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Simultaneous production of two different gel-forming exopolysaccharides by an *Alteromonas* strain originating from deep sea hydrothermal vents

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Exopolysaccharide production by the marine bacterium *Alteromonas* sp. strain 1644 was shown to be stimulated by restricted growth conditions and was optimized in nitrogen limited fed-batch cultures. Exopolysaccharides were either partly secreted in the medium or stayed firmly cell-associated. The cell-polysaccharide associations could be destroyed by dialysis against distilled water, allowing polysaccharide purification. The chemical and rheological characterization of this last polysaccharide showed that it was different from the secreted polysaccharide that has been previously described (polysaccharide 1644). At low ionic concentration (below 0.03 M whatever the nature of the ions), solutions of this new polysaccharide had very low viscosities. However, at higher ionic concentration, it formed a gel or exhibited in solution at low polymer concentration an unusually high temperature dependent viscosity. This behaviour was also dependent on the nature of the ions and the following sequences for cations and anions were $NH4^+ > Mg^{2+} > Na^+ > Li^+ > K^+ > TMA^+$ and $Br^- > NO_3^- > SO_4^{2-} > Cl^- > I^-$ respectively. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

The polysaccharide 1644 is produced by a strain of *Alteromonas sp.* which was isolated from samples collected in deep sea near a hydrothermal vent (Vincent, 1993; Vincent *et al.*, 1994). This polysaccharide has been selected among several marine bacterial exopolysaccharides because of its unique rheological behavior (Guezennec *et al.*, 1994). In the presence of divalent ions, purified polysaccharide 1644 forms a very strong elastic gel which can be stretched reversibly without any damage (Bozzi *et al.*, 1995a) The chemical structure of polysaccharide 1644 has recently been determined (Dubreucq, 1996). The repeating unit consist of 4 neutral and 3 acidic sugars; an unusual dicarboxylic acid sugar (3-O-[(R)-1-carboxyethyl]-D-glucuronic acid) is located on a side chain composed of 3 sugars (Dubreucq *et al.*, 1996). The presence of this dicarboxylic acid is thought to explain the high affinity of the polysaccharide for divalent cations. Rheological studies suggest that the strength and the elasticity of 1644 gels are due to a low degree of crosslinking which could be related to the high affinity of the polysaccharide for divalent ions (Bozzi *et al.*, 1995b).

The physiology of exopolysaccharide production by *Alteromonas sp.* strain 1644 is not well understood (Vincent, 1993). On one hand, polysaccharide 1644 is secreted in the medium but a large exopolysaccharide fraction remain cell-associated and is not recovered by common purification processes. On the other hand, polysaccharide 1644 is mainly produced at the beginning of the stationary phase of growth, suggesting that polysaccharide synthesis is induced by restricted growth conditions. The initial goal of this study was to

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increase the production yield of polysaccharide 1644. We first show that the yield and the reproducibility of polysaccharide production can be improved by using a fermentation strategy based on a nitrogen growth-limitation. Then we described a method to recover and purify the cell-associated polysaccharide fraction. However, we also found that this polysaccharide was completely different from polysaccharide 1644. This new polysaccharide was characterized and shown to exhibit very unusual gelling properties.

MATERIALS AND METHODS

Organism and culture conditions

Alteromonas sp. strain 1644 (CNCM no I-1282) was obtained from P. Vincent, CNRS, Station Biologique de Roscoff (France). Routine culture were performed at 25°C on marine medium which contained: peptone, 4 $g l^{-1}$; yeast extract, 1 $g l^{-1}$; sea salt (sigma S 9883), 30 $g l^{-1}$; MOPS (3-N-(morpholino)propanesulfonic acid), 3 $g l^{-1}$. The defined mineral medium contained: sea salt, 30 $g l^{-1}$; MOPS, 3 $g l^{-1}$; KH_2PO_4, 2 mM; vitamin solution, 10 $ml l^{-1}$; trace mineral solution, 10 $ml l^{-1}$. The concentration of ammonium chloride and of fructose are further indicated in the text. Vitamin and trace mineral solution were prepared as described (Zeikus & Wolfe, 1972). Phosphate and fructose were autoclaved separately.

Polysaccharide fermentations were performed in two-litre reactors. The pH was initially adjusted to 7.0 and then maintained at 6.6 by automatic addition of 2.5 N NaOH. The stirring rate was 1000 rpm and the air flow rate was 0.5 $l min^{-1}$. Foaming was avoided by addition of silicon oil.

