



Bacteria producing L-asparaginase isolated from Peruvian saline environments

Bacterias productoras de L-asparaginasa aisladas de ambientes salinos peruanos

Jeanett Montes; Pamela Elizabeth Canales*; Juan Carlos Flores-Santos; Stephy Saavedra; Abad Hurtado-Gómez; Cynthia Esquerre-Huallpa; Amparo Iris Zavaleta

Laboratorio de Biología Molecular, Facultad de Farmacia y Bioquímica, Universidad Nacional Mayor de San Marcos, Lima, Perú.

* Corresponding author: pamela.canales@unmsm.edu.pe (P. E. Canales).

ORCID ID of the authors

J. Montes:  <https://orcid.org/0000-0003-0981-8274>

P. E. Canales:  <https://orcid.org/0000-0001-6019-8846>

J.C. Flores-Santos:  <https://orcid.org/0000-0001-5632-7203>

S. Saavedra:  <https://orcid.org/0000-0002-4867-7622>

A. Hurtado-Gómez:  <https://orcid.org/0000-0002-4278-243X>

C. Esquerre-Huallpa:  <https://orcid.org/0000-0002-2727-8884>

A. I. Zavaleta:  <https://orcid.org/0000-0003-3844-7185>

ABSTRACT

L-asparaginase (EC 3.5.1.1) hydrolyzes L-asparagine in L-aspartic acid and ammonia. Its efficiency is subject to its kinetics and specificity on the substrate, characteristics that vary from one source to another. Thus, microorganisms from saline environments constitute a phylogenetic and metabolically heterogeneous group for the search of new enzymes. Therefore, the objective of this study was the phenotypic and genotypic characterization of bacteria with L-asparaginase activity isolated from Maras, Pilluana and Chilca salterns. The 24 evaluated bacteria were classified as 38% Gram-negative bacilli and 54% positive; and 8% Gram-positive cocci. The majority grew in 5% salt water, pH 7.0 and 37 °C, all assimilated glucose. Of the 24 bacteria that produced L-asparaginase in solid medium, enzymatic activity was determined in submerged cultures by the Nessler method in 14 of them. The CH11, M62, M64, M68, and P19 strains identified as *Bacillus* sp., by partial sequencing of the 16S ribosomal gene, presented higher L-asparaginase activity and instability due to the presence of proteases. Saline environments bacteria are potential sources for the prospective production of L-asparaginase to use them as a therapeutic agent and in the food industry.

Keywords: L-asparaginase; saline environments; *Bacillus*; *Enterobacter*; *Halomonas*.

RESUMEN

L-asparaginasa (EC 3.5.1.1) hidroliza L-asparagina en ácido L-aspártico y amonio. Su eficiencia está supeditada a su cinética y especificidad sobre el sustrato, características que varían de una fuente a otra. Así, los microorganismos de ambientes salinos constituyen un grupo filogenético y metabólicamente heterogéneo para la búsqueda de nuevas enzimas. Por ello, el objetivo de este estudio fue la caracterización fenotípica y genotípica de bacterias con actividad L-asparaginasa aisladas de las Salinas de Maras, Pilluana y Chilca. Las 24 bacterias evaluadas fueron clasificadas como bacilos Gram negativos 38% y positivos (54%); y cocos Gram positivos (8%). La mayoría creció en agua de sales 5%, pH 7,0 y 37 °C, todas asimilaron glucosa. De las 24 bacterias productoras de L-asparaginasa en medio sólido, se determinó la actividad enzimática en cultivos sumergidos por el método de Nessler en 14 de ellas. Las cepas CH11, M62, M64, M68 y P19 identificadas como *Bacillus* sp., por secuenciamiento parcial de los genes ribosómicos 16S, presentaron mayor actividad L-asparaginasa e inestabilidad por presencia de proteasas. Estas bacterias de ambientes salinos son fuentes potenciales para la producción prospectiva de L-asparaginasa de uso como agente terapéutico y en la industria alimentaria.

Palabras clave: L-asparaginasa; ambientes salinos; *Bacillus*; *Enterobacter*; *Halomonas*.

Recibido: 02-04-2021.

Aceptado: 10-05-2021.



