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MICROBIOLOGY

Biotechnological potential of microorganisms isolated from the salar del hombre muerto, Argentina

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Abstract: Bacterial strains were isolated from soil and aqueous solution samples from the Salar del Hombre Muerto, Argentina. A total of 141 strains were characterized and the tolerance to sodium chloride was evaluated. We performed a screening to search for molecules of biotechnological interest: carotenoids (11%), emulsifiers (95%), and exopolysaccharides (6%), and to assess the production of enzymes, including proteolytic (39%), lipolytic (26%), hemolytic (50%), and catalase activities (99%); 25 bacterial strains were selected for further studies. Some of them produced biofilms, but only *Bacillus* sp. HA120b showed that ability in all the conditions assayed. Although 21 strains were able to form emulsions, the emulsifying index *Kocuria* sp. M9 and *Bacillus* sp. V3a cultures were greater than 50% and, emulsions were more stable when the bacteria grew in higher salt concentrations. Only pigmented *Kocuria* sp. M9 showed lipolytic activity on olive oil medium and was able to produce biofilms when cultured without and with 4 M of NaCl. Yellow pigments, lipase activity, and biosurfactant production were observed for *Micrococcus* sp. SX120. Summarizing, we found that the selected bacteria produced highly interesting molecules with diverse industrial applications and, many of them are functional in the presence of high salt concentrations.

Key words: Halotolerant, enzymes, carotenoids, bioemulsifiers, exopolysaccharides.

INTRODUCTION

The physicochemical conditions of an extreme environment are severe and unusual, lethal for some living beings (Antranikian et al. 2005). Among extreme environments, those characterized by high amounts of salt are known as hypersaline and located in different geographic areas around the world. In the Puna ecoregion, there is an area known as the Lithium Triangle which includes all the salt flats (*salar* in Spanish) within the triangle defined Salar de Uyuni (Bolivia), Salar de Atacama (Chile), and Salar del Hombre Muerto (Argentina), which contains between 50-85% of the soluble lithium chloride reserves in continental brines (Flexer et al. 2018). Although lithium is exploited there, sodium chloride (NaCl) is the predominant salt of the brine (Flexer et al. 2018, Martínez et al. 20219a). These environments exhibit multiple extreme conditions, such as salt concentrations several times higher than seawater, high altitude and UV radiation, wide temperature amplitudes between day and night, extreme pH values, low nutrient content, and oxygen availability (DasSarma & Arora 2002, Ventosa et al. 2015). Despite the inhospitable conditions, many microorganisms, called extremophiles, develop there (Macelroy 1974, Elleuche et al. 2015). Those who thrive in saline environments are called halophiles (they require salts to grow) and

halotolerant (they do not require salt but can grow under saline conditions) (Setati 2010).

Halophiles and halotolerant microorganisms have strategies that allow them to cope with osmotic stress by maintaining high intracellular salt concentrations and/or by synthesizing compatible solutes that allow them to balance their osmotic pressure (Yin et al. 2015). These microorganisms are of interest due to their potential industrial applications (Oren 2002, Wang et al. 2014, Nercessian et al. 2015). It is expected that the proteins they produce have different and extraordinary characteristics compared to those produced by non-halophilic microorganisms since halophiles and halotolerant organisms must maintain their catalytic properties at high salt concentrations (Ortega et al. 2015).

As a general adaptation mechanism, cells that are subjected to environmental stresses counteract them by stimulating different pathways and synthesizing specific molecules, such as antioxidant enzymes, carotenoids, exopolymeric substances (EPS), or by producing biofilms and biosurfactants (Irazusta et al. 2013, Fu et al. 2014, Moraga et al. 2017, Moshabaki et al. 2018, Martínez et al. 2019a). Many of the molecules produced by halophilic microorganisms are currently applied in biotechnological processes (Kumar et al. 2012). Recently, the use of microorganisms as a source of biotechnological products was presented as an interesting and feasible alternative (Akinsemolu 2018). Halophilic microorganisms can also be used for bioremediation of environments contaminated with hydrocarbons and heavy metals, and in agriculture to reduce salt concentrations of hypersaline soils (Sei & Fathepure 2009, Al-Mailem et al. 2010, Najera-Fernández et al. 2012, Erdogmus et al. 2013, Al-Mailem et al. 2013). Other applications of these microorganisms or their molecules are in the food industry (Amoozegar et al. 2003, Chakraborty et al. 2009), in dye production (Kolekar et al. 2008), in the pharmaceutical industry (Shivanand & Jayaraman 2009) and in the manufacture of antibiotics (Shinde & Thombre 2016), to name a few.

The search for bacteria with new or improved capabilities in their essential functions is crucial for the development of cleaner technologies, which have several advantages and can be used in the industrial production of various molecules of biotechnological interest. The aims of this work were: i) to isolate microorganisms from the Salar del Hombre Muerto, ii) to characterize them and to evaluate their tolerance to salt, and iii) to search for molecules with biotechnological interest.

MATERIALS AND METHODS

Isolation of microorganisms

Aqueous solutions collected from a salt pond (brine) (AS: 25° 28´ 17.2" S, 67° 5´ 45.3" W), and soil samples from two different sites (Site 1-S1: 25 ° 28' 49.4" S, 67° 0.6´ 21.7" W and Site 2-S2: 25° 27' 32.9" S, 67° 0.6' 12.7" W) from the Salar del Hombre Muerto were used for the isolation of microorganisms (Martínez et al. 2019a, Yañez-Yazlle et al. 2021) (Figure 1).