Analytical techniques

Growth was followed by measuring the culture turbidity at 540 nm. Fructose concentrations were quantified by determining the reducing sugar with the dinitrosalicylic method (Miller, 1959). Uronic acid concentrations were quantified with the 3-hydroxydiphenyl-sulfuric acid method (Blumenkranz & Asboe-Hansen, 1973). Ammonium concentration was enzymatically determined with glutamate dehydrogenase using a kit from Boehringer Mannheim.

Polysaccharide quantification

Culture samples (0.5 ml) withdrawn from the fermenter were diluted (1:20) with 1% NaCl and centrifuged at 35000*g* for 30 min. The supernatants were dialysed overnight against 1% NaCl to eliminate residual fructose which could interfere with the dosage of uronic acid. Free polysaccharide (corresponding to the polysaccharide 1644) was quantified by measuring the uronic acid concentration in the supernatant using purified polysaccharide 1644 as a standard. The pellet containing the bacterial cells and the cell-associated polysaccharide was suspended in distilled water. The polysaccharide fraction associated with the cells (corresponding to the polysaccharide 1644B) was quantified by determining the uronic acid concentration using purified polysaccharide 1644B as standard.

Purification of polysaccharide 1644B

One litre of culture medium was diluted (1:3) with distilled water and centrifuged for 2 h at 20000*g*. The gelatinous pellet containing the bacterial cells and the polysaccharide 1644B was suspended in 1.5 l of 1% NaCl and centrifuged again for 1 h at 20000*g* (this step was necessary to eliminate residual polysaccharide 1644 present in the pellet and NaCl was added to maintain the polysaccharide 1644B in the gel phase). The supernatant was eliminated and the gelatinous pellet was poured directly into dialysis bags and dialysed overnight against distilled water. The pellet was then suspended in 1 l of distilled water and centrifuged for 1 h at 20000*g*. The supernatant containing the polysaccharide 1644B was recovered and filtered through Millipore filters (0.8 μm). After addition of 30 $g l^{-1}$ of NaCl, the polysaccharide was precipitated by addition of ethanol (50% v/v). The precipitate was successively washed with ethanol–water mixtures from 70% to 100% (v/v) and dried under vacuum for 48 h at 30°C.

Chemical analysis of polysaccharide 1644 and 1644B

Monosaccharides resulting from methanolysis (0.5 M methanolic HCl, 24 h, 80°C) were analyzed by gas chromatography/mass spectrometry (GC/MS) as pertrimethylsilylated methyl glycosides (Kamerling *et al.*, 1975). Gas chromatography analysis were performed on Delsi DI 700 gas chromatograph equipped with a fused silica OV 101 capillary column (0.3 mm i.d. × 24 m), using helium as carrier gas at a pressure of 0.15 atm. The following temperature program (120–240°C at 2°C min^{-1}) was used. GC/MS analyses were carried out on a Nermag R10-10 spectrometer. MS analyses were carried out in chemical ionisation mode using ammonia as a reactant gas.

Rheological characterization

The rheological behavior of solutions of polysaccharide 1644B was studied by flow measurements using a LS30 viscometer (Contraves, Switzerland) equipped with a Haake thermostat and a Carrimed CS50 (Dorking, UK) equipped with a Rheo 1000C system and 5.0

software. The various geometries used were cones: 4 and 6 cm diameter, 4° cone angle end, 6 cm diameter, 1° cone angle. To obtain a network at very low polysaccharide concentration, the solution of polysaccharide 1644B needed to be prepared as follows: the polysaccharide was first dissolved in pure deionized water and the salt solution was poured very slowly with gentle stirring into the polysaccharide solution. The polysaccharide and salt concentrations were adjusted in order to obtain the required final concentrations of salt and polysaccharide after addition of the same volume of each solution.

RESULTS AND DISCUSSION

Optimization of exopolysaccharide production

In contrast to some bacterial polysaccharides such as xanthan or gellan which are synthesized during active bacterial growth (Lobas *et al.*, 1992; Moraine & Rogovin, 1973), polysaccharide 1644 is only produced during a growth-restricted phase that follows the initial exponential growth phase. To improve the production yield, our fermentation strategy was to control the growth by employing a nitrogen-limitation. As the complex medium, which has been previously used for polysaccharide 1644 production, contains high concentration of various nitrogen sources (peptone and yeast extract), we change to a mineral defined medium containing ammonium chloride as the sole nitrogen source.

