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Exopolysaccharide production by *Volcaniella eurihalina*

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Abstract. The application of microbial extracellular polymers began in the 1960s, and since then there has been a remarkable increase in their commercial use. They are used in the food, textile, pharmaceutical, agricultural, paint and petroleum industries. *Volcaniella eurihalina*, a moderately halophilic eubacterium, produces an extracellular polysaccharide whose physical and chemical properties could be of interest for various industrial applications. The aim of this investigation is to analyze the different environmental parameters which influence the production of polysaccharide, and to study its chemical composition and the rheological properties of its solutions.
Key words. EPS; exopolysaccharides; biopolymers; moderately halophilic eubacteria.

Introduction

Volcaniella eurihalina is a halophilic eubacterium described by us in 1990 and named after B. Elazari Volcani, the microbiologist who first described halophilic bacteria from the Dead Sea[12]. This microorganism is a nonmotile Gram-negative rod, a chemoorganotroph with a respiratory type of metabolism using oxygen as the terminal electron acceptor. It grows optimally at salt concentrations of 5–10% (wt/vol) and therefore, according to Kushner's classification of the halophilic microorganisms, it can be regarded as a moderate halophile[6].

V. eurihalina strains produce extracellular polysaccharidic substances (EPS) in very large amounts. The presence of these exopolymers was first suggested by the typically large, mucous colonies formed. It was confirmed by scanning electron microscopy and transmission electron microscopy using ruthernium red, a specific stain for acidic polysaccharides (figs 1, 2 and 3).

EPS layers have several important functions in bacteria. They are being extensively studied nowadays because of their unique physical and chemical properties, which have led to numerous successful applications in industry. For example, they are used as thickening, gelling or suspending agents, or protective colloids. The production of microbial EPS for commerical use offers several potential advantages compared to isolation from plants and seaweed. These include the wide diversity of polymers produced by microorganisms, the fact that production is relatively independent of climatic conditions, the possibility of modification of the polymer composition and therefore of its physical properties, etc[10]. However, it is evident that not all the microorganisms that produce polysaccharides are suitable for use in practice, and many of the products obtained have only restricted usefulness. There are two major constraints: legislative and financial. Consequently, the search for new EPS should be continued.

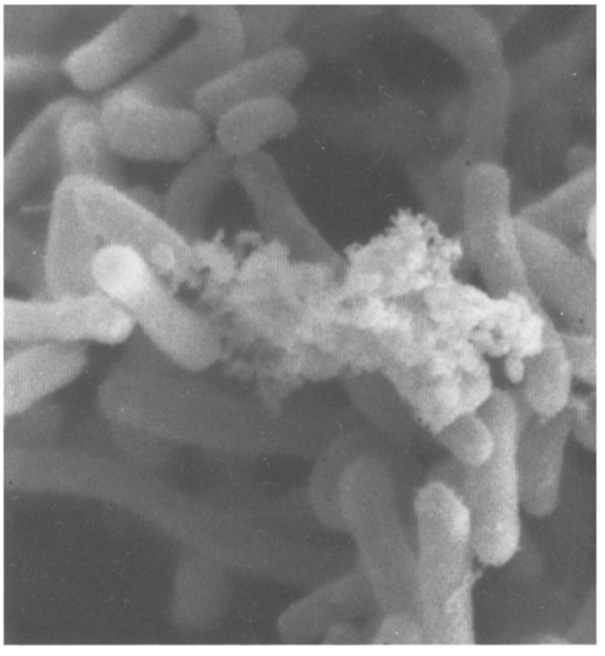

Figure 1. Scanning electron micrograph showing *Volcaniella eurihalina* cells attached to each other by means of an amorphous substance. Magnification × 10,000.

The present work is focused on the analysis of the different chemical and physical parameters which influence the production of polysaccharide by *V. eurihalina*, and on the preliminary study of its chemical composition and its rheological properties, as all these properties are of paramount importance in determining possible applications for the EPS in industry. After a screening program involving a total of 28 strains of *V. eurihalina*, strain F2-7 was selected as an exopolysaccharide producer, because of its particular growth requirements and other properties, which will be described later. This strain was isolated from hypersaline soil near Alicante in southern Spain[12].