Esta obra está publicada bajo la licencia [CC BY-NC 4.0](https://creativecommons.org/licenses/by-nc/4.0/)

INTRODUCTION

L-asparaginase (EC 3.5.1.1) hydrolyzes L-asparagine in L-aspartic acid and ammonium, it is present in bacteria, yeast, fungi, mammals, among others. It is used as a chemotherapeutic agent in the treatment of acute lymphoblastic leukemia. The L-ASNase (L-asparaginase) formulations correspond to type II L-ASNases from *Escherichia coli* and *Erwinia chrysanthemi* in their native or pegylated forms. However, these enzymes have adverse effects such as hypersensitivity, pancreatitis, and neurotoxicity (Shrivastava et al., 2016; Muneer et al., 2020). Therefore, there is a pressing search for new sources of L-ASNase to overcome existing limitations.

In this regard, obtaining L-ASNase from microorganisms that inhabit saline environments is an attractive alternative due to the structural modifications of their proteins. Which allow them to adapt to variable temperatures, pH and salinity that could give them new properties and kinetic characteristics. These environments are widely distributed in various geographical regions. They contain microorganisms belonging to different taxonomic groups with wide metabolic diversity and biotechnological potential (Ventosa et al., 1998; Oren, 2010; Corral et al., 2019).

In general, the enzymes of saline environments microorganisms present greater stability under extreme conditions of pH and temperature. Which is why they are considered enzymes with catalytic

attractiveness under demanding reaction conditions, and they also serve as a model to improve the genetic characteristics of existing ones. Various authors have shown the great versatility of these organisms in the production of hydrolases such as proteases, lipases, amylases, cellulases, inulinases, pullulanases, pectinases and nucleases (Sánchez-Porro et al., 2003; Delgado-García et al., 2012; Ruginescu et al., 2019).

Additionally, the great demand for therapeutic enzymes with better kinetic and immunogenic profiles such as L-ASNase puts enzymes from saline environments microorganisms in the spotlight. Thus, L-ASNases isolated from *Bacillus* sp. BCCS 034 (Ebrahiminezhad et al., 2011), *Bacillus aryabhatai* (Shirazian et al., 2016), *Halomonas elongata* (Ghasemi et al., 2017), *Marinobacter* sp., *Paracoccus* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Vibrio* sp., among others (Zolfaghar et al., 2019) indicate appropriate characteristics for use in thermoprocesses in the food industry, and as a therapeutic and diagnostic agent. In this regard, obtaining new bacterial sources that produce L-ASNase allows us to have alternatives for the productive sectors that use this enzyme. Consequently, the purpose of the study is to characterize L-ASNase-producing bacteria from three Peruvian saline environments characterized by different climates and altitudes.

MATERIALS AND METHODS

Samples and isolation

The study was carried out using 24 bacteria isolated from the muds of Maras at 3380 m (Cusco), Pilluana at 200 m (San Martín) and Chilca salterns at 3 m (Lima) altitude, which belong to the strains collection of the Molecular Biology Laboratory of the Faculty of Pharmacy and Biochemistry of the Universidad Nacional Mayor de San Marcos. In the reactivation of the bacteria, a 5% Salt Water (SW) medium supplemented with 0.5% yeast extract was used (Dyall-Smith, 2006). The composition of 5% SW (g l^{-1}) was NaCl 40.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 5.83, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 5.0, KCl 1.17, NaBr 0.13, CaCl_2 0.083, NaHCO_3 0.03. Solid SW medium was prepared by adding 15 g l^{-1} of agar.

Preselection of bacteria with L-ASNase activity in solid medium

Bacteria were grown on agar with modified M-9 medium according to Gulati et al. (1997), supplemented with 5% SW, and incubated at 37 °C for 48 h. L-asparaginase activity was detected by the formation of pink halos around the colonies. Likewise, the modified Czapek Dox medium (Gulati

et al., 1997) with the following composition (g l^{-1}) was prepared: Na_2HPO_4 6.0, KH_2PO_4 2.0, NaCl 10.0, L-asparagine 10.0 (Sigma-Aldrich, MO, USA), D-glucose 2.0, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, pH 6.0. The medium was supplemented with 2% agar and 0.007% bromothymol blue (Sigma-Aldrich, MO, USA). L-asparaginase activity was detected by the formation of a blue halo around the growth.

