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Bacterial exopolysaccharides produced by newly discovered bacteria belonging to the genus *Halomonas*, isolated from hypersaline habitats in Morocco

S Bouchotroch[1], E Quesada[1], I Izquierdo[2], M Rodríguez[2] and V Béjar[1]

[1]*Microbial Exopolysaccharide Research Group, Department of Microbiology, Faculty of Pharmacy, University of Granada, 18071 Granada, Spain;* [2]*Department of Organic Chemistry, Faculty of Pharmacy, University of Granada, 18071 Granada, Spain*

We have studied the exopolysaccharides (EPS) from a new group of moderately halophilic bacteria belonging to the genus *Halomonas*. The quantity of EPS produced, its chemical composition and physical properties depend greatly upon the bacterial strain. The majority of the polymers produced viscous solutions and/or emulsified different hydrocarbon compounds. The most interesting strain, S-30, produced EPS at 2.8 g/l with a maximum viscosity of 23.5 Pa·5 and exhibited pseudoplastic behavior. This EPS emulsified five hydrocarbons more efficiently than did four control surfactants tested. Its monosaccharide composition was glucose:galactose:manose:glucuronic acid in equimolar ratio. Some two-thirds of the strains tested emulsified crude oil better than control surfactants did. There are many potential industrial applications for polysaccharides with these qualities. *Journal of Industrial Microbiology & Biotechnology* (2000)**24,** 374–378.

Keywords: exopolysaccharides; *Halomonas*; halophilic bacteria; viscosifier; crude-oil emulsifier

Introduction

For several decades, interest has been shown in the capacity of microorganisms to produce exopolysaccharides (EPS) because these biopolymers often possess advantageous qualities not shared by many polysaccharides currently in use, which are generally extracted from plants or marine algae. EPS have a wide range of uses in many sectors of industry as thickening, gelling, suspendant and surfactant agents [13,29,31,32].

This study is part of a wide research program aimed at discovering and identifying microorganisms of biotechnological importance in hypersaline habitats. Hypersaline habitats are characterized by their high salt concentration, intense solar radiation and low oxygen content and often harbour microorganisms of considerable interest to biotechnology. We have reported EPS production by strains of *Halomonas eurihalina*, a moderately halophilic bacterium isolated from saline habitats [3,22]. These strains produced 0.8–1.6 g/l of EPS. The most notable characteristics of these polymers are their capacity to increase the viscosity of solutions at low pH values and to emulsify several hydrocarbon compounds [8,9,17].

We describe here the production of extracellular polysaccharides by a new group of microorganisms belonging to the genus *Halomonas* [5], isolated from a solar saltern in Morocco. We also report on the chemical and rheological properties of the polymers and their emulsifying activity.

Correspondence: Professor E Quesada, Departamento de Microbiología, Facultad de Farmacia, Campus Universitario de Cartuja s/n, 18071 Granada, Spain
E-mail: e.quesada@ugr.es
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Materials and methods

Bacterial strains

From more than 500 strains of halophilic microorganisms that we isolated in northern Morocco, we selected for this study 32 strains from three different salterns, located at Asilah (10 strains), Larache (13 strains) and Souk el Arbaa (9 strains). These strains appear to constitute new taxa of moderately halophilic bacteria [5].

Culture medium

To collect the EPS, the selected bacterial strains were grown for 5 days (shaken at 100 rpm, 32°C) in 200-ml Erlenmeyer flasks, each containing 100 ml of MY complex medium [19], supplemented with a sea-salt solution [25] to reach 7.5% (w/v) salt concentration.

We described the methods for isolation and purification of EPS in previous papers [22,23]. Briefly, the cultures are centrifuged and the polymers precipitated from supernatants with three volumes of cold ethanol before being ultracentrifuged, dialysed against distilled water and lyophilized.

Analytical procedures

Colorimetric analysis: We made the following colorimetric analyses: total carbohydrates [14], proteins [6], uronic acids [4] and acetyl residues [18].