With an initial ammonium chloride concentration of 1.1 gl^{-1}, the cells grew exponentially up to an OD of 7.0 (Fig. 1). Then, because of ammonium exhaustion, growth slowed down. However, although no nitrogen source was available, the OD of the culture continued to increase linearly until all the fructose has been consummed. Microscopic examination and counting by plating indicated that this increase in OD correlated with the number of viable cells. Accumulation of storage granules of poly-hydroxybutyrate, which could have explain this increase of biomass in absence of nitrogen source (Kragelund & Nybroe, 1994), was not observed. Both soluble and cell-associated polysaccharide fractions started to be produced only 15 h after the end of the exponential phase. Because of this delay the final polysaccharide yield was poor. As this delay has not been observed in complex media, we made the hypothesis that the growth limitation by ammonium was too sudden and that a complete privation of nitrogen could be unfavorable for the synthesis of the protein machinery necessary to build polysaccharides. We thus adopted a slightly different strategy. The initial ammonium chloride concentration was lowered to 0.4 gl^{-1} and after the initial exponential phase, an ammonium chloride solution was continuously fed into the medium to maintain a small level of protein synthesis (Fig. 2). During the entire feeding phase, the ammonium did not accumulate indicating that growth was nitrogen-limited. In these conditions, exopolysaccharide production started as soon as the ammonium has been depleted and continued almost linearly until the end of the fermentation. Soluble and cell-associated polysaccharides were simultaneously produced and their final yields were 7.5 gl^{-1} and 5.0 gl^{-1}, respectively. These yields are 50% higher than those we have previously obtained with the complex medium. In addition this fermentation protocol reduces the variations in polysaccharide yield which are observed with complex media from one fermentation to an other.

Recovery and purification of the cell-associated polysaccharide

After centrifugation of the medium, bacterial cells were recovered in a large gelatinous pellet whose volume

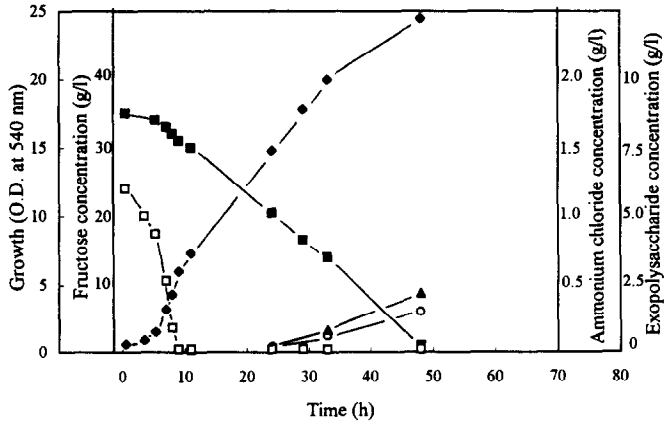

Fig. 1. Exopolysaccharide production by *Alteromonas sp.* strain 1644 in batch culture with a limited initial supply of 1.1 gl^{-1} of ammonium chloride: (◆) bacterial growth; (■) fructose consumption; (▲) soluble exopolysaccharides; (○) cell-associated exopolysaccharides; (□) ammonium choride.

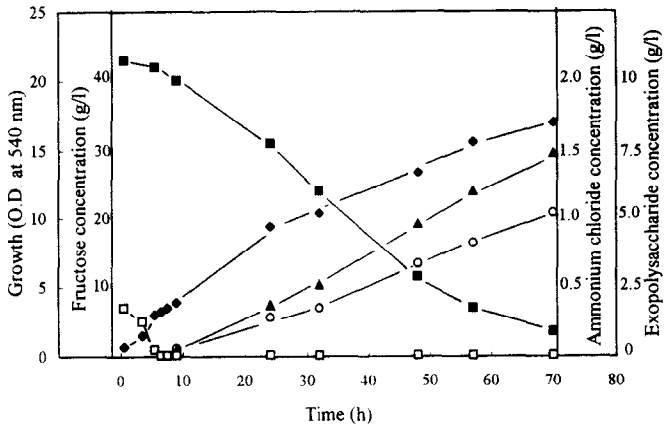

Fig. 2. Exopolysaccharide production by *Alteromonas sp.* strain 1644 in fed-batch culture with a continuous supply of ammonium after the exhaustion of the small initial amount of ammonium chloride (0.4 $g l^{-1}$) present in the starting medium: (◆) bacterial growth; (■) fructose consumption; (▲) soluble exopolysaccharides; (○) cell-associated exopolysaccharides; (□) ammonium choride.

represent up to 25% of the initial medium volume. Determination of uronic acid content indicated that this pellet contain high concentration of acidic polysaccharide (see above). All attempts to solubilize this cell-associated polysaccharide by physical (sonication) or chemical treatment (pH, EDTA) were unsuccessful. We finally found that dialysis of the pellet against distilled water resulted in a complete loss of its gelatinous nature. Centrifugation of the dialysed pellet produced a compact pellet suggesting that it was made only of bacterial cells. All the acidic polysaccharide fraction was recovered in the supernatant. This supernatant had no apparent viscosity but its supplementation with 3% sea salt (Sigma S9883) resulted immediately in the formation of a gel having the same appearance that the original gelatinous pellet. This rheological behavior is completely different from this of polysaccharide 1644. This strongly suggests that the cell-associated polysaccharide is different from polysaccharide 1644. This new polysaccharide was further referred as polysaccharide 1644B.

