Isolation and characterization of halophilic bacteria producing exopolymers with emulsifying and antioxidant activities

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ABSTRACT

Halophilic bacteria are considered a great source of new strains producing novel exopolymers with functional properties. In this work we isolated ten halophilic strains producing exopolymers from different hypersaline environments in Morocco. Phenotypic characterization showed that the strains were moderately halophilic, mesophilic and neutrophilic with the ability to produce some hydrolytic enzymes. Strains identification based on 16S rRNA gene sequences comparison showed that nine strains, designed as N1, N2, N5, N7, N8, N9, N10, N11 and N12 belong to *Halomonas* genus and one strain, designed as N4, to *Marinobacter* genus. The majority of the strains showed high levels of exopolymer production. The study of emulsifying and antioxidant activities revealed that all the polymers have an interesting emulsifying and antioxidant activities with the polymer from *Marinobacter* sp. N4 forming the highest and most stable emulsions and exhibiting the best antioxidant activity in comparison with other exopolymers produced by *Halomonas* strains. The obtained results demonstrate the great potential of exopolymers from halophilic bacteria to be applied as emulsifying and antioxidant agents in food, cosmetics and oil industries.

Keywords: Halophilic bacteria, exopolymer, antioxidant activity, emulsifying activity.

1. Introduction

Halophilic bacteria are microorganisms that inhabit hypersaline environments like salt mines, salt pans and marine ecosystems. They have adapted to this kind of biotopes by developing various strategies in order to survive the osmotic stress induced by high salt concentration (Gunde-Cimerman et al., 2018; Barozzi et al., 2018).

In the last decade this type of extreme microorganisms has gained a lot of attention from the research community as a source of new biomolecules that have interesting biotechnological applications and that can compete with chemical products (Enache et al., 2015). In fact, halophilic bacteria can be used for open and continuous fermentation process due to their ability to grow at high salt concentrations which minimize the cost of production (Tan et al., 2011; Yin et al., 2015). In addition, these bacteria can be cultivated using recycled sea water which make them one of the suitable platform strains that can be used in the next generation industrial biotechnology where an economy of energy and water is needed (Chen and Jiang, 2018).

Microbial exopolymers are a group of high molecular weight molecules that can have various applications in different industrial fields. They provide many advantages over other types of polymers such as safety, biodegradability and sustainable production (Rehm, 2010). The research for new bacteria producing exopolymers with challenging properties has become the aim of many research papers because a number of industries try to use natural polymers in their combinations in order to follow the new customers tendency of using biological and natural products (Poli et al., 2010; Finore et al., 2014; Hussain et al., 2017).

Among the multiple applications of exopolymers, emulsifying and antioxidant activities are two important properties that are suitable for application in food and cosmetic industries since this type of productions need natural emulsifying agents to stabilize their mixtures and antioxidants to protect their products from oxidation (Poli et al., 2010; Carocho et al., 2018; Yildiz and Karatas, 2018). Antioxidants are also much needed in pharmaceutical industry to be applied as radical scavengers to protect the body from free radicals that can cause different chronic diseases (Carocho et al., 2018).

In this work we focus on the isolation of exopolymer producing halophilic bacteria from different hypersaline environments in Morocco and on the study of the emulsifying and antioxidant activities of the produced polymers.

2. Material and methods

2.1 Sample collection

Four natural hypersaline environments in Morocco where chosen for this study. Samples of soil, wetland and water were collected from a saltern and natural saline soil situated in douar Marigha (Ouirgane, Al Haouz province), natural saline soils in doaur Hjar Melaghi and douar Halhal (Ouezzane province) and from salt mine in Oued Amlil (Taza province) (Figure 1).

2.2 Culture media and strains isolation

The samples were diluted in 5% (w/v) sea salts solution, transferred to plates containing MY agar medium (Moraine and Rogovin, 1966) supplemented with different concentrations of sea salts solution (1, 2, 3, 5, 7.5, 10, 15, 20, 25 and 30% w/v) (Rodriguez-Valera et al., 1981) and incubated at 37ºC for 7 days. Exopolymer producing bacteria were selected based on the mucoid aspect of their colonies. Selected strains were transferred to new plates of the same medium and stored aerobically at 4ºC and as glycerol solution 20% at -80ºC.

2.3 Phenotypic characterization

Salt tolerance was determined using solid MY medium at different sea salts concentration 0–30% (w/v). Growth at different pH 5–10 and different temperatures 4–45ºC was studied on solid MY medium supplemented with 5% (w/v) sea salts solution. Other phenotypic characteristics were studied using the methods previously described (Ventosa Ucero et al., 1982; Quesada et al., 1983; Mata et al., 2002). Susceptibility to antibiotics was tested according to the method described by Bauer et al. (1966).