Physiological characteristics

Gram-staining was performed and the morphological characteristics of the selected colonies were determined. In addition, the growth capacity of the bacteria was evaluated at salt concentrations of 0.9, 5, 10, 15 and 20%, w/v; at pH of 5, 6, 7, 8 and 9, and at temperatures of 4, 20, 30, 37 and 42 °C. All tests were performed in medium SW supplemented with 0.5% yeast extract and incubated at 37 °C for 48 h. In addition, the ability to assimilate glucose, fructose, lactose, and maltose was evaluated. For this last test, the isolates were cultured in 5% SW supplemented with 0.1% yeast extract and the carbohydrate to be evaluated at 0.2% (w/v) (Mata et al., 2002).

Biochemical tests

For the catalase test, the bacteria were cultured in 5% SW supplemented with 0.5% yeast extract and incubated at 37 °C for 24 h. A small bacterial culture sample was removed on a sterile slide and drops of 3% (v/v) hydrogen peroxide were added. The formation of bubbles indicated the production of catalase (Miao et al., 2014). For the production of H₂S and gas, TSI agar supplemented with 5% SW was employed using the conditions described above. Blackening and bubble formation or rupture of the culture medium indicated the production of H₂S and gas, respectively.

Amplification of 16S ribosomal gene and molecular analysis

Bacteria DNA was extracted using organic solvents as described by Flores-Fernandez et al. (2019). The 16S rRNA gene was amplified using universal primers specific for the Bacteria domain 16SBF 5'-AGAGTTTGATCATGGCTCAG-3' and 16SBR 5'-GGTTACCTGTTCAGACTT-3'. The final volume of the amplification reaction was 25 µl, which contained 20 µM of each primer, 200 µM of each dNTP, 50 mM KCl, 10 mM Tris/HCl, 0.1% (v/v) Triton X-100, 1.5 mM MgCl₂, 1.5 U Taq DNA polymerase and 50 ng of DNA. The reaction conditions were 94 °C for 4 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 1 min and 72 °C for 45 s, with a final extension at 72 °C for 7 min. Then, the PCR products were separated by electrophoresis on 1% agarose gel. DNA 1 kb ladder (Invitrogen, Waltham, USA) was used as a molecular weight marker and was stained with ethidium bromide for the visualization of the fragments.

The 16S ribosomal gene was partially sequenced by Macrogen (Rockville, USA). The nucleotide sequences obtained were compared to those deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) using the BlastN algorithm.

Assay in submerged culture of bacteria producing L-ASNase

The bacteria were cultured at 37 °C for 24 h, in Czapek Dox modified medium supplemented with 0.009% phenol red (Sigma-Aldrich, MO, USA) or 0.007% bromothymol blue at pH 5.6. Afterwards, the L-ASNase activity was considered positive if the phenol red or bromothymol blue media changed to

pink or blue, respectively. Furthermore, the intensity of the colour was measured by spectrophotometry at 560 and 620 nm; and the final pH of the cultures. The positive strains with the highest L-ASNase activity were selected for further studies.

Quantification of L-ASNase activity by the Nessler method

The ammonium released from the hydrolysis of L-asparagine was quantified using the Nessler reagent as described by Imada et al. (1973) with the modifications described below. The crude extract was obtained from the bacterial isolates cultured in the medium proposed by Qeshmi et al. (2015). The reaction was carried out in two stages. In the first, the reaction mixture containing 250 µl of 50 mM Tris buffer (pH 8.6), 225 µl of ultrapure water, 25 µl of 189 mM L-asparagine and 25 µl of the sample (crude extract) was incubated at 37 °C for 30 min. Then, 25 µl of 1.5 mM trichloroacetic acid (TCA) was added to the mixture and centrifuged at 2000 g for 2 min at 4 °C. In the second stage, 1.075 ml of ultrapure water and 125 µl of Nessler's reagent (Merck, Darmstadt, Germany) were added to 50 µl of the supernatant from the first stage. The absorbance at 436 nm was measured in a multiwell plate reader (model Infinite M200PRO, brand TECAN). The amount of ammonia was determined from the standard curve prepared with different concentrations of ammonium sulfate. The L-ASNase activity was expressed in U ml⁻¹. One unit (U) of L-ASNase was defined as the amount of enzyme that liberates 1 µmol of ammonia per min.