Briefly, liquid samples were inoculated in Yeast Peptone Dextrose (YPD) and YPDsalt (YPD with 12.5% of salt brine) media at 30 °C and 150 rpm until growth was observed. For soil samples, a microbial suspension was prepared by adding the soil to either sterile 1% hexametaphosphate (XE) or distilled water (WE), and the suspension was shaken at 200 rpm for microbial detachment. Aliquots of these suspensions (100 µL) were inoculated on Petri dishes containing Agar Plate Count media (APC, Britania), prepared with 12.5% of brine from the salt flat (APCsalt) or soil extract media. This was prepared by mixing 500 g of soil

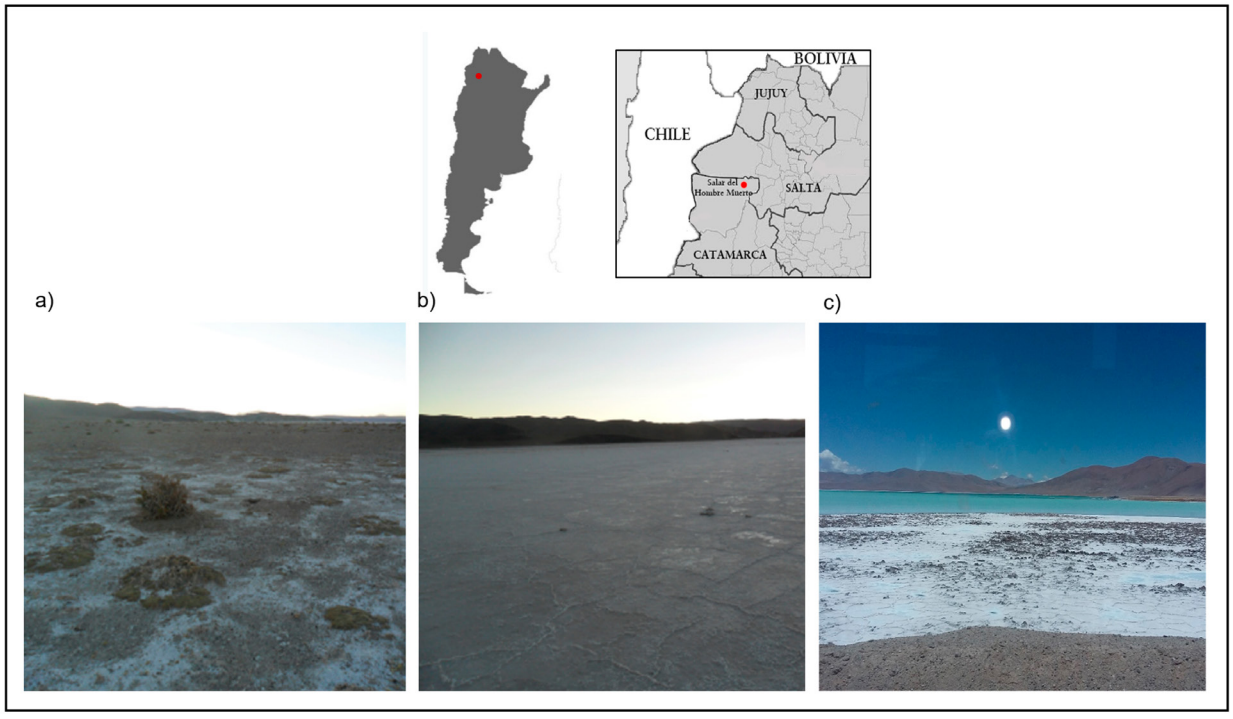

Figure 1. Sampling collation in the salt flat "Salar del Hombre Muerto", Catamarca Province, Argentina. Sampling points for soil (S1: a; S2: b) and salt pond (brine) for aqueous solutions samples (c).

with 1 L of distiller water in a 2 L Erlenmeyer flask; the suspension was sterilized in an autoclave for 15 min, decanted for 1 h, filtered (Hamaki et al. 2005, Lutton et al. 2013), and 15 g/L agar (Britania) was added. The inoculated plates were incubated at 23 °C for 72 h.

Pure cultures of each isolate were stored in glycerol at -20 and -80 °C in triplicates. Some bacteria evaluated in this study were previously identified by 16S rDNA sequencing and sequences were uploaded in the GenBank database (Martínez et al. 2019a).

Tolerance to NaCl and bacteria characterization

Bacteria able to grow in presence of salts (Martínez et al. 2019a) and isolated using soil extract media were evaluated according to their tolerance against NaCl. One colony of each strain grown in APCsalt media was added to tubes containing 5 mL of defined medium (g/L: glucose 10.0; L-asparagine 0.5; K_2HPO_4 0.5, $FeSO_4.7H_2O$ 0.001; $MgSO_4$ 0.02) supplemented with 1 M (58.5 g/L), 2 M (116.9 g/L) (also found as DM2M), 3 M (175.4 g/L) or 4 M (233.8 g/L) (also found as DM4M) of NaCl and without salt addition (DM). Tubes were incubated at 30 °C and 200 rpm for 24 h. Growth was evaluated through optical density at 600 nm (OD_{600}) in a spectrophotometer (Biotraza model 752); pellet formation was registered when observed. All experiments were performed in duplicate.

Strains were characterized macroscopically according to colony color, shape, border, and texture and microscopically by Gram staining.

Extracellular enzymatic activities

The development of hydrolysis halos was indicative of extracellular enzymatic activities. The halo diameters were measured from the edge of the colony until the halo´s further limit. The assay was performed in duplicates and the reported diameter is the average of the measurements.