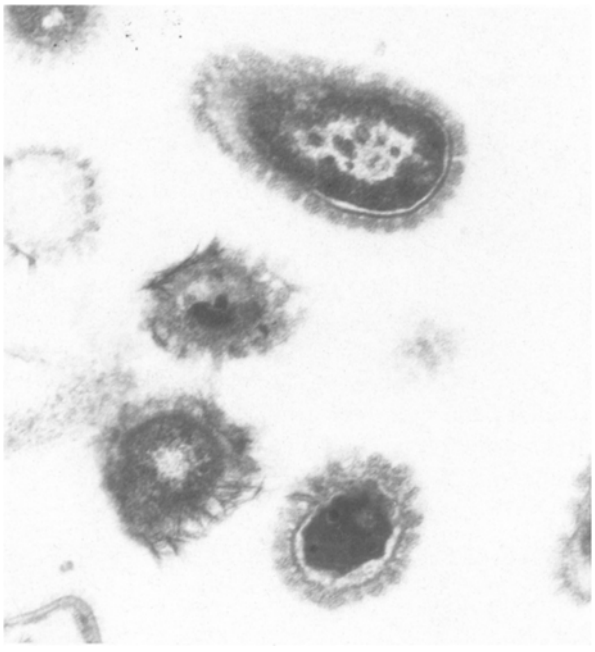

Figure 2. Transmission electron micrograph of a thin section of *Volcaniella eurihalina* cells showing the fibrous appearance of the EPS associated with the cell surface. Magnification × 40,000.

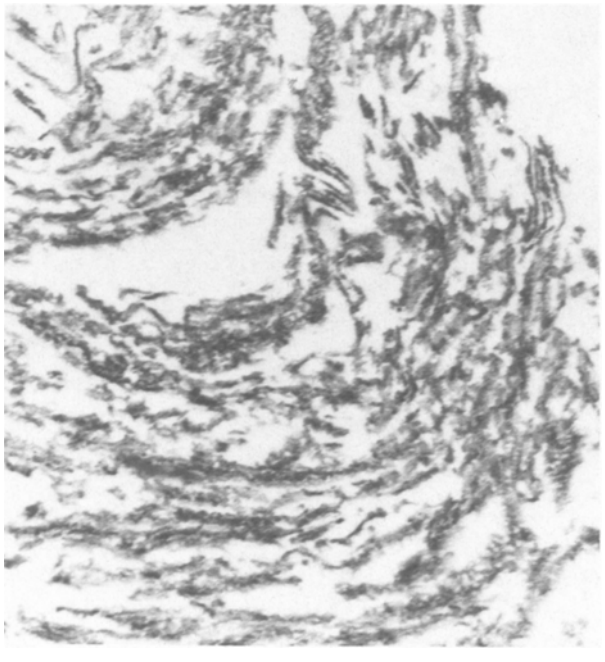

Figure 3. Transmission electron micrograph of a thin section of purified *Volcaniella eurihalina* EPS. Magnification × 40,000.

Productivity studies

Although most polysaccharide-synthesizing microorganisms produce EPS under almost all culture conditions which permit growth, the environmental variables needed to be optimized in order to give maximal production. As a starting point, exopolysaccharide production was investigated using MY medium[9]. This medium contains (%, wt/vol): glucose, 1; yeast extract, 0.3; malt extract, 0.3; and proteose-peptone, 0.5. A balanced mixture of sea salts[13] was added to give a final salt concentration of 7.5% (wt/vol). Using this basic medium, different modifications of the growth conditions were tested. We studied different incubation times (1–14 days), salt concentrations (2.5, 7.5, 10, 15 and 20%, wt/vol), and incubation temperatures (22, 32 and 42 °C). The pH of all media were adjusted to 7.2 with 1M NaOH. Media were distributed in 30 ml portions into 500-ml Erlenmeyer flasks, and sterilized by autoclaving at 110 °C for 30 min. Media were inoculated with 1 ml of a 2-day culture grown in the same medium ($OD_{520} = 2.5$) and incubated at 32 °C without shaking. The technique used in the isolation and purification of the EPS was as follows. First of all, cultures were centrifuged at 36,000 *g* in a Sorvall RC-5B refrigerated centrifuge for 60 min. The EPS were precipitated from the supernatants with 3 volumes of cold ethanol, resuspended in distilled water, and purified by ultracentifugation at 226,000 *g* for 90 min in a Beckman L8-M ultracentrifuge. They were then dialyzed against distilled water, and finally lyophilized and the yield determined gravimetrically. This purified material was used for analytical and rheological studies.

