Halotolerant bacteria in the São Paulo Zoo composting process and their hydrolases and bioproducts

Lilian C.G. Oliveira1, Patricia Locosque Ramos1,3, Alyne Marem1, Marcia Y. Kondo1, Rafael C.S. Rocha1,4, Thiago Bertolini1, Marghuel A.V. Silveira2, João Batista da Cruz3, Suzan Pantaroto de Vasconcellos2,3, Luiz Juliano1,3, Debora N. Okamoto1

1Departamento de Biofísica, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil.
2Departamento de Ciências Biológicas, Universidade Federal de São Paulo, Diadema, SP, Brazil.
3Laboratório de Microbiologia Aplicada, Fundação Parque Zoológico de São Paulo, São Paulo, SP, Brazil.
4Koppert Biological Systems, Itapetininga, SP, Brazil.

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Abstract

Halophilic microorganisms are able to grow in the presence of salt and are also excellent source of enzymes and biotechnological products, such as exopolysaccharides (EPSs) and polyhydroxyalkanoates (PHAs). Salt-tolerant bacteria were screened in the Organic Composting Production Unit (OCPU) of São Paulo Zoological Park Foundation, which processes 4 ton/day of organic residues including plant matter from the Atlantic Rain Forest, animal manure and carcasses and mud from water treatment. Among the screened microorganisms, eight halotolerant bacteria grew at NaCl concentrations up to 4 M. These cultures were classified based on phylogenetic characteristics and comparative partial 16S rRNA gene sequence analysis as belonging to the genera Staphylococcus, Bacillus and Brevibacterium. The results of this study describe the ability of these halotolerant bacteria to produce some classes of hydrolases, namely, lipases, proteases, amylases and cellulases, and biopolymers. The strain characterized as of Brevibacterium avium presented cellulase and amylase activities up to 4 M NaCl and also produced EPSs and PHAs. These results indicate the biotechnological potential of certain microorganisms recovered from the composting process, including halotolerant species, which have the ability to produce enzymes and biopolymers, offering new perspectives for environmental and industrial applications.

Key words: halophilic, protease, lipase, amylase, cellulase.

Introduction

The biocatalysts required in several industrial processes exhibit optimal activities at high ranges of salt concentration, pH and temperature. Halophiles are excellent sources of such enzymes and are found in nearly all major microbial clades, including prokaryotic (Bacteria and Archaea) and eukaryotic forms; two categories have been defined: halotolerant microorganisms that are adapted in live at high salinity, and halophiles that require salinity for growth. Halotolerant species tend to live in areas of salinity, such as hypersaline lakes, coastal dunes, saline deserts and salt seas (Ventosa and Nieto, 1995).

Halophilic enzymes perform the same enzyme function as their non-halophilic counterparts but require 1-4 M salt concentrations for their full activity and stability. In addition, these enzymes typically demonstrate a large excess of acidic amino acids compared to basic residues (Enache and Kamekura, 2010).

Proteases constitute approximately 66% of the total enzymes employed in biotechnological and commercial...
processes (Gupta et al., 2002), and the moderately halo-
philic aerobic bacteria of genera Bacillus, Pseudomonas,
Halomonas and Serratia are important sources of proteases
(Ventosa et al., 1998). Amylases are extensively studied
due to their potential application in the food, detergent, pa-
paper and pharmaceutical industries, representing approxi-
mately 25% of the total enzymes in the industrial market.
The extracellular production of β-amylase by halophilic
Halobacillus sp. LY9 and of two α-amylases from
Chromohalobacter sp. has been reported (Li and Yu, 2011;
Prakash et al., 2009). Cellulases also have industrial appli-
cation, including the generation of bioethanol and in the
textile industry, and a halotolerant cellulase was character-
ized in a soil metagenome analysis (Voget et al., 2006).
Lipolytic enzymes are of particular industrial interest, and
their identification in halophilic bacteria has been reported
and recently reviewed (Gomez et al., 2012). Exopoly-
saccharides (EPSs) and polyhydroxyalkanoates (PHAs) are
biotechnological products that were identified and pro-
duced from halophilic/halotolerant microorganisms (Legat
et al., 2010; Litchfield, 2012).