Active protease production

Proteolytic activity was assayed on Nutrient Agar (NAM; Britania) plates supplemented with 65 g/L of NaCl, containing 10% v/v skimmed milk. Plates were incubated at 30 °C for 48 h. Clear halos around the colonies were taken as evidence of proteolytic activity in the evaluated conditions, as they are indicative of casein lysis (Paterson & Bridge 1994).

Lipolytic activity

Lipolytic activity was analyzed on NAM plates supplemented with three different compounds in separate experiments: 1% v/v Tween 80, 1% v/v olive oil, and 5% v/v egg yolk (Hasan et al. 2009). The plates were incubated at 30 °C for 48 h. Precipitation or opalescence around the colony indicated lipolytic activity.

Catalase activity

This activity was evaluated through the addition of a drop of hydrogen peroxide (H_2O_2 10 vol.) to one bacterial colony. The immediate formation of bubbles indicated positive catalase activity (Shangari & O'Brien 2006).

Extracellular polymeric substances production

Biosurfactants production

Blood Agar media (Britania) supplemented with 5% v/v of human blood was used for the detection of hemolytic activity. Plates were incubated at 30 °C for 48 h (Hasan et al. 2009). Considering halo and bacterial growth in this culture media, hemolysis was classified as alpha, beta, and gamma. Alpha hemolysis corresponded to the development of dark and greenish color under and around the colonies, indicating partial lysis of the red blood cells. The formation of transparent halos was classified as beta hemolysis, and indicated total lysis of red blood cells. Finally, the lack of halos was considered as gamma hemolysis, since bacterial growth was observed even though there was no lysis of red blood cells.

Bioemulsifiers production

The ability to produce emulsifiers was evaluated in the strains grown in DM, DM2M, and DM4M at 30 °C and 200 rpm for 48 h. The assay consisted of mixing equal volumes of kerosene and liquid culture of each strain in the vortex (Labnet S0200) for 2 min. The emulsion was stabilized for 1 h before measurement. Tween-80 and water were used as positive and negative controls, respectively. The emulsion index (EI) was calculated as follows:

$$EI(\%) = \frac{he}{ht} x\,100$$

being *he* the height of the formed emulsion and *ht* the total liquid height. The emulsion stability and the index were determined after 24 h (Sarafin et al. 2014, Nercessian et al. 2015, Gomes et al. 2018).

Biofilm formation

The ability of the isolates to form biofilms in 96-well sterile plates when grown in DM, DM2M, and DM4M at 30 °C for 48 h, were evaluated. For that, the colorimetric assay using crystal violet was developed as described by Merritt et al. 2011.

Pigment production

Pigment production was evaluated by colonies color observation on DM plates. Pink, orange, and yellow colonies were considered as probable carotenoid-producing bacteria.

RESULTS

Tolerance to NaCl and morphological characterization

The Salar del Hombre Muerto represents a natural source of extraordinary microorganisms able to produce biomolecules with great potential. A total of 141 strains were isolated from soil and aqueous solutions samples and characterized through macroscopic and microscopic observation (Table I). Most bacteria presented colonies with whole borders, creamy texture and were rod-shaped, Gram-positive cells.

Table I. Macroscopic and microscopic characterization by observation of colony´s shape, border, and texture and by Gram staining using pure cultures grown on APCsalt.

Characteristics			N
Shape	Irregular		18
	Circular		34
	Concentric		2
	Fusiform		33
	Puntiform		54
Border	Whole		137
	Irregular		4
Texture	Creamy		67
	Hard		10
	Elastic		26
	Thick		38
Gram stain	Bacilli	Pos	45
		Neg	36
	Coccus	Pos	24
		Neg	8
	Filaments	Pos	5
		Neg	1
	Not classified		22

Sixteen (out of 141) of the isolated bacteria produced pigments between orange and yellow, compatible with the production of carotenoids. The rest (89%) formed white or beige colonies (Figure 2a). Most of the strains were obtained from soil samples (103 strains vs 38 from aqueous solutions) and hexametaphosphate was more effective than water for bacteria isolation (Figure 2a).

For bacterial selection and as an initial screening to determine salt tolerance, growth was evaluated in a medium without salt (DM), and adding of 2M (DM2M) and 4M (DM4M) of NaCl (Figure 2b). Forty-three strains were unable to grow in the tested media under these conditions. Of the remaining 98 strains, 53 grew in all the evaluated media and 32 of them in both DM2M and DM4M. It is important to note that more than 90% of the strains grew in the presence of salt and 40 strains (from 98) did it only in presence of high salt concentration (2 and 4 M of NaCl).

Enzymes, molecules, and metabolites produced by halotolerant bacteria

Production of different molecules, including enzymes, was evaluated after 48 h of incubation. From 141 strains, 81% grew on skim milk and 48% of them (39% of the total) presented extracellular proteolytic activity on skimmed milk (Figure 3a). Regarding lipolysis, the strains showed different behavior in the evaluated media, being 30 strains (21% of the total) able to produce a precipitation halo on media supplemented with Tween 80; six were positive on egg yolk, and only one strain showed lipolytic activity on olive oil (Figure 3b). It must be considered, however, that most of the strains were able to grow on the used media but did not produce a precipitation halo around colonies.

