the log phase after 24 h of cultivation in LB added with NaCl at either 2.5% or 5.0%, and by the second day in LB added with 0.5% NaCl. Elevated amount of NaCl (10.0%) retarded the bacterial proliferation. Moreover, they differed in growth pattern, which revealed a poor growth of *H. blutaparionensis* in LB medium containing 0.5%, 5.0% or 10.0% NaCl and an abundant proliferation when LB plus 2.5% NaCl was used (Figure 1, inset).

To adapt to saline conditions, bacteria have developed various strategies to maintain cell structure and function. Studies of such bacteria are of great importance, as

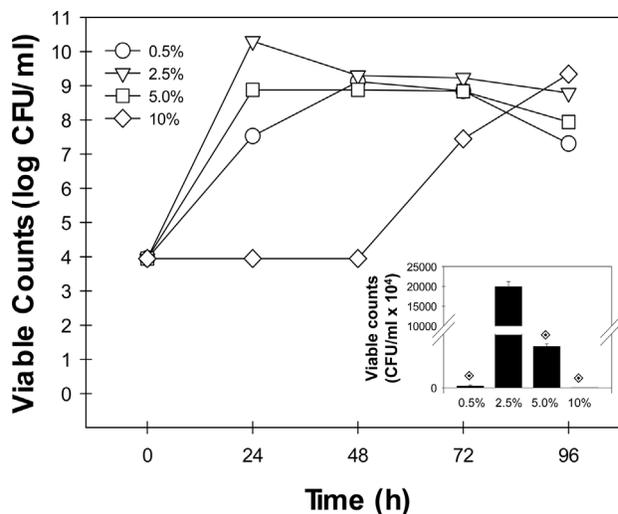


Figure 1 - Growth curve profiles of *H. blutaparionensis* cultured in LB medium supplemented with different concentrations of NaCl (0.5%, 2.5%, 5.0% and 10.0%) at 32 °C up to 96 h. The inset emphasizes the difference among the abundant bacterial proliferation in the presence of 2.5% NaCl when compared to the other systems after 24 h of *in vitro* growth. The values represent the mean of three independent experiments performed in triplicate. *P* values of 0.05 or less were considered statistically significant (\diamond , Student's *t*-test).

they may produce compounds of industrial interest, such as extracellular hydrolytic enzymes that have diverse potential usage in biomedical science and chemical industries (Ventosa and Nieto, 1995; Margesin and Schinner, 2001; Mellado and Ventosa, 2003). Proteases are the largest selling industrial enzymes. Their sale is projected to increase further in the coming years with anticipated applications in protein processing, peptide synthesis and detergent formulations. However, the above-mentioned applications necessitate the proteases to be stable in salts, organic solvents and alkaline pH, respectively (Gupta *et al.*, 1999, 2002a, 2002b). In this context, halotolerant and/or halophilic microorganisms are valuable source of novel enzymes, including proteases, for using in different industrial practices. Taking this finding in mind, the production of proteases by *H. blutaparionensis* was initially investigated onto LB agar plates supplemented with both NaCl and gelatin during a period of 96 h of growth (Figure 2). The gelatin degradation was observed in bacterial cells grown under different concentration of salt, ranging from 0.5% to 10% NaCl, in a similar way (Figure 2). As it is well-known, the stability and activity of the enzyme in the presence and absence of salt make it quite interesting for wider applications in biotechnological processes (Rao *et al.*, 1998; Gupta *et al.*, 1999, 2002a, 2002b).

After these first results, LB broth supplemented with 2.5% NaCl was chosen to carry out the further experiments. In this context, the secretion of proteolytic enzymes was examined in the culture supernatants of *H. blutaparionensis* along 96 h of *in vitro* growth. A simple inspection of the overlay gels showed that the secreted proteases were more

