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COMPARATIVE METHODS FOR ISOLATION OF *Volcaniella eurihalina* EXOPOLYSACCHARIDE

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SUMMARY

The best isolation procedure to obtain the EPS of Volcaniella eurihalina was centrifugation at 36,000 x g for 60 min and precipitation with cold ethanol after tangential filtration with 100,000 D ultrafilters. Ion chromatography showed that this EPS contained glucose, rhamnose and mannose in a molar ratio of 3.2: 1.1: 1, respectively.

INTRODUCTION

The growth of bacteria is often accompanied by the production of polysaccharides which are found outside the cell wall. These exopolysaccharides (EPS) may be found as a capsule attached to the bacteria or they may be released to the environment as slime or both. A polysaccharide may be important to the bacterium but it may have commercial value as well. Some polysaccharides are known to have gelling, emulsifying or thickening properties, and also may represent a source of certain important monosaccharides (Sutherland, 1990).

Volcaniella eurihalina is a moderately halophilic bacterium described by us in 1990 (Quesada et al., 1990). This microorganism produces an EPS in large amounts (Quesada et al., 1993). The EPS of *V. eurihalina* has potential commercial interest because of its sulphate group content and on account of the relatively high viscosity of its solutions at low pH values.

The quantity and quality of EPS produced and the ease of harvest and processing of the exopolysaccharide are important factors for industrial applications. There are many methods for recovery EPS, such as extraction with NaOH (Savidge and Colvin, 1985), EDTA (Mian et al., 1978), NaCl (Read and Costerton, 1987), heating (Allen et al., 1987) or filtration (Cerning et al., 1988); and still more, the precipitation can be carried out using different organic and inorganic compounds (Beech et al., 1991; Meade et al., 1994; Mody et al., 1989).

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The aim of this work was to determine the best extraction and purification method in order to reach the highest product yield with the lowest cost. Also we have determined the sugar composition of *V.eurihalina* EPS obtained by the method finally selected.

EXPERIMENTAL METHODS

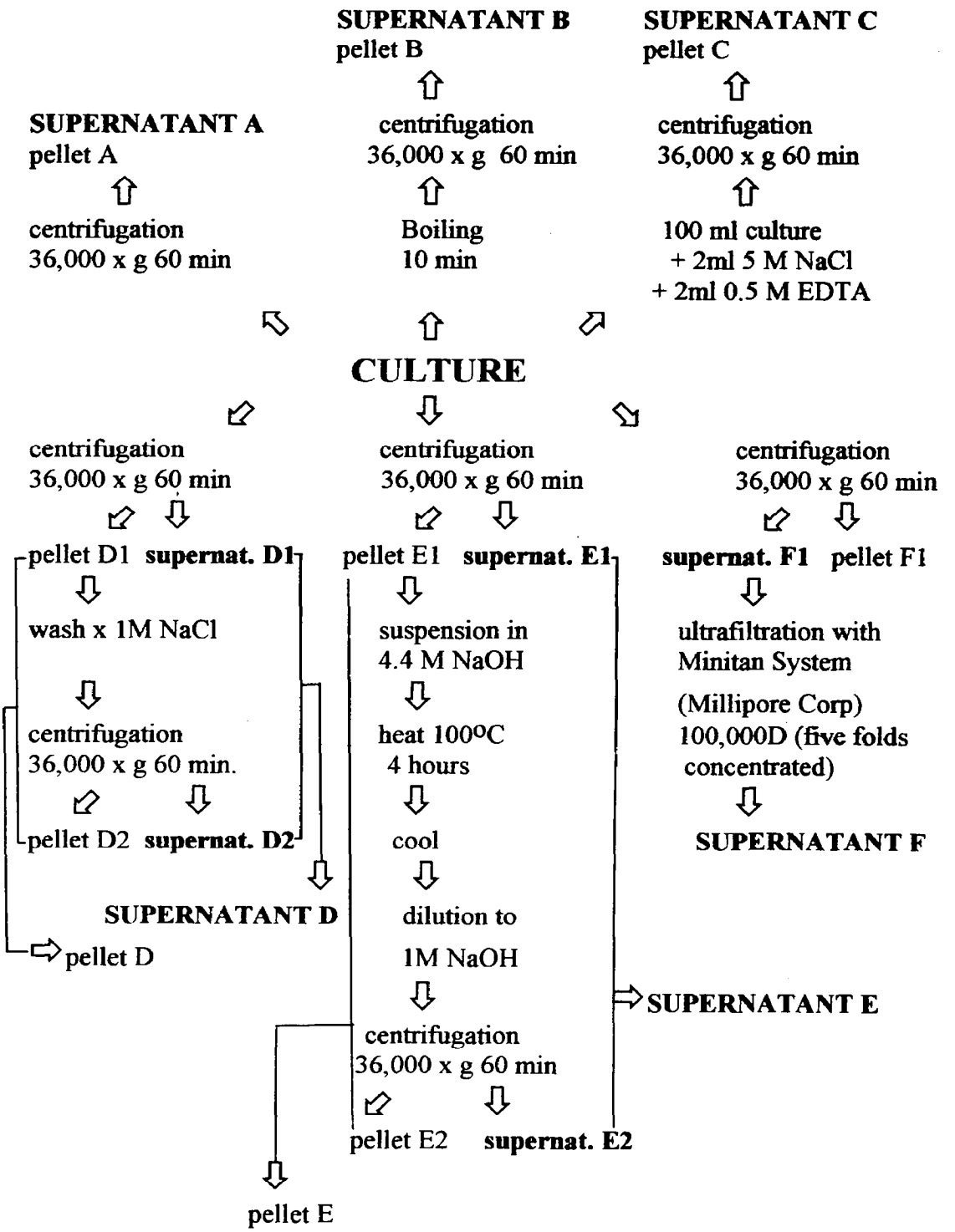
Bacterial strain and growth method. *Volcaniella eurihalina* is a moderately halophilic bacteria which grows optimally at salt concentrations of 5-10 % (wt/vol) (Quesada et al., 1990). The strain F2-7 has been used through this study.