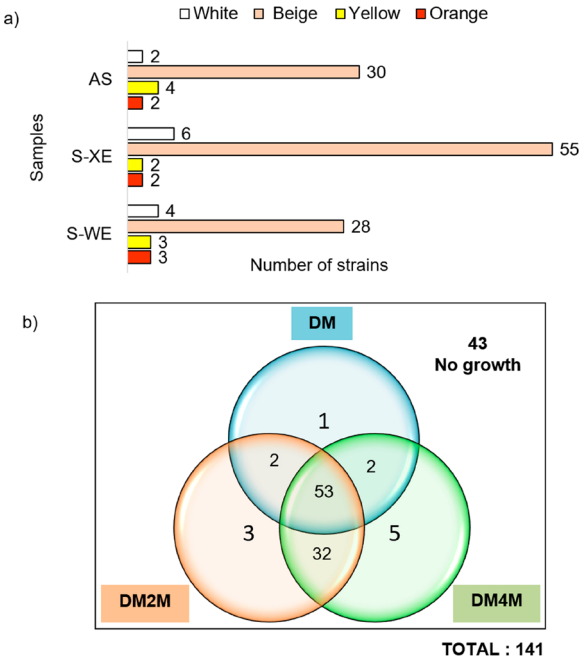

Figure 2. Bacteria isolated from the Salar del Hombre Muerto (141 in total): a) Number of isolates obtained from aqueous solution (AS) and soil (S) samples using water (S-WE) or hexametaphosphate (S-XE) as extractor agent, according to the colony´s color; b) Evaluation of NaCl tolerance; strains were cultured in defined medium (DM) and defined medium supplemented with 2 M (DM2M) and 4 M (DM4M) of NaCl at 30 °C and 200 rpm for 24 h.

Most of the bacteria under study were able to grow in Blood Agar (93%). An important fraction of them (40%) did not show hydrolysis halo (gamma hemolysis), while the 37% totally lysed red blood cell forming a clear halo around the colonies and the 23% did it partially, being classified as beta and alpha-hemolytic, respectively (Figure 4a).

Almost all supernatants from culture media of the strains evaluated were able to produce emulsions with kerosene (134 out 141; 95%, Figure 4b). The production was enhanced when there was NaCl in the media and it was especially stable at the maximum salt concentration (4 M NaCl). In this way, from the 31 bacteria grown in DM with the ability to produce stable emulsion after the first hour, 55% of them were stable after 24 h. Similar results were observed for strains grown in DM2M, where from 42 positive emulsifier strains, 23 shown a stable emulsion after one day at room temperature. The maximum number of strains with the ability to produce emulsifiers was obtained when cultured with 4 M of NaCl; furthermore, 64% of the emulsions formed (assessed after one hour) were still stable after 24 h.

Selection of bacterial strains with biotechnological potential

Considering the growth in different media, particularly in those with high concentrations of NaCl, and also the ability to produce molecules with biotechnological potential, 25 strains out of the initial 141 were selected.

Growth of the selected bacteria was further evaluated using growth media prepared with 1, 2, 3, and 4 M of NaCl (DM1M, DM2M, DM3M, and DM4M, respectively) and without salt (DM) (Figure 5). Growth was evaluated, and some particularities were observed, for example, strains HA16, HA120b, HA120c, HX131, HX135, SA35, SA312, SA11, and SX132 grew forming pellets when salt concentration increased. Instead, for other strains like HA116, SA129b, SX120, SX139, M12, and V3a, growth could be measured by turbidity in all the evaluated media, and from these, only SX120 showed higher OD in higher NaCl concentrations. Strains SX120, M12, SA211 developed a yellow phenotype in the evaluated conditions, whereas SA120b was orange. Interestingly, SA211, SX120, and M12 were the only pigmented strains that lysed Tween-80. Out of the yellow isolates, only M9 tested positive for lipase activity in Olive oil agar, in addition to emulsion formation with kerosene, and this strain also formed biofilms in DM and DM4M (Figure 5).

Biofilm production was assessed in DM, DM1M, DM2M, DM3M, and DM4M. Although most strains did not produce biofilms, the salt-free

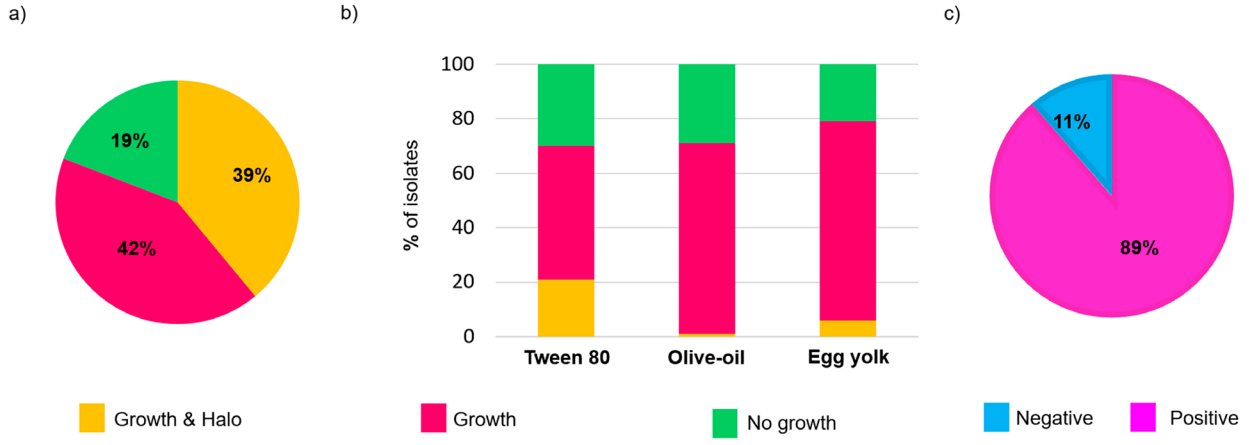

Figure 3. Growth and production (halo) of (a) protease on skim milk, (b) lipase on three different substrates (Tween 80, olive oil, and egg yolk) and (c) catalase activity by the 141 strains isolated. Assays were performed in Petri dishes with Nutrient Agar, supplemented with the corresponding substrate, incubated at 30 °C for 48 h .

condition was the best for it (24% of the strains) and only *Bacillus* sp. HA120b showed that ability in all the evaluated conditions (Figure 5).