Figure 4 shows the relationship between the yields obtained in MY medium and incubation time, and also the kinetic relationships between the amounts of EPS, optimal densities of cultures, and numbers of viable cells per ml (obtained by plate count using MY medium solidified with 1.6% wt/vol agar). EPS synthesis started early during growth, rising as the number of viable cells increased, and reaching a maximum at the stationary phase. Although the largest amount of EPS, 2.8 g/l of culture medium, was obtained after a period of 8 days, significant quantities of EPS were precipitated from the fifth day onwards. We therefore selected an incubation

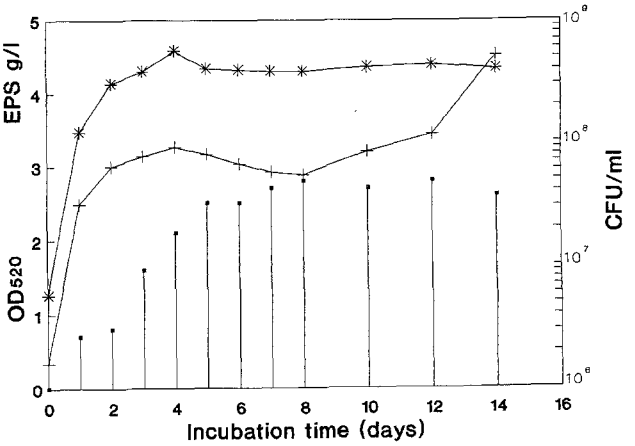

Figure 4. Influence of incubation time on EPS production. Symbols: —■—, EPS dry weight; —+—, optical density at 520 nm (OD_{520}); —*—, number of colony forming units per milliliter (CFU/ml).

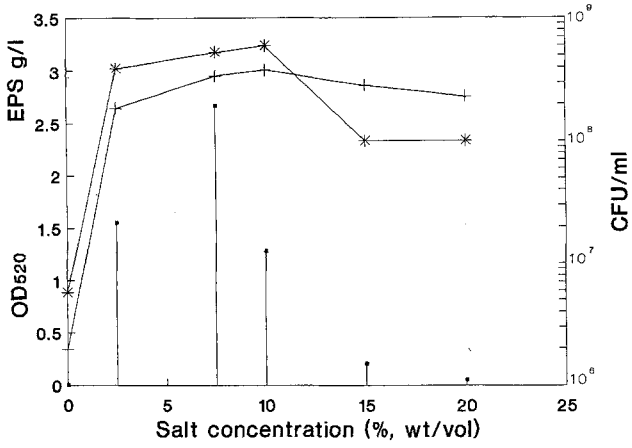

Figure 5. Influence of salt concentration on EPS production. Symbols: —■—, EPS dry weight; —┼—, optical density at 520 nm (OD_{520}); —∗—, number of colony forming units per milliliter (CFU/ml).

period of five days to save time. Generally speaking, the phase of growth during which polysaccharides are produced varies from one microorganism to another. Synthesis of the polymers may be growth-associated, as in the case of pullunan or alginates, or it may occur only when growth has ceased, as in the case of curdlan[14].