In this sense, the Organic Composting Production Unit (OCPU) of SPZPF is a potential source of microorgan-
isms, as demonstrated by an OCPU metagenomic analysis,
which revealed a diversity of biomass degradation func-
tions and organisms (Martins et al., 2013). The composting
process is predominantly aerobic, with organic residues be-
ing degraded by microorganisms, generating a humus-like
material. In recent years, composting has attracted attention
as a viable and environmentally adequate alternative for the
treatment of organic waste. The initial phase of composting
is thought to be the most dynamic part of the process and is
characterized by a rapid increase in temperature, a large
change in pH, and the degradation of simple organic com-
ounds (Schloss et al., 2003). A detailed comparison of the
bacterial diversity from different composting plants
revealed a large difference at both the species and strain
levels (Partanen et al., 2010).

This paper reports the screening of the composting
process of OCPU at SPZPF for bacteria in the presence of a
range of NaCl concentrations and also the evaluation of
their potential for the production hydrolases and bio-
polymers. To date, the microbial diversity of this ecosys-
tem has not been explored, particularly with regard to the
screening of halotolerant microorganisms.

Material and Methods

Bacterial strains and isolation of DNA

Composting process

The composting process was conducted in the SPZPF
OCPU in 2.5 x 2.0 x 1.6 m (lengthxwidthxheight) cells, as
shown in Figure 1. The piles were formed by organic resi-
dues including food, droppings and excreta, the beds of na-
tive and exotic wild animals, carcasses and wood chips
from gardening. The pile has decomposition phases that
were considered active degradation (before aeration) and
mature compost (after aeration). Pile aeration was achieved
by the mechanical turning of the material after 50 to 60 days
of composting. The temperature of the pile was monitored
at five different points (four sides and one center). The av-
erage temperature of the pile at the time of collection was
50 °C.

Microbial isolation

Compost samples (10 g) were collected from the piles
and diluted in 90 mL of sterile water. Serial dilutions (-2, -4
and -6) were performed and spread on agar plates of two se-
lective halophilic media: JCM nº 377 medium (10% [w/v]
NaCl, 0.5% [w/v] casamino acids, 0.5% [w/v] yeast extract,
0.2% [w/v] KCl, 0.3% [w/v] sodium citrate, 2% [w/v] MgSO$_4$·7H$_2$O, 0.036% [w/v] FeCl$_2$·4H$_2$O, 0.00036% [w/v] MnCl$_2$·4H$_2$O and 2% [w/v] agar, pH 7.2) and YPC medium (0.5% [w/v] yeast extract, 0.1% [w/v] peptone and 0.1% [w/v] casamino acids with 60% [v/v] of salt water solution 24% [w/v] NaCl, 3% [w/v] MgCl$_2$·6H$_2$O, 3.5% [w/v] MgSO$_4$·7H$_2$O, 0.1% [w/v] KCl, 20 mM Tris HCl pH 7.5 and 3 mM CaCl$_2$) and incubated at 30, 37 and 42 °C. After 24 or 48 h, the colonies were selected and transferred separately to obtain purified colonies.

Screening of secreted extracellular hydrolytic activities

Enzymatic agar plate assays were performed to detect the presence of extracellular hydrolases. All media were adjusted to pH 7.3, and NaCl was added to obtain a salt concentration in the range of 0-4 M. The composition of the media used is described below.

**Determination of extracellular amylase activity**

Amylolytic activity on plates was determined qualitatively using a previously described method (Pascon et al., 2011), which was modified for halophilic microorganisms by adding NaCl in the medium. After incubation at 37 °C for 5 days, the plates were exposed to iodine crystals for 5 min to reveal the starch degradation zone that indicates amylolytic activity.

**Determination of extracellular protease activity**

The cultures were screened in JCM nº 377 medium and YPC medium supplemented with 1% skim milk for the determination of protein hydrolytic activity. Clear zones around the colonies after 7 days were taken as evidence of proteolytic activity.

**Determination of extracellular lipase activity**

Lipase production by the isolated microorganisms was evaluated in nutrient agar tributyrin medium (NAT), which consisted of 1.3% nutrient broth, 1% tributyrin and 2% agar (Ben-Gigirey et al., 2000). After incubation at 37 °C for 7 days, the hydrolytic zones around the bacterial colonies were considered an indication of lipase production.