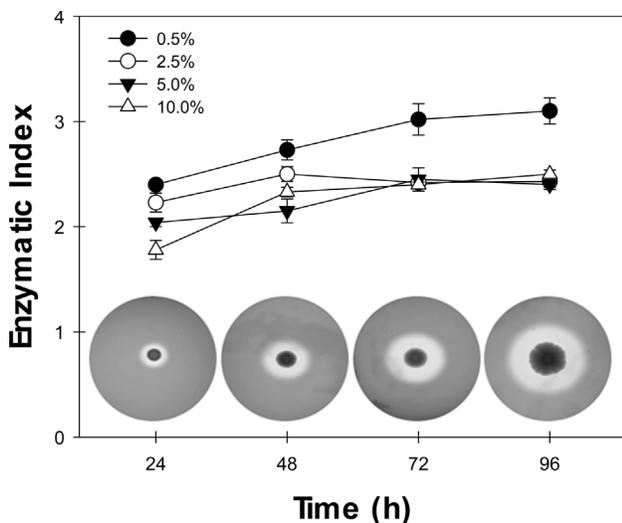


Figure 2 - Protease production by *H. blutaparonensis* cultured in LB solid medium supplemented with different concentrations of NaCl (0.5%, 2.5%, 5.0% and 10.0%) at 32 °C up to 96 h. The enzymatic index was expressed as the ratio of colony diameter and the diameter of colony plus the clear zone. The values represent the mean \pm standard error of three independent experiments performed in triplicate. The inset exemplifies a representative image of the results, showing the gelatin degradation in LB medium added of 0.5% NaCl.

active under neutral-alkaline pH range (Figure 3). At least five major proteolytic bands were observed with molecular masses ranging from 80 to 30 kDa. We also observed that different proteolytic expression by *H. blutaparonensis* was induced depending on culture age: proteases of 80, 70, 55 and 50 kDa were more pronounced in culture supernatant harvested after two days of cultivation (stationary phase) at neutral pH, while the 30 kDa protease had its production enhanced along the 96 h of growth with noticeable activity at alkaline pH (Figure 3). Similarly, maximum protease production in stationary phase is well documented in *Pseudoalteromonas* sp. strain CP76 (Sanchez-Porro *et al.*, 2003) and *Bacillus sphaericus* (Singh *et al.*, 2004).

Aiming to characterize these proteases more fully, we tested their activities in gelatin-containing gels in the presence of proteolytic inhibitors. The results showed that the extracellular proteolytic profile of *H. blutaparonensis* was composed exclusively by serine-type proteases, displaying total sensibility to PMSF (Figure 4). Conversely, 1,10-phenanthroline (a zinc metalloprotease inhibitor), E-64 (a cysteine protease inhibitor) and pepstatin A (an aspartic protease inhibitor) did not significantly interfere with the secreted proteolytic activities (Figure 4). Interestingly, EDTA (a calcium metalloprotease inhibitor/chelating agent) partially inhibited the proteases released by *H. blutaparonensis* (Figure 4). Likewise, Karbalaei-Heidari *et al.* (2009) purified a 36 kDa alkaline extracellular protease from *Halobacillus karajensis* strain MA-2 sensible to classical serine protease inhibitors (PMSF and Pefabloc SC) and EDTA, indicating that it probably belongs to the subclass of

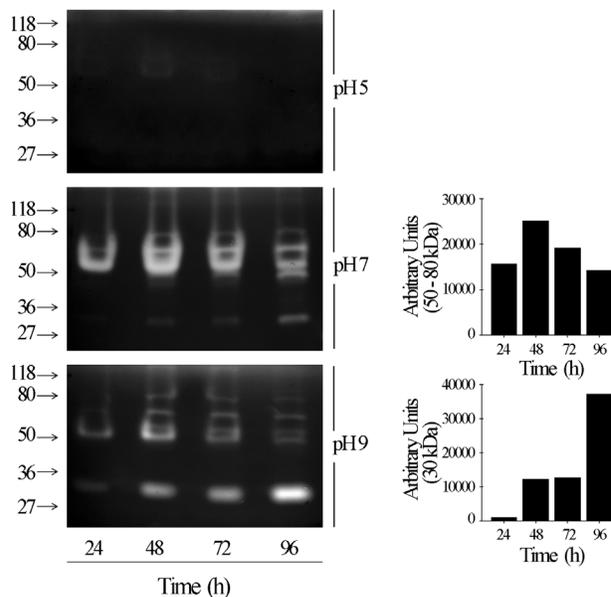


Figure 3 - Effect of pH and culture age on the extracellular protease profile of *H. blutaparonensis* cultured in LB medium supplemented with 2.5% NaCl at 32 °C. Proteolytic profiles were evidenced by gelatin-SDS-PAGE. The gel strips were incubated in different buffer systems: 20 mM sodium phosphate buffer (pH 5 and 7) and 20 mM glycine-NaOH (pH 9). The numbers on the left indicate the apparent molecular masses of protein standards, expressed in kilodaltons. The graphics on the right represents the densitometric analyses of the proteolytic halos, expressed in arbitrary units of proteolytic activity.

serine metalloproteases. This inhibition profile is commonly observed in many members of the subtilase superfamily, including subtilisins, which are one of the most valuable industrial enzymes and have been extensively studied in terms of molecular structure, protein engineering and their application (Rao *et al.*, 1998; Gupta *et al.*, 2002a). Gram-positive bacilli are well-known sources for high productivity of subtilisins (Sanchez-Porro *et al.*, 2003).

