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Effect of Growth Conditions on the Rheological Properties and Chemical Composition of *Volcaniella eurihalina* Exopolysaccharide

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ABSTRACT

The exopolysaccharide produced by *Volcaniella eurihalina*, an halophilic eubacterium, under different environmental and nutritional conditions, is studied. *V. eurihalina* synthesizes an acidic heteropolysaccharide, composed by rhamnose, glucose, and mannose, as well as amino sugars, uronic acids, and acetyl and sulphate residues. This composition varies depending on the nutrients of culture medium. Viscosity and pseudoplasticity of the polymer solutions are also influenced by the nutritional conditions in which the microorganism was grown.

Index Entries: Exopolysaccharides; halophilic bacteria; *Volcaniella eurihalina*.

INTRODUCTION

The growth of bacteria is often accompanied by the production of polysaccharides found outside the cell wall. These exopolysaccharides (EPS) can take the physical form of capsules attached to the cells or may be released to the environment as extracellular slimes. In chemical terms,

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the EPS are either homopolymers or heteropolymers, and may carry a variety of noncarbohydrate substituents such as ester-linked acetate, pyruvate, and others (*1*).

Microbial EPS are an attractive alternative to traditional gums extracted from plants and seaweeds, as they are not vulnerable to adverse crop conditions or pollution, show a wide range of physical properties, and their chemical composition can be changed in accordance with the growth conditions (*2–4*). Some polysaccharides have gelling properties, others are emulsifying or suspending agents or protective colloids, and still others may be useful owing to their biological properties (*1,5*). All these features allow microbial EPS to be useful in many applications in food, in the oil industry, and in medicine.

Volcaniella eurihalina is a moderately halophilic eubacterium, which grows optimally at salt concentrations of 5–10% (w/v) (*6*). This microorganism synthesizes an extracellular polysaccharidic substance (*7*), which, in solution, shows a pseudoplastic non-newtonian behavior quite resistant to high ionic strength and thermostable. Moreover, it is able to form high viscosity solutions like a gel at low pH values even in the presence of inorganic salts (*8*). From this point of view, this EPS would be valuable for use in various industrial applications, e.g., in the food industry for salad sauces or citric desserts where the pH is usually acid or in oil-recovery because of its stability to temperature and ionic strength. The objective of this work is focused on the study of chemical composition and physical properties of the extracellular polysaccharide produced by *V. eurihalina* strain F2-7, under different environmental and nutritional conditions. Our aim is to determine to what extent those conditions have an influence on the chemical composition and therefore on the physical properties of the polymer, to improve their characteristics to a particular end-use.

MATERIALS AND METHODS

Microorganism

V. eurihalina strain F2-7 was used through this study. This is a moderate halophile, with optimal growth at a total salt concentration of 7.5% (w/v). Other features of this microorganism have been described previously (*6*).

Growth Media and Cultivation Conditions

The complex medium used was MY (*9*) modified by adding the appropriate balanced mixture of sea salts (*10*) to give the final salt concentration of 7.5% (w/v); its composition is the following: 10.0 g/L glucose; 5.0 g/L

proteose-peptone (Difco); 3.0 g/L yeast extract (Difco); 3.0 g/L malt extract (Difco); 51.3 g/L NaCl; 13.0 g/L $MgSO_4 \cdot 7H_2O$; 9.0 g/L $MgCl_2 \cdot 6H_2O$; 1.3 g/L KCl; 0.2 g/L $CaCl_2 \cdot 2H_2O$; 0.15 g/L NaBr; and 0.05 g/L $NaHCO_3$.

As a synthetic medium, we used the NH. This medium is based in that originally developed by Ng and Hu (*11*), in which the following changes were made: Ethanol was substituted by glucose, $CoCl_2 \cdot 6H_2O$ by $CoNO_3 \cdot 6H_2O$ and NaCl was added to get a suitable osmolarity; its composition is the following: 10.0 g/L Glucose; 2.4 g/L KH_2PO_4; 5.6 g/L K_2HPO_4; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 4.0 g/L $(NH_4)_2SO_4$; 50.0 g/L NaCl; and 3.5 mL of a trace solution containing: 0.368 g/L $CaCl_2 \cdot 2H_2O$; 0.642 g/L $CaSO_4 \cdot 5H_2O$; 0.604 g/L Na_2MoO_4; 0.594 g/L $NO_3Co \cdot 6H_2O$; 0.422 g/L $MnSO_4 \cdot H_2O$; 0.718 g/L $ZnSO_4 \cdot 7H_2O$; and 0.696 g/L $FeSO_4 \cdot 7H_2O$.