**Determination of extracellular cellulase activity**

Cellulase activity was screened on a solid medium containing carboxymethyl cellulose (CMC) (Rohban et al., 2009). After incubation at 37 °C for 7 days, the plates were flooded with 0.1% Congo red solution. The clear zone around colonies indicated cellulolytic activity.

Screening of polyhydroxyalkanoates and exopolysaccharides

Detection of polyhydroxyalkanoate (PHA)-producing microorganisms

The isolates were evaluated in mineral medium (Schlegel et al., 1970) with 2.5 M NaCl and containing glucose, xylene or octanoic acid as the carbon source. Glucose is known to be a carbon source for the production of short-chain-length PHAs, whereas octanoic acid produces medium-chain-length PHAs. Sugarcane bagasse contains xylose, and its excess is a promising substrate for producing by-products, such as second-generation bioethanol and PHAs (Lopes et al., 2009). After 24 h of incubation (30 °C), the isolated strains were evaluated for their ability to grow on these carbon sources; the isolates were stained with Sudan Black B after 72 h to verify their potential to produce PHAs.

Detection of exopolysaccharide (EPS) producers

The isolates were cultured in Bushnell Haas Salt Medium (50 mL) containing 2.5 M NaCl with glycerol as sole the carbon source for the microbial growth. After incubation for 5 days at 30 °C in a rotary shaker (150 rpm), the cultures were centrifuged at 8,200 x g for 15 min (4 °C). The emulsification index (E24) of the supernatant was evaluated according to the method described by Fleck et al. (2000) using hexadecane as a hydrophobic model compound. The chemical composition of EPSs precipitated from the supernatant with ethanol up to 70% was dialyzed against pure water, and carbohydrates, proteins and uronic acids were quantified in the retained high molecular weight fraction, as reported (Tanasupawat et al., 2010).

Bacterial identification

Mass spectrometry

The isolated microorganisms were treated with ethanol/formic acid for content extraction, following a previously described protocol (Pascon et al., 2011). Measurements were conducted with a Microflex LT mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.0, Bruker Daltonics) in the positive linear mode (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.6 kV; lens voltage, 7.5 kV; mass range, 2000 to 20 000 Da). For each spectrum, 240 shots in 50-shot steps from different positions of the target spot (automatic mode) were collected and analyzed. The spectra were internally calibrated using Escherichia coli ribosomal proteins. The raw spectra were imported into the BioTyper software (version 2.0, Bruker Daltonics) and processed by standard pattern matching with default settings; the results were reported in a ranking table.
Amplification and sequencing of 16S rRNA gene fragment

DNA (30-50 ng) from each strain was incubated in a 50-µL reaction mixture containing 2 mM MgCl₂, 200 µM dNTPs, 0.3 µM universal primer 27f (5-AGAGTTGATCCTGGCTCAG-3), 0.3 µM 1525r (5-AAGGAGGTGWTCCARCC-3) and 2 U Taq DNA polymerase (Invitrogen) in the recommended buffer. Amplification was performed in a Veriti 96 well Thermal Cycler (Applied Biosystems) with an initial temperature at 94 °C for 2 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min. A final extension at 72 °C was included for 10 min. The PCR products were purified with a GFX PCR DNA and gel band purification kit (GE Healthcare), and the sequence analysis was performed using a 3500 Genetic Analyser Sequencer (Applied Biosystems). Subsequently, 5.0 µL purified PCR product was mixed with 4.0 µL of BigDye v. 3.1 (Applied Biosystems) and 1.0 µL sequencing primer (0.5 µmol). The primers used in the sequencing reactions were 27f (Dojka et al., 1998), 782r (5ACACGGATCTAATCCTGT3) (Chun and Goodfellow, 1995) and 1401r (5CGGTGTGTACAAGACC C3) (Nübel et al., 1996). The sequencing program consisted of 25 cycles at 95 °C for 20 s, 50 °C for 15 s and 60 °C for 60 s. The 16S rRNA gene sequence of all the analyzed strains was compared to bacterial sequences deposited in GenBank. Sequences with similarity were retrieved, and the consensus sequences were aligned using CLUSTALW with MEGA 5.05. EzTaxon tools (http://147.47.212.35:8080/) were further employed to confirm the similarities, and phylogenetic trees were constructed based on neighbor-joining, maximum-likelihood and maximum-parsimony methods. The resulting tree topologies were evaluated by a bootstrap analysis based on 1000 replicates.

