Journal of Industrial Microbiology & Biotechnology (1998) 20, 205–209

http://www.stockton-press.co.uk/jim

Effect of cations, pH and sulfate content on the viscosity and emulsifying activity of the *Halomonas eurihalina* exopolysaccharide

C Calvo[1], F Martinez-Checa[2], A Mota[1], V Bejar[2] and E Quesada[2]

[1]*Water Institute;* [2]*Department of Microbiology, Faculty of Pharmacy, University of Granada 18071, Spain*

The effects of monovalent and divalent cations on the rheological behavior of *Halomonas eurihalina* exopolysaccharide (EPS) were studied. Sodium, potassium, magnesium and calcium were added and the relative abilities to increase viscosity were as follows: KCl > NaCl > $MgCl_2$ > $CaCl_2$. The highest viscosity value was measured in acidic 10^{-4} M KCl, in which a gel formed. A loss of sulfate content seemed to correlate with the increase of viscosity. *H. eurihalina* produced EPS in all growth media. Addition of hydrophobic substrates to culture media produced changes in chemical composition and emulsifying activity of the EPS. Xylene was the most effectively emulsified substance and the EPS produced on tetradecane and on corn oil the most active emulsifier.

Keywords: exopolysaccharide; rheology; emulsifier

Introduction

Halomonas eurihalina is a moderate halophile isolated from hypersaline soils [16,24]. This species produced extracellular polymeric substances (EPS) [22], with the chemical composition and physical properties of heteropolysaccharides [5].

Microbial polysaccharides that are useful in industry show much diversity, which implies the possibility of finding specific biopolymers for specific end-uses. To establish the utility of a new polymer, it is necessary to know its chemical composition and physical properties as well as the influence of physical and chemical factors on its behavior [6,13,20,26,28].

The exopolysaccharides produced by *H. eurihalina* have interesting properties, such as the increase in viscosity in acidic solutions, the ability to emulsify hydrocarbons and the presence of sulfate groups. These polymers have been the focus of several studies [4,10,14,22,23]. The present study was undertaken to establish the effects of the addition of monovalent and divalent cations on the EPS solutions, the addition of hydrophobic substances to culture media production on polysaccharide sulfate content, and whether variations in the sulfate content would influence viscosity or emulsifying activity of the biopolymers.

Materials and methods

Microorganism

The organism used in this study was *H. eurihalina* strain H96, a moderately halophilic bacterium, with optimal growth at a total salt concentration of 7.5% (w/v) [24].

Correspondence: Dr C Calvo, Departamento de Microbiología, Facultad de Farmacia, Universidad de Granada, Campus Universitario de Cartuja s/n, 18071 Granada, Spain
Received 25 July 1997; accepted 30 January 1998

Culture media

The complex medium used was MY [17], modified by adding a mixture of sea salt [25] to give the final total salt concentration of 7.5% (w/v); its composition was as follows (g L^{-1}): glucose 10 (Panreac, Barcelona, Spain); proteose peptone 5.0 (Difco, Detroit, USA); yeast extract 3.0 (Difco); malt extract 3.0 (Difco); NaCl 51.3; $MgSO_4 \cdot 7H_2O$ 13.0; $MgCl_2 \cdot 6H_2O$ 9.0; KCl 1.3; $CaCl_2 \cdot 2H_2O$ 0.2; NaBr 0.15; $NaHCO_3$ 0.05. The pH was adjusted to 7.2 with NaOH.

To study the influence of hydrocarbons on the emulsifying activity of the EPS, glucose as carbon source was substituted by the following hydrophobic substrates: *n*-tetradecane, *n*-hexadecane, *n*-octane, xylene, petrol, crude oil, corn oil (vegetable oil) and two mineral oils (light white oil and heavy white oil) supplied by Sigma Co.

Production and isolation of EPS

As previously described [22], 500-ml Erlenmeyer flasks containing 100 ml of medium were inoculated with a suitable inoculum (1 ml, $OD_{520nm} = 2.5$) made in the same medium and incubated at 32°C for 8 days.