The degradation of different serine protease substrates was employed using the supernatant fluid of *H.*

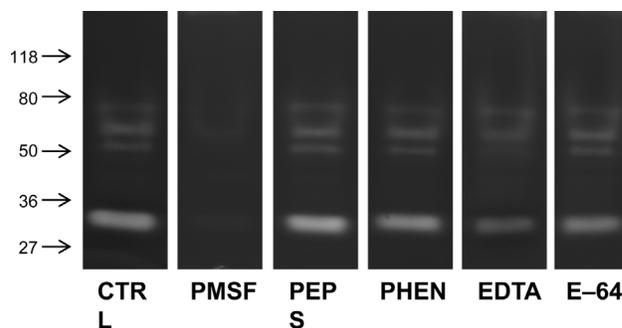


Figure 4 - Effect of proteolytic inhibitors on the proteases secreted by *H. blutaparonensis*. The gel strips, containing the supernatant of cells cultured in LB medium added of 2.5% NaCl for 96 h, were incubated in 20 mM glycine-NaOH (pH 9) in the absence (CTRL) or in the presence of the following inhibitors: 10 mM PMSF, 10 μ M pepstatin A (PEPS), 10 mM 1,10-phenanthroline (PHEN), 10 mM EDTA and 10 μ M E-64. The numbers on the left indicate the apparent molecular masses of protein standards, expressed in kilodaltons.

blutaparionensis (Figure 5). To this end, we performed kinetic assays using chromogenic peptide substrates specific to elastase, trypsin and chymotrypsin. The serine proteases secreted by *H. blutaparionensis* showed elevated degradation rate on the chymotrypsin substrate when compared to trypsin or elastase substrates (Figure 5). As expected, PMSF was able to completely abolish the cleavage of the chromogenic chymotrypsin substrate (data not shown).

In industrial applications, use of crude enzyme is preferred over purified preparation. This is to avoid the cost of purification and make the processes commercially viable. The applications of crude proteases have been highlighted in several processes, for instance, the crude protease from the extreme halophilic archaea *Halobacterium* sp. SP1 (Akolkar *et al.*, 2008) that is stable over a broad pH range and high salt concentration has been effectively used in order to accelerate the fish sauce fermentation process (Akolkar *et al.*, 2010). Joshi *et al.* (2007) described the production of alkaline serine protease stable in the presence of NaCl, SDS and acetone by a moderately halotolerant strain of *Bacillus cereus* (designated MTCC 6840) isolated from Lake Nainital, Uttaranchal State, India. Herein, the preliminary characterization of the crude proteases secreted by *H. blutaparionensis* was undertaken more specifically to assess the novel features of enzyme in terms of its catalytic behavior and stability in distinct organic solvents and different concentrations of salt (Figure 6). The proteases of *H. blutaparionensis* were highly stable in the presence of NaCl up to 20% along 60 min of experimentation (Figure 6A). Regarding the effects of organic solvents, the proteolytic activity showed high stability in ether, isooctane and cyclohexane (at 25% v/v) after 60 min (Figure 6B); however,

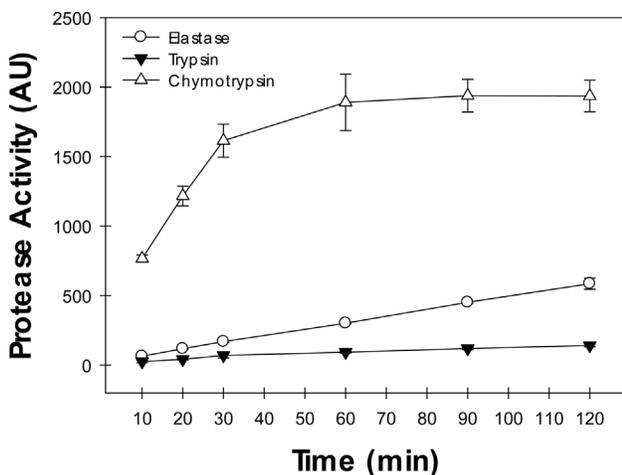


Figure 5 - Time course of hydrolysis of chromogenic substrates from serine proteases secreted by *H. blutaparionensis*. The chromogenic substrates used are specific to measure the proteolytic activity of elastase (*N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide), trypsin (*N*-Benzoyl-Phe-Val-Arg-*p*-nitroanilide) and chymotrypsin (*N*-Succinyl-Ala-Ala-Pro-Phe-Phe-*p*-nitroanilide). The enzymatic activities, expressed in arbitrary units (AU), were determined at 37 °C for 120 min under alkaline conditions of pH (pH 9.0). The values represent the mean \pm standard error of three independent experiments performed in triplicate.