The pH was adjusted at 7.2 with 1*M* NaOH. Both media were distributed in 500-mL Erlenmeyer flasks, each containing 100 mL and sterilized by autoclaving at 112°C for 30 min (NH medium requires the separate preparation of phosphate buffer, which is added to the other components after the sterilization process).

In each experiment, media were inoculated with a suitable inoculum (1 mL, OD_{520} = 2.5) made in the same medium and the flasks were incubated in steady conditions at 32°C and for 8 d, unless otherwise stated.

By using MY medium, we obtained EPS samples from cultures incubated at different temperatures (22, 32, and 42°C), from cultures with different total salt contents (2.5, 5, 7.5, 10, 15, and 20% w/v), and from cultures with various concentrations of glucose (0, 1, 2, 4, and 8%, w/v). All these EPS samples were chemically analyzed and subjected to rheological studies.

NH medium was used to study the influence of the following factors: The relation C/N 40, 20, 10, 5, and 2.5 (1% w/v glucose and the appropriate amount of ammonium sulphate), limitations of sulfur ($[NH_4]_2SO_4$ was substituted by NH_4Cl), magnesium (0.005%, w/v $MgSO_4 \cdot 7H_2O$), and phosphorus (phosphate buffer was substituted by tris-hydrochloride buffer and 0.05% w/v KH_2PO_4 was added to NH medium); we also tested 15 substrates that can be used by *V. eurihalina* strain F2-7 as the sole source of carbon and energy, as glucose replacements in the chemically defined medium NH (L-alanine, L-arabinose, citrate, D-galactose, D-gluconate, L-glutamic acid, L-histidine, *meso*-inositol, lactose, maltose, D-mannitol, D-mannose, L-rhamnose, D-salicin, or L-serine [Sigma]). As in the case of samples from MY medium, all polymers obtained under the different conditions assayed in NH media were chemically and rheologically studied.

Methods for isolation and purification of the EPS have been described previously (*7,12*) and they include centrifugation of cultures, tangential filtration (Minitam System, 100,000 D ultrafilters), precipitation with 3 vol of cold ethanol, ultracentrifugation, dialysis against distilled water, and lyophilization. Both lyophilized EPS and dry cell weight were determined gravimetrically in each experiment.

Analytical Procedures

The colorimetric analyses carried out were the following: total carbohydrates (*13*), proteins (*14*), hexosamines (*15*), glucose (*16*), uronic acids (*17*), sulfates (*18*), acyl residues (*19*), and ashes.

Thin-Layer Chromatography (TLC)

Neutral sugar composition of pure EPS was analyzed by TLC in cellulose as described before (*7*). EPS samples were hydrolyzed in 0.5*N* sulfuric acid. Plates were developed in a mixture of butanol/pyridine/water (Sigma) (6:4:3) and stained with alkaline silver nitrate. Standard sugars (Sigma) were used for identification.

Gas Chromatography-Mass Spectrometry (GC-MS)

We used the trimethylsilyl (TMS) reagent from Sigma (*20*) for the preparation of the TMS derivatives. Gas chromatography was performed on an HP-5890 gas chromatograph coupled with a mass spectrometry HP-5988-A detector. The ion source was at 200°C. The carrier gas was He. An SPB1 capillary column (30 m × 0.2 mm) was operated with a temperature gradient (80 to 300°C; 10°C per min). Sugars were identified by their retention times with those of standard sugars and by mass spectrometry.

Nuclear Magnetic Resonance (NMR)

In order to determine the presence of acetyl residues, hydrolyzed EPS samples were each dissolved in D_2O and ^1H NMR spectra were recorded in a Bruker AM-300 spectrometer at 24°C.