2.4 Strains identification

DNA extraction, PCR amplification, and sequencing of the 16S rRNA gene were performed using previously described methods (Miñana-Galbis et al., 2007). Pairwise sequences similarity values between the obtained 16S rRNA sequences and reference sequences were calculated by

the GenBank database obtained from the National Centre of Biotechnology Information database using the BLAST search. Phylogenetic analyses were made using the MEGA software version 6 using neighbor joining method with bootstrap values based on 1000 replications.

2.5 Exopolymers production and determination of total carbohydrates contents

The production was carried out on the complex media MY supplemented with 5% (w/v) sea salt solution. The cultures were incubated for 7 days at 30ºC with orbital shaking at 100 rpm. The extraction of exopolymers was done as follows: the culture was centrifuged at 7.000 rpm for 1 h; the polymer was then precipitated from cell-free supernatant with three volumes of cold ethanol 96% (v/v) and kept at 4ºC for 24 h. The precipitated polymer was then recuperated by centrifugation at 7.000 rpm for 10 min, rinsed with water, centrifuged at 7.000 rpm for 1 h, lyophilized and finally weighted. The total content of carbohydrates in the polymers was estimated following the phenol-sulphuric acid method modified by Chaplin (Dubois et al., 1956; Chaplin, 1982), using glucose as standard.

2.6 Emulsifying activity of exopolymers

Emulsifying activity was studied following the procedure previously described (Cooper and Goldenberg, 1987). Briefly, mixtures of equal volumes of various hydrophobic substrates and exopolymers solutions (0.5% w/v) in distilled water were vortexed for 2 min and allowed to stand for 24 h. Emulsifying activity was expressed as the percentage of the total height occupied by the emulsion after 24 h. The hydrophobic substrates tested were sunflower (commercial brand), paraffin oil and diesel. Tween 80 and xanthan gum were used as controls.

2.7 Antioxidant assays

2.7.1 Total antioxidant assay

Total antioxidant activity was determined by the method of Prieto et al. (1999). The antioxidant activity is revealed when the polymer reduces Mo (VI) to Mo (V) and a green phosphate/Mo (V) complex at acid pH is formed. Exopolymers solutions at 2.5 mg/ml were mixed with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95ºC for 90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a control. L-

ascorbic acid was used as standard. The antioxidant activity was expressed as ascorbic acid equivalent.

2.7.2 DPPH free radical Scavenging

The capacity of scavenging DPPH free radical was studied following the method of Zhang et al. (2013). Briefly, 2 ml ethanolic DPPH radical solution (0.05 mM) was mixed with 1 ml of exopolymers solutions at concentration of 2.5 mg/ml. After mixing vigorously, the mixture was incubated in the dark for 30 min at room temperature. The blanks contained only ethanol and the controls included deionized water and DPPH solution. L-ascorbic acid was used as standard. The absorbance of the samples was measured in triplicate at 517 nm after centrifugation at 8.000 rpm for 10 min. The scavenging ability was defined as: Scavenging activity (%) = [1 − (Asample − Ablank)/Acontrol] × 100.

3. Results and discussion

3.1 Phenotypic characterization

After processing all the samples, a total of 193 halophilic strains were isolated. Ten colonies were selected on the basis of their distinctive morphology and their mucoid aspect that indicated the capacity of exopolymer production.

The isolated strains were short rods, Gram-negative moderately halophilic bacteria (Table 1). They grew optimally at 5–10% (w/v) of total salt. No growth was observed at concentrations under 2–3% or higher than 20% with the exception of one isolate that grew at concentration up to 25%. The strains were catalase- and oxidase- positive, neutrophilic and mesophilic bacteria that grew at pH from 6 to 8 and temperature between 20 to 37 ºC with the exception of some isolates that showed growth at 4 and 40 ºC and pH up to 9. Some isolates have shown their ability to produce hydrolytic enzymes (lipase, gelatinase and tyrosinase) which demonstrates their potential for other biotechnological applications as source of hydrolytic enzymes (Ali Amoozegar et al., 2007; Beygmoradi and Homaei, 2017; Menasria et al., 2018).