The efficiency of polymer synthesis was also affected by other environmental variables. Figure 5 shows the results obtained when we used MY media containing different salt concentrations. EPS synthesis increased with increasing salt concentration, up to 7.5% (wt/vol) of salts, when it was drastically reduced. Differences in the incubation temperature had little effect on the yields. Finally, we also studied how the stirring/aeration conditions influenced EPS production, since aeration of the culture medium is an important requirement for most polysaccharide-synthesizing bacteria, whether they are aerobes or facultative anaerobes[14]. We therefore cultivated cultures in a rotatory shaker at different values of revolution per min. Surprisingly, in all cases yields were less than those obtained in unshaken conditions. For example, at 200 rpm, the amount of EPS was reduced almost by half, to 55%. This result is anomalous in the sense that bacterial growth was favored by stirring.

At present we are carrying out other experiments on the optimization of production using different culture media and various sources of carbon and energy, and are studying how the pH of the culture medium influences the process. We are also trying other methods of isolation and purification, designed to improve product recovery.

Analysis of polysaccharide composition

Samples of the EPS obtained under optimal conditions for EPS production (MY medium; 7.5% wt/vol salts; 32 °C; 5–10 days of incubation in unshaken flasks) were analyzed in order to confirm the polysaccharidic nature of the polymer. The studies carried out were colorimetric assays and thin layer chromatography (TLC) in cellulose.

Colorimetric analysis

	% of total weight of polymer	Reference
Carbohydrates	42	3
Proteins	15	7
Hexoses	40	4
Uronic acids	4	2
Acyl residues	0.4	8

The table shows the results of colorimetric analysis, expressed as percentages of total EPS dry weight. These values are the means of at least five determinations. The total amount of carbohydrate was only 42% of the purified polymer dry weight. EPS with higher and lower percentages of carbohydrates have been described. For example, the EPS of *Pseudomonas aeruginosa* only contains 37% of carbohydrate[5], but *Rhizobium meliloti* produces an EPS with 79% total carbohydrates[1]. Our result could be due to the presence of uronic acids, which make exopolysaccharides resistant to acid hydrolysis[11]. The EPS preparations contained a certain amount of protein. Protein is usually considered as a normal contaminant of EPS preparations[15]. However, the presence of several amino acids has recently been described in some EPS. For example, serine is found in the EPS of *Escherichia coli* strain K-40, and L-glutamic acid has been reported in *Klebsiella aerogenes* type 82 EPS[14]. Further work to determine the composition of the protein fraction is in progress.

We used TLC in cellulose to identify the neutral sugar of the EPS. Purified polysaccharide was hydrolysed in 0.5 N sulphuric acid at 100 °C for 16–24 h. Then it was neutralized with Amberlite MB-1A and the solution was lyophilized. A suitable amount of EPS was dissolved in distilled water and applied to TLC plates. The plates were developed for about 24 h in a mixture of butanol/pyridine/water (6:4:3) and stained with alkaline silver nitrate. The purified EPS contained at least four components which chromatographed identically with glucose, galactose, mannose and rhamnose, which are also quite frequent in other EPS studied.

At present we are doing other experiments in order to confirm these findings, such as using other hydrolysis procedures, solvents or stains. Preliminary gas-chromatography mass-spectrometry studies indicate the presence of other monosaccharides and monosaccharide derivatives.

Rheological studies

As is known, end-use applications of most exopolysaccharides are almost exclusively based upon the rheolog-

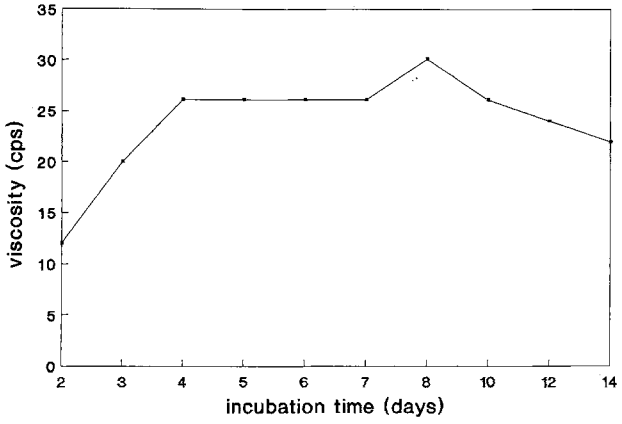

Figure 6. Effect of incubation time on the viscosity of *Volcaniella eurihalina* EPS.