Rheological Studies

Lyophilized samples obtained under the aforementioned culture conditions were dissolved in distilled water to give 1% (w/v) solutions. Viscosity measurement of the solutions was determined with a Brookfield viscometer LTV fitted with a small samples adapter (Brookfield Engineering Laboratories, MA). Determinations were made at room temperature (25°C) and at different shear rates. Additionally, in samples extracted from NH media, the influence of pH on viscosity was studied using 1% (w/v) citric-acid bisodic phosphate buffer solutions. The range of pH tested varied from 2 to 8.

RESULTS AND DISCUSSION

The conditions under which microbial polysaccharides are produced and their effect on chemical composition and physical properties of the polymers change widely between the microorganisms. Although the composition and amount of polymers synthesized are genetically determined,

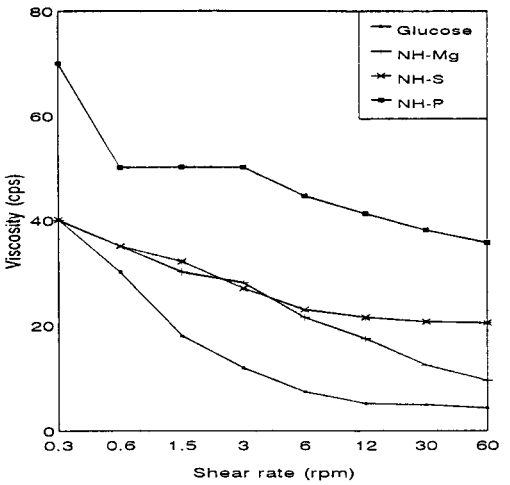

Fig. 1. Rheological properties of the EPS produced by *Volcaniella eurihalina* strain F2-7 under different culture conditions.

it is possible to modify both by changing nutritional and environmental culture conditions. In many cases, EPS production is favored by an excess of the carbon source, high ratio of carbon to nitrogen, and low temperatures. Both exopolysaccharide yield and chemical composition may be influenced by the carbon source and limiting nitrogen, phosphorus, and sulfur (*1,21–23*).

According to what has been stated above, we have determined if both chemical composition and physical properties of the *V. eurihalina* EPS were influenced by the various conditions assayed.

The amount of extracellular polysaccharide formed by *V. eurihalina* in MY medium was quite influenced by the cultural conditions, as we have reported before (*7*); EPS produced seems to be mostly linked to the total biomass, yields ranging from 0.20 to 1.9 g/L of culture medium. The organism was nutritionally nondemanding and the EPS was also formed in the chemically defined medium (0.21–0.72 g of EPS/liter of culture medium; 0.20–0.25 g of EPS/gram of dry cell weight) for all different nutritional conditions tested in this study, including a wide range of carbon substrates. Polysaccharide production was favored by a high carbon/nitrogen ratio in the growth medium, the EPS yield being the greatest. Increased exopolysaccharide was not observed under conditions of phosphate, sulfur, or magnesium limitations.

With respect to the rheological studies, although viscosity and pseudoplasticity of polymers isolated from NH media were similar to those produced by *V. eurihalina* in MY media, it is noteworthy that the ability of MY exopolysaccharides to produce high viscosity solutions at low pH (*8*) had not been found in any EPS isolated from NH media. On the other hand, viscosity appeared to be influenced by limitation of nutrients; thus, polymers obtained in sulfur-, magnesium-, and phosphorous-deficient cultures tend to have higher viscosity but lower pseudoplasticity than those isolated from NH medium without nutrient limitation. (Fig. 1).

Table 1
Chemical Analysis of the EPS Produced by *Volcaniella eurihalina* Under Different Cultural Conditions

	MY[a]	NH[b]	NH-P	NH-S	NH-Mg
Carbohydrates	34.5	10.0	13.0	12.0	18.0
Proteins	14.1	8.9	11.5	12.1	9.1
Acetyls	0.6	0.8	1.3	1.1	1.0
Uronic acids	1.5	0.9	2.2	1.1	3.0
Hexosamines	2.4	2.3	3.9	2.6	1.5
Glucose[c]	35.5	10.4	10.8	7.7	8.5
Sulphates	2.7	2.0	ND	ND	ND
Ashes	12.0	19.0	ND	ND	ND

Results are expressed as percentages of total dry weight of the polymer.
[a]Mean values obtained under several conditions assayed in MY medium (different incubation temperatures, salt contents, and glucose concentrations).
[b]Mean values obtained under the several conditions assayed in NH medium (different relations of C/N and different carbon sources).
[c]Expressed as percentage of total carbohydrates.
NH-P, phosphorus deficient medium; NH-S, sulfur deficient medium; NH-Mg, magnesium deficient medium; ND, not determined.