Also, 84% (21 out of 25) of the selected strains were able to form emulsions in presence of kerosene when grown in DM. However, only five strains showed the maximum emulsion index (EI: 30-60%). Furthermore, all of the evaluated strains, except for HA116, tested positive for catalase activity.

DISCUSSION

In recent years, alternatives for the production of different molecules generated by extreme microorganisms are being investigated. Among them, halophiles are a group of great interest due to their high potential in enzymes and molecules synthesis that can be used in different industries under conditions that would inhibit their activities if they were produced by non-halophilic organisms (Margesin & Shinner 2001, Menasria et al. 2019, Ruginescu et al. 2020). Saline environments harbor a great variety of halophiles, with peculiarities that make them extremely interesting in diverse biotechnological applications, such as the production of thermostable and hydrolytic enzymes, EPS, biosurfactants, antibiotics, and biopesticides, among other molecules (Margesin & Shinner 2001, Amoozegar et al. 2007, Moshabaki et al. 2018, Safarpour et al. 2018, Menasria et al. 2019). Furthermore, halophilic microorganisms are also been studied in decontamination processes, since they have been proved to biodegrade glyphosate (Sharifi et al. 2015), polycyclic aromatic hydrocarbons (Daane et al. 2001) and reduce chromate (Margesin & Shinner 2001), to mention a few.

Pigment production and potential applications

Pigments are known to be involved in processes that allow cellular survival even in environments with high levels of UV radiation. Microorganisms display several responses to light, which allows them not only to save energy from it, but also to protect them from its damages. The pigmented molecules protect the cells by absorbing the energy that would produce irreversible damage to the cellular genetic information, acting as a protective agent, thus they are essential in microbial survival in high-altitude environments. The Salar del Hombre Muerto, as previously mentioned, is located in

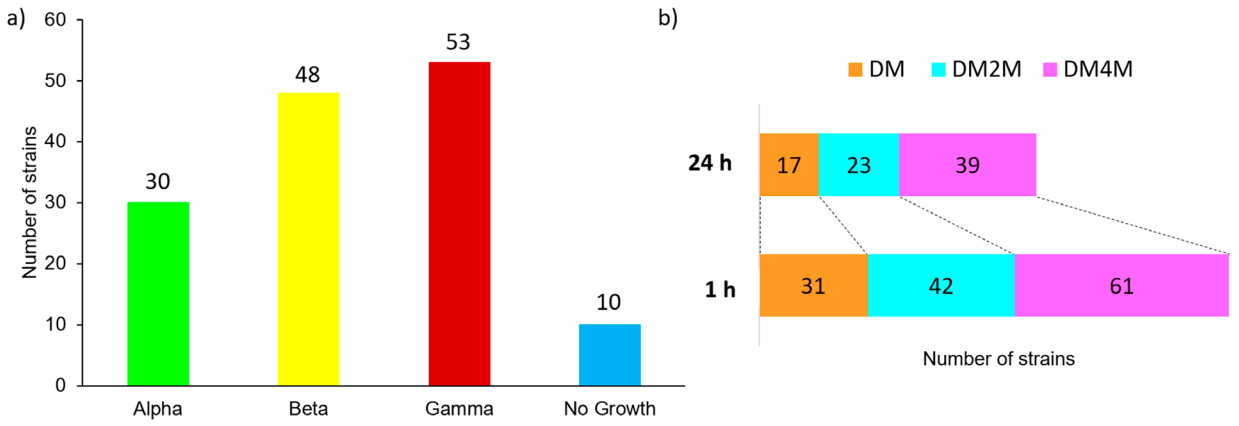

Figure 4. Strains with hemolytic and emulsifying activities from a total of 141 evaluated. a) Strains able to grow and to have alpha, beta and gamma hemolytic activity on blood agar, after incubation at 30 °C for 48 h. b) Strains that formed emulsions with kerosene (134 out of 141); stability of the emulsion was assessed after 1 h and 24 h. Bacteria were cultured on defined media (DM) without salt, and with the addition of 2 M (DM2M) and 4 M (DM4M) of NaCl, at 30 °C and 200 rpm for 48 h.

