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Research article

Bioprospection of proteases from *Halobacillus andaensis* for bioactive peptide production from fish muscle protein

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ABSTRACT

Background: Biologically active peptides produced from fish wastes are gaining attention because their health benefits. Proteases produced by halophilic microorganisms are considered as a source of active enzymes in high salt systems like fish residues. Hence, the aim of this study was the bioprospection of halophilic microorganisms for the production of proteases to prove their application for peptide production.
Results: Halophilic microorganisms were isolated from saline soils of Mexico and Bolivia. An enzymatic screening was carried out for the detection of lipases, esterases, pHB depolymerases, chitinases, and proteases. Most of the strains were able to produce lipases, esterases, and proteases, and larger hydrolysis halos were detected for protease activity. *Halobacillus andaensis* was selected to be studied for proteolytic activity production; the microorganism was able to grow on gelatin, yeast extract, skim milk, casein, peptone, fish muscle (*Cyprinus carpio*), and soy flour as protein sources, and among these sources, fish muscle protein was the best inducer of proteolytic activity, achieving a protease production of 571 U/mL. The extracellular protease was active at 50°C, pH 8, and 1.4 M NaCl and was inhibited by phenylmethylsulfonyl fluoride. The proteolytic activity of *H. andaensis* was used to hydrolyze fish muscle protein for peptide production. The peptides obtained showed a MW of 5.3 kDa and a radical scavenging ability of 10 to 30% on 2,2-diphenyl-1-picrylhydrazyl and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and a ferric reducing ability of plasma.
Conclusion: The use of noncommercial extracellular protease produced by *H. andaensis* for biologically active peptide production using fish muscle as the protein source presents a great opportunity for high-value peptide production.
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1. Introduction

Hypersaline environments such as saline soils provide ecosystems with high microbial diversity, especially for halophilic microorganisms that are able to grow at a high salt concentration. Cuatro Cienegas Basin is an environment with a great biological endemism; this place is considered a Precambrian park where ancient microorganisms have been conserved. Moreover, the soil has a low nutrient content and moderate salinity and is considered an extremophilic soil [1]. On the other hand, Sayula and San Marcos lakes are salty; however, there are no studies on their halophilic microbiota. The interest in the exploration of saline environments has been focused on a search for halophilic microorganisms with the capacity to produce salt-tolerant metabolites [2]. The enzymes from halophilic microorganisms are the most studied metabolites. Efforts have been directed to understand their unusual characteristics at the molecular level, including their stability and activity under extreme salinity. The aim of these studies is to discover the enzymes for the development of novel biocatalytic technologies with low water activity systems [3,4].

Hydrolases are the most studied halophilic enzymes, and proteases are the most important hydrolytic enzymes studied extensively; nearly 60% of commercially available enzymes are proteases [4,5,6]. The application of proteases is extensive; they are used in laundry, food, leather, brewing, photography, and other industries. However,

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current studies of proteases have been re-evaluated because of the high demand for better pharmaceutical and nutritional products, particularly aiming to obtain biologically active peptides by hydrolysis of protein sources [7].

Efforts to generate biologically active peptides for therapeutic applications have considered the use of various waste materials as the substrates for protease and peptide production. Fish wastes have recently been considered an interesting protein source for peptide production owing to their importance in human nutrition and their potential to become health-promoting peptides. Recent advances in biotechnological processes are focused on the development of an economical and highly efficient process for fish waste exploitation [8,9].

Halobacillus andaensis has been poorly studied; thus far, there are no reports on their ability to produce proteases. Moreover, only a few papers deal with the protease and peptide production using halophilic microorganisms, providing an opportunity to study halophilic/halotolerant proteases and their biotechnological potential. Thus, the aim of this study was to investigate the use of halophilic microorganisms isolated from saline soils for the production of hydrolases, and the specific goal was to explore *H. andaensis* protease and its biotechnological application for the production of biologically active peptides.

2. Materials and methods

2.1. Sample collection and bacterial isolation

Soil samples were collected from saline soils of three sites in Mexico, including Cuatro Ciénegas Basin in Coahuila state (27°00′ N and 101°48′ 49″ W) and Sayula and San Marcos lakes in Jalisco state (19°54′24″ N and 103° 27′39″ W; 20°20′49.14″ N and 103°33′42.13″ W, respectively). Additionally, salt crystals were used from Salar de Uyuni, Bolivia (20° 08′02″ S and 67° 29′21″ W). A suspension was prepared by shaking the soil sample (1 g) and sterile saline solution (10 mL) in a 50 mL tube for 1 min. The dilution was incubated for 1 h at room temperature until the sediment was obtained. One milliliter of the suspension was inoculated in 10 mL of the ATCC® 2185 culture medium. Microbial growth was observed by turbidity (after 48 ± 24 h) at 37°C with stirring at 180 rpm. Then, microorganisms were isolated by the serial dilution technique (10^{-1} to 10^{-4}); each dilution was inoculated in agar plates with ATCC® 2185 and incubated at 37°C for 24–72 h. After microorganisms have grown, their pure cultures were obtained by subculturing in agar plates.

2.2. Identification of isolated microorganisms

The 16S rRNA gene was used for microorganism identification. DNA extraction was performed by cell lysis; lysates were prepared by thermal shock at 95°C for 10 min. One microliter of the cell lysate was used for amplification of a 1300 bp fragment using bacterial forward and reverse primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′). The following conditions were used for bacteria: initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, and a final extension at 72°C for 5 min [10]. For archaea, the forward and reverse primers PARCH340F (5′-CCC TAG GGG GYG CAS CAG-3′) and ARCH915R (5′-GTG CTC CCC CGC CAA TTC CT-3′) were used under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 53.5°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min [11]. The consensus sequences were obtained using CLC Main Workbench software (QIAGEN Bioinformatics©), and then, sequences were analyzed using the BLAST search for homology in the 16S ribosomal RNA sequences (Bacteria and Archaea) in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). Finally, a phylogenetic tree was constructed using MEGA software version 7.0 using the maximum likelihood method and 1000 bootstraps.