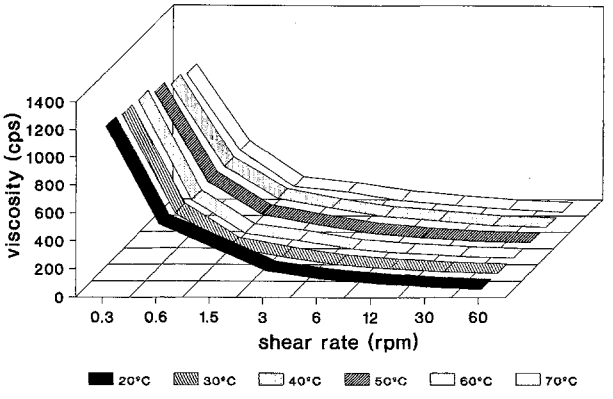

Figure 8. Viscosity of *Volcaniella eurihalina* EPS as a function of shear rate at different temperatures.

ical properties they confer on their solutions. Therefore we have also carried out some initial studies concerning the rheological properties of the polymer solutions.

Lyophilized samples of EPS obtained under different culture conditions were dissolved in distilled water to give 1% (wt/vol) solutions. Viscosity was measured with a Brookfield viscometer LVT fitted with a small sample adaptor. Measurements were done at 25 °C with a shear rate of 1.98 sec^{-1}, unless otherwise stated.

Figure 6 shows the viscosities of 1% (wt/vol) EPS solutions obtained after different incubation times. These values were quite similar from the fourth to the tenth day of incubation, ranging from 20 to 30 centipoises. Figure 7 shows the effect of EPS concentration on the solution viscosities; this rheogram indicates a relatively high viscosity increasing with the concentration. Finally, *V. eurihalina* EPS solutions exhibit pseudoplastic behavior, as is shown in figure 8. Pseudoplasticity, which is another important characteristic for some applications, is indicated when the viscosity decreases as the shear rate is increased[10]. These data were obtained by analyzing 2% (wt/vol) EPS solutions at the different shear rates and temperatures indicated in the illustration.

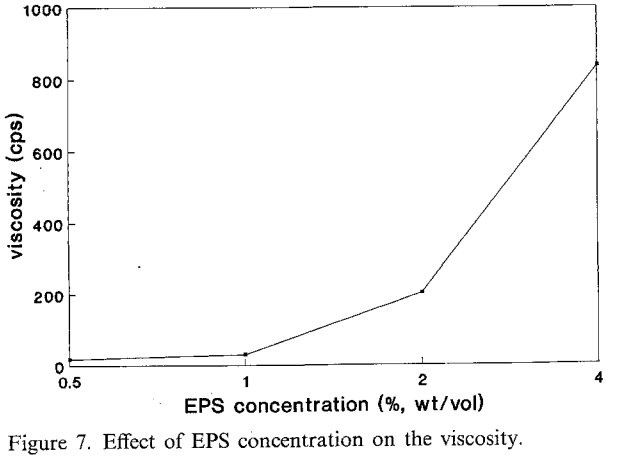

Figure 7. Effect of EPS concentration on the viscosity.

Conclusions

Volcaniella eurihalina strain F2-7, the microorganism used in this study, offers specific advantages for biotechnological use. It grows rapidly and produces polysaccharide in a relatively short period of time; it is not pathogenic to humans or to economically important animals or plants; it is capable of growing on a relatively cheap culture medium and the cultures are difficult to contaminate due to their high salt content. It is almost certain that the strain F2-7 only produces one exopolysaccharide, and it does not use substrate for the synthesis of large amounts of intracellular storage products such as poly-β-hydroxybutyric acid or glycogen. Furthermore, and above all, *V. eurihalina* strain F2-7 synthesizes an extracellular polysaccharide which produces solutions of relatively high viscosities. Some studies carried out very recently proved that the solution viscosities are resistant to high ionic strength, quite thermostable, and show the highest viscosities at acid pH (unpublished observations). This EPS could therefore be valuable for use for various industrial applications.

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