Cell-free supernatant fluids were obtained by centrifugation of cultures at 36 000 × *g* for 60 min. The EPS was precipitated with three volumes of cold ethanol, suspended in distilled water and purified by ultracentrifugation (226 000 × *g* for 60 min) and dialyses against distilled water. Then it was lyophilized.

Chemical analysis

EPS extracted from each culture medium was subjected to colorimetric analysis of proteins [9], total carbohydrates [11], uronic acids [7] and acyl residues [15].

Cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and sulfate as anionic components were determined with a Dionex DX-300 Gradient Chromatography System with chemical suppression of the eluent conductivity. For cations, the eluent was 18 mM HCl acid, whereas the regenerant (chemical suppressor) was a 10-mM tetrabutylammonium hydroxide solution. For anions, the eluent was 1.7 mM Na_2CO_3/$NaHCO_3$. Finally, a 35-mM H_2SO_4 was used as acidic regenerant. These assays were carried out at the Water Institute of Granada University.

Rheological studies

NaCl, KCl, $MgCl_2$ and $CaCl_2$ were dissolved in deionized water to a final concentration of 10^{-4} to 10^{-1} M. EPS lyophile (10 mg ml^{-1}) was dissolved in these solutions and for comparison EPS was also dissolved in deionized water without addition of salt. The rheological behavior of these solutions was studied at neutral and at pH 3. Solutions were acidified with 1 N HCl.

Viscosity measurements were determined with a Bohlin CSR-10 rheometer at 25°C [10].

To study if acidification of EPS salt solutions produces changes of chemical composition, all solutions were dialyzed against distilled water for 48 h, lyophilized and tested as previously described.

Emulsifying activity

A standard emulsification assay described by Navon-Venezia *et al* [18] was used. Samples (2.5 mg EPS) were introduced into a 125-ml flask containing 7.5 ml TM buffer (20 mM tris-hydroxymethyl aminomethane, pH 7.2 plus 10 mM $MgSO_4$), added to 0.1 ml of the hydrophobic substrate: *n*-tetradecane, *n*-hexadecane, *n*-octane, xylene, petrol, crude oil, corn oil (vegetable oil) and the two mineral oils (light white oil and heavy white oil) supplied by Sigma Co. Samples were incubated at 30°C with reciprocal shaking, 160 rpm, for 1 h. Then, turbidity was determined in a Perkin Elmer spectrophotometer at 660 nm.

Results

To study the effect of monovalent and divalent cations on viscosity, EPS was dissolved in NaCl, KCl, $MgCl_2$ and $CaCl_2$ at between 10^{-1} to 10^{-4} M, stored overnight at 4°C and the rheology of neutral and acid solutions (pH near 3) was measured (Table 1). The viscosities of EPS salt solutions at neutral pH ranged from 100 to 200 cps at shear stress between 20 to 30 Pa (with the exception of EPS in high Mg^{2+} and Ca^{2+} solution). After acidification, the effect of monovalent and divalent cations on the viscosity of EPS was noticeable. At several salt concentrations, viscosity of monovalent chlorides was more than 1000 cps, at shear stress superior to 50 Pa.

To find out the rheological behavior of these polymer solutions, viscosity was measured at different shear stresses and the relationship between shear rate and shear stress was established. All EPS solutions showed a non-Newtonian behavior as the solutions' viscosity was influenced by shear rate. The relationship between shear stress and shear rate was that of a Bingham plastic fluid. These materials exhibit an infinite viscosity when a low stress is applied.

Acidic potassium solutions were the most viscous and data corresponding to their rheology can be seen in Figure 1. When mathematical expression of Bingham fluid was applied to these data (Shear Stress = Shear Stress$_o$ + A × Shear Rate), the coefficient of correlation was near 1 and