2.3. Screening of strains for extracellular hydrolytic activities

Detection of extracellular hydrolases was performed by monitoring the formation of hydrolysis halos in selective media. The ATCC 2185 agar medium with the following composition was used (g/L): $MgSO_4*7H_2O$ (20), sodium citrate (3), KCl (2), tryptone (5), yeast extract (3), mineral solution (0.1 mL/L) (g/L), $ZnSO_4*H_2O$ (1.32), $MgSO_4*H_2O$ (0.34), Fe $(NH_4)_2SO_4*6H_2O$ (0.82), and $CuSO_4*5H_2O$ (0.14). To explore the hydrolytic activities of both halophilic and halotolerant microorganisms, the culture medium was supplemented with 1.4 M, 2.8 M, and 4.2 M NaCl at pH 7.5 and 9.5. Selective substrates were prepared by adding (1% *w*/*v*) casein, chitin, trioctanoin, tributyrin, or polyhydroxybutyric acid to the ATCC medium. The extracellular hydrolases assayed included proteases (acid casein), chitinases (colloidal chitin), lipases and esterases (trioctanoin and tributyrin with 0.001% of phenol red), and pHB depolymerases (polyhydroxybutyric acid at 0.33% (*w*/*v*)). Each strain was inoculated in the selective medium and incubated at 37°C, and the hydrolysis halo formation was measured every 24 h; in the case of lipase and esterase activity, the hydrolysis was verified on the basis of the red color of the halo turning yellow. Statistical analysis was run using Statgraphics Centurion XVI version 16.1.18 software (Statgraphics Centurion for Windows, Statpoint Technology, Inc., USA). A categorical data analysis was performed to evaluate the occurrence of extracellular hydrolase producers by relating the genera with the three salt concentrations used. Additionally, the frequency of three hydrolase activities tested was determined according to the site sampled. The best extracellular hydrolase producer was selected depending on the halo size. The selected strain was deposited in an international type culture collection with accession number CM-CNRG TB65.

2.4. Selection of protein sources for protease production

Protease production by the selected strain (*H. andaensis*) was performed in 250 mL flasks. Various protein sources (1% *w*/*v*) were used, including gelatin, yeast extract, skim milk, casein, peptone, fish muscle (*Cyprinus carpio*), and soy flour. These protein sources were added as a nitrogen source substitute to the ATCC 2185 medium. The strain was incubated at 37°C at 200 rpm for 96 h, and every 12 h, a cell-free supernatant was obtained. The proteolytic activity was determined according to the modified method of Chuprom et al. [12] using azocasein as the substrate. The reaction mixture was prepared containing 0.02 mL of crude enzyme and 0.5 mL of 0.5% (*w*/*v*) azocasein in 0.05 M Tris–HCl buffer (pH 7.5) with 1.4 M NaCl. The reaction mixture was incubated at 50°C for 1 h under shaking at 900 rpm. The reaction was stopped by adding 0.25 mL of 20% (*w*/*v*) trichloroacetic acid and incubated at −20°C for 5 min. The precipitate was removed by centrifugation at 6500 rpm for 10 min. A volume of 500 μL of the supernatant was mixed with 200 μL of 1 M NaOH. The absorbance was measured at 366 nm using a UV–Vis spectrophotometer. One unit (U) of protease activity was defined as the amount of the enzyme that produces a change of 0.01 in the absorbance under reaction conditions.

2.5. Biochemical characterization of produced protease

Zymography of protease produced during the growth of *H. andaensis* was performed using polyacrylamide gels (12%) according to the method described by Garcia-Carreño et al. [13]. After electrophoresis (15 mA, 2 h), the gel was incubated at 50°C for 1 h in 50 mM Tris–HCl (pH 7.5) with 1.4 M NaCl containing 3% (*w*/*v*) of sodium caseinate. Other protein sources such as gelatin, fish muscle, soy flour, and skim milk were used for obtaining zymograms at 3% (*w*/*v*). The enzyme susceptibility to inhibitors was evaluated by incubating 0.02 mL of crude extract with 1 mM PMSF, EDTA, and benzamidine; then, the gel

was developed as described above. The gels were stained with Coomassie brilliant blue R-250. The development of a clear zone on the blue background of the gel indicated the presence of proteolytic activity. To determine the molecular weight of the proteases in the crude enzymatic extract, SDS-PAGE gels were run using low-range molecular weight markers from BIORAD®. The effect of pH on the activity of protease produced by *H. andaensis* was determined using a universal buffer of pH range from 5 to 13. The effect of temperature on protease activity was assayed from 30 to 80°C at pH 8.0. The effect of salt on protease activity was studied by adding NaCl or KCl to the reaction mixture at the final concentrations of 0.4 to 2.8 M. Activities were determined as a percentage of the measured activity versus maximal activity.

2.6. Hydrolysis of fish muscle (C. carpio) using bacterial extracellular protease

Fish muscle of *C. carpio* was hydrolyzed using the enzyme extract from *H. andaensis*: 20 μL of enzyme extract was mixed with 0.5 mL of 50 mM Tris–HCl buffer, pH 8, and 0.5 mL of 2% fish muscle, and the mixture was incubated at 50°C [14]. Samples were collected at 2 and 4 h and were inactivated at 95°C for 5 min. The samples were centrifuged at 10,000 rpm for 10 min at 4°C. The hydrolysates were analyzed by 12% SDS-PAGE.

2.7. Characterization of fish muscle hydrolysates

For molecular weight distribution, fish muscle hydrolysates after 2 and 4 h of the reaction were analyzed by Tricine SDS-PAGE according to Haider et al. [15] using a 4% stacking gel and 15% resolving gel. The hydrolysates were mixed with the loading buffer (1:1 ratio) and heat-denatured at 95°C for 5 min. Protein bands were stained using a silver staining commercial kit (AMRESCO®). The molecular weight (MW) of the hydrolysates was determined using a polypeptide marker set (Bio-Rad®) consisting of triosephosphate isomerase (26.6 kDa), myoglobin (16.9 kDa), α-lactalbumin (14.4 kDa), aprotinin (6.5 kDa), oxidized insulin β-chain (3.5 kDa), and bacitracin (1.4 kDa). Different assays for radical scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing ability of plasma (FRAP) were performed to study the antioxidant properties of the hydrolysates; assays were performed according to Alemán et al. [7].