a high-altitude site, in continuous exposure to high levels of UV radiation. The ability to produce pigments detected in the microbial organisms isolated from this site would be directly related to their ability to colonize highly irradiated environments. Carotenoids produced by microorganisms can be used in the food, pharmaceutical, cosmetic and medical industries, therefore, these organisms represent an attractive alternative to the conventional pigments usually obtained from petrochemicals (Amoozegar et al. 2007, Delgado-García et al. 2015, Ruginescu et al. 2020). The main advantage of using microorganisms as a source of carotenoids is the cost reduction in the production process by optimizing it and using industrial by-products as nutrient sources for microbial development (Marova et al. 2012). In this study, 16 isolates synthesized pigments compatible with the production of carotenoids: seven strains were orange-pink and nine showed the presence of yellow pigment. Some of the studied strains were previously identified (Martínez et al. 2019a) and one of them, *Micrococcus luteus* SA211, had its genome completely sequenced (Martínez et al. 2019b). Five colored strains were selected from the pigmented group to be evaluated regarding other relevant characteristics, among other 20 non-pigmented strains (Figure 5). The selected group included three pigmented strains that belong to *Micrococcus* genus (SA211, M12, and SX120) and two to *Kocuria* genus (SA129b and M9). It is interesting to mention that these bacteria belong to the phylum Actinobacteria, which was extensively studied for its ability to produce biological active natural compounds (Margesin & Shinner 2001). Other authors described the ability to produce rare carotenoids from strains closely related to the previously mentioned genera. In this way, Strand et al. (1997) (Donio et al. 2013) characterized *Micrococcus roseus* strain MTCC 678 as able to produce bacteriorhodopsin, the main carotenoid responsible for the red color found in halophilic Achaea of the genera *Halobacteriaceae* and *Haloferacaceae* (Margesin & Shinner 2001, Donio et al. 2013). It should also be considered that the production of bacteriorhodopsin can be easily improved by optimizing environmental culture conditions like temperature, pH, salt concentrations, and light incidence, among others (Delgado-García et al. 2015). The optimization and production

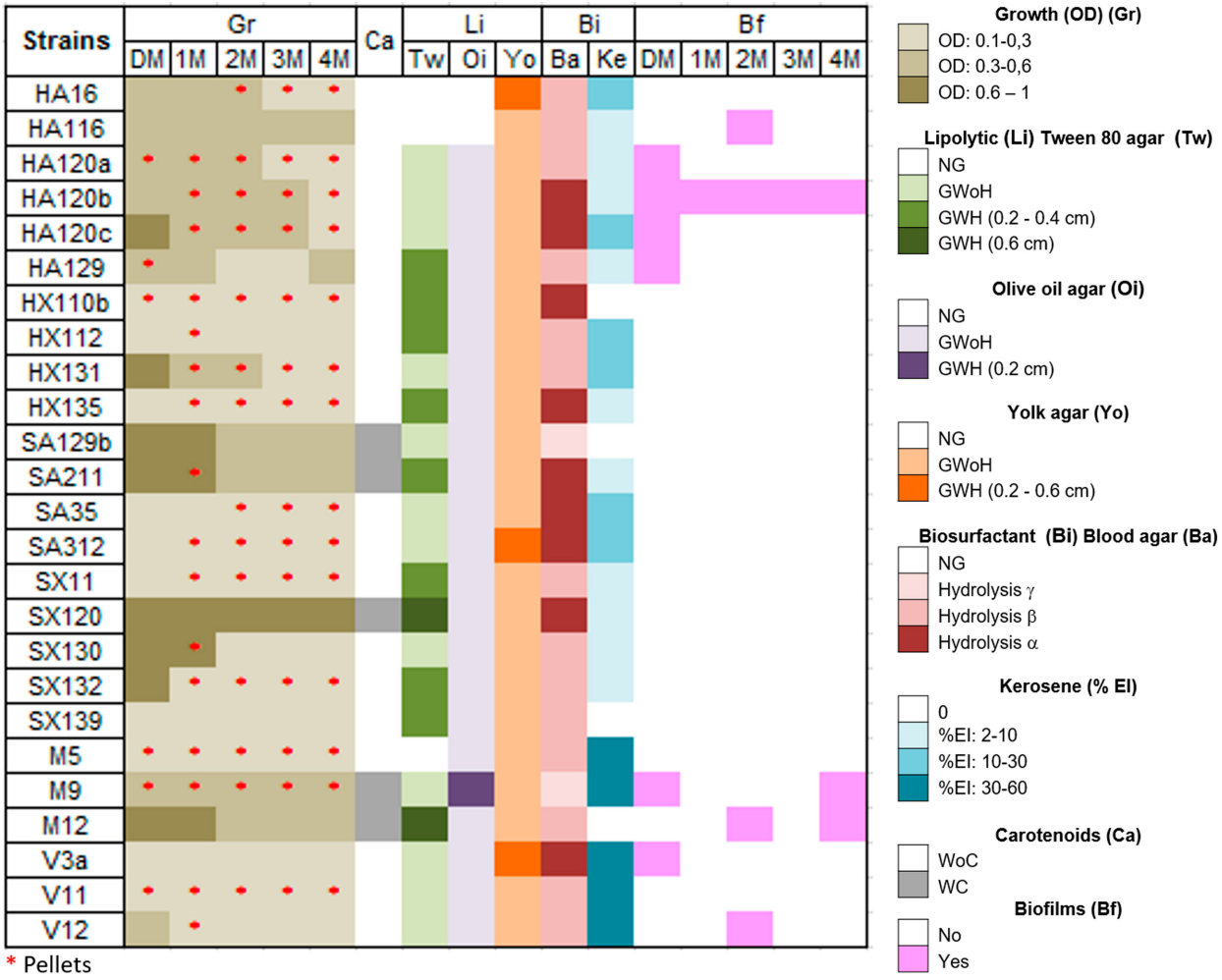

Figure 5. **Characteristics of the 25 selected strains. Growth (Gr) was measured as optical density at 600 nm (OD600) or as pellets at different concentrations of NaCl (defined medium without salt (DM), supplemented with 1, 2, 3, and 4 M of NaCl (DM1M, DM2M, DM3M, and DM4M, respectively). Presence of pigments (Ca) was determined by observation of colonies in APC-salt plates. Proteolytic activity (Pr) was assayed on media supplemented with skimmed milk (no growth (NG), growth without halo (GWoH) and growth with halo (GWH), were evaluated using different distance between colonies and halo). Lipase activity (Li) was assayed on media supplemented with Tween 80 (Tw), olive oil (Oi) and egg yolk (Yo) (no growth (NG), growth without halo (GWoH) and growth with halo (GWH), were evaluated using different distance between colonies and halo). Production of biosurfactants (Bi) using their ability to produce γ, β, and α hemolysis in agar blood medium (Ba) and bioemulsifiers (Ke) with different emulsifier index (IE) and biofilms production (Bf) using colorimetric assay with crystal violet.**

of this compound on a high scale may be of enormous interest since the current trend is leading to the search and use of renewable and environmentally friendly alternatives.