Table 1 Effect of cations on viscosity of EPS solutions

Salt solution (M)		Shear stress (Pa)[a]	Shear rate (L s^{-1})	Viscosity (cps)[b]
Neutral K	10^{-4}	30	138	213
	10^{-3}	30	138	213
	10^{-2}	30	179	165
	10^{-1}	30	300	98
Acidic K	10^{-4}	57	11	5026
	10^{-3}	62	27	2313
	10^{-2}	50	36	1375
	10^{-1}	50	37	1329
Neutral Na	10^{-4}	30	195	151
	10^{-3}	30	146	201
	10^{-2}	30	172	171
Acidic Na	10^{-4}	65	92	708
	10^{-3}	65	60	1084
	10^{-2}	65	75	865
	10^{-1}	65	196	332
Neutral Mg	10^{-4}	20	204	100
	10^{-3}	20	140	146
	10^{-2}	20	240	85
	10^{-1}	20	306	63
Acidic Mg	10^{-4}	25	194	129
	10^{-3}	30	25	1191
Neutral Ca	10^{-4}	20	129	158
	10^{-3}	20	118	172
	10^{-2}	20	285	72
	10^{-1}	12	246	49
Acidic Ca	10^{-4}	35	108	327
	10^{-3}	35	203	174
	10^{-2}	12	88	136

[a] Pascals; [b] centipoises.

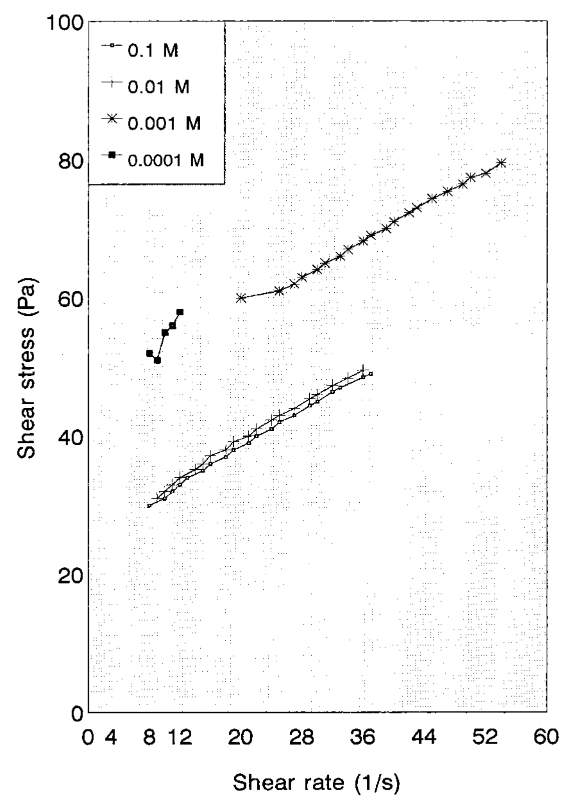

Figure 1 Rheological behavior of acidic EPS solutions at different potassium chloride salt concentrations.

the limiting shear stress (stress yield) and the A (viscosity divided by the Newtonian conversion factor) values were similar with the exception of KCl 10^{-4} M solution (Table 2).

It would be of interest to determine whether the ionic content of EPS varied with acidification of EPS salt solutions. Therefore, each of the above-mentioned solutions was extensively dialyzed against distilled water for 48 h and lyophilized. The compounds were dissolved in deionized water and the most viscous solutions were studied.

Sulfates played an important role in physical properties of *H. eurihalina* EPS. Thus, a decrease from 14% to 6.9% of sulfate content was detected in the lyophiles coming from gel-like systems. With respect to cation percentages, a slight loss of sodium, potassium and magnesium was detected; in contrast, no significant change in calcium was found. Carbohydrates, proteins, uronic acids and acetyls were not modified.

H. eurihalina was able to grow in all culture media tested with a similar rate, indicating that none of these substrates was toxic and that the cells have the ability to produce EPS in all media. To better evaluate the influence of hydrocarbons on bacterial growth and on EPS produced, we have also assayed the MY complex medium (control), without addition of carbohydrate or hydrocarbon as carbon source. The yield production varied from 0.8 to 1.2 g L^{-1} and crude and corn oils were the most suitable substrates for production.

The emulsifying activity of exopolysaccharides was tested on each of the hydrophobic substances used as carbon source (Table 3). Xylene was the substrate most effectively emulsified followed by tetradecane and corn oil; in contrast, other substrates were poorly or not at all emulsified. As has been reported [13], specificity was modified by culture conditions. Some EPS showed selective activity, as the polymer synthesized on xylene and on mineral light oil reacted differently compared with tetradecane.