3. Results

3.1. Isolation and identification of microorganisms

A total of 32 strains were isolated, and 30 strains were identified as bacteria and two as archaea. The predominant genera in the isolates were *Alkalibacillus* spp. (8 strains, GenBank accession nos. MF595089, MF595092, MF595093, MF595095, MF595097, MF595098, MF595099, and MF595102), *Marinococcus* spp. (10 strains, GenBank accession nos. MF595086, MF595087, MF595088, MF595096, MF595100, MF595101; MF595102, MF595107, MF595109, and MF595110), and *Halobacillus* spp. (7 strains, GenBank accession nos. MF595085, MF595090, MF595104, MF595105, MF595106, MF595108, and MG001320). Other genera isolated were *Salicola* sp. (1 strain, GenBank accession no. MG77049), *Aquisalibacillus* sp. (1 strain, GenBank accession no. MF595091), *Aidingimonas* sp. (1 strain, GenBank accession no. MG001319), and *Bacillus* spp. (2 strains, GenBank accession nos. MF595084 and MF595094) (Fig. 1). Phylum Firmicutes was predominant in this study and was represented particularly by the family Bacillaceae containing five genera: *Alkalibacillus* sp., *Marinococcus* sp., *Halobacillus* sp., *Aquisalibacillus* sp., and *Bacillus* sp. Other genera were detected, including *Aidingimonas* and *Salicola* belonging to the family *Halomonadaceae* of the phylum Proteobacteria. The 16 rRNA gene sequences of the isolated halophilic bacteria exhibited >97% similarity with those of other published species according to the NCBI database. Phylogenetic analysis based on 16S rRNA according to the molecular identification of the strains revealed a total of seven well-supported clades by genus and indicated division into two clades based on the phylum Firmicutes and class γ-Proteobacteria (Fig. 1). Some genera studied had a common ancestor, such as *Marinococcus* sp. with *Bacillus* sp., *Halobacillus* sp. with *Alkalibacillus* sp., and *Aidingimonas* sp. with *Salicola* sp. In particular, the strain 4C (MF595101) is separated between the clades with a high phylogenetic distance compared with the analyzed sequences, although the sequence was similar (99%) to that of *Bacillus qingdaonensis* according to the NCBI database.

Based on the 16S rRNA gene, archaea isolated from Salar Uyuni have high similarity (<97%) with *Halorubrum tebenquichense* (accession number MG001322) and *Halobacterium salinarum* (accession number MG001321) according to the NCBI database. Phylogenetic analysis based on 16S rRNA revealed two well-supported clades based on genus and on the families *Halobacteriales* and *Haloferacales* (Fig. 2). The bacterium isolated in this study from the crystals of Salar de Uyuni was identified as *Salicola marasensis* (SU6) (Fig. 1); this bacterium belongs to γ-Proteobacteria, the second most abundant bacterial phylum present at this location.

3.2. Screening for extracellular hydrolytic activities

Detection of extracellular hydrolases was performed by hydrolysis halo formation. The tested strains formed hydrolysis halos in the cases of casein (proteases), tributyrin (esterases), and trioctanoin (lipases), while in the cases of chitin (chitinases) and polyhydroxybutyric acid (PHB depolymerases), there was no hydrolysis halo. Lipase and esterase activities were detected between 24 and 48 h of incubation at pH 7.5, and protease activity was detected between 48 and 168 h of incubation at pH 7.5. Six strains were able to produce esterase, lipase, and protease at three salt concentrations evaluated (Table 1). The strains isolated from Cuatro Cienegas Basin displayed greater hydrolytic capacity, being positive for lipases, esterases, and proteases (Fig. 3B). However, the hydrolysis halo formed by the protease activity was larger (0.7–1.6 cm) than the esterase and lipase halos (<0.7 cm); thus, protease production was selected for further studies. *Bacillus halochares*, *B. qingdaonensis*, *S. marasensis*, *Aidingimonas halophila*, and *Aquisalibacillus elongatus* had hydrolysis halos for one or two substrates at three salt concentrations tested (Table 1). The genus distribution of hydrolytic activity producers in the sampled sites was not associated with salt concentration according to the chi-square test ($P = 0.99$ and $P = 0.94$, respectively). However, the frequency of hydrolytic activity production according to salt concentration revealed that *Marinococcus* sp. was a producer of three hydrolytic activities (lipase, esterase, and protease) at three salt concentrations tested. The genera *Halobacillus* sp. and *Alkalibacillus* sp. exhibited two out of three hydrolytic activities tested at three salt concentrations. *Salicola* sp., *Aidingimonas* sp., and *Aquisalibacillus* sp. displayed only one hydrolytic activity at 1.4 M or 4.2 M NaCl (Fig. 3A). On the other hand, strains isolated from Cuatro Cienegas Basin and San Marcos Lake were producers of esterases and proteases, while the production of lipases and esterases was more frequent than that of proteases in strains isolated from Sayula Lake. In the case of Salar de Uyuni strains, only *S. marasensis* was positive for lipases and proteases (Fig. 3B). According to hydrolysis halo formation, *H. andaensis* (strain MQ), *Marinococcus luteus* (5S), and *Halobacillus dabanensis* (strain 5C) were the best producers of proteolytic activity at all salt concentrations tested (1.4, 2.8, and 4.2 M NaCl), with 1.4 M being the best salt concentration for hydrolysis halo production using casein as the substrate. *H. andaensis* was selected for further studies because of its larger halo production (1.2–1.6 cm) than those of *H. dabanensis* (0.5–1 cm) and *M. luteus* (0.8–1 cm).