As shown in Table 1, all the strains were susceptible to amoxicilline/clavulanic acid, ceftriaxone, norfloxacin, polymixin B, rifampicin, chloramphenicol, ciprofloxacin and gentamycin and resistant to oxacillin, with the exception of strain N12 that showed an

intermediate response. Other antibiotics had different response depending on the strain. Resistance to antibiotics can be attributed to many factors. A recent study of halophilic bacteria resistance to antibiotics has reported that two drug resistant strains that belong to *Halomonas* and *Marinobacter* genera contain both plasmids and efflux pumps which are considered as the most plausible mechanism that can be conferring them resistance to antibiotics (Shinde and Thombre, 2016).

3.2 Strains identification

The comparison of 16S rRNA gene sequence of the isolates with reference sequences revealed that strains belong to two genera: *Halomonas* genus (*Halomonadaceae* family) and *Marinobacter* genus (*Alteromonadaceae* family) with 99% similarity to the closely related species. The phylogenetic tree (Figure 2) showed the high species diversity of the *Halomonas* genus and its domination in number of bacterial isolates: *H. stenophila* (6 isolates), *H. rifensis* (1 isolate), *H. Ventosae* (1 isolate) and *H. koreensis* (1 isolate) whereas the only *Marinobacter* isolate was affiliated to *M. adhaerens*. These results are in concordance with other research papers that focused on the extensive research of new exopolysaccharide producing halophilic bacteria from hypersalins environments in Spain and Morocco and resulted in the isolation and description of several strains belonging to the *Halomonas* genus (Bouchotroch et al., 2001; Martínez-Checa et al., 2005; Llamas et al., 2011; Amjres et al., 2011).

Even though our isolates share a high similarity with the type strains previously described, 16S rRNA is a highly conserved sequence within the *Halomonas* family species and closely related species cannot be distinguished (de la Haba et al., 2012). Thus, our strains are considered new isolates with a great potential to be producing novel exopolymers.

3.3 Exopolymers production and total carbohydrates content

The results of exopolymers production by the isolated strains when grown in MY medium are shown in Table 2. The highest yield was obtained from strain N8 which produced 5.82 g/l. Other strains have shown good yields varying from 1.285 to 5.438 g/l with the exception of strains N1 and N4 that produced the lowest quantities (0.55 and 0.498 g/l, respectively).

166 Generally, our isolates produced high quantities of exopolymers in comparison with other
167 halophilic closely related strains like *H. ventosae* Al12^T and Al16 that excreted 0.2835 and
168 0.2895 g/l respectively and *H. anticariensis* strains FP35^T and FP36 with about 0.2965 and
169 0.4995 g/l respectively (Mata et al., 2006). Strains N8, N9 and N10 were good exopolymer
170 producers (>5 g/l) and better than *H. almeriensis* M8^T (1.7 g/l), *H. stenophila* HK30 (3.89 g/l)
171 and *H. nitroreducens* WB1 (<1.4 g/l) (Llamas et al., 2012; Amjres et al., 2015; Chikkanna et al.,
172 2018), however, they didn't reach the level of production of *H. xianhensis* SUR308 which
173 produced 1.7 g/l when grown in malt extract–yeast extract medium supplemented with 2.5%
174 NaCl, 0.5% casein hydrolysate and 3% glucose (Biswas et al., 2015).

175 The carbohydrates content of the produced exopolymers varies from a strain to another
176 (Table 2). Strains N1, N4, N5 and N7 produced exopolymers with low carbohydrates fraction
177 varying from 17.42 to 18.88 % (w/w), while strains N2, N8, N9, N10, N11 and N12 produced
178 polymers rich with carbohydrates ranging between 25.71 and 48.68% (w/w). Exopolymers are
179 known to be composed mostly of carbohydrates; however, the presence of other organic fractions
180 such as of uronic acids, proteins, amino acids, ester-linked substituents and pyruvate ketals have
181 been reported (Raj et al., 2018).

182 3.4 Emulsifying activity

183 The emulsifying activity of all the polymers at a concentration of 0.5% (w/v) against three
184 different hydrophobic compounds, namely cosmetic oil, food oil and hydrocarbon is shown in
185 Table 3. All the polymers showed an interesting emulsifying activity against the three different
186 hydrophobic phases.

187 The exopolymers from members of *Halomonas* genus were able to produce better emulsion
188 than Tween 80 in the case of paraffin oil, with the exception of the exopolymer from strain N1
189 that produced a lower emulsion. The exopolymer produced by strain N4 gave the best results
190 among all the biopolymers studied in the present work. It exhibited high emulsifying activity
191 against the three hydrophobic substrates. The obtained emulsions were fairly very stable after 24
192 h. The polymer´s emulsifying behavior was better than xanthan gum in the case of diesel oil and
193 better than the chemical control Tween 80 in the case of paraffin oil.