Quantitative assay of proteolytic activity

The method described by Coêlho et al. (2016) using azocasein as substrate (Sigma-Aldrich, MO, USA) was employed with the following modifications. The crude enzyme extract was obtained from the bacterial isolates grown in the medium proposed by Qeshmi et al. (2015). The enzyme extract (500 µl) was mixed with 500 µl 0.6% w/v azocasein (dissolved in 50 mM Tris-HCl, pH 8.5) and incubated for 30 min at 37 °C. Then, 500 µl of 10% TCA was added and centrifuged at 2000 g for 5 min. Finally, 100 µl of the supernatant was mixed with 100 µl of 0.5 N NaOH and the absorbance was read at 430 nm in the plate reader.

RESULTS AND DISCUSSION

Preselection of bacteria producing L-ASNase

Preselected bacteria with L-asparaginase activity were 24, of which 12, 11 and 1 came from Maras, Pilluana and Chilca salterns, respectively. These bacteria were named M54, M55, M57, M62, M64, M67, M68, M72, M74, M75, M76, M84, P2, P4, P6, P7, P13, P16, P17, P19, P20, P23, P31, and CH11; where the first letter represents the name of the saline, followed by the strain number.

Saline environments are variable among themselves in parameters such as salinity, ionic composition, temperature, pH, and nutrients. Which mainly depend on the geographical location, climate and season (Bell, 2012).

They harbour a great diversity of microorganisms that, unlike their counterparts that live in non-extreme conditions, possess enzymes capable of being active under adverse conditions. Likewise, the enzymes of these organisms generally possess

particular structures as an adaptation strategy to extreme conditions (Ventosa et al., 1998; Gomes & Steiner, 2004; Enache & Kamekura, 2010). Consequently, these proteins could present new immunological or catalytic properties important for the treatment of acute lymphoblastic leukemia (Ebrahiminezhad et al., 2011), or the thermo-processing of foods (Batoool et al., 2016).

The present study describes bacteria with L-ASNase activity isolated from saline environments located in different geographical, climatic and altitude regions. Thus, Maras salterns generally support low temperatures, high radiation; and they present saline concentrations influenced by underground spring waters in the Andes (Maturrano et al., 2006). Pilluana salterns, located in a tropical zone, contain sodium and chloride as the majority ions (Valencia, 2000). And like Maras salterns, they are used as sources of salt for food consumption. On the contrary, Chilca salterns, located on the coast, contain a high concentration of sodium chloride and sulfate (Chacón, 1980) and are traditionally used for medicinal baths. This diversity of environments harbours bacteria that, due to their adaptation mechanisms, could show enzymes with novel characteristics for biocatalysis at an industrial level.

Although these three salterns have been reported as sources of halophilic microorganisms with biotechnological potential (Chávez-Hidalgo, 2010), the L-ASNase activity in bacteria from these environments has not been explored. In this regard, for example, previous studies in Iran described saline environments in which a varied number of bacteria with L-ASNase activity were isolated. Thus, Barati et al. (2016) reported 38 from diverse saline environments. Ebrahiminezhad et al. (2011) described 11 from Maharloo salt lake; and Zolfaghar et al. (2019) reported 29 bacteria between halophilic and halotolerant from different saline areas of Iran.

Phenotypic and molecular characterization of bacteria producing L-ASNase

Table 1 shows that 92% of the bacteria were identified as bacilli, 38% Gram-negative, 54% Gram-positive; and 8% Gram-positive cocci. Also, 88% were catalase-positive, 75% and 38% produced H₂S and gas, respectively. Besides, 54% grew in a temperature range between 4 and 37 °C; and 21%, in the range of 4 to 42 °C. Regarding saline tolerance, 29% showed the ability to grow from 0.9 to 20% of salts, while 50% grew between 0.9 and 15%. Regarding the pH, 96% grew in the range of 5 to 9. In the assimilation of sugars, all the strains were able to use glucose and 21% of the isolates used glucose, lactose, maltose, and fructose. Likewise, the analysis of the partial nucleotide sequences of the 16S ribosomal genes showed similarity with members of the genera *Bacillus* (13), *Enterobacter* (5), *Halomonas* (2), *Marinobacter* (1), and *Staphylococcus* (2) (Table 1). Of the studied bacteria, 54% belongs to the genus *Bacillus*, which groups together a wide collection of bacilli ubiquitous in nature that use various carbon sources, with predominance in saline

environments, and industrially relevant (Canales et al., 2014; Harwood et al., 2018; Ortiz & Sansinenea, 2019). It is evident in the characteristics of the strains (Table 1) that they phenotypically and metabolically correspond to a heterogeneous group, as has already been described for this genus (Slepecky & Hemphill, 2006).