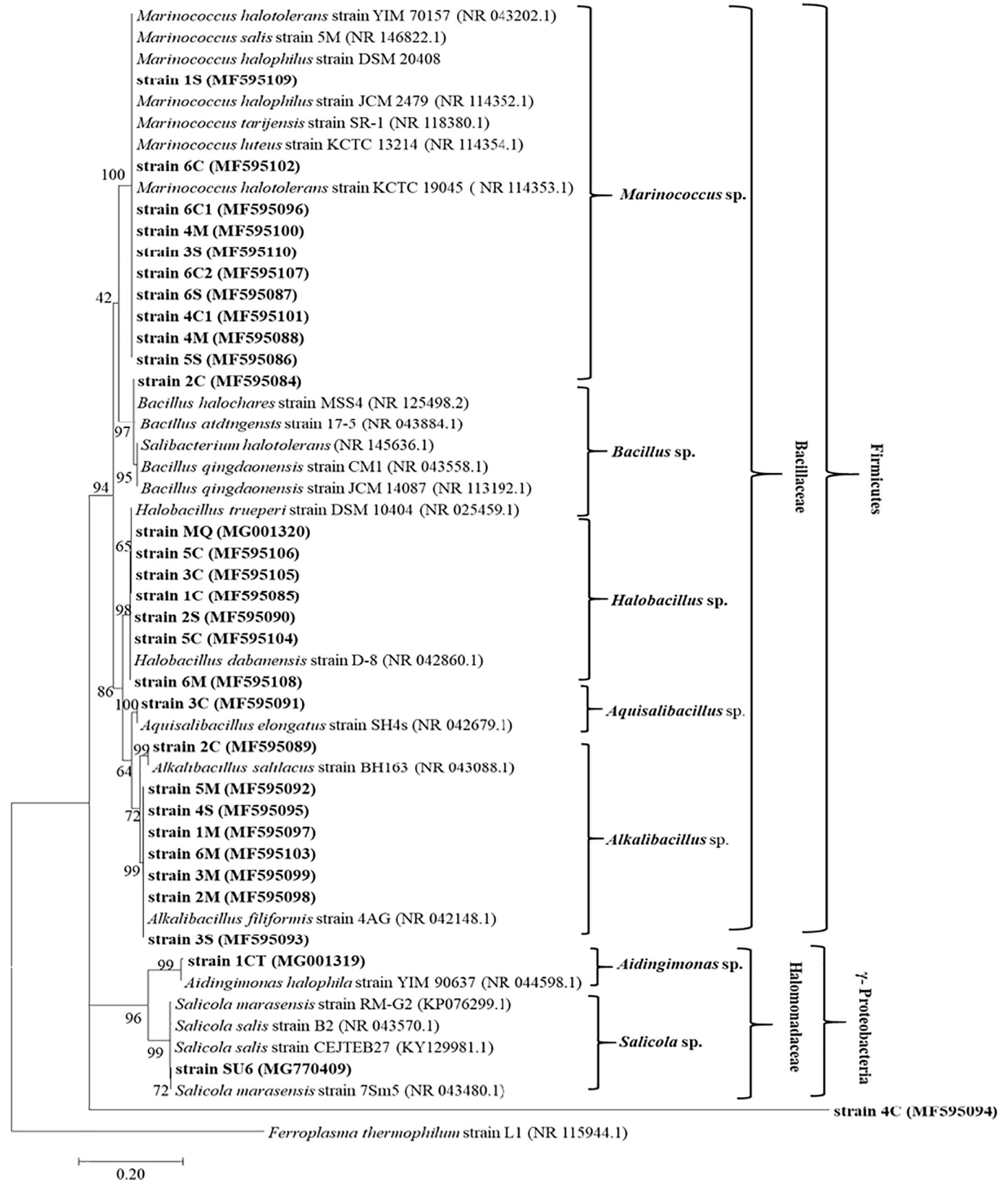

Fig. 1. Maximum likelihood tree showing the evolutionary relationship among halophilic and halotolerant bacteria based on the 16S rRNA gene. The rooted phylogenetic tree was inferred using the maximum likelihood method based on the Tamura-Nei model with bootstrap confidence values (%) based on 1000 replications. A discrete gamma distribution was used to model evolutionary rate differences among the sites, and the rate variation model allowed for some sites to be evolutionary invariable. The branch lengths were measured as the number of substitutions per site (0.20). *Ferroplasma thermophilum* strain L1 (NR 115944.1) was used as the outgroup. Monophyletic clades are grouped according to the genus, taxonomy family, and phylum. The accession numbers of the isolated strains are indicated in parenthesis.

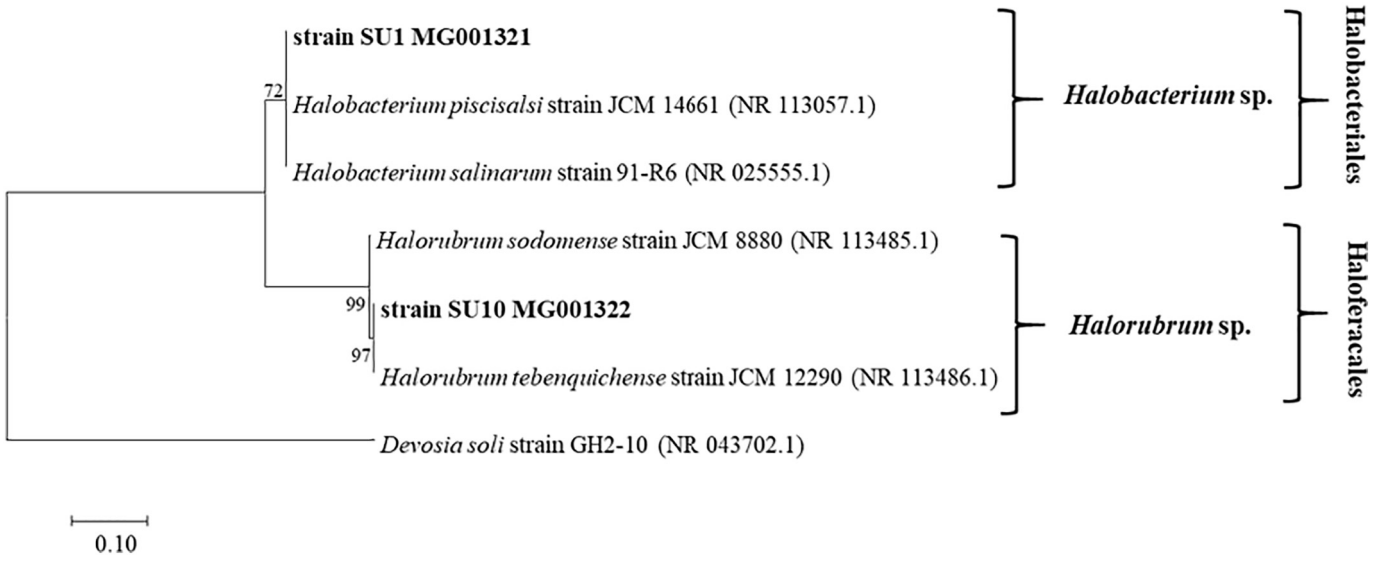

Fig. 2. Maximum likelihood tree showing the evolutionary relationship among archaea based on the 16S rRNA gene. The rooted phylogenetic tree was inferred using the maximum likelihood method based on the Tamura-3-parameter model with bootstrap confidence values (%) based on 1000 replications. A discrete gamma distribution was used to model evolutionary rate differences among the sites and the rate variation model allowed for some sites to be evolutionary invariable. The branch lengths were measured as the number of substitutions per site (0.10). *Devosia soli* strain GH2-10 (NR 043702.1) was used as the outgroup. The accession numbers of the isolated strains are indicated in parenthesis.

3.3. Protease activity from H. andaensis

Submerged cultures of *H. andaensis* isolated from Cuatro Cienegas Basin and selected for their hydrolysis halo were evaluated for protease production using seven protein sources. *H. andaensis* was able to use six out of seven protein sources evaluated. Among the protein sources tested, fish muscle, soy flour, and skim milk were the best inducer of protease production by *H. andaensis* (571, 392, and 342.6 U/mL, respectively) (Fig. 4A). The culture of *H. andaensis* in fish muscle as a protein source had a growth rate of 0.97 h^{-1} (Fig. 4B). Proteolytic activity was detected at 36 h in the early exponential phase and 60 h in the early lag phase, reaching the maximum of proteolytic activity, and this level was maintained until 80 h of growth.