Nucleotide sequence accession numbers

The sequenced strains SR5-6, SR5-7, SR5-12, YPC-6, YPC-8, YPC-11, YPC-13 and YPC-15 were deposited in GenBank under accession numbers JX154082, JX154083, JX154084, JX154085, JX154086, JX154087, JX154088 and JX154089, respectively.

Results and Discussion

Identification of halophilic strains

The isolated bacteria were obtained from the composting process during the turning stage (60th day). Eight out of eleven halophilic isolates in 2.5 M NaCl from the composting process were subjected to a MALDI-TOF mass spectrometry analysis, which indicated that the genera of all isolates were Gram positive, which was confirmed by Gram staining. These procedures ensured and confirmed the purity of the isolates. The 16S rRNA gene sequences of eight strains (> than 1300 bp) were compared with those previously deposited in GenBank. The neighbor-joining and maximum-likelihood trees showed the taxonomic position of these strains, which were affiliated with Bacillus, Staphylococcus and Brevibacterium genera (Figure 2).

Strain SR5-7 showed high 16S rRNA gene sequence similarity to Bacillus when compared with the 184 different species of this genus. However, based on the similarity matrix of the 16S rRNA gene, this isolate did not show 100% similarity with any of the species already reported. The species of Bacillus described as halophilic to date are as follows: B. hemicentroti (Chen et al., 2011); B. humanensis (Chen et al., 2011); B. xianensis (Sanchez-Porro et al., 2003; Schlegel et al., 1970); B. alkalophilic (Zhang et al., 2012); B. halochares (Pappa et al., 2010); B. chungangensis (Cho et al., 2010) and B. subtilis (Takenaka et al., 2011). Thus, the possibility that a new species of halophilic Bacillus was isolated from a compost process is noteworthy.

The YPC-11 strain was identified as Brevibacterium avium (100% similarity). An EzTaxon analysis confirmed that this strain shared 100% 16S rRNA gene sequence similarity with B. avium and 99.97% with Brevibacterium epidermidis, the only halotolerant bacterium (Nagata and Wang, 2005) described in the genus Brevibacterium.

Strains SR5-12, SR5-6, YPC-6, YPC-8, YPC-13 and YPC-15 were classified as members of the Staphylococcus genus. The isolates SR5-12 and SR5-6 showed 100% 16S rRNA gene sequence similarity with S. lentus and S. sciuri, respectively, a result that was confirmed by EzTaxon. However, to date, these species have not been described as high salt concentration-tolerant bacteria, with the only species of Staphylococcus known as halophilic being Staphylococcus equorum (Essghaier et al., 2009).

All the selected isolates were deposited at the São Paulo Zoo Park Culture Collection (SPZSP-CCol).

Salt tolerance and growth of halophilic isolates

All the bacteria isolated in 2.5 M NaCl were tested for their ability to grow at different salt concentrations. A slowing of bacterial growth was observed in the presence of high salt concentrations, as indicated by the time (in days) required for detecting the presence of bacteria in the culture medium (Table 1). Staphylococcus strains SR5-12, YPC-6, SR5-6 and YPC-8 showed similar growth behavior from 0 to 4.0 M NaCl. Strain YPC-13 had the slowest growth at high salinity, and strain YPC-15 grew preferentially at 2.5 M NaCl or higher. Bacillus strains SR5-7 and YPC-11 (affiliated with B. avium) exhibited a preference for growing in a culture medium containing 0.5 to 2.0 M NaCl but failed to grow in 4.0 M NaCl. It is important to note that although all of these bacteria tolerated high salinities (2.5 M NaCl or higher), they are not strictly halophilic bacteria. According to Kushner (1978), bacteria that are able to grow in the absence of salt as well as in the presence of relatively high salt concentrations are designated halotolerant or ex-
tremely halotolerant if growth extends above 2.5 M. Based on this classification, seven out of the eight isolated microorganisms isolated from composting process were halotolerant or extremely halotolerant. It should be noted that the salt requirement and tolerance of many species vary according to the growth conditions, such as temperature and medium composition.