As observed in this work, the production of L-ASNase is not exclusive to the *Bacillus* genus, for example Barati et al. (2016) found *Halomonas* and *Aidingimonas* as the largest producers of L-ASNase in their study of hypersaline environments in Iran. Besides, Sudha et al. (2017) and Han et al. (2014) describe the production of L-ASNase by *Enterobacter* and *Staphylococcus* strains isolated from salty seafood. Likewise, Zolfaghar et al. (2019) mention the genera *Bacillus*, *Halomonas*, *Marinobacter*, among others as producers of L-ASNase. Microorganisms can inhabit environments with different characteristics if they manage to adapt to their conditions (De Wit & Bouvier, 2006). Therefore, it is not surprising that as studies elucidating the microbial diversity of different geographic areas and samples increase (Muneer et al., 2020), new L-ASNase-producing species will be described.

Selection of bacteria producing L-ASNase by submerged culture

The methods for the selection of microorganisms producing L-ASNase are based on the detection of ammonia after the hydrolysis of L-asparagine by L-ASNase, which increases the pH in the culture medium.

In this regard, in the preselection of bacteria with L-ASNase activity, two types of semi-quantitative tests were carried out using the indicator dyes phenol red or bromothymol blue to better discriminate colour change and intensity (Gulati et al., 1997; Mahajan et al., 2013). The L-ASNase production of Maras, Pilluana and Chilca salterns bacteria in submerged culture was evidenced by a colour change with two pH indicators (Table 2). The cultures that presented the highest absorbance at 560 nm and 620 nm were selected, when phenol red and bromothymol blue were used, respectively (Truppo & Turner, 2010). Thus, of the 24 strains evaluated, 14 were considered the best producers of L-ASNase (Table 2).

L-ASNase and proteolytic activities of bacterial strains

The quantification of the L-ASNase and proteolytic activities of the bacteria selected from the submerged cultures is presented in Figure 1, where the *Bacillus* sp. M68, M64, M62, P19 and CH11 produced higher activity of both L-ASNase and proteases. However, of these five, the P19 strain showed the lowest proteolytic activity (Figure 1b). It should be noted, in the search for bacteria that produce L-ASNase, an important factor to consider is the presence of extracellular proteases, which influences the decrease in activity and stability *per se* of the L-ASNase secreted into the culture medium.

Table 1
Biochemical and microbiological tests of bacteria with L-asparaginase activity

| Test | Isolate | | | | | | | | | | | | | | | | | | | | | | | | |
|--------------------------|---------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---|
| | M84 | M76 | M75 | M74 | M72 | M68 | M67 | M64 | M62 | M57 | M55 | M54 | CH11 | P31 | P23 | P20 | P19 | P17 | P16 | P13 | P7 | P6 | P4 | P2 | |
| Cellular morphology | ba | ba | ba | m | h | ba | nd | ba | ba | h | e | ba | ba | ba | e | b | ba | e | e | s | ba | ba | ba | s | |
| Gram staining | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | b | c | b | b | b | c |
| Catalase | + | + | + | - | - | + | - | + | + | - | - | + | + | + | - | - | + | - | - | + | + | + | + | + | |
| H ₂ S | - | + | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | - | - | + | |
| Gas | - | + | - | - | + | - | - | - | + | - | - | + | - | - | + | + | + | - | - | - | - | + | - | - | |
| Optimum range of growth: | | | | | | | | | | | | | | | | | | | | | | | | | |
| Temperature (°C) | 4-37 | 4-42 | 4-42 | 4-42 | 4-37 | 4-37 | 4-37 | 4-37 | 4-37 | 4-37 | 4-42 | 4-42 | 30-42 | 20-37 | 4-37 | 4-37 | 20-42 | 20-37 | 4-37 | 4-37 | 4-37 | 30-37 | 4-37 | 30-37 | |
| pH | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-8 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | 5-9 | |
| Salts (%) | 0.9-15 | 0.9-20 | 0.9-15 | 0.9-20 | 0.9-15 | 0.9-15 | 0.9-15 | 0.9-20 | 0.9-15 | 0.9-20 | 0.9-15 | 5-20 | 0.9-20 | 0.9-15 | 0.9-10 | 0.9-10 | 0.9-15 | 0.9-10 | 0.9-15 | 0.9-20 | 0.9-15 | 0.9-15 | 0.9-20 | 0.9-10 | |
| Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| Lactose | - | - | + | - | + | - | - | - | - | - | - | + | - | - | + | - | - | - | + | + | - | - | - | + | |
| Maltose | - | - | + | + | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + | - | - | - | + | |
| Fructose | - | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | |

ba: *Bacillus* sp., e: *Enterobacter* sp., h: *Halomonas* sp., m: *Marinobacter* sp., s: *Staphylococcus* sp., b: bacillus, c: coccus, nd: not determined.