Halophilic bacteria are a great source of exopolymers with emulsifying activity. Many studies have demonstrated the potential of some exopolysaccharides produced from halophilic bacteria to be used as emulsifying agent (Mata et al., 2008; Llamas et al., 2012; Amjres et al., 2015; Chikkanna et al., 2018), however, to the best to our knowledge, this is the first time that an exopolymer from *Marinobacter* genus has been studied for its application as emulsifier agent.

The results obtained in this study prove the potential application of these biopolymers and specially the one produced by *Marinobacter* sp. N4 in food, cosmetics and oil industries as biological emulsifying agents with the multiple advantages they offer over other chemical products such as safety, biodegradation and stable cost and supply (Gugliandolo et al., 2014; Hussain et al., 2017).

3.5 Antioxidant activity

3.5.1 Total antioxidant activity

The total antioxidant activity based on the reduction of Mo (VI) to Mo (V) by the exopolymers and subsequent formation of a green phosphate Mo (V) complex at acidic pH is shown in Figure 3. The results showed that all the exopolymers exhibited an antioxidant activity and were able to reduce Mo (VI) to Mo (V). The highest activity was obtained with the polymer produced by *Marinobacter sp.* N4 which was equivalent to 68.94 µg/ml of ascorbic acid at a concentration of 2.5 mg/ml.

Among the strains of *Halomonas* genus, the polymer from the isolate N5 exhibited the best activity where 2.5 mg/ml was equivalent to 27 µg/ml of ascorbic acid, whereas the polymer from isolate N1 gave the lowest equivalence value of 14 µg/ml of ascorbic acid.

Generally, all the exopolymers had low equivalence values of ascorbic acid which means that they have moderate reduction ability. A similar study of an exopolysaccharide from a halophilic bacteria *Labrenzia* sp. has also reported low equivalence values of ascorbic acid (Priyanka et al., 2014).The total antioxidant activities obtained in the present study were higher compared to the polymer produced by the extremely halophilic archaea *Haloterrigena turkmenica* (5mg/ml EPS equivalents 2 µg/ml of ascorbic acid) (Squillaci et al., 2016). The obtained data demonstrate the ability of the polymers to change the oxidation state of molecules which is one of the mechanisms responsible for free radical neutralization (Lü et al., 2010).

3.5.2 DPPH free radical scavenging

DPPH free radical scavenging ability of the exopolymers is shown in Figure 4. All the polymers were able to exert a scavenging activity; however, some of them were more efficient than others. The exopolymer produced by *Halomonas* sp. N10 exhibited a more powerful activity than other *Halomonas* exopolymers with an activity of 33.85%. Whereas, the exopolymer produced by *Marinobacter* sp. N4 was the most effective among all the tested polymers with an activity of 72.75%; which is higher than the activity obtained by several exopolymers produced from the halophilic bacterium *H. nitroreducens* (<70%) (Chikkanna et al., 2018) and archaeon *Haloterrigena turkmenica* (<40%) (Squillaci et al., 2016) when tested at the same concentration. The scavenging activity was even better than that of the polymer produced by non halophilc bacteria *Lactobacillus plantarum* (72% at 4mg/ml) (Wang et al., 2017).

The antioxidant activity of these exopolymers may be due to their content in some functional groups such as sulfate that could be playing an important role in scavenging and chelation reactions (Qi et al., 2006; Rocha De Souza et al., 2007; Priyanka et al., 2014).

Lipid oxidation is a major problem that causes the loss of food quality. It also can cause the formation of potentially toxic reaction products, such as carcinogenic or inflammation-inducing substances. Thus, the control of lipid oxidation in oil-in-water emulsion is considered a major challenge because some emulsifiers can accelerate the process of lipids oxidation (McClements and Gumus, 2016). However, our exopolymers have shown their ability to exhibit both emulsifying and antioxidants activities which proves their potential to be used as emulsifiers that can prevent lipid oxidation in food industry.

Conclusion

In this work, we isolated and identified 10 halophilic strains that produce exopolymers with antioxidant and emulsifying activities. The majority of the isolates yielded high levels of exopolymer. The polymer from *Marinobacter* sp. N4 was the most efficient among all the polymers in emulsifying different mixtures of water and hydrophobic substrates and maintaining stable emulsions. Furthermore, it had the best total antioxidant activity and was the most powerful in scavenging DPPH free radicals which prove its potential to be applied in food, cosmetics and oil industries as safe, natural and biodegradable antioxidant an emulsifying agent.

