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Effects of yeast extract on the production and the quality of the exopolysaccharide, zooglan, produced by *Zoogloea ramigera* 115SLR

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Abstract Although many studies have examined the influence of culture conditions on the production and composition of polysaccharides, little is known about the factors influencing the quality of exopolysaccharides (EPS). In this work we studied the effect of yeast extract on the production, composition and molecular weight of the EPS zooglan produced by *Zoogloea ramigera* 115SLR. This bacterium was grown on a new completely defined synthetic medium and on a medium containing yeast extract. Growth and polysaccharide production performances were comparable on the two media with a glucose to exopolysaccharide conversion yield of 35% (g/g). The polysaccharides produced on these two media have an identical composition but a different molecular weight and molecular weight distribution. The yeast extract medium leads to a more homogeneous polysaccharide solution.

Introduction

Zoogloea ramigera 115 (ATCC 25935), a gram-negative, floc-forming bacterium, produces an extracellular polysaccharide, zooglan. *Zoogloea ramigera* was first isolated from waste water and has been extensively examined as a bioflocculating organism for the control of biological oxygen demand (Friedman and Dugan 1968). Zooglan is able to bind metals including Cd, Co, Cu, Cr, Fe, Ni, Pb and Zn (Norberg 1984; Sag and Kutsal 1989a, b; Sag et al. 1995a, b). The presence of negatively charged groups in the polysaccharide backbone may explain this property. Although the complete structure of this exopolysaccharide is not yet known, the proposed structure contains glucose and galactose in a molar ratio of 2:1, in a main chain predominantly β1-4 linked. Branching occurs at the galactose units and consists of short β1-4 and β1-6 linked glucose chains. The exopolysaccharide (EPS) is modified by the addition of pyruvate, acetate and succinate as side-groups (Ikeda et al. 1982; Franzen and Norberg 1984; Troyano et al. 1996).

Zooglan shows pseudoplastic behavior with yield stress and high chain-stiffness values (Rha et al. 1989) close to those of xanthan gum. Its viscoelastic properties enable zooglan to be used as a gellifier, stabilizer and lubricant and its combined properties of surface activity, acid stability and salt compatibility make it a good stabilizer/emulsifier when used in oil-in-water emulsion systems (Stauffer et al. 1980).

Since the presence of the capsular polysaccharide in this strain makes physiological studies difficult, a slime-forming mutant, *Z. ramigera* 115SLR (ATCC 53590), derived from *Z. ramigera* 115 mutagenized with nitrosoguanidine, was developed in our laboratory (Easson 1987). This mutant is non-capsule-forming, non-floc-forming and produces an exopolysaccharide that does not remain bound to the cell wall and is consequently released into the culture broth.

Although many studies have examined the influence of culture conditions on the production and composition of polysaccharides such as xanthan, including nutritional studies (Garcia-Ochoa et al. 1992; Molina et al. 1993; Prell et al. 1995; Lo et al. 1997) and studies on the effects of temperature (Shu and Yang 1990) or oxygenation rate (Flores et al. 1994), and the effect of the culture mode (Tait et al. 1986), little is known about the effect of fermentation conditions on the quality of the product, such as its molecular weight distribution. It is known that the molecular weight of a polysaccharide has a direct impact on its rheological behavior, specifically its viscosity (Ash 1985; Rizzoti 1994), and that the molecular weight and its distribution are perhaps the most important properties for the commercial suitability of a given

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polymer (Gilbert 1995). Controlling the quality of gums could have an important impact on industrial processes. Herbst et al. (1988) were the first to report the relationship between xanthan molecular weight and culture conditions. In that study, the mean molecular weight was found to increase during the exponential growth phase and then was relatively constant during the following 20 h. Silman et al. (1990) showed that the nature of the carbon source could also have an impact on the molecular weight of the polymers. Thus they found that starch-grown cultures of *Aureobasidium pullulans* often produced higher yields of lower-molecular-weight pullulan than did glucose or sucrose-grown cultures. Nevertheless, these results appear to be strain-dependent. The influence of process parameters such as oxygenation conditions (Flores et al. 1994; Suh et al. 1990) and agitation rate (Peters et al. 1989) on xanthan molecular weight has also been reported. To date, no studies have demonstrated the effect of medium composition on product quality in terms of molecular weight distribution.