3.4. Characterization of proteolytic crude extract

Crude extracts collected during the growth of *H. andaensis* using fish muscle as a protein source were tested for proteolytic activity by the zymogram method. Proteolytic activity was detected by the presence of diverse bands using the extracts collected at various times. However, an intense band was observed at the bottom of the gel from 36 to 80 h (Fig. 5A). The start (36 h) and end (72 h) of the log phase were selected for analysis of inhibitor susceptibility, and the activity band observed at the bottom of the gel was inhibited by PMSF (Fig. 5B). The proteolytic activity was detected by zymograms of five evaluated substrates. A single band was observed when sodium caseinate was used as a substrate (Fig. 5C). The same band was present in the tests of other substrates. Discrete bands of high molecular weight were detected when gelatin was used as a substrate. According to SDS-PAGE, the apparent molecular mass of the intense band was between 14.4 and 21.5 kDa (Fig. 5C).

The extracellular protease activity of *H. andaensis* preferred neutral-alkaline pH, having a relative activity above 80% over a wide range of pH values from 7 to 10 with an optimal activity at pH 8 (Fig. 6A). According to the results shown in Fig. 6B, optimal activity of *H. andaensis* protease was observed at 50°C, and approximately 60% of the activity was retained at 60°C. The effect of salt on *H. andaensis* protease activity was studied, and enzymatic activity was optimal at 1.4 M NaCl (Fig. 6C); the protease activity was also observed at 0.4 M NaCl with a relative activity of 80%. The extracellular protease activity was similar when KCl was used. In addition, a decrease in the activity (60%) was observed when both NaCl and KCl were at 2.8 M. Halotolerance of the proteolytic activity was evident, but the enzyme is not salt dependent because the activity was observed even with no salt present in the hydrolysis reaction.

Table 1
Hydrolysis halo formation in media with 4.2, 2.8, and 1.4 M NaCl.

Strains	Proteases	Lipases	Esterases
Bacteria			
Halobacillus andaensis (4 strains)	+	−	+
Halobacillus dabanensis (3 strains)	+	−	+
Alkalibacillus salilacus (1 strain)	+	+	+
Alkalibacillus filiformis (7 strains)	+	+	+
Marinococcus luteus (10 strains)	+	+	+
Bacillus quingdaonensis (1 strain)	−	+	−
Bacillus halochares (1 strain)	−	+	−
Aquisalibacillus elongates (1 strain)	+	+	+
Salicola marasensis (1 strain)	+	+	+
Aidingimonas halophila (1 strain)	+	+	+
Archaea			
Halorubrum tebenquichense (1 strain)	−	−	−
Halobacterium salinarum (1 strain)	−	−	−
Total 32 strains	28 strains	14 strains	28 strains

3.5. Hydrolysis of fish muscle (C. carpio) using H. andaensis protease

The hydrolysis of fish muscle flour using *H. andaensis* protease revealed a decrease in abundant protein bands at 2 h of the reaction time, especially pronounced in proteins with molecular weights of 25, 49, and 106 kDa. At 4 h of the reaction time, the disappearance of abundant protein bands is clearly visible (Fig. 7A). The hydrolysates had peptides with molecular weights ranging between 3.4 and 26.5 kDa, although the hydrolysate obtained at 4 h had an intense band corresponding to a peptide with a molecular weight of 5.3 kDa (Fig. 7B). The radical scavenging activity of the hydrolysates showed an inhibition capacity between 10 and 20% in the case of both DPPH and ABTS; inhibition was higher with DPPH and in the hydrolysate at 4 h of the reaction time, indicating that the peptides with low molecular weight have a higher antioxidant capacity (Fig. 7C). Another method for measuring antioxidant potential is the FRAP

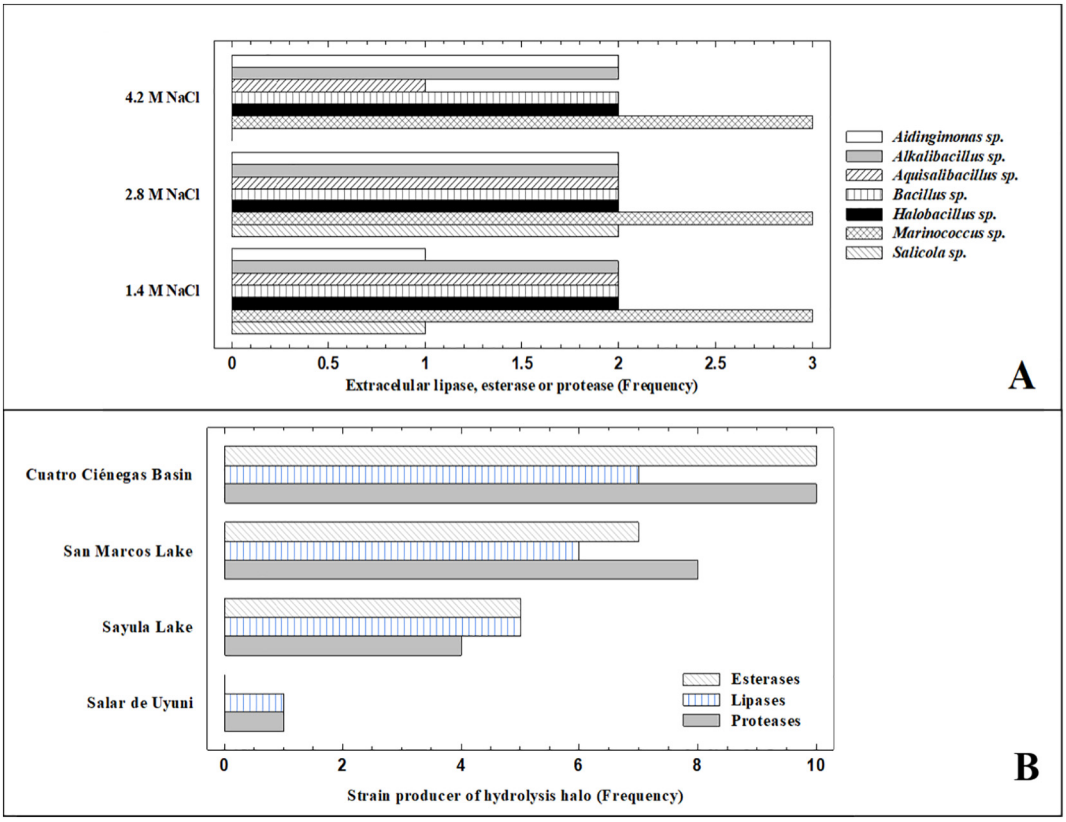

Fig. 3. Categorical analysis of hydrolytic activities based on hydrolysis halo formation. A: Frequency analysis of hydrolysis activity (esterase, lipase, and protease) by genus at three salt concentrations tested. B: Frequency analysis of three hydrolytic activities evaluated in the strains isolated from four sampled sites.