Sugar analysis: We methanolysed the EPS (12 mg) with 3% (v/v) methanolic HCl (2 ml) at 80°C for 7 h. An aliquot (0.5 ml) was concentrated, co-distilled with methanol and trimethylsilylated. It was then analyzed by gas–liquid chromatography (GLC) (program A).

Samples were hydrolysed with 4 M trifluoroacetic acid at 110°C for 20 h. The free sugars were then transformed into peracetylated alditols by reduction with $NaBH_4$ followed by acetylation with acetic anhydride:sulfuric acid (15:2 v/v) [16] at room temperature overnight. The mixture was analyzed by GLC (program B) and gas chromatography-mass spectrometry (GC-MS).

Unambiguous identification of sugars was by GLC and GC-MS with sugar standards. GLC runs were performed on a Hewlett-Packard 5890 A instrument fitted with a flame ionization detector and equipped with an HP-5 column with an He flow of 1.1 ml/min, split ratio 100:1. The temperature program used was A: 200–250°C at 5°C/min, B: 210°C for 10 min and from 210°C to 250°C at 10°C/min. GC-MS was performed on a FISONS VG, PLATFORM II instrument equipped with an SP-2380 column and with an He flow of 1.5 ml/min. The temperature program was 125°C for 2 min and from 125°C to 270°C at 5°C/min.

Rheological studies

Lyophilized EPS were dissolved in distilled water to give 1% (w/v) solutions. Viscosity was measured with a Bohlin Rheometer CSR-10 at 25°C with shear stresses of between 3 and 30 Pa.

Emulsification assay

The emulsifying activity of the EPS was determined by a modified version of the procedure described by Cooper and Goldenberg [12]. Equal volumes of the different EPS solutions [1% (w/v) in distilled water] and various hydrocarbons were added to 12-mm-diameter glass tubes. The mixtures were mixed vigorously using a vortex and allowed to stand for 24 h. Emulsifying activity was expressed as the percentage of the total height occupied by the emulsion. The hydrocarbons used were *n*-octane (Sigma), *n*-hexadecane (Sigma), *n*-tetradecane (Sigma), crude oil (heavy Iran type) and mineral oil (Sigma). We used Tween 20, Tween 80, Span 60 and Triton X-100 (Sigma) as control surfactants.

Results

EPS production varied widely from strain to strain (Table 1). All bacteria synthesized more than 0.3 g EPS/l culture medium. Eighteen strains produced more than 1 g/l, and strain S-30 achieved a maximum of 2.8. The gross chemical composition of the EPS is set out in Table 1. The results are expressed as percentages of total dry weight of the polymers. Carbohydrates made up between

Table 1 Productivity, chemical composition and viscosity of the EPS

Strains and origin	EPS production		Chemical composition				Viscosity at 4.5 Pa shear stress
	g EPS/l	g EPS/g DCW	CH	Pr	UA	Acet	
Asilah							
S-1	1.2	0.23	19.6	2.2	3.0	0.7	0.33[a]
S-5	0.6	0.20	34.7	1.6	5.9	0.6	0.045
S-7	1.1	0.42	47.0	0.7	5.3	1.9	0.064
S-10	1.1	0.18	40.6	3.7	6.7	1.4	0.19
S-30	2.8	0.26	40.9	5.3	9.1	0.5	23.48
S-31	1.5	0.28	60.2	0.3	16.3	0.03	10.98
S-32	1.7	0.26	37.0	4.1	4.1	0.2	6.18
S-33	1.2	0.07	26.5	0.7	5.1	0.1	0.022
S-35	2.7	0.26	49.4	0.3	2.7	0.1	0.89
S-36	1.3	0.08	48.6	0.3	6.3	0.1	0.071
Larache							
S-14	1.5	0.22	37.5	1.6	4.5	0.3	0.09
S-19	1.0	0.14	33.9	1.0	4.2	1.7	0.13
S-24	1.0	0.20	30.8	0.6	6.1	0.2	1.8
S-25	0.9	0.07	37.9	0.2	4.1	0.1	0.27
S-26	0.9	0.26	47.6	1.0	5.0	1.5	0.09
S-27	0.3	0.10	45.3	1.0	3.4	0.2	0.024
S-28	2	0.26	36.4	0.5	3.1	0.2	0.24
S-38	1.6	0.17	6.6	0.5	1.3	0.1	0.43
S-41	2.2	0.20	17.6	0.7	4.2	ND	0.72
S-42	0.6	0.05	22.8	1.2	2.4	0.7	0.073
S-43	0.4	0.34	32.8	0.4	5.6	0.9	0.93
S-45	0.5	0.17	24.4	1.4	9.2	0.6	0.035
S-46	1.3	0.27	25.1	0.5	6.0	0.7	0.79
Souk el Arbaa							
S-6	0.7	0.10	58.4	0.7	5.0	1.3	0.055
S-34	1.4	0.15	29.9	1.3	3.7	0.1	0.027
S-39	0.7	0.14	13.3	0.9	2.5	0.3	0.021
S-40	0.6	0.41	14.0	1.3	1.3	0.2	0.021
S-44	0.9	0.45	8.4	0.8	2.8	0.3	0.023
S-47	0.5	0.13	19.9	0.7	3.5	0.6	0.026
S-48	1.4	0.30	46.6	1.4	4.0	3.0	0.089
S-49	0.4	0.10	33.3	1.3	1.6	2.3	0.043
S-50	0.5	0.14	43.8	1.4	3.2	0.9	0.082