Because the chemical composition of polymers synthesized may be influenced by nutritional and environmental culture conditions [19,27], we determined that the presence of hydrophobic substances in the culture media correlates with changes in relative composition of the exopolysaccharides (Table 4). When comparing to exopolysaccharide synthesized on MY medium [5], an increase of uronic acids and sulfate contents, with a decreasing percentage of carbohydrates was detected on all polymers produced on media supplied from hydrophobic substances.

The viscosity of these EPS solutions, with a high content of sulfates, was always less than 60 cps at a shear stress of 5 Pa. These data demonstrated that the increasing of sulfate groups is correlated with a loss of viscosity. Low viscous emulsions are generally required in oil recovery industry since they are easily pumped [2].

Table 2 Rheology of acidic EPS potassium chloride solutions

KCl solutions (M)	SS_0 (Pa)[a]	A[b]	Correlation coefficient
0.1	25	0.67	0.999
0.01	25	0.69	0.996
0.001	46	0.63	0.996
0.0001	34	1.95	0.837

[a] Stress yield; [b] conversion factor.
Data correspond to application of Bingham plastic equation.

Discussion

The effect of cations on the rheological behavior of bipolymer solutions has been extensively described [8,29]. Monovalent and divalent cations cause an increase in viscosity [21]. The optimal salt concentration, for viscosity of *H. eurihalina* seems to be 10^{-3} M, with slightly higher viscosities for monovalent cation solutions. On the other hand, all these solutions were more viscous than solutions of the EPS in deionized water (61.8 cps at 20 Pa of shear stress); but it was potassium solutions which yielded maximal viscosity (Table 1 and Figure 1). In 10^{-4} M KCl, the EPS solution became exceedingly viscous or gelled, so that with the measuring system used in our assays, the viscosity could not be measured at the optimal conditions. On the other hand, viscosities could be determined only at low concentrations of Mg^{2+} and Ca^{2+} because at high concentrations the polysaccharide precipitated. This explains the absence of such data in Table 1.

The growth of microorganisms on hydrocarbons is often associated with the production of surface-active compounds, which are useful to emulsify these hydrophobic substances in the growth medium, enhancing their uptake [3,13]. Since *H. eurihalina* produces exopolysaccharides with emulsifying activity, it is possible that some of these substances are used as carbon and energy source by this microorganism. Currently HPLC chromatography assays are being performed in order to establish the ability of several *H. eurihalina* strains to degrade some hydrophobic compounds.

In comparison to EPS synthesized on MY medium [4], chemical composition of exopolysaccharides was strongly influenced by the presence of hydrophobic substrates in the culture medium; a low content of carbohydrate and an augmentation of sulfates and uronic acids was detected. The decrease in carbohydrates has already been observed in the EPS synthesized by *H. eurihalina* strain F2-7 growing on phosphorus, sulfur or magnesium-deficient medium [5]. Highly sulfated polysaccharides have been described in some marine bacteria and some archaebacteria [1,12]. Concentrations of sulfates ranged from 2 to 21% in the polymers synthesized by deep sea bacteria [12]. Furthermore, high sulfate-containing polymers, along with high uronic acid-containing polysaccharides are of great interest because of their heavy metal-binding capability [12]. Thus, these new biopolymers could be expected to also have applications in the field of biodetoxication and wastewater treatment.

Acknowledgements

This work was supported by grants from Spanish Ministerio de Educacion y Cultura (CICYT BIO 095-0497) and Junta de Andalucıa.

Table 3 Emulsifying activity of *H. eurihalina* EPS produced on control medium and on medium added with organic solvents