Chemical characterization of polysaccharide 1644B.

Chemical analysis of purified 1644B confirmed that it is really different from polysaccharide 1644 (Table 1). In contrast with the latter, it contained rhamnose and mannose but not the specific dicarboxylic sugar (3-O-[(R)-1-carboxyethyl]-D-glucuronic acid) and galacturonic acid.

Rheological properties of the network formed from polysaccharide 1644B solutions

We have characterized the flow behavior of dilute and semi-dilute solutions of polysaccharide 1644B as a function of polymer and salt concentrations. We have also tested the influence of the temperature and of the nature of the salt (anions and cations) on the viscosity.

In Fig. 3, we give an example of the flow curves obtained for different salts. No thixotropic effect was observed in the range of shear rates and polymer concentration investigated in this work. These results show that a very low concentration of polysaccharide 1644B (0.05 $g l^{-1}$) resulted in a 100-fold increase in the solvent viscosity at low shear rates ($10^{-2} s^{-1}$). This unusually high viscosity at low polymer concentration will be referred to as the "1644B effect". The magnitude of this "effect" depended on the nature of the ions. The following sequences were obtained for the cations:

$$NH_4^+ > Mg^{2+} > Na^+ > Li^+ > K^+ > TMA^+$$

(tetramethylammonium)

and for the anions:

$$Br^- > NO_3^- > SO_4^{2-} > Cl^- > I^-$$

The appearance of the "effect" was also dependent on salt concentration. Whatever the nature of the salt, the effect was not observed below a minimal salt concentration of 0.03 M (Fig. 4). Temperature also had a peculiar influence on the "effect". A decrease in the viscosity was observed when the temperature increased from 35°C to 60°C. Opposite behavior was observed above 60°C (Fig. 5).

The influence of polymer concentration on the viscosity level was investigated in 0.1 M NH_4NO_3 at two different shear rates (Fig. 6). At low shear rate, the viscosity increased concurrently with the augmentation of the polymer concentration to reach a plateau at a concentration of around 1 $g l^{-1}$. In contrast, at higher shear rate a continuous increase in the viscosity was observed up to a polymer concentration equal to 7 $g l^{-1}$. Below this concentration, no thixotropic effect was observed for the shear rates used. The plateau

Table 1. Molar composition of polysaccharides 1644 and 1644B

Polysaccharide	Glc	Gal	Man	Rha	GlcUA	GalUA	X[a]	%methanolyse
1644	0.92	1.0	0	0	0.7	0.26	0.34	36
1644B	2	1	1.2	0.7	1.1	0	0	31

Molar ratios were calculated as a function of the internal standard myo-inositol on the basis of one galactose residu
[a] X = 3-O-[(R)-1-carboxyethyl]-D-glucuronic acid.

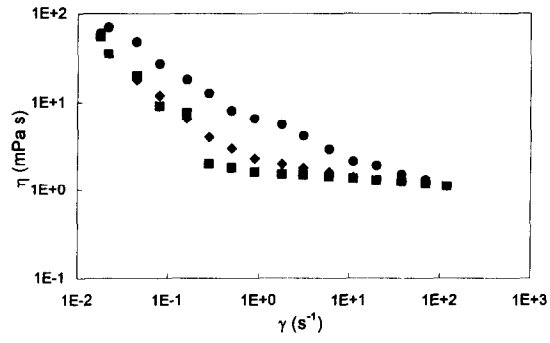

Fig. 3. Viscosity of a 1644B solution ($C_p = 0.05$ g l^{-1})as a function of shear rate in different salts (0.1 M) and at 25°C.(●) NH_4NO_3; (■) $LiNO_3$; (◆) $NaNO_3$.