(ferric reducing antioxidant power) assay shown in Fig. 7D; the hydrolysates obtained at 2 h had a higher antioxidant capacity.

4. Discussion

The microorganisms isolated from Cuatro Cienegas Basin and Sayula and San Marcos lakes were mainly bacteria. The family Bacillaceae is the most abundant family, including *Alkalibacillus* sp., *Marinococcus* sp., and *Halobacillus* sp. as the predominant genera. Bacteria belonging to the family Bacillaceae are the most abundant bacteria in the natural environments including salt lakes, salterns, etc. On the other hand, the family Halomonadaceae comprises principally marine and moderately halophilic microorganisms that can grow in a salt range of 7.5–10% (*w*/*v*), but there are some strains that are able to grow at 15–20% of salt. Some halomonas can be found in various saline environments such as soils, salty foods, and saline lakes. Some members of both Bacillaceae and Halomonadaceae families had been isolated from diverse saline environments such as Razzaza Lake [16], South African saltpan [17], Valanthakad mangroves, India rish [18], and Lunsu, India [19], including *Bacillus* and *Halobacillus* as the most reported genera; in saline-alkaline environments, there are reports of the phylum Firmicutes and predominantly the family Bacillaceae, with

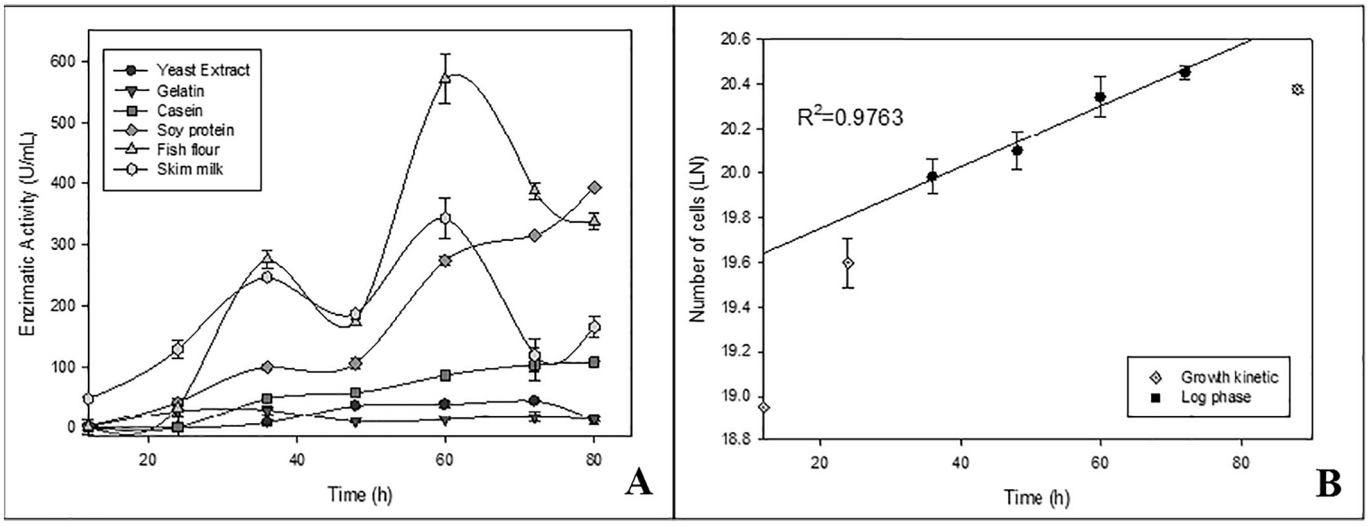

Fig. 4. Proteolytic activity of *Halobacillus andaensis* (strain MQ). A: Effects of protein sources on the proteolytic activity of *H. andaensis*. B: Growth kinetics of *H. andaensis* in fish muscle as a protein source.

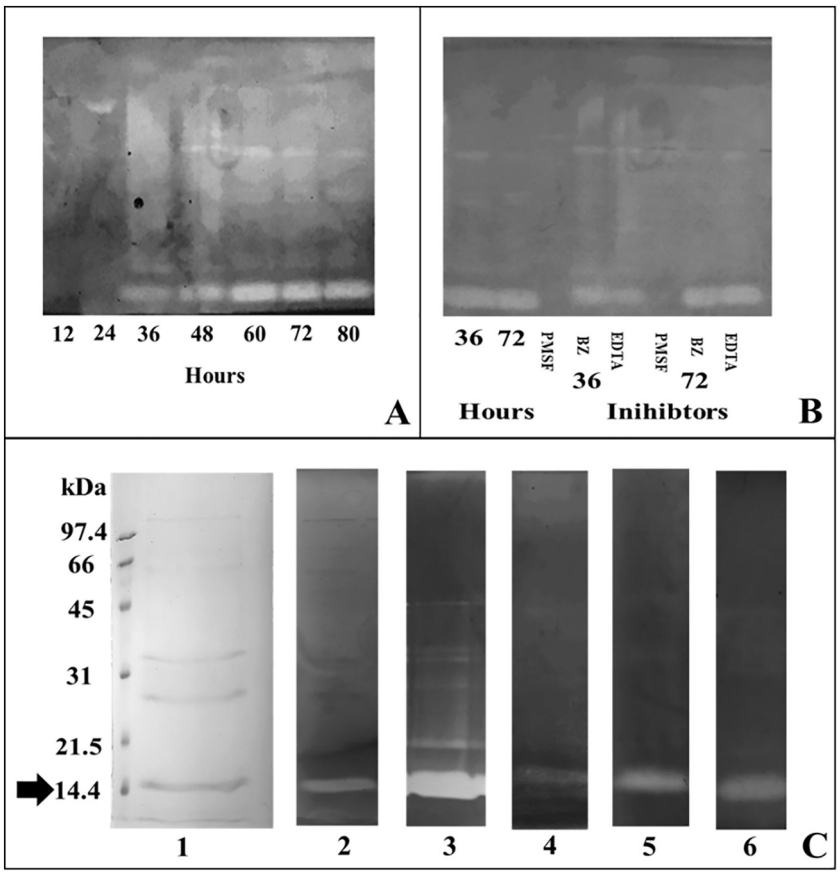

Fig. 5. Zymogram analysis of a crude enzymatic extract of *Halobacillus andaensis* MQ. A: Zymography of the enzymatic crude extract obtained during the growth of *H. andaensis* (strain MQ) using fish muscle as a protein source. B: Inhibitor susceptibility of the enzymatic crude extract at 36 h and 72 h of growth of *H. andaensis* (PMSF = phenylmethylsulfonyl fluoride, EDTA = edetic acid, BZ = benzamidine). C: SDS-PAGE, zymography and apparent molecular mass of proteolytic activity of *H. andaensis*. 1: low-range molecular weight standards (BIORAD ®). 2–6: zymography of proteolytic activity of *H. andaensis* (strain MQ) using various protein sources (3% *w*/*v*). 2: skim milk, 3: gelatin, 4: fish muscle, 5: soy flour, 6: sodium caseinate.