The complex medium used was MY (Moraine and Rogovin, 1966); this medium was supplemented with 7.5 % (wt/vol) sea salts (Rodriguez-Valera et al., 1981) to get a suitable osmolarity. Cultures were grown in 500 ml Erlenmeyer flasks containing 100 ml of medium at 32°C for 8 days in steady conditions. They were inoculated with a suitable inoculum (1 ml, OD_{520}= 2.5) made in the same medium.

Isolation of EPS. The procedures for isolation of EPS are illustrated schematically in scheme 1. After incubation time, all cultures were joined and an aliquot from the mixture was used to carry out each extraction method. All pellets were lyophilized to determine the dry cell weight. Supernatants were processed as following: All EPS were precipitated from supernatants with 3 volumes of cold ethanol. Supernatants obtained by methods A and F were also precipitated with other compounds including acetone, isopropanol or $(NH_4)_2SO_4$ (Rosenberg et al., 1988). Next, the precipitated polymers were resuspended in distilled water and purified by ultracentrifugation (226,000 x g for 60 min). Then, they were dialyzed for 24 hours against running distilled water, frozen and lyophilized. Finally, they were determined gravimetrically.

Chemical analysis. EPS extracted by each method above described was subjected to colorimetric analysis, including the following determinations: proteins (Lowry et al., 1951), carbohydrates (Dubois et al., 1956) and uronic acids (Blumenkrantz and Asboe-Hansen, 1973). Additionally, the sugar composition of samples obtained by method F was studied by ion chromatography. This was performed on a Dionex (QIC) ion chromatography system equipped with a 25 μl loop and at an eluent flow rate of 1 ml min^{-1}. The detector was a pulsed amperometric detector with a gold electrode. An anionic-exchange column Carbo Pac PA-1 equipped with a Carbo Pac PA-1 guard column was used. EPS samples were previously hydrolyzed with 0.5N H_2SO_4 and neutralized with Amberlite MB-1A. Standard sugars were used to determine retention times.