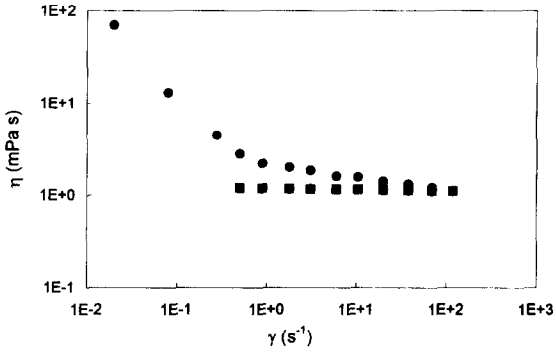

Fig. 4. Viscosity of a 1644B solution ($C_p = 0.05$ g l^{-1}) as a function of shear rate in different concentrations of $LiNO_3$ at 25°C. (●) 0.1 M; (□) 0.03 M; (+) 0.01 M.

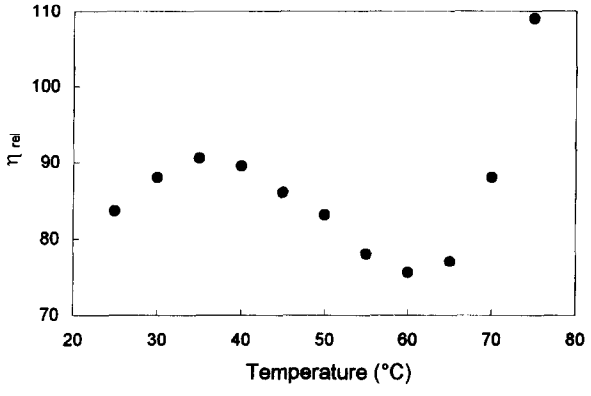

Fig. 5. Relative viscosity as a function of temperature for a 1644B solution ($C_p = 0.25$ g l^{-1}) in 0.1 M NaCl at a shear rate equal to 1 s^{-1}

found in the viscosity, at low shear rate, between 1 and 7 g l^{-1} could be due to an increase in the intermolecular interactions leading to microgel formation compared to loose associations below 1 g l^{-1} giving high shear rate effect. Over a polymer concentration of 7 g l^{-1}, it was no longer possible to obtain reproducible flow curves. The structure of the gels was destroyed during the measurement and the

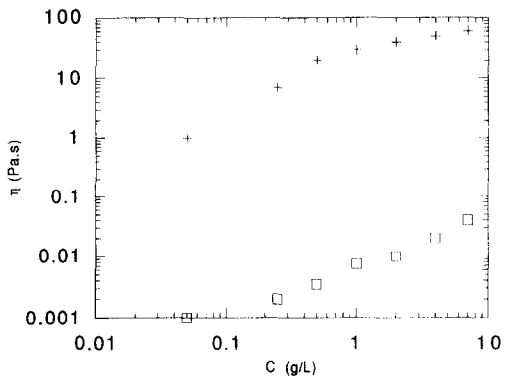

Fig. 6. Influence of 1644B concentration on the viscosity of the solution in 0.1 M NH_4NO_3 at 25°C. (+) at a shear rate equal to $10^{-2}\,s^{-1}$; (□) at a shear rate equal to $10^{2}\,s^{-1}$.

gels became turbid due to the increase in the chain associations and there to a decrease in polymer solubility.

The general behavior of polysaccharide 1644B described above is very different from that of polysaccharide 1644 (Bozzi *et al.*, 1995b). This last polysaccharide forms a very elastic and resistant gel only in the presence of divalent counterions which act as crosslinking agents. In contrast, the gel formed from polysaccharide 1644B is very loose and does not need divalent counterions. The nature of the intermolecular interactions involved is not well understood at present. But the increase in viscosity at high temperatures could suggest hydrophobic interactions. More experiments and a knowledge of the chemical structure are needed to clarify these very unusual properties which lead to very viscous solution at very low polymer concentration and shear rate, looking like a gel. In fact, loose interchain interactions stabilized by secondary forces must be formed as a precursor of the gel.

CONCLUSION

Both chemical and rheological characterizations of the cell-associated polysaccharide fraction clearly demonstrate that this fraction was made of a polysaccharide which is different from the soluble polysaccharide 1644 which has already been characterized (Bozzi *et al.*, 1995a; Bozzi *et al.*, 1995b). Only a few reports on simultaneous production of two different exopolysaccharides by the same microorganism are known (Christensen *et al.*, 1985). This may be attributed to the difficulty in separating possible mixture of exopolysaccharides. In our case, the separation of the two polysaccharides produced by strain 1644 has been greatly facilitated by their different gelling properties. Low concentration solutions of polysaccharide 1644B have a very high viscosity compared with xanthan. Possible applications of this unique property are under investigation.

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