EPS	Substrates				
	Xylene	Hexadecane	Corn	Petrol	Tetradecane
Tetradecane	0.85 ± 0.1	0.01 ± 0.001	0.03 ± 0.001	0.06 ± 0.003	1.16 ± 0.02
Corn oil	0.68 ± 0.03	0.02 ± 0.003	0.03 ± 0.001	0.08 ± 0.002	0.03 ± 0.001
Hexadecane	0.53 ± 0.003	0	0.07 ± 0.003	0.02 ± 0.002	0
Heavy	0.37 ± 0.01	0.03 ± 0.002	0.13 ± 0.003	0.04 ± 0.001	0.05 ± 0.004
Crude	0.38 ± 0.003	0.01 ± 0.001	0.26 ± 0.002	0.1 ± 0.006	0.16 ± 0.015
Petrol	0.19 ± 0.006	0.05 ± 0.003	0.06 ± 0.002	0.02 ± 0.001	0.01 ± 0.045
Light	0.11 ± 0.010	0.12 ± 0.006	0.02 ± 0.001	0.02 ± 0.002	0.39 ± 0.025
Xylene	0.02 ± 0.104	0.09 ± 001	0.06 ± 0.002	0.03 ± 0.001	0.56 ± 0.046
Octane	0.01 ± 0.005	0.04 ± 0.001	0.34 ± 0.008	0.01 ± 0.001	0.04 ± 0.003
Control	0.09 ± 0.002	0	0.01 ± 0.001	0.02 ± 0.001	0

Results are expressed as absorbance at 660 nm.

Table 4 Chemical composition of EPS produced on culture media supplemented with hydrocarbons

	EPS				
	Tetradecane	Corn[a]	Hexadecane	Crude	Heavy[b]
Protein	11 ± 1	6 ± 0.6	11 ± 1	7 ± 0.5	10 ± 0.1
Carbohydrates	21 ± 1.3	23 ± 1.1	27 ± 0.9	20 ± 1	19 ± 1
Uronic acids	6 ± 0.1	6 ± 0.1	7 ± 0.3	7 ± 0.1	5 ± 0.03
Acetyls	0.5 ± 0.01	1.6 ± 0.2	0.4 ± 0.01	0.3 ± 0.02	0.5 ± 0.03
Sulfates	30 ± 1.3	35 ± 1	29 ± 1	35 ± 1.4	29 ± 0.5
Na^+	17 ± 0.6	18 ± 0.5	20 ± 0.5	20 ± 0.6	20 ± 0.6
Mg^{2+}	3 ± 0.01	2 ± 0.1	3 ± 0.03	2 ± 0.2	3 ± 0.02
Ca^{2+}	3 ± 0.1	5 ± 0.2	2 ± 0.1	2 ± 0.2	4 ± 0.1

Results are expressed as percentage of total dry weight of the polymer; values are means of at least three determinations.
[a] Vegetable oil; [b] mineral oil.

References

1 Anton J, I Meseguer and F Rodriguez-Valera. 1988. Production of an extracellular polysaccharide by *Haloferax mediterranei*. Appl Environ Microbiol 54: 2381–2386.

2 Banat IM, N Samarah, M Murad, R Horne and S Banerjee. 1991. Biosurfactant production and use in oil tank clean-up. World J Microbiol Biotechnol 7: 80–88.

3 Banat IM. 1993. The isolation of a thermophilic biosurfactant producing *Bacillus* sp. Biotechnol Lett 15: 591–594.

4 Bejar V, C. Calvo, J Moliz, F Díaz-Martínez and E Quesada. 1996. Effect of growth conditions on the rheological properties and chemical composition of *Volcaniella eurihalina* exopolysaccharide. Appl Biochem Biotechnol 59: 77–86.

5 Bejar V, I Llamas, C Calvo and E Quesada. 1998. Characterization of exopolysaccharides produced by 19 halophilic strains of the species *Halomonas eurihalina*. J Biotechnol (in press).

6 Bertrand JC, P Bonin, M Goutx, M Gauthier and G Mille. 1994. The potential application of biosurfactants in combatting hydrocarbon pollution in marine environments. Res Microbiol 145: 39–81.

7 Blumenkratz N and G Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. Anal Biochem 54: 484–489.

8 Bozzi L, M Milas and M Rinaudo. 1996. Solution and gel rheology of a new polysaccharide excreted by the bacterium *Alteromonas* sp strain 1644. Int J Biol Macromol 18: 83–91.

9 Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

10 Calvo C, MR Ferrer, F Martínez-Checa, V Béjar and E Quesada. 1995. Some rheological properties of the extracellular polysaccharide produced by *Volcaniella eurihalina* F2–7. Appl Biochem Biotechnol 55: 45–54.