Table 2
Production of L-asparaginase in submerged culture according to pH indicator

| Strain code | bromothymol blue | | | phenol red | | |
|-------------|---------------------|---------------|-------------------|---------------------|---------------|-------------------|
| | pH _{final} | colour change | OD ₆₂₀ | pH _{final} | colour change | OD ₅₆₀ |
| M84 | 7.9 | + | 0.23 | 7.3 | + | >3.00 |
| M76 | 8.2 | + | 0.22 | 8.4 | + | >3.00 |
| M75 | 6.5 | - | 0.04 | 6.6 | + | 0.92 |
| M74 | 8.1 | + | 0.16 | 8.0 | + | >3.00 |
| M72 | 8.5 | + | 0.31 | 8.0 | + | >3.00 |
| M68 | 7.6 | + | 0.14 | 7.9 | + | >3.00 |
| M67 | 7.9 | + | 0.13 | 8.1 | + | >3.00 |
| M64 | 8.1 | + | 0.22 | 8.1 | + | 2.74 |
| M62 | 8.3 | + | 0.46 | 8.2 | + | >3.00 |
| M57 | 8.1 | + | 0.30 | 7.0 | + | 2.08 |
| M55 | 7.0 | - | 0.07 | 6.5 | + | 0.92 |
| M54 | 7.7 | + | 0.17 | 7.3 | + | >3.00 |
| P31 | 8.3 | + | 0.25 | 8.1 | + | >3.00 |
| P23 | 6.7 | - | 0.06 | 6.4 | + | 0.74 |
| P20 | 6.7 | - | 0.05 | 6.5 | + | 0.62 |
| P19 | 8.2 | + | 0.21 | 7.7 | + | >3.00 |
| P17 | 6.6 | - | 0.00 | 6.4 | + | 0.84 |
| P16 | 6.7 | - | 0.00 | 6.3 | + | 0.52 |
| P13 | 6.4 | - | 0.00 | 7.4 | + | 0.47 |
| P7 | 6.8 | - | 0.06 | 7.5 | + | 0.56 |
| P6 | 6.8 | - | 0.00 | 6.5 | + | 0.42 |
| P4 | 7.9 | + | 0.18 | 7.3 | + | >3.00 |
| P2 | 6.5 | - | 0.00 | 7.3 | + | 0.37 |
| CH11 | 8.2 | + | 0.19 | 8.1 | + | >3.00 |

Initial pH of cultures: 5.6.

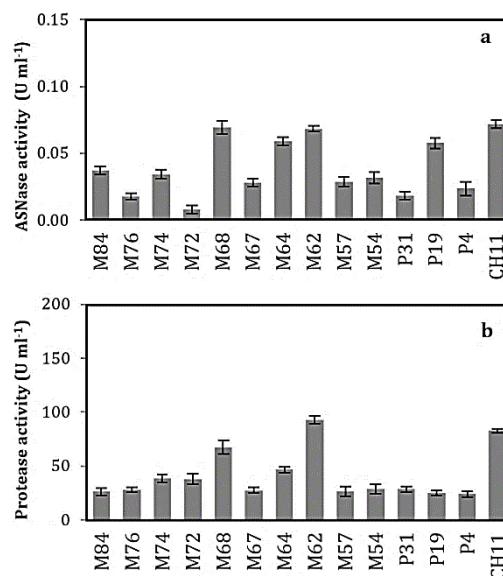


Figure 1. L-asparaginase (a) and protease (b) activities of bacterial strains.

In this regard, it has been described that species of the genus *Bacillus* are protease producers (Bhunia et al., 2012), as was found in the members of this genus M68, M64, M62, P19 and CH11. These data are important to design cultivation strategies that reduce or avoid the production of proteases. We suggest cultivation strategies that include changing or adjusting the concentrations and the relationship of the C/N sources. Additionally, include mixtures of protease inhibitors in the purification processes. Likewise, molecular biology and genetic engineering techniques may be used for gene cloning.