Scheme 1.- Different methods for the isolation of EPS



RESULTS AND DISCUSSION

The attainment of optimal conditions for EPS recovery is a time consuming task with many variables under study. In this work we have evaluated different methods for *V. eurihalina* EPS extraction. Our aim has been to increase both quality and quantity of the purified polymer.

In table 1, we have represented the results obtained using the different extraction procedures and ethanol precipitation of the EPS. In these experiments we use aliquots of the mixture of cultures, for this reason the dry weight of cells must be similar. However, when we carried out NaOH extraction (method E) the dry weight was significant lower probably due to cellular lysis. This argument explains, in this case, the high efficiency of extraction. Extraction with EDTA (method C), did not cause lysis of the cells and could be effective for separation bacterial cells and exopolysaccharide, since quelating agents eliminate polymer-cell cross-linking by divalent cations (Mian et al., 1978). This, surely, is the reason why extraction C is the most efficient method in spite that, differences are not very significant. With respect to method F, the Minitan system allowed to save a large amount of precipitating agents; moreover , the ultrafiltration decreased the protein content associated with the polymer. Protein, carbohydrate and uronic acid contents were measured as specific indicators of the polymer purity (Fazio et al., 1982). With the exception of method E, the values obtained were quite similar for all extraction method studied.

Table 1.- Characteristics of EPS obtained from MY medium by different extraction procedures.

Method	EPS[a]	Dry cell weight[a]	Productivity	CH[b]	Pr[b]	UA[b]
A	0.88	2.69	32.71	34.3	15.3	1.5
B	0.96	2.54	37.79	33.5	15.6	1.3
C	1.05	2.72	38.60	33.8	15.4	1.3
D	1.03	2.76	37.31	36.1	19.2	1.3
E	1.12	0.99	113.13	40.6	25.1	1.4
F	0.99	2.69	36.80	39.0	12.0	1.6

[a] expressed in g/l

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

CH = carbohydrates; Pr = proteins; UA = uronic acids

In table 2 we have compared EPS obtained by methods A and F when we used different precipitating agents. Isopropanol, acetone and ammonium sulphate were more effective as precipitating agents than ethanol for EPS extracted by method A. However, these compounds precipitated proteins in a high percentage; moreover, ammonium sulphate precipitated carbohydrates in a low level. In method F, in which the Minitan system allow to remove some small peptides and other impurities from the culture, differences were not so pronounced. As in most polysaccharides described, some of the proteins are surely contaminants of EPS preparations, even so, a fraction of the protein content detected could be close-associated to the EPS, seeing that high percentages are found in all samples studies (see table 1 and 2). That happens to emulsan, a EPS synthesized by *Acinetobacter calcoaceticus* (Rosenberg, 1986)

Table 2.-Characteristics of EPS obtained by extraction methods A and F and with different precipitating agents.

Precipitating agent	EPS[a]	Productivity	CH[b]	Pr[b]	UA[b]
Method A					
Ethanol	0.88	32.71	34.3	15.3	1.5
Acetone	2.13	79.18	31.3	31.1	1.3
Isopropanol	1.94	72.12	31.1	28.5	1.0
Ammonium sulphate	1.78	66.17	9.2	28.0	0.4
Method F					
Ethanol	0.99	36.80	39.0	12.0	1.6
Acetone	1.12	41.63	35.6	19.3	1.4
Isopropanol	0.78	28.99	37.0	18.0	1.4
Ammonium sulphate	0.80	29.74	13.0	18.2	0.6

[a] expressed in g/l

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

CH = carbohydrates; Pr = proteins; UA = uronic acids

In these experiments the dry cell weight is the same (2.69 g/l).

From all these results we have deduced that the best procedure to obtain the EPS of *V. eurihalina* was centrifugation at 36,000 x g for 60 minutes, followed of tangential filtration with 100,000 D ultrafilters of the supernatants (method F), and then precipitation with cold ethanol. This method is simple, economical and rapid, yieldind a good recovery and a clean single-step preparation of EPS. Finally, once selected the conditions above described, we have analyzed the sugar composition of the polymer by ion chromatography. EPS of *V. eurihalina* F2-7 is composed at least by three monosaccharides, glucose, rhamnose and mannose in a molar ratio of 3.2:1.1:1, respectively.

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