Several bacteria of *Bacillus*, *Halobacillus* and *Staphylococcus* have been found in saline environments, such as Salt Plains National Wildlife Refuge, Great Salt Plains of Oklahoma, a Bolivian hypersaline lake, deep-sea sediments and tropical marine sediments (Ventosa et al., 1998). Some species of *Bacillus* sp. are salt tolerant and are important degraders of organic pollutants. Examples include *Bacillus cereus*, which degrades 1,3-dichlorobenzene derivatives from town-gas industrial influent (Wang et al., 2003), and *Bacillus subtilis*, which degrades p-aminobenzene from textile industry wastewater (Zissi et al., 1997).

**Hydrolytic activities of halotolerant isolates**

The SR5-6, SR5-7, SR5-12, YPC-8, YPC-11 and YPC-15 strains were found to be moderate halophilic microorganisms and showed combined cellulolytic, amyloytic, lipolytic and proteolytic activities (Table 1). These strains have potential biotechnological applications with respect to their ability to produce different hydrolases (Rohban et al., 2009). In contrast, no hydrolytic activity was observed for YPC-6 and YPC-13.

Only YPC-11 (affiliated with *B. avium*) presented amylase and cellulase hydrolytic activities from 0 to 4 M NaCl. Members of the genus *Bacillus* are well known enzyme producers, and many industrial processes utilize species belonging to this genus for the commercial production

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**Figure 2** - Phylogenetic tree showing the position of the halotolerant isolates, as based on a partial 16S rRNA gene sequence comparison obtained by neighbor-joining and maximum-likelihood trees. The nucleotide sequence accession numbers were deposited in GenBank, as described in Material and Methods.
Table 1 - Different types of hydrolytic activities, amylase (A), cellulase (C), lipase (L) and protease (P), found in the eight strains isolated from OCPU at the 0 - 4.0 M NaCl concentration range.

| [NaCl] | Days | Hydrolase | Days | Hydrolase | Days | Hydrolase | Days | Hydrolase | Days | Hydrolase | Days | Hydrolase | Days | Hydrolase |
|--------|------|-----------|------|-----------|------|-----------|------|-----------|------|-----------|------|-----------|------|-----------|
|        |      |           |      |           |      |           |      |           |      |           |      |           |      |           |
| 0 M    | 1    | Nd        | 1    | L, P      | 1    | Nd        | 2    | L         | 2    | A, C      | 2    | Nd        | 4    | A, C, L   |
| 0.5 M  | 1    | L         | 1    | L, P      | 1    | L         | 2    | L         | 2    | A, C      | 2    | Nd        | 0    | Nd        |
| 1.0 M  | 1    | Nd        | 1    | L         | 1    | Nd        | 2    | L         | 2    | A, C      | 2    | Nd        | 0    | Nd        |
| 1.5 M  | 1    | Nd        | 1    | L         | 1    | Nd        | 2    | L         | 2    | A, C      | 2    | Nd        | 0    | Nd        |
| 2.0 M  | 1    | Nd        | 1    | L         | 1    | Nd        | 2    | L         | 3    | A, C      | 3    | Nd        | 1    | A, C      |
| 2.5 M  | 1    | Nd        | 2    | Nd        | 2    | Nd        | 2    | L         | 4    | A, C      | 7    | Nd        | 2    | A, C      |
| 3.0 M  | 2    | Nd        | 2    | Nd        | 2    | Nd        | 2    | Nd        | 7    | A, C      | 7    | Nd        | 2    | A, C      |
| 3.5 M  | 4    | Nd        | 2    | Nd        | 3    | Nd        | 3    | Nd        | 21   | A, C      | 21   | Nd        | 3    | A, C      |
| 4.0 M  | 7    | Nd        | -    | Nd        | 4    | Nd        | 4    | Nd        | 21   | A, C      | 21   | Nd        | 3    | A, C      |

The assays were performed as described in Material and Methods. Days: Time in days that visible growth was observed, considering zero to be the day of inoculation. (Nd): Hydrolytic activity not detected.