11 Dubois M, KA Gilles, JK Hamilton, PA Rebers and F Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356.

12 Guezennec JC, P Pignet and G Raguenes. 1994. Preliminary chemical characterization of unusual eubacterial expolysaccharide of deep-sea origin. Carbohydrate Res 24: 287–294.

13 Kosaric N. 1992. Biosurfactants in industry. Pure Appl Chem 64: 1731–1737.

14 Martínez-Checa F, C Calvo, MA Caba, MR Ferrer, V Béjar and E Quesada. 1996. Efecto de las condiciones nutricionales sobre la viscosidad y capacidad emulgente del biopolímero V2–7 de *Volcaniella eurihalina*. Microbiología SEM 12: 55–60.

15 McComb EA and RM McCready. 1957. Determination of acetyl in pectin and in acetylated carbohydrate polymers. Hydroxamic acid reaction. Anal Chem 29: 819–821.

16 Mellado E, ERB Moore, JJ Nieto and A Ventosa. 1995. Phylogenetic interferences and taxonomic consequences of 16S ribosomal DNA sequences comparison of *Chromohalobacter marismortui*, *Volcaniella eurihalina* and *Deleya salina* and reclassification of *V. eurihalina* as *Halomonas eurihalina* comb nov. Int J Syst Bacteriol 45: 712–716.

17 Moraine RA and P Rogovin. 1966. Kinetics of polysaccharide B-1459 fermentation. Biotechnol Bioeng 8: 511–524.

18 Navon-Venezia S, Z Zosim, A Gottlieb, R Legmann, S Carmell, EZ

Ron and E Rosenberg. 1995. Alasan, a new bioemulsifier from *Acinetobacter radioresistens*. Appl Environ Microbiol 61: 3240–3244.
19 Novak JS, SW Tanenbaum and JP Nakas. 1992. Heteropolysaccharide formation by *Arthrobacter viscosus* grown on xylose and xylose oligosaccharides. Appl Environ Microbiol 58: 3501–3507.
20 Pace GW. 1987. Microbial gum. In: Basic Biotechnology (Bulock J and Kristiansen B, eds), pp 449–462, Academic Press, London.
21 Parker DL, BR Schram, JL Plude and RE Moore. 1996. Effect of metal cations on the viscosity of a pectin-like capsular polysaccharide from the cyanobacterium *Microcystis flos-aquae* C3-40. Appl Environ Microbiol 62: 1208–1213.
22 Quesada E, V Bejar and C Calvo. 1993. Exopolysaccharide production by *Volcaniella eurihalina*. Experientia 49: 1037–1041.
23 Quesada E, A del Moral and V Bejar. 1994. Comparative method for isolation of *Volcaniella eurihalina* exopolysaccharide. Biotechnol Techn 8: 701–706.
24 Quesada E, MJ Valderrama, V Bejar, A Ventosa, MC Gutierrez, F Ruiz-Berraquero and A Ramos-Cormenzana. 1990. *Volcaniella eurihalina* gen nov sp nov, a moderately halophilic nonmotile gram-negative rod. Int J Syst Bacteriol 40: 261–267.
25 Rodrıguez-Valera F, F Ruiz-Berraquero and A Ramos-Cormenzana. 1981. Characteristics of the heterotrophic populations in hypersaline environments of different salt concentration. Microb Ecol 7: 235–243.
26 Roller S and ICM Dea. 1992. Biotechnology in the production and modification of biopolymers for foods. Crit Rev Biotech 12: 261–277.
27 Schelbenbogen K, RG Zytner, H Lee and JT Trevors. 1994. Enhanced removal of selected hydrocarbons from soil by *Pseudomonas aeruginosa* UG2 biosurfactants and some chemical surfactants. J Chem Tech Biotech 59: 53–59.
28 Sutherland IW. 1990. Biotechnology of Microbial Exopolysaccharides. Cambridge University Press, Cambridge.
29 Thibault JF and M Rinaudo. 1985. Interactions of mono- and divalent counter ions with alkali- and enzyme-deesterified pectins in salt-free solutions. Biopolymers 24: 2131–2144.