CONCLUSIONS

Microorganisms adapted to saline environments are capable of resisting extreme conditions in more than one environmental parameter. Thus, the bacteria in the present study resist a wide range of pH, temperature and salt concentration. In addition, they all assimilated glucose which would make them adaptable to various sources that contain this monosaccharide. Likewise, since they come from saline environments, their nutritional

requirements are not demanding, which is convenient in large-scale bioprocesses, and there is the possibility that they have new immunological properties. It should be noted, of the 14 strains that produce L-asparaginase in liquid medium, five are potential sources for the prospective production of L-asparaginase, and it is recommended to continue with their study and optimization of production.

ACKNOWLEDGMENT

This work has been financed with the agreement N° 169-FONDECYT-2017; and project VRIP-

A17040034a. We thank Nataly Allasi Canales for the English language review.

REFERENCES

- Barati, M., Faramarzi, M. A., Nafissi-Varcheh, N., Khoshayand, M. R., Tehrani, M. H. H., Vahidi, H., & Adrang, S. 2016. L-asparaginase activity in cell lysates and culture media of halophilic bacterial isolates. *Iranian Journal of Pharmaceutical Research*, 15(3), 435-440.
- Batool, T., Makky, E. A., Jalal, M., & Yusoff, M. M. 2016. A comprehensive review on L-asparaginase and its applications. *Applied Biochemistry and Biotechnology*, 178(5), 900-923.
- Bell, E. (Ed). 2012. *Life at Extremes: Environments, Organisms and Strategies for Survival* (Vol. 1). Wallingford: CABI.
- Bhunia, B., Basak, B., & Dey, A. 2012. A review on production of serine alkaline protease by *Bacillus* spp. *Journal of Biochemical Technology*, 3(4), 448-457.