Table 2 - Analysis of isolates for the ability to produce EPSs in mineral medium and PHAs using different carbon sources in the presence of 2.5 M NaCl.

|        | SR-6 (Staphylococcus sp.) | SR-7 (Bacillus sp.) | SR-12 (S. lentus) | YPC-6 (Staphylococcus sp.) | YPC-8 (Staphylococcus sp.) | YPC-11 (B. avium) | YPC-13 (Staphylococcus sp.) | YPC-15 (Staphylococcus sp.) |
|--------|--------------------------|---------------------|-------------------|---------------------------|---------------------------|-------------------|-----------------------------|-----------------------------|
| EPS (E24) | 58%                     | 50%                 | 51%               | 53%                       | 50%                       | 55%               | 55%                         | 55%                         |
| PHA     | Nd                       | Xylose (+)          | Nd                | Nd                        | Xylose (+) Octanoic acid (+) | Octanoic acid (+) | Glucose (+)                 |                             |

(E24): Emulsification indexes of the isolates in a mineral medium containing 2.5 M NaCl, as described in Materials and Methods.
(+): indicates that PHAs were detected when using the indicated carbon source.
(Nd): PHA production not detected.
of enzymes (Vasconcellos et al., 2011). The strain SR5-7 (affiliated with Bacillus) produced lipase and protease in 2.0 M and 0.5 M NaCl, respectively. It is interesting to note that the lipase producers reported thus far are limited to representatives of the genera Salinispira, Halomonas and Bacillus-Salibacillus (Sanchez-Porro et al., 2003).

Polyhydroxyalkanoate (PHA) producers

All the isolates were evaluated using a medium with nitrogen limitation and different carbon sources (Table 2). The isolates grew better with glucose as the sole carbon source compared to xylose and octanoic acid. The isolates YPC-13 (affiliated with Bacillus sp.), SR5-7 (affiliated with Bacillus sp.) and YPC-15 (affiliated with Staphyloccocus sp.) accumulated PHAs in presence of octanoic acid, xylose and glucose, respectively. The genus Bacillus is known as a producer of PHAs (Lopes et al., 2009), and Staphylococcus epidermidis, which was isolated from sesame oil, presented the ability to produce poly-3-hydroxybutyrate (Wong et al., 2000). The strain YPC-11 (affiliated with B. avium) was detected as a potential producer of biopolymers using octanoic acid and xylose. This result is in accordance with the previous observation that Brevibacterium casei (SRKP2 strain) could produce PHAs in a medium containing dairy industrial waste, yeast extract and seawater (Pandian et al., 2009). Halotolerant microbes are important for the biotechnology industry due to their advantages for use in sterilization processes and the control of contaminants; the PHA-producing halophilic microorganisms have recently been reviewed (Polli et al., 2011). The production of PHAs using xylose is an alternative strategy to produce economically competitive PHAs using agro-industrial products such as sugarcane molasses and bagasse (Gomez et al., 2012).

EPS production and emulsification potential

Microbial exopolymers (EPSs) correspond to compounds produced by microorganisms to solubilize essential nutrients for their survival or to promote their adherence onto surfaces (Ron and Rosenber, 2002). The use of glycerol as a sole carbon source and 2.5 M NaCl resulted in EPS values up to 60% of the emulsification index (E24) of hexadecane (Table 2). A colorimetric analysis showed that the biosurfactant produced by the evaluated halotolerant strains were mainly composed of carbohydrates (95%) but also contained proteins (0.5%) and uronic acids (4.5%) in their composition. A similar EPS composition was also reported in halophilic Archaea strains (Polli et al., 2011).

Conclusion

Screens for halotolerant or halophilic microorganisms in non-saline environments are scarce as is the detection of extracellular enzymes. This study found eight isolates from an organic residue composting process that showed the ability to tolerate a wide range of salinity. Some of these strains presented combined hydrolytic ability in the presence of NaCl. The possibility of these microorganisms, particularly YPC-11 (affiliated with B. avium), to produce EPSs and PHAs in the presence of 2.5 M NaCl can offer new biotechnological and bioremediation perspectives for the treatment of oilfield wastes as well as in MEOR (microbial-enhanced oil recovery) processes. The performance of the halotolerant isolates in the present work were not compared to other already known and classic halophilic microorganisms, and this should be performed in future work.

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