To determine if these changes in the rheological properties are owing to modifications in the chemical compositions of the EPS, purified polymers were subjected to quantitative analysis using different chemical determinations. First, we have to make a special point of the uniformity of the results obtained with samples extracted from MY and NH media, respectively (Table 1). The EPS of strain F2-7 obtained under all the conditions investigated in each medium were indistinguishable on the basis of the chemical studied carried out, although different yields were obtained. However, some medium-to-medium variations were encountered. Table 1 only shows these conditions under which differences were found. Columns 1 and 2 represent the mean values obtained under the several conditions assayed in media MY and NH, respectively, except for media limited in phosphorus, sulphur and magnesium, that correspond with columns 3, 4, and 5, respectively. All EPS contained less carbohydrates than the predicted amounts. This low content of carbohydrates has also been observed in a number of other EPS (*24,25*). A considerable part of the polymer was not accounted for with the quantitative analysis carried out. This could be owing to the presence of some water bound to the hydroscopic molecule, even though all samples were dried to constant weight by lyophilization. However, this also could be owing to the existence of other types of compounds not analyzed. The halophilic nature of *V. eurihalina* could be the reason for the existence of unusual components undetected by the methods used. On the other hand, the presence of uronic acids, together with amino sugars, makes EPS resistant to acid

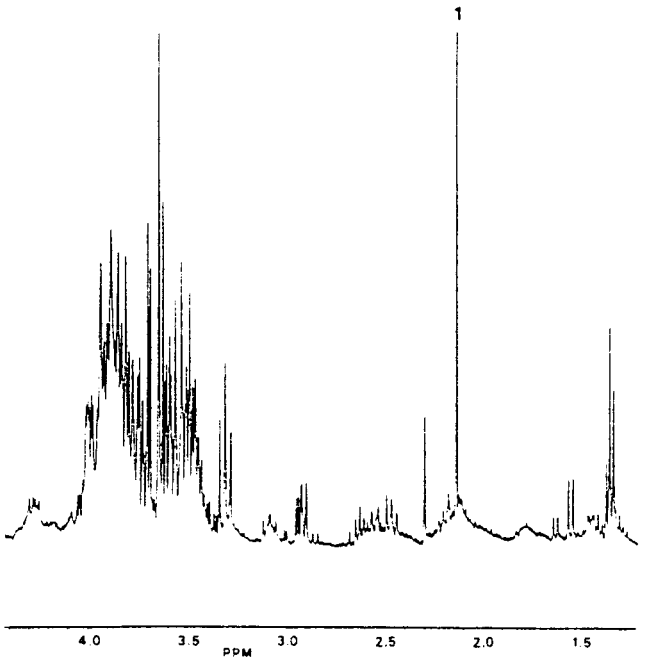

Fig. 2. ^{1}H-NMR spectra of hydrolyzed EPS of *Volcaniella eurihalina* strain F2-7. Peak 1 indicated acetyl residues.

hydrolysis and decreases reactivity with H_2SO_4 reagent (*26*). This fact can also explain the low carbohydrate content detected by colorimetric analysis.

Concerning other components, we point out the presence of sulfates in the polymers under study. These substituents are quite unusual in other bacterial EPS studied, with the exception of polysaccharides of some marine bacteria and some archaeobacteria, which are like *V. eurihalina*, halophilic microorganisms (*24,27,28*). Sulphated EPS are of great interest because of their unique biological properties. They may be used as blood anticoagulants, as antitumor and antiviral agents, as vaccines, and in other applications (*1,29*).

The percentage of acetyls by weight, determined by RMN (Fig. 2) and colorimetric assay (Table 1), was found to be higher in EPS from NH medium than in those from MY medium, reaching the greatest values in EPS extracted from nutrient-deficient NH media. A number of other bacteria also synthesize exopolysaccharide with variable degree of acylation (*23,30,31*). EPS with different acylation patterns often show similar viscosity (*32*), although, in some exceptions, acetyls may modify viscosity of polymer solutions (*30*). In our case, acetyl content of different EPS can be positively correlated with the viscosity of their solutions.