[a]Viscosity in Pa·5.
DCW=Dry cell weight; CH=Carbohydrates; Pr=Proteins; UA=Uronic Acids; Acet=Acetyls; ND=not detected.

6.6% and 60.2% (w/w) (strains S-38 and S-31), whilst proteins and acetyls accounted for less than 2% (w/w) in the majority of strains. Using Blumenkrantz and Asboe-Hansen's method [4], we detected uronic acid in all the polymers studied, particularly in S-30 and S-45 [9.2% and 9.1% (w/w), respectively]. A percentage of the polymer was not detected by the colorimetric methods used, which might be due to the existence of inorganic compounds linked to the EPS, just as has been found with the expolysaccharides from *H. eurihalina* strains [3] and from some other bacterial sources [24]. To evaluate the rheological properties of the EPS, we measured their viscosity at shear stresses ranging from 3 to 30 Pa. Table 1 shows the viscosity values at a shear stress of 4.5 Pa. EPS solutions from 18 strains (all those isolated from Souk el Arbaa and some from the other two habitats) gave low viscosity values (less than 0.1 Pa) whilst 11 EPS gave intermediate values (from 0.1 to 1.8 Pa). The polymers from strains S-30, S-31 and S-32 were the most viscous (23.48, 10.98 and 6.18 Pa, respectively). All EPS exhibited pseudoplastic behavior, their viscosity decreased concomitantly with an increase in shear stress. In Figure 1, we represent the pseudoplastic behaviour of the EPS that produced the most viscous solutions.

One of the most suitable ways of screening potential biosurfactant-producing microorganisms is to use Cooper and Goldenberg's [12] method to estimate the percentage of emulsification after 24 h. In this way, we observed that 21 strains produced EPS that emulsified at least one of the hydrocarbons tested (crude oil) to a greater extent than did the controls. Six of these strains produced EPS capable of emulsifying two or more hydrocarbons (Table 2). The best emulsifiers were those from strains S-32 (four hydrocarbons) and S-30 (all five hydrocarbons assayed). The most salient feature of both these EPS is that once the emulsions were formed, they were not broken down by coalescence of oil droplets.