In order to study the effect of nutrients on the production, composition and molecular weight distribution of the exopolysaccharide zooglan, we first developed a synthetic medium that contains no complex additives, such as yeast extract. In the present work, we report the production of zooglan by *Z. ramigera* 115SLR growing on this completely defined medium. We then examined the production, composition and molecular weight distribution of zooglan produced in the new defined medium and a medium containing yeast extract. Whereas production and composition of the exopolysaccharide were comparable in the two media, the molecular weight and its distribution were affected by the composition of the medium.

Materials and methods

Bacterial strains and growth media

Zoogloea ramigera 115SLR is a mutant derived from *Z. ramigera* 115 (ATCC 25935). The organism was grown at 30 °C on a Luria-Bertari medium (LB) rifampicin/agar plate containing 50 μg/ml rifampicin. Yeast extract medium (YEM) for exopolysaccharide production has the following composition: 15 g/l glucose, 0.01 g/l yeast extract, 0.2 g/l $MgSO_4$, 2 g/l K_2HPO_4, 1 g/l KH_2PO_4 and 1 g/l NH_4Cl (Norberg and Enfors 1982).

The defined medium, basal medium for zooglan production (BMZP), developed in this study has the same composition as YEM except that the yeast extract is replaced by a mixture of vitamins: panthothenic acid, pyridoxamine, folic acid, riboflavin and cyanocobalamin, 3 mg/l each; 2 mg/l thiamine and 3 μg/l biotin. Both YEM and BMZP have a $C_{Glc}/N_{ammonium}$ ratio of 23 and an osmolality of 265 mosmol/kg. The osmolarity of the medium was measured with a model 3Mo micro-osmometer (Advanced Instruments Inc., Norwood, USA).

Fermentation conditions

Starter cultures were prepared by inoculating 5 ml YEM or BMZP with one colony of *Z. ramigera* 115SLR from an LB/rifampicin/agar plate. These cultures were incubated for 2 days at 30 °C and 200 rpm. the 500-ml conical flasks, containing 50 ml YEM or BMZP, were inoculated with 1 ml starter culture. Cultures were incubated at 30 °C and 200 rpm for 24 h and then used as a 5% (v/v) inoculum for 2 l BMZP or YEM medium in a 4-l Chemap CMF100 reactor (ChemapAG, Volketswil, Switzerland). Fermentation was carried out at 30 °C with an aeration rate of 0.45 vvm and an agitation rate of 1500 rpm. The pH was maintained at 7 with NaOH.

Determination of biomass, glucose and ammonium

For determination of biomass, samples were collected from the fermentor cultures and centrifuged at room temperature at 20 000 *g* for 15 min. When the cultures reached high viscosity, the samples were diluted with two volumes of hot (50 °C) water before centrifugation. Cell pellets were washed twice with water and the cell dry weight was determined gravimetrically.

For the determination of glucose and ammonium, samples were collected and filtered through 0.2-μm Acrodisc filters (Gelman Sciences, Ann Arbor, USA). Glucose concentration was determined by HPLC (Hewlett Packard model 1050, Waldbronn, Germany) using an Aminex HPX-87H column (Bio-Rad, Hercules, USA). Sample analysis was performed at 40 °C with 5 mM sulfuric acid as the mobile phase at a flow rate of 0.6 ml/min. The detection was performed with a refractive-index detector (Hewlett Packard model 1047 A). Ammonium analyses were performed by a UV method (Boehringer Mannheim ammonia kit, Indianapolis, USA).

Preparation of exopolysaccharide samples

To remove cells, centrifugation was used under the same conditions as described for biomass determination. EPS was precipitated with two volumes of isopropanol at −20 °C and then recovered by centrifugation (4000 g for 15 min). EPS pellets were dissolved in water, reprecipitated by isopropanol, redissolved in water, dialyzed (12–14 kDa cut-off) against deionized water for 3 days (with three changes of deionized water per day) and lyophilized. The EPS concentration was calculated after weighing the lyophilisate.