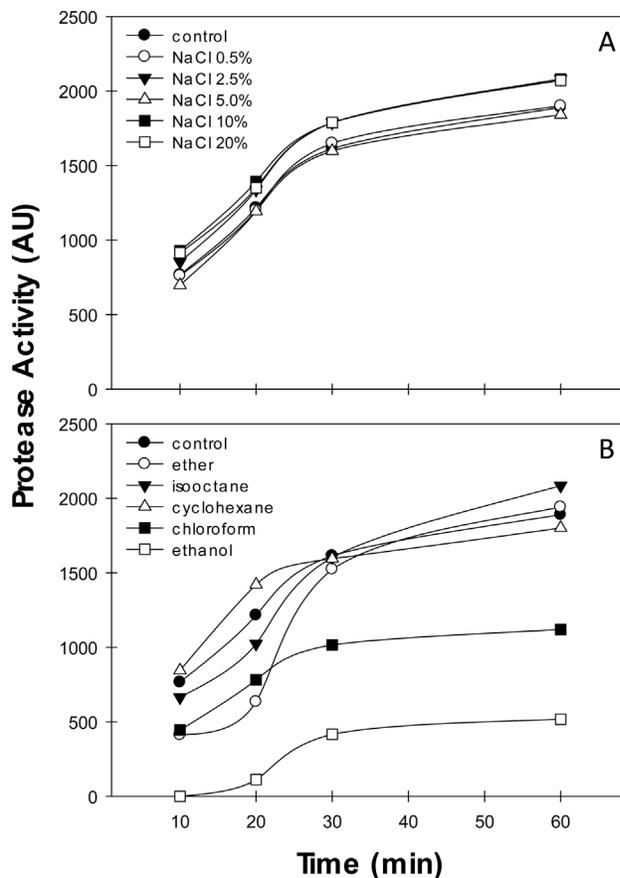


Figure 6 - Effect of salt concentration (A) and organic solvents (B) on the time course of hydrolysis of chymotrypsin chromogenic substrate from serine proteases secreted by *H. blutaparionensis*. The proteolytic activities were measured in the absence (control) or in the presence of different concentrations of NaCl (0.5, 2.5, 5, 10 and 20%) or organic solvents at 25% v/v (ether, isooctane, cyclohexane, chloroform and ethanol). The enzymatic activities, expressed in arbitrary units (AU), were determined at 37 °C for 60 min under alkaline conditions of pH (pH 9.0). The values represent the mean of three independent experiments performed in triplicate.

chloroform and ethanol reduced the enzymatic activity around 40% and 70%, respectively (Figure 6B).

In conclusion, this is the first study describing the protease production by the moderately halophilic bacterium *H. blutaparionensis*. Some of the features of the secreted proteases by this new bacterial species, such as production in the absence and presence of different salt concentrations, hydrolytic activity in a wide pH range (especially neutral-alkaline pHs) and stability in salt and organic solvents are credentials to future biochemical studies. So, upcoming works are in progress in our laboratory in order to purify some of the extracellular proteases produced by *H. blutaparionensis*, aiming to test their application in some industrial/biotechnological processes.

Acknowledgments

This study was supported by grants from the Brazilian Agencies: Conselho Nacional de Desenvolvimento

Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho de Ensino e Pesquisa para Graduados da Universidade Federal do Rio de Janeiro (CEPG-UFRJ). André L.S. Santos and Lucy Seldin are supported by CNPq and FAPERJ fellowships. Anderson F. Santos, a postgraduate student of Programa de Pós-Graduação em Bioquímica - Instituto de Química - Centro de Tecnologia - UFRJ, is supported by a CAPES fellowship.