However, its yield is still inadequate for wide applications in industrial sectors. Further studies should be carried on in order to get a higher production through optimization of culture conditions and control of growth parameters in a fermenter.

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Conflicts of interest

The authors declare that they have no conflict of interest.

263 Tables:

264 Table 1: Salt, pH and temperature ranges for growth, biochemical characteristics and
265 susceptibility to antibiotics of the isolated strains.

	N1	N2	N4	N5	N7	N8	N9	N10	N11	N12
Salt range (% w/v)	2-15	2-25	2-20	2-20	3-20	3-20	2-20	2-20	3-20	2-20
pH range	6-8	6-9	6-9	6-8	6-8	6-8	6-8	6-8	6-8	6-9
Temperature range (°C)	15-40	10-37	4-40	15-37	15-37	15-37	15-37	15-37	15-37	15-37
Hydrolysis of:										
Gelatin	+	–	–	+	–	–	–	–	–	_
Starch	–	–	–	–	–	–	–	–	–	_
Casein	–	–	–	–	–	–	–	–	–	_
Tween 20	+	–	+	–	+	+	–	–	–	+
Tween 80	–	–	+	–	–	–	–	–	–	_
DNA	–	–	–	–	–	–	–	–	–	_
Tyrosine	+	–	–	–	+	+	–	–	–	+
Lecithin	–	–	+	–	–	–	–	–	–	_
Urea	+	–	–	–	–	–	–	–	–	_
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	–	+	+	+	+	+	+	+	+	+
Nitrite reduction	–	+	–	–	–	–	–	–	+	–
Acid from glucose	–	–	–	–	–	–	–	–	–	_
VP/RM	–	–	–	–	–	–	–	–	–	_
Indole production	–	–	–	–	–	–	–	–	–	_
Nitrate respiration	–	+	–	–	–	–	–	–	–	_
Growth on MacConkey agar	–	+	–	–	–	–	–	–	–	_
Growth on cetrimide agar	–	–	–	–	–	–	–	–	–	_
Susceptibility to antibiotics:										
Amoxicillin/clavulanic acid [30 µg]	S	S	S	S	S	S	S	S	S	S
Ampicillin [10 µg]	I	S	S	S	S	S	S	S	I	S
Ceftriaxone [30 µg]	S	S	S	S	S	S	S	S	S	S
Doxycycline hydrochloride [30 µg]	R	R	S	R	R	S	S	S	S	S
Norfloxacin [10 µg]	S	S	S	S	S	S	S	S	S	S
Oxacillin [5 µg]	R	R	R	R	R	R	R	R	R	I
Penicillin G [6 µg]	R	S	S	S	S	S	S	S	R	S
Polymixin B [300 µg]	S	S	S	S	S	S	S	S	S	S
Rifampicin [30 µg]	S	S	S	S	S	S	S	S	S	S
Spectinomycin [100 µg]	R	S	S	S	S	S	S	S	S	S

Cefuroxime [30 µg]	R	R	R	R	R	I	I	I	R	S
Chloromphenicol [30 µg]	S	S	S	S	S	S	S	S	S	S
Ciprofloxacin [5 µg]	S	S	S	S	S	S	S	S	S	S
Gentamycin [30 µg]	S	S	S	S	S	S	S	S	S	S
Pristinamycin [15 µg]	I	I	I	R	S	S	S	S	S	S

Table 2: Yield in exopolymers and exopolymers carbohydrates contents

Strains	Yield in exopolymer g/l	Exopolymer carbohydrates content % (w/w)
N1	0.55	17.86
N2	2.726	48.68
N4	0.498	17.64
N5	1.285	17.42
N7	3.839	18.88
N8	5.82	37.26
N9	5.026	29.03
N10	5.438	25.71
N11	2.571	39.56
N12	4.448	39.94

Table 3: Emulsifying activity of the exopolymers produced by the isolated strains

Hydrophobic substrate	Emulsifying activity %[a]											
	N1	N2	N4	N5	N7	N8	N9	N10	N11	N12	Tween 80	Xanthan
Paraffin oil	18.23	39.72	52.89	39.65	29.45	32.88	45.65	29.45	32.79	40.54	23.6	87.9
Sunflower oil	26.18	31.36	56.28	38.41	30.3	30.07	33.33	23.83	26.84	27.57	56	62.6
Diesel oil	21.21	27.36	58.66	28.99	37.73	28.9	25.72	30.76	10.16	11.49	68	56.06

[a] Expressed as the percentage of the total height occupied by the oil–water emulsion after 24 h; 0.5% w/v exopolymer and xanthan gum or the chemical surfactants was used as emulsifier. Each value represents the average of three measurements.