Alkalibacillus being one of the most predominant genera [20]. Salar de Uyuni is a heterogeneous and complex ecosystem where bacterial and archaeal communities are present, especially halophilic and halotolerant phylotypes [21]. The family Halobacteriaceae is one of the most abundant prokaryotic communities in Salar de Uyuni where the occurrence of *Halobacterium* sp. has been shown. Additionally, *Halorubrum* sp. has been reported in the soil samples.

Bacteria isolated from Cuatro Cienegas Basin are able to produce hydrolytic enzymes assayed in this study. There are few reports on enzyme production by bacteria originating from this location; Delgado-García et al. [22] reported the production of xylanases, chitinases, DNases, and amylases. Studies using strains isolated from Salt Lake Razzaza in Iraq report the production of proteases and lipases at alkaline pH by *Halobacillus* sp. and *Bacillus* sp. [16]. Additionally, the presence of Gram-positive bacteria as the highest group of bacteria was revealed in this study as having the largest hydrolysis halos. Studies in bacteria isolated from Howz-e Soltan revealed *Halobacillus* sp. as the predominant genus with amylase, protease, and inulinase hydrolytic activities [23]. There are few reports of haloalkaliphiles isolated from the soda lakes with the ability to produce hydrolysis. *Alkalibacillus* sp. is one of the genera of moderately salt-tolerant organisms of the group of γ-Proteobacteria present in the soda lakes, particularly isolated from Sayula and San Marcos; this genus has high production of lipase, esterase, and protease, and the latter activity was reported in other studies [20].

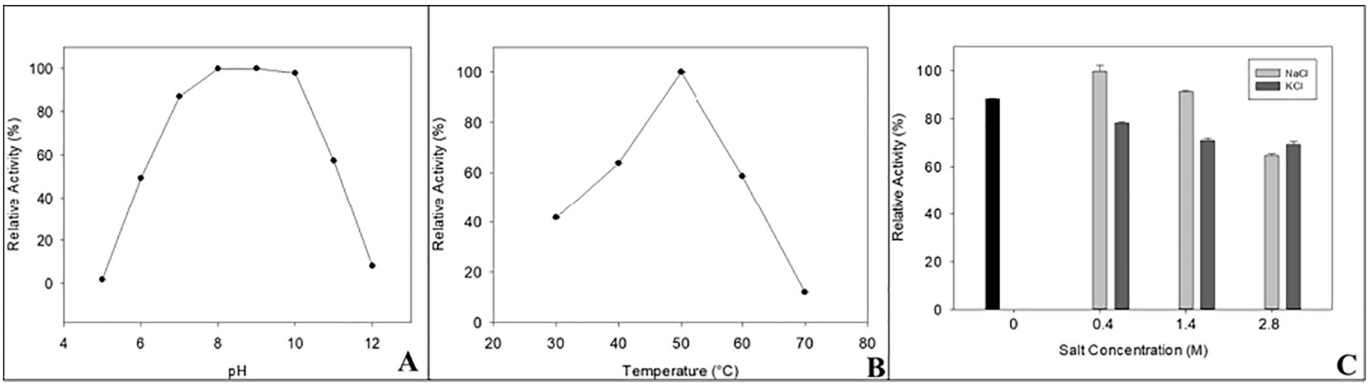

Fig. 6. Effects of pH (A), temperature (B), and salt (C) on *H. andaensis* (strain MQ) extracellular protease activity.