Hydrolytic enzyme synthesis and potential in biotechnological industries

Other products of great interest from a biotechnological perspective are the hydrolytic enzymes synthesized by halophilic microorganisms (Edbeib et al. 2016, Menasria et al. 2019, Drissi Kaitouni et al. 2020). Extreme microorganisms synthesize these proteins to get the most advantages of the available resources present in the different biotopes (Menasria et al. 2019, Drissi Kaitouni et al. 2020). The halotolerant of enzymes synthesized by halophilic microorganisms allows their exploitation

under wide ranges of pH, temperature, and salinity, representing an important advantage in comparison to the conditions under which conventional enzymes usually work (Marova et al. 2012, Menasria et al. 2019, Drissi Kaitouni et al. 2020). Enzymes including amylases, lipases, and proteases are being widely studied for their application in biotechnological processes, such as the food, leather, oil, pharmaceutic and textile industries, in biosynthetic processes, and also for environmental bioremediation (Torregrosa-Crespo et al. 2018, Menasria et al. 2019). Furthermore, biopolymer-degrading enzymes have also gained importance for their potential use in the treatment of oilfield waste *in situ*. Having such potential applications, their search and study have been encouraged and have increased considerably in the last years, finding interesting hydrolytic abilities in halotolerant and halophilic bacteria (Torregrosa-Crespo et al. 2018, Ruginescu et al. 2020). It has also been reported that the environmental isolates belonging to the *Bacillus* and the *Micrococcus* genera are well known for their ability to produce hydrolytic enzymes (Torregrosa-Crespo et al. 2018, Menasria et al. 2019). It is interesting to emphasize that strains HA120a, HA120b, HA120c, SA35, SX130, and V3a were identified in a previous study as *Bacillus* strains (Martínez et al. 2019a) widely known for their abilities to hydrolyze a wide variety of compounds (Menasria et al. 2019, Drissi Kaitouni et al. 2020). In the present study, the mentioned strains were able to grow in presence of Tween-80, olive oil, and yolk but only V3a produced a hydrolysis halo in yolk agar (Figure 5), indicating a lipolytic activity for this specific compound. Additionally, strains HA120a, HA120b, and HA120c produced active proteases in the casein medium (Figure 5). These results are indicative of the potential of the strains to synthesize hydrolytic enzymes. Lipolysis and proteolysis were verified in the growth media used in this study, but it is indicative of a high potential of the strains to hydrolyze other compounds with similar structures. Also, strains M12 and SA211, classified as *Micrococcus* strains (Martínez et al. 2019b), tested positive for both lipolytic and proteolytic activity in Tween-80 and casein medium respectively, providing further evidence of the high potential for enzymatic hydrolysis of Gram-positive bacteria. The selected isolates would then, not only have the ability to synthesize lipases and proteases but also have an interesting potential for the synthesis of other enzymes of interest out of the scope of this paper, such as pullulanase, gelatinases, cellulases, nucleases, and amylases, widely described in Gram-positive bacteria (Sánchez-Porro et al. 2003, Torregrosa-Crespo et al. 2018, Menasria et al. 2019, Drissi Kaitouni et al. 2020). It should also be considered that most of the strains tested positive for at least one of the hydrolytic activities evaluated, showing the plasticity of these strains, which would allow their survival and growth in different environments.

Microorganisms, in general, can synthesize hydrolytic enzymes intracellularly or extracellularly. If the final purpose is to apply them in a specific process that does not require the presence of the microorganism, extracellular synthesis is preferred, since it represents lower production costs and higher purity of the final product. However, optimization of the enzyme production may be achieved by cloning the gene of interest and expressing it in a different system, for instance, in *Escherichia coli*. In the present study, the evaluated enzymatic activities occurred extracellularly (the formation of hydrolysis halos was detected in growing cultures, without previous bacterial lysis) representing an additional advantage of our selected group of microorganisms for enzyme production and use. Enzyme synthesis and activity conditions could

be optimized to achieve the maximum efficiency in each.

Proteases have been deeply studied for industrial applications and the stability and wide range of conditions under which halophilic enzymes can act turns them into great candidates as alternative to conventional processes. Lipolytic enzymes have broad substrate specificity and are being currently used as detergent additives, in the food and paper industries and selection processes for fine chemicals. Being obtained from an extreme organism, these enzymes have a huge potential for application in a wide range of conditions.

In addition to the hydrolytic enzymes previously discussed, catalase activity was also evaluated. Elyasifar et al. 2019 isolated seven halophilic bacteria and all of them tested positive for catalase activity. Considering that this enzyme is involved in cell protection from oxidative damage by reactive oxygen species (ROS), it would have the main role in the cellular response to environmental stress.