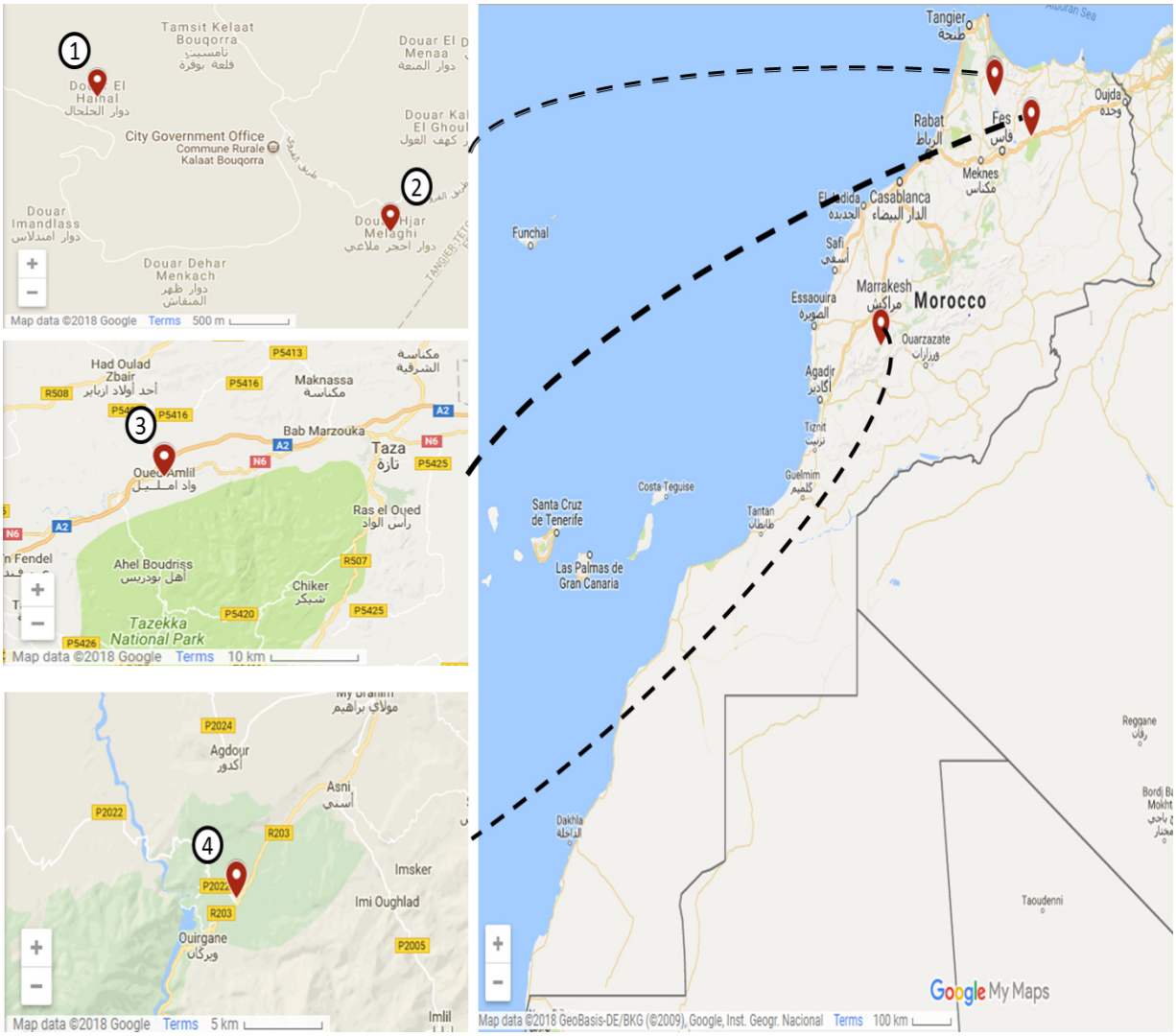
Figure captions:

Figure 1: Sampling locations map of the studied hypersalins environments. 1. Douar El Halhal, Ouezzane province; 2. Douar Hjar Melaghi, Ouezzane province; 3. Oued Amlil, Taza province; 4. Douar Marigha, Ouirgane, Al Haouz province.

Figure 2: Phylogenetic tree showing relationship between the studied isolates and closely related species. The tree is based on neighbor joining algorithm. Bar: 2% sequence divergence. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at the branch points.