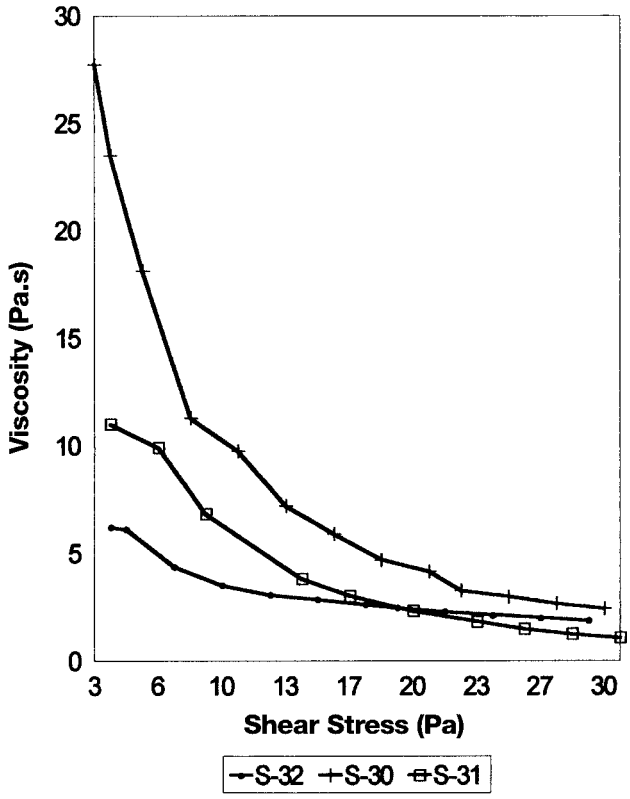

Figure 1 Rheological behaviour of EPS S-30, S-31 and S-32.

Table 2 Emulsifying activity (%) of *Halomonas* strains

Strains and origin	Crude oil	Mineral oil	Octane	Hexadecane	Tetradecane
Asilah					
S-1	20.8	6.8	8.5	8.5	8.5
S-5	**27.3**	56	11.9	35.6	59.3
S-7	**35**	22	22	10.3	8.8
S-10	14.3	4.4	22	4.4	3.7
S-30	**70.2**	**83**	**62.7**	**78**	**71.2**
S-31	**30**	4.4	11.8	7.4	5.9
S-32	**65**	19.5	**62.3**	**65**	**63.6**
S-33	18.2	9.1	13	10.4	9.1
S-35	**36.4**	3	7.4	4.4	7.4
S-36	**75**	44.1	20.6	29.4	23.5
Larache					
S-14	**37.7**	57.1	24.7	14.3	15.6
S-19	**75.3**	**64.4**	45.8	18.6	57.6
S-24	**44.2**	10.4	23.4	9.1	10.4
S-25	**54.5**	10.2	8.5	5.1	8.5
S-26	14.3	29.4	44	10.3	7.4
S-27	**36.4**	33	10	50	36
S-28	**73**	7.8	5.2	54	10.4
S-38	19.5	36.8	55.9	22	4.4
S-41	**65**	3.9	9.1	6	7.8
S-42	22	27.7	30.8	4.6	10
S-43	**41.6**	5.9	4.4	5.9	5.9
S-45	9.1	42.4	3.4	5.1	8.5
S-46	9.1	26.5	55.9	5.9	5.9
Souk el Arbaa					
S-6	16.9	49.2	49.2	17	23
S-34	**74**	**67.8**	45.8	8.5	11.9
S-39	**45.5**	35.9	47.7	6.2	15.4
S-40	**37.7**	42.4	15.2	5.1	6.8
S-44	19.5	11.8	19.1	5.9	5.9
S-47	**60.3**	36.8	**60.3**	11.8	5.9
S-48	**58.8**	**60.3**	22	25	10.3
S-49	**28.6**	5.9	57.3	14.7	17.6
S-50	7.8	56	10.2	6.8	10.2
Biosurfactants controls					
Tween 20	23.4	48	58.4	52	61
Tween 80	23.4	58.4	55.8	57.1	58.4
Triton X-100	15.1	55.9	58.8	58.8	60.3
Span 60	0	2.9	26.5	8.8	58.8

Bold numbers indicate higher emulsifying activities than controls.

The polymers with the best rheological properties and emulsifying activity, EPS S-30, S-31 and S-32, were chemically characterized by GLC and GC-MS. Our analyses indicated the presence of glucose, manose and galactose in EPS S-31 and S-32 in ratios of 1:4:2.5 and 5:1:1.6, respectively. EPS S-30 was composed of glucose, manose, galactose and glucuronic acid in equimolar ratio.