- Canales, P. E., Chávez-Hidalgo, E. L., & Zavaleta, A. I. 2014. Caracterización de bacterias halófilas productoras de amilasas aisladas de las Salinas de San Blas en Junín. *Revista Colombiana de Biotecnología*, 16(2), 150-157.
- Coêlho, D. F., Saturnino, T. P., Fernandes, F. F., Mazzola, P. G., Silveira, E., & Tambourgi, E. B. 2016. Azocasein substrate for determination of proteolytic activity: reexamining a traditional method using bromelain samples. *BioMed Research International*, 2016, 1-6.
- Corral, P., Amoozegar, M. A., & Ventosa, A. 2019. Halophiles and their biomolecules: recent advances and future applications in biomedicine. *Mar Drugs*, 18(1), 1-33.
- Chacón, G. 1980. Acción bactericida de la laguna minero-medicinal "Santa Cruz de las Salinas" Chilca, Lima-Perú. *Revista Peruana de Biología*, 2(1), 8-51.
- Chávez-Hidalgo, E. L. 2010. Bacterias halófilas moderadas con actividad lipolítica aisladas de las Salinas de Pilluana- San Martín. Thesis of Master. Universidad Nacional Mayor de San Marcos, Lima, Peru.
- De Wit, R., & Bouvier, T. 2006. 'Everything is everywhere, but, the environment selects', what did Baas Becking and Beijerinck really say? *Environmental Microbiology*, 8(4), 755-758.
- Delgado-García, M., Valdivia-Urdiales, B., Aguilar-González, C. N., Contreras-Esquivel, J. C., & Rodríguez-Herrera, R. 2012. Halophilic hydrolases as a new tool for the biotechnological industries. *Journal of the Science of Food and Agriculture*, 92(13), 2575-2580.
- Dyall-Smith, M. 2006. The halohandbook. Melbourne: University of Melbourne.
- Ebrahiminezhad, A., Rasouli-Amini, S., & Ghasemi, Y. 2011. L-asparaginase production by moderate halophilic bacteria isolated from Maharloo Salt Lake. *Indian Journal of Microbiology*, 51(3), 307-311.
- Enache, M., & Kamekura, M. 2010. Hydrolytic enzymes of halophilic microorganisms and their economic values. *Romanian Journal of Biochemistry*, 47(1): 47-59.
- Flores-Fernandez, C. N., Chávez-Hidalgo, E., Santos, M., Zavaleta, A.L., & Arahall, D.R. 2019. Molecular characterization of protease producing *Idiomarina* species isolated from Peruvian saline environments. *Microbiology and Biotechnology Letters*, 47(3), 401-411.
- Ghasemi, A., Asad, S., Kabiri, M., & Dabirmanesh, B. 2017. Cloning and characterization of *Halomonas elongata* L-asparaginase, a promising chemotherapeutic agent. *Applied Microbiology and Biotechnology*, 101(19), 7227-7238.
- Gomes, J., & Steiner, W. 2004. The biocatalytic potential of extremophiles and extremozymes. *Food Technology and Biotechnology*, 42(4), 223-235.
- Gulati, R., Saxena, R., & Gupta, R. 1997. A rapid plate assay for screening L-asparaginase producing microorganisms. *Letters in Applied Microbiology*, 24(1), 23-26.
- Han, S., Jung, J., & Park, W. 2014. Biochemical characterization of L-asparaginase in NaCl-tolerant *Staphylococcus* sp. OJ82 isolated from fermented seafood. *Journal of Microbiology and Biotechnology*, 24(8), 1096-1104.
- Harwood, C. R., Mouillon, J. M., Pohl, S., & Arnau, J. 2018. Secondary metabolite production and the safety of industrially important members of the *Bacillus subtilis* group. *FEMS Microbiology Reviews*, 42(6), 721-738.
- Imada, A., Igarasi, S., Nakahama, K., & Isono, M. 1973. Asparaginase and glutaminase activities of microorganisms. *Journal of General Microbiology*, 76(1), 85-99.
- Mahajan, R. V., Saran, S., Saxena, R. K., & Srivastava, A. K. 2013. A rapid, efficient and sensitive plate assay for detection and screening of L-asparaginase-producing microorganisms. *FEMS Microbiology Letters*, 341(2), 122-126.
- Mata, J. A., Martínez-Cánovas, J., Quesada, E., & Béjar, V. 2002. A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Systematic and Applied Microbiology*, 25(3), 360-375.
- Maturrano, L., Santos, F., Rossello-Mora, R., & Anton, J. 2006. Microbial diversity in Maras salterns, a hypersaline environment in the Peruvian Andes. *Applied and Environmental Microbiology*, 72(6), 3887-3895.
- Miao, C., Jia, F., Wan, Y., Zhang, W., Lin, M., & Jin, W. 2014. *Halomonas huangheensis* sp. nov., a moderately halophilic bacterium isolated from a saline-alkali soil. *International Journal of Systematic and Evolutionary Microbiology*, 64(3), 915-920.
- Muneer, F., Siddique, M. H., Azeem, F., Rasul, I., Muzammil, S., Zubair, M., Afzal, M., & Nadeem, H. 2020. Microbial L-asparaginase: purification, characterization and applications. *Archives of Microbiology*, 202(5), 967-981.
- Oren, A. 2010. Industrial and environmental applications of halophilic microorganisms. *Environmental Technology*, 31(8-9), 825-834.
- Ortiz, A., & Sansinenea, E. 2019. Chemical compounds produced by *Bacillus* sp. factories and their role in nature. *Mini Reviews in Medicinal Chemistry*, 19(5), 373-380.
- Qeshmi, F.I., Rahimzadeh, M., Javadpour, S., & Poodat, M. 2015. Intracellular L-Asparaginase from *Bacillus* sp. PG02: purification, biochemical characterization and evaluation of optimum pH and temperature. *American Journal of Biochemistry and Biotechnology*, 12(1), 12-19.
- Ruginescu, R., Purcarea, C., Dorador, C., Lavin, P., Cojoc, R., Neagu, S., Lucaci, I., & Enache, M. 2019. Exploring the hydrolytic potential of cultured halophilic bacteria isolated from the Atacama Desert. *FEMS Microbiology Letters*, 366(17), fnz224.
- Sánchez-Porro, C., Martín, S., Mellado, E., & Ventosa, A. 2003. Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *Journal of Applied Microbiology*, 94(2), 295-300.
- Shirazian, P., Asad, S., & Amoozegar, M. A. 2016. The potential of halophilic and halotolerant bacteria for the production of antineoplastic enzymes: L-asparaginase and L-glutaminase. *EXCLI Journal*, 15, 268-279.