References

- Akolkar AV, Deshpande GM, Raval KN, Durai D, Nerurkar AS, Desai AJ (2008) Organic solvent tolerance of *Halobacterium* sp. SP1(1) and its extracellular protease. *J Basic Microbiol* 48:1-5.
- Akolkar AV, Durai D, Desai AJ (2010) *Halobacterium* sp. SP1(1) as a starter culture for accelerating fish sauce fermentation. *J Appl Microbiol* 109:44-53.
- Alvarez VM, Von der Weid I, Seldin L, Santos ALS (2006) Influence of growth conditions on the production of extracellular proteolytic enzymes in *Paenibacillus peoriae* NRRL BD-62 and *Paenibacillus polymyxa* SCE2. *Lett Appl Microbiol* 43:625-630.
- Barbosa DC, Von der Weid I, Vaisman N, Seldin L (2006) Halotolerant spore-forming Gram-positive bacterial diversity associated with *Blutaparon portulacoides* (St. Hill.) Mears, a pioneer species in Brazilian coastal dunes. *J Microbiol Biotechnol* 16:193-199.
- Bernardi H, Seeliger U (1989) Population biology of *Blutaparon portulacoides* (St. Hill.) Mears on southern Brazilian backshores. *Ciência & Cultura* 41:1110-1113.
- Ferreira EO, Dias DAA (2000) Methylendioxyflavonol from aerial parts of *Blutaparon portulacoides*. *Phytochemistry* 53:145-147.
- Frazier C (1926) A method for the detection of changes in gelatin due to bacteria. *J Infect Dis* 39:302-309.
- Fujinami S, Fujisawa M (2010) Industrial applications of alkaliphiles and their enzymes - Past, present and future. *Environ Technol* 31:845-856.
- Gupta R, Gupta K, Saxena RK, Khan S (1999) Bleach-stable, alkaline protease from *Bacillus* sp. *Biotechnol Lett* 21:135-138.
- Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol* 59:15-32.
- Gupta R, Beg QK, Khan S, Chauhan B (2002) An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl Microbiol Biotechnol* 60:381-395.
- Heussen C, Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing SDS and copolymerized substrates. *Anal Biochem* 102:196-202.
- Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. *Microbiol Mol Biol Rev* 63:735-750.
- Joo HS, Kumar CG, Park GC, Paik SR, Chang CS (2003) Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. *J Appl Microbiol* 95:267-272.
- Joshi GK, Kumar S, Sharma V (2007) Production of moderately halotolerant, SDS stable alkaline protease from *Bacillus cereus* MTCC 6840 isolated from Lake Nainital, Uttaranchal State, India. *Braz J Microbiol* 38:773-779.
- Karan R, Khare SK (2011) Stability of haloalkaliphilic *Geomicrobium* sp. protease modulated by salt. *Biochemistry (Mosc)* 76:686-693.
- Karbalaee-Heidari HR, Amoozegar MA, Hajighasemi M, Ziaee AA, Ventosa A (2009) Production, optimization and purification of a novel extracellular protease from the moderately halophilic bacterium *Halobacillus karajensis*. *J Ind Microbiol Biotechnol* 36:21-27.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275.
- Margasin R, Schinner R (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. *Extremophiles* 5:73-83.
- Mellado ME, Ventosa A (2003) Biotechnological potential of moderately and extremely halophilic microorganisms. In: Barredo JL (ed) *Microorganisms for health care, food and enzyme production*. Research Signpost, Kerala, p.233-256.
- Namwong S, Hiraga K, Takada K, Tsunemi M, Tanasupawat S, Oda K (2006) A halophilic serine proteinase from *Halobacillus* sp. SR5-3 isolated from fish sauce: purification and characterization. *Biosci Biotechnol Biochem* 70:1395-1401.
- Ng TK, Kenealy WR (1986) Industrial applications of thermostable enzymes. In: Brock, T.D. (Ed.), *T.G.M. and Applied Microbiology*. John Wiley, p.197-205.
- Phrommao E, Rodtong S, Yongsawatdigul J (2011) Identification of novel halotolerant bacillopeptidase F-like proteinases from a moderately halophilic bacterium, *Virgibacillus* sp. SK37. *J Appl Microbiol* 110:191-201.
- Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev* 62:597-635.
- Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ (2008) MEROPS: the peptidase database. *Nucleic Acids Res* 36:320-325.
- Sanchez-Porro C, Mellado E, Bertoldo C, Antranikian G, Ventosa A (2003) Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium *Pseudoalteromonas* sp. strain CP76. *Extremophiles* 7:221-228.
- Schallmey M, Singh A, Ward OP (2004) Developments in the use of *Bacillus* species for industrial production. *Can J Microbiol* 50:1-17.
- Seldin L, Van Elsas JD, Penido EGC (1983) *Bacillus* nitrogen fixers from Brazilian soils. *Plant Soil* 70:243-255.
- Setyorini E, Takenaka S, Murakami S, Aoki K (2006) Purification and characterization of two novel halotolerant extracellular proteases from *Bacillus subtilis* strain FP-133. *Biosci Biotech Biochem* 70:433-440.
- Singh J, Vohra RM, Sahoo DK (2004) Enhanced production of alkaline proteases by *Bacillus sphaericus* using fed-batch culture. *Process Biochem* 39:1093-1101.
- Ventosa A, Nieto JJ (1995) Biotechnological applications and potentialities of halophilic microorganisms. *World J Microbiol Biotechnol* 11:85-94.