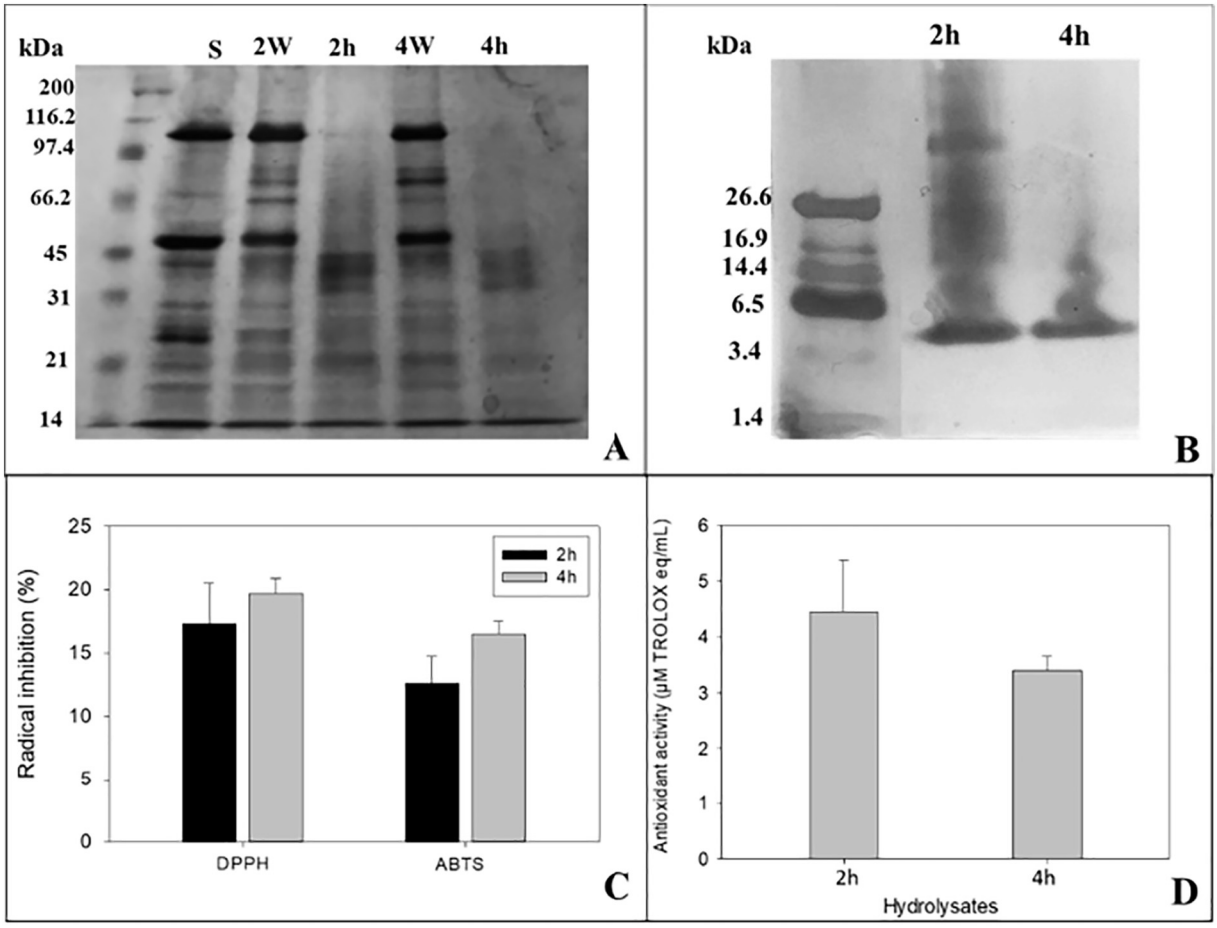

Fig. 7. Hydrolysis of fish muscle (*Cyprinus carpio*) using extracellular protease extract from *Halobacillus andaensis* (strain MQ). A: SDS-PAGE of fish muscle hydrolysis by extracellular protease extract using broad-range molecular weight standards (BIORAD®) (S = substrate not hydrolyzed; 2 W, 4 W = substrate incubated at 50°C without enzyme; 2 h, 4 h = substrate incubated at 50°C with enzyme). B: Tricine-SDS-PAGE of peptide profile from fish muscle hydrolysates (2 h, 4 h = fish muscle hydrolysates obtained at various times) using polypeptide SDS-PAGE molecular weight standard (BIORAD ®). C: Antioxidant activity of fish muscle hydrolysates obtained at various times of hydrolysis as DPPH and ABTS radical scavenging capacity. D: Fe reducing power (FRAP) capacity of hydrolysates from fish muscle obtained at various times of hydrolysis.

In general, protease production by bacteria is not growth associated and occurs at the mid-exponential phase, with an important decrease in protease activity during the stationary phase [24]. A similar behavior was shown in the case of *H. andaensis* protease production when fish muscle protein was used as a nitrogen source (Fig. 4A). Production of proteases during the stationary phase has been reported in *Halobacillus* sp. CJ4, *Halobacillus blutaparonensis*, and *Halobacillus karajensis* [25,26,27]. The growth of *H. andaensis* in fish protein was successful; we suggested that the strain was adapted to use this nitrogen source, as the strain was isolated from Cuatro Cienegas Basin, where the microbial communities are fundamental for the aquatic ecosystem by generating a symbiosis, especially with fish [1]. According to the zymogram with the inhibitors, the enzyme activity expressed in the most intense band was inhibited by PMSF (Fig. 5B), an inhibitor of serine proteases. This suggests that the protease produced by *H. andaensis* is a serine protease. Serine proteases belong to a group of enzymes produced by diverse moderately halophilic bacteria and haloarchaea including members of *Halobacillus* sp. However, some of these serine proteases have been reported as serine metalloproteinases; this inhibition profile is observed in the members of the subtilase superfamily including subtilisin, one of the most used industrial enzymes [26,27]. There are reports of some proteases with variable molecular weight; however, the common range is approximately 30–80 kDa in the genus *Halobacillus*. However, the intense band observed in the zymograms of *H. andaensis* protease has an apparent MW of approximately 14 kDa similar to that of the protease expressed by the *Halobacillus* sp. strain CJ4 [25]. When we performed an SDS-PAGE of the denatured protease produced by *H. andaensis*, the molecular weight was 40 kDa, an average reported for similar proteases from *Halobacillus* [24].

Protease activity from *H. andaensis* hydrolyzed diverse protein substrates according to the zymograms and growth characteristics (Fig. 4 and Fig. 5). The use of broad protein sources of animal and vegetal origin as the substrate has important implications in biotechnology applications and especially in bioactive peptide production. On the other hand, the extracellular protease produced by *H. andaensis* was active in a broad range of alkaline pH and high NaCl concentration with great opportunities for potential applications. In particular, the haloalkaline property can be used in the treatment of saline water or wastes with protein residues and a high salt content where the pH fluctuations are crucial for an industrial process [25,28,29]. The ability of the extracellular protease from *H. andaensis* to retain approximately 60 and 40% of its enzymatic activity at 40 and 30°C, respectively (Fig. 6B), and its neutral-alkaline nature are important features for the detergent industry [30,31]. The protease retains approximately 40% of enzymatic activity at 2.8 M NaCl and KCl (Fig. 6C), suggesting a possibility to be active in a nonaqueous medium. The extracellular protease of *H. andaensis* was able to hydrolyze gelatin (Fig. 5C), and this property is desirable for the recovery of silver from photographic films [3].

Wastes generated in the fish industry have a great potential to produce high-value hydrolysates to be used in the food industry. Peptides from fish muscle are receiving recent attention because of their antioxidant, antihypertensive, immunomodulatory, and antimicrobial activities [32]. Usually, the commercial protease Alcalase® is used for

fish protein hydrolysis and *Catla catla*, *C. carpio*, and *Oreochromis niloticus* are used to obtain bioactive peptides [14,33,34]. In this work, we propose the use of extracellular protease from *H. andaensis* in the *C. carpio* muscle protein hydrolysis; it was possible to obtain small bioactive peptides (5.3 kDa) at 2 h of incubation. Low-molecular-weight peptides were obtained in *O. niloticus* hydrolysis by Alcalase®; these small peptides are characteristic of fish protein hydrolysates and have a high nutritional value [34]. Antioxidant scavenging reported by the peptides from fish enzymatic hydrolysates is over 30% of radical inhibition [7,35]. Peptides from *C. carpio* obtained at 4 h of hydrolysis showed better radical inhibition than peptides generated at 2 h of hydrolysis, indicating that peptides of low molecular weight have stronger antioxidant activity. Currently, the study of unexplored regions offers a great biotechnological potential and not only expands the known microbial diversity but also investigates applications of their biomolecules to improve industrial processes with the impact on human health. Although studies of the application of halophilic enzymes are scarce, the efforts to apply these enzymes to diverse processes are increasing. The use of noncommercial extracellular protease produced by *H. andaensis* (strain MQ) for biologically active peptide production using fish muscle as a protein source represents a great opportunity for high-value peptide production.

Declarations of interest

None.

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Supplementary data

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