Figure 3: Total antioxidant activity of the exopolymers synthesized by the isolated strains.

Figure 4: DPPH free radical scavenging activity of the exopolymers synthesized by the isolated strains.



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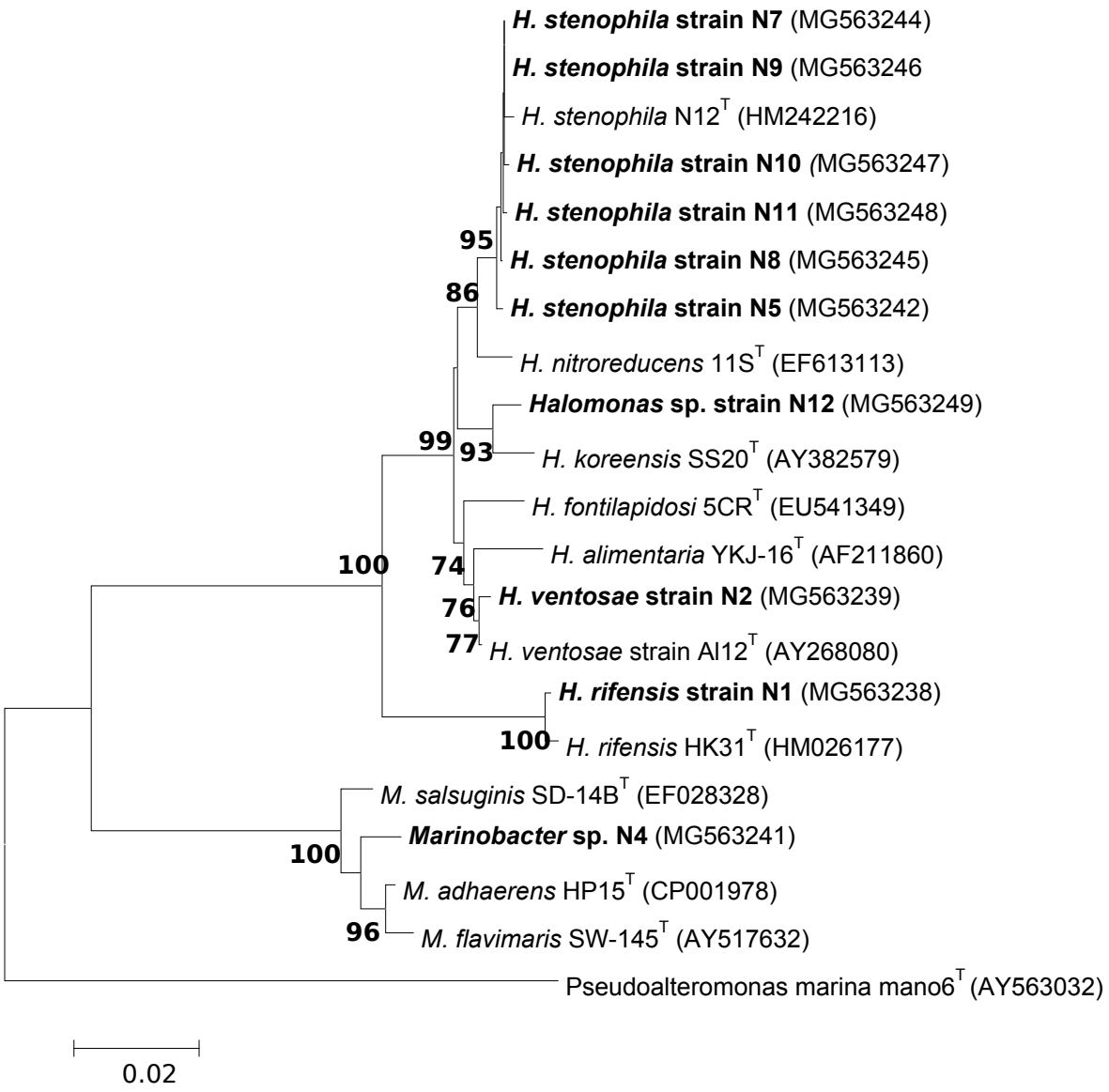