Discussion

Certain moderately halophilic microorganisms found in hypersaline habitats have already proved to have interesting properties

capable of being used in industry. They include a taxonomically diverse array of bacteria and some archaea [33] and grow best at NaCl concentrations between 0.5 and 2.5 M. The 32 strains selected for this study belong to the genus *Halomonas* and all produce extracellular polymers. Recently, we suggested that these microorganisms should be classified as new species of *Halomonas* [5] and are currently engaged in establishing their definitive classification. On comparing the G+C composition and carrying out DNA–DNA hybridization of these strains with validly published *Halomonas* species phenotypically related to them, our results [5] showed that these 32 strains constitute at least a new species, and thus we are currently engaged in characterizing their 16S rRNA genes to resolve their definitive classification.

A comparison of their EPS indicates that their characteristics (yield, chemical composition and physical properties) vary from strain to strain. This feature has been observed previously for this genus by other authors on comparing various polymers synthesized by different strains of a single species [7,28].

Although most EPS-producing microorganisms can synthesize their polymers under almost all growth-permitting culture conditions, it is possible and obviously desirable to optimize the environmental variables to achieve maximum production. As a starting point, we chose those culture conditions (aeration, pH, temperature and time) that we found in previous studies to be ideal for *H. eurihalina* [22], a halophilic bacterium closely related to our newly discovered isolates.

The chemical characterisation of halophilic EPS provides valuable information about the relationship between the composition of the polysaccharides and their physical properties (Table 1). In general, the carbohydrate content of the polymers was low; a feature that has also been observed in other EPS [1,2]. This may be due to the presence of uronic acid, which renders the EPS resistant to acid hydrolysis with H_2SO_4, which we used in our colorimetric analyses [21]. This feature may also explain the fact that a part of the polymer remained unaccounted for in the quantitative analysis carried out. Uronic acids confer anionic characteristics upon the EPS and are responsible for two of their most important biotechnological applications: their use in the biodetoxification of environments polluted with heavy metals, and in waste water treatment [15].

Proteins play an important role in the emulsifying activity of emulsan [27], liposan [11], alasan [20] and manoproteins [10]. EPS from our halophilic strains had protein concentrations ranging from 0.3% to 5.3% (w/w), particularly the polymers from strains S-30 and S-32, which, as might be expected, were also the best bioemulsifiers (Table 2). Emulsan, a biosurfactant obtained from *Acinetobacter calcoaceticus*, is a complex of polysaccharides and proteins. This compound emulsifies mixtures of water and aliphatic, aromatic or cyclic hydrocarbons but cannot emulsify pure hydrocarbons [26]. Strains S-30 and S-32 produced EPS capable of emulsifying pure hydrocarbons (tetradecane, hexadecane and octane) at higher percentages than the controls. Furthermore, 66% of the EPS from these *Halomonas* strains gave stable emulsions from crude oil whilst this substrate was only slightly emulsified by the controls. The potential use of these EPS in the oil industry is apparent: for cleaning sludge in storage tanks and mobilising oil in contaminated water and sludge, for example. It is noteworthy that the EPS from strains S-30 and S-32 had higher emulsifying activity than the chemical surfactants used as controls (Table 2).

As already mentioned, the EPS gave solutions with different viscosity values, but three polymers (S-30, S-31 and S-32) produced highly viscous solutions (Table 1). Some authors reported that the degree of acetylation and its pattern may modify the viscosity potential of EPS [30]. In our case, however, the acetyl content of different EPS did not correlate with the viscosity of their solutions; EPS with different acetyl contents often yielded a similar viscosity. Moreover, polymer S-30, which produced the highest viscosity value, had just 0.5% (w/w) of acetyl residues (Table 1).

In conclusion, our data suggest that at least three of the 32 *Halomonas* strains selected for this study showed considerable promise for industrial use. Strains S-30, S-31 and S-32 synthesized EPS that produced highly viscous solutions, and furthermore, S-30 and S-32 showed interesting emulsifying activity with potential for use in the oil industry. These polymers presented the same neutral monosaccharides: glucose, manose and galactose although in different proportions. The polymer S-30 also had glucuronic acid in equimolar proportion with respect to the above neutral monosaccharides. Furthermore, its high uronic acid content makes it a useful acidic EPS with potential to bind heavy metals and cations.

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