Extracellular polymeric compounds (EPS), their properties, and importance

Halophilic bacteria can synthesize large amounts of EPS to protect themselves from the hostile surrounding environment (More et al. 2014, Wang et al. 2019, Ibrahim et al. 2020). It is also of major interest to characterize molecules such as exopolysaccharides and biosurfactants as a consequence of their multiple applications, including synthesis of biomaterials, biomedicine, in water treatment, removal of toxic compounds, soil remediation, and food industry (Poli et al. 2011, Wang et al. 2019) and their advantages, such as biodegradability, high efficiency, and non-toxicity, to name a few (More et al. 2014). Surfactants, or surface-active agents, are molecules that reduce surface and interfacial tensions due to their amphiphilic and hydrophilic properties and emulsifiers are surfactants that stabilize emulsions. Some microorganisms can synthesize these biopolymers and secret them into the environment. EPS may vary in chemical composition, molecular weight, and therefore, in the functions, they have to depend on the environmental conditions, including salt concentration, carbon and nitrogen sources, pH, and temperature values (More et al. 2014, Moshabaki et al. 2018). These compounds would be naturally involved in intercellular communication, molecular recognition, biofilm formation, flocculation, adhesion, protective barrier for cells, water retention, and one more interesting feature to consider is that they are environmentally friendly, being one of their major advantages in comparison to traditional polymers (More et al. 2014, Wang et al. 2019). As previously discussed, bacteria from the *Bacillus* genera have many abilities, including hydrolytic enzyme synthesis, and are also able to produce EPS of different compositions depending on the carbon source. It is known, for instance, that *Bacillus subtilis* produces poly-gamma-glutamate, which is an anionic homo-polyamide that forms capsular or slime (More et al. 2014). Furthermore, glycoprotein production in EPS is very diverse and has been associated with Gram-positive organisms, including *Bacillus* strains (More et al. 2014). The different EPS composition would allow interaction with specific elements, which is a property that has a high potential for heavy metal bioremediation (Wang et al. 2019, Ibrahim et al. 2020). It is also known that minor changes in EPS composition affect its structure and chemical properties, being necessary for the optimization of its production for a specific purpose. Bacterial EPS were proved to have biosurfactant properties, and as described by Ibrahim et al. 2020, their low toxicity, high activity, and biodegradability turn them into promising alternatives to conventional surfactants, as

they can be used in bioremediation and several industries (Muntaha & Khan 2015, Nercessian et al. 2015, Ibrahim et al. 2020). In our study, we detected the ability of the *Bacillus* strains (HA120a, HA120b, and HA120c) to develop between 2 and 30% EI (Figure 5), whereas, for V3a, this value was increased to an EI between 30 and 60%. It should be considered that only four of the evaluated strains did not form emulsions when the supernatant of the cultures was mixed with kerosene, indicating the absence of bioemulsifier production. Furthermore, five of the 25 strains formed emulsions in the highest EI values (30 – 60% EI). *Kocuria* sp. M9 was also included in these five strains, indicating that this microorganism that belongs to a different genus (still a Gram-positive bacterium) can develop stable bioemulsifiers. This behavior was already described by Sarafin et al. 2014, who detected the capacity of *Kocuria marina* BS-15 to form emulsions with kerosene at lower values than those developed by our strain. Another component of EPS is biosurfactants, which can disperse hydrophobic substances in the medium. It has been reported that *Bacillus subtilis* can produce surfactin, which contributes to its capacity to colonize environments and gives an advantage in the competition for resources (More et al. 2014). Biosurfactant production was verified in this study by the lysis of red cells, which was detected in blood agar growth. All of the strains identified as *Bacillus* were able to lyse red cells, suggesting the production of biosurfactant. In general, 23 out of the 25 evaluated strains tested positive for the qualitative study of biosurfactant production. These compounds are suitable for different applications such as humidification, detergency, foam flotation, emulsification, and oil recovery, among others (Romano-Armada et al. 2020). Most surfactants used for domestic and industrial work are synthetic and dispersed in the environment generating serious pollution problems (Strand et al. 1997).

EPS synthesis is also related to the ability of cells to attach to different surfaces. When this phenomenon occurs in natural niches, cellular development forms a protected environment that allows their growth and resistance to environmental disturbances. EPS form a porous matrix that surrounds cells, which lose mobility but gain the ability to exchange genetic material between the different species that form the biofilm (Calegari-Santos et al. 2016). Some authors demonstrated the ability to isolate and characterize moderate halophilic bacteria with EPS production capacity in Indian salt mines (Edbeib et al. 2016). Curiously, *Halomonas* species were isolated and identified for their abilities to produce EPS (Sánchez-Porro et al. 2003). In the same way some halotolerant bacteria able to produce biofilm only in the presence of salt were isolated in this work. In other cases, like in *Bacillus* sp. HA120b, the production of these substances was obtained under all the evaluated conditions, including the absence of salt in the culture media (Figure 5). Thus, it is expected that the microbial EPS produced by the bacteria isolated from the Salar del Hombre Muerto, could present a wide range of environmental, agricultural, and industrial applications including the production of cosmetics, pharmaceuticals, and food, and in biolixiviation. Nevertheless, since information regarding the EPS synthesis process is still limited, optimization of EPS production can be approached using genetically modified strains of specific characteristics (regarding chemical characteristics, which are tightly related to their properties, as mentioned before).

To conclude, some of the bacteria studied showed the ability to produce more than one compound of interest (enzymatic or polymeric) that could have interesting applications in

different industries related to the production of pigments, biosurfactants, enzymes, and/or EPS. Besides, many of them are also functional in the presence of high concentrations of salts. To the best knowledge, this is the first study that describes biotechnological potential applications from microorganisms isolated from the Salar del Hombre Muerto, demonstrating the great possibilities that this niche offers in the search and characterization of enzymes and molecules with unique characteristics.

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