The study of some specific components of the EPS was carried out using TLC in cellulose and next confirmed by GC-MS. The composition and molar ratios of neutral sugar constituents in the EPS purified from MY and NH media are presented in Table 2. No discernible differences

Table 2
Relative Sugar Composition of Exopolysaccharide Produced by *Volcaniella eurihalina*

Culture medium	Molar ratios			
	Glucose	Mannose	Rhamnose	UC
MY medium[a]	2.9	1.5	1	ND
NH-Alanine	1	≤0.1	2.7	1.8
NH-Citrate	1	≤0.1	6	1
NH-Glucose	1	≤0.1	2.9	1.6
NH-Maltose	1	≤0.1	2	0.1
NH-Mannose	1	≤0.1	3	0.3
NH-Mannitol	1	≤0.1	7	0.1
NH-Salicin	1	≤0.1	2.8	0.1

[a]Values are the means of results obtained under all conditions assayed in MY medium. UC, unknown component; ND, not detected.

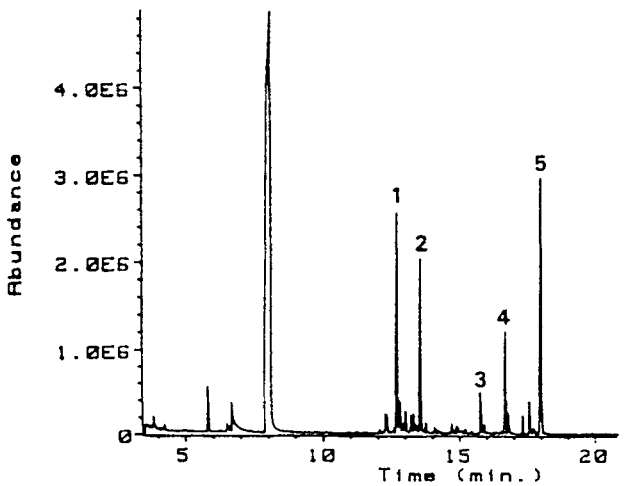

Fig. 3. Gas chromatography analysis of the TMS derivatives of the neutral sugars from the EPS synthesized by *Volcaniella eurihalina* strain F2-7 cultivated in NH medium with glucose as sole source of carbon and energy. Peaks corresponding to both anomeric forms, rhamnose (1,2) and glucose (3,4) and an unknown component (UC-5).

about the relative monosaccharide composition were found, under the deficient conditions studied in MY medium. However, there were some variations in the nature and relative amounts of neutral sugar components between NH and MY media. Thus, three major carbohydrate components, glucose, mannose, and rhamnose (molar ratio 2.9:1.5:1) were present in all polysaccharides extracted from MY medium, regardless of culture conditions. However, in all NH media, the EPS contained rhamnose and glucose, mannose in a low proportion, and an additional unknown component, designated in Fig. 3 as UC, detected at 18 min retention time. The identity

of the component giving rise to this peak is presently under investigation. Some sugars produced two peaks because of the occurrence of both anomeric forms. Table 2 also shows some examples of EPS extracted from NH media containing different carbon sources. As can be seen, the EPS varied in their relative sugar composition, although rhamnose was always the major component of the polymer.

Rheological behavior shown by polysaccharides in aqueous solution depends on sugar residues, linkage pattern, and occurrence of intra and intermolecular interactions (*33*). Certainly the most remarkable aspect shown still now by V2-7 polysaccharide is its ability to produce gel-like systems in acidic conditions (*8*), ability lacked in the EPS obtained from NH media. Differences between monosaccharide composition of EPS from NH and MY media could first explain the different behavior of EPS in solutions at acidic pH. Moreover, acetyl and uronic acid contents could also affect the crosslinking density and thus, modify the rheological properties of the polymer.

We are in agreement with Bryan et al. (*2*), which suggested that researchers studying EPS should carefully examine the growth conditions that are being used. It is important to define those conditions affording not only maximum production but also optimal chemical composition of the exopolysaccharide since it may determine its physical and biological properties.

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