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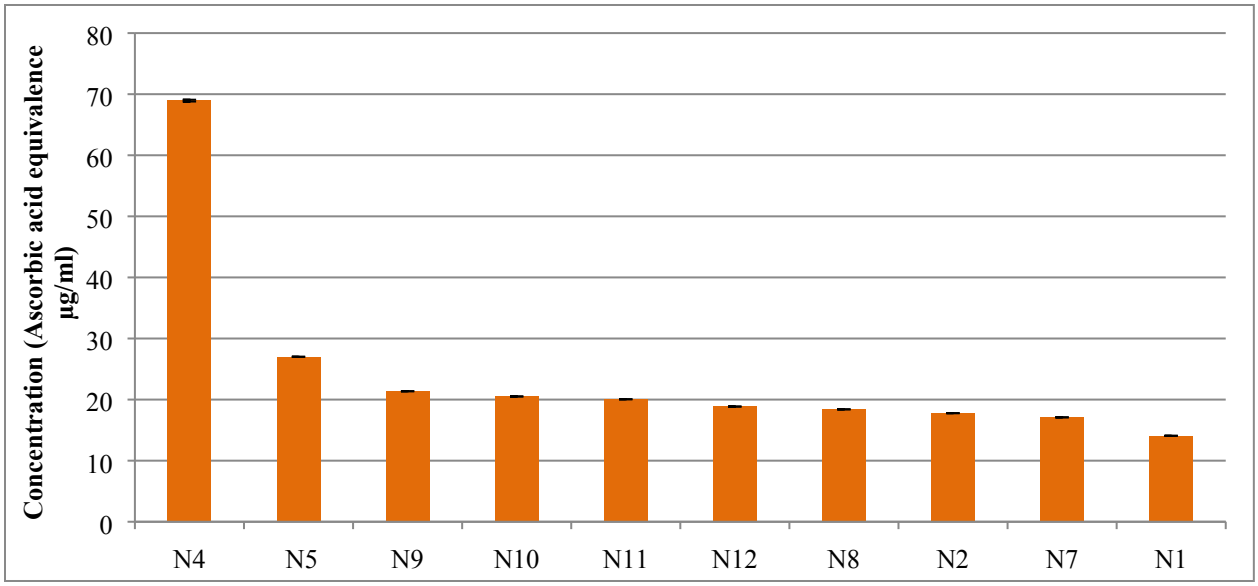

299

300 Figure 3: Total antioxidant activity of the exopolymers synthesized by the isolated strains.

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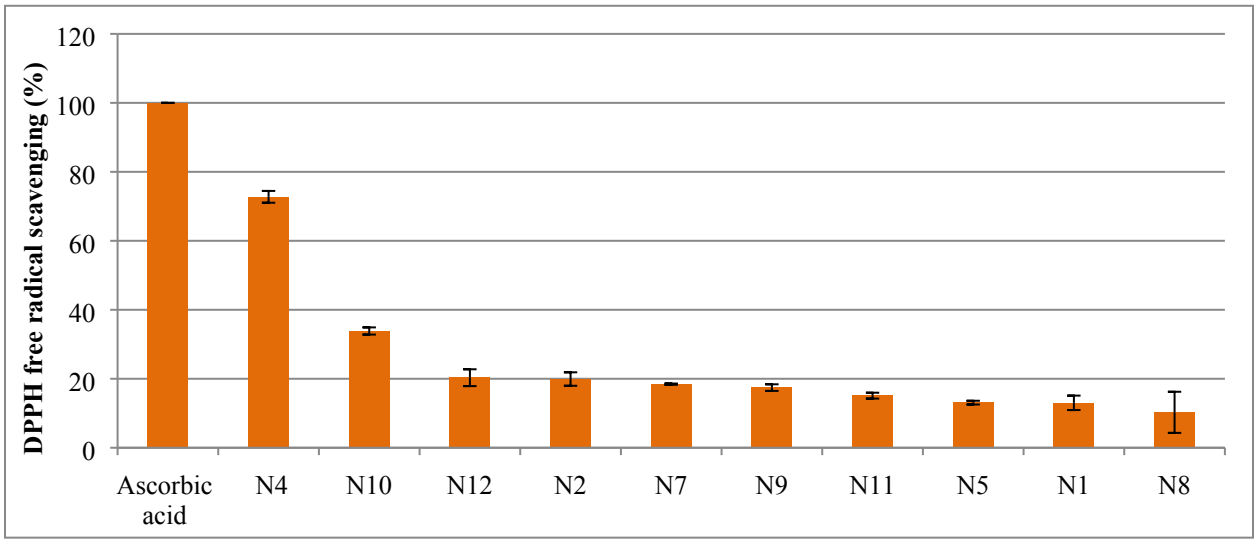

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303 Figure 4: DPPH free radical scavenging activity of the exopolymers synthesized by the isolated
304 strains.

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