Compositional analyses of the EPS

A 10-mg sample of lyophilized exopolysaccharide was dissolved in 5 ml water and hydrolyzed by the addition of 55 μl 99% trifluoroacetic acid (Aldrich, Milwaukee, USA) and incubation at 120 °C. Samples were collected after 6 h, 12 h and 24 h of hydrolysis and filtered through 0.22-μm Millex-GV filters (Millipore, Bedford, USA). Glucose, galactose and pyruvic acid concentrations were determined by HPLC (Hewlett Packard model 1050) with an Aminex HPX-87H column (Bio-Rad). Sample analysis was performed at 40 °C with 5 mM sulfuric acid as the mobile phase at a flow rate of 0.6 ml/min. The detection was performed with a refractive-index detector (Hewlett Packard model 1047 A) for sugar analysis and a UV detector (Hewlett Packard model 35900) at 220 nm for organic acid analysis (Troyano et al. 1996). Acetic and succinic acid concentrations were determined spectrophotometrically, using Boehringer Mannheim kits.

Molecular mass measurements

Light-scattering analyses were performed in a Dawn-F multi-angle laser photometer ($\lambda = 623.8$ nm; Wyatt Technology Corporation, Santa Barbara, USA) at ambient temperature. The flow-type light-scattering measurements were conducted in a gel-permeation chromatography system (GPC). The GPC system was composed of an HPLC pump (Waters model 501; Waters, Milford, USA), a UK6 injector (Waters), a column incubator (Waters) and a differential refractometer (Waters model 410). A pulse-dampener was also used in-line. A light-scattering detector was placed in-line following the GPC column. The sample was effectively divided into fractions by the column and light-scattering intensities were mea-

sured for each fraction. The measurement of light scattering with the GPC system was calibrated and normalized against a poly(ethylene oxide) standard (Tosoh Corp., Tokyo, Japan). The calibrated concentration detector (differential refractometer) followed the scattering cell to measure the concentration of each species of different molecular weight. The molecular weight of each fraction was calculated by the Debye method (Debye 1947). An Ultrahydrogel linear GPC column (Waters) was used for the sample analyses. The temperatures of the column and detector were maintained at 30 °C and 35 °C respectively, at a flow rate of 0.5 ml/min. The Waters 410 differential refractometer was calibrated by the method described by Huglin (1989) to measure both the dn/dc of the samples and the instantaneous concentrations of the sample eluting from the column. The change in the refractive index with respect to the change in zooglan concentration (dn/dc) was determined to be 0.152 with this system.

Results

In order to obtain a synthetic medium that could be used for nutritional studies of the production of exopolysaccharide, we formulated a defined medium by replacing the yeast extract of the standard medium (YEM) (Norberg and Enfors 1982) with a cocktail of vitamins. *Zoogloea ramigera* 115SLR was grown at 30 °C on the new defined medium (BMZP) containing 15 g/l glucose as carbon source.

We monitored the cultures and determined the kinetics of growth, substrate consumption and exopolysaccharide production (Fig. 1). The exponential cell growth rate was 0.18 h^{-1}. During this exponential growth, the bacteria consumed two-thirds of the glucose and produced 4.4 g/l exopolysaccharide with an overall specific productivity of 0.13 g EPS g dry weight^{-1} h^{-1}. The glucose was completely exhausted after 60 h of fermentation. During the stationary phase, the growth and EPS production were reduced and we observed an EPS production of 0.6 g/l. When the glucose to EPS conversion yield remained at 30%, the specific productivity was dramatically decreased (0.012 g EPS g biomass^{-1} h^{-1}).

In order to determine whether the composition of the EPS varied during the fermentation, we withdrew samples and subjected them to compositional analyses (Fig. 1). The exopolysaccharide contains glucose, galactose, acetic acid, pyruvic acid and succinic acid. The ratio of glucose to galactose remained constant at around 1.9–2 during the fermentation. Acetic, pyruvic and succinic acid in the exopolysaccharide also remained constant.

The performance of the strain was tested under the same culture conditions on the YEM. EPS production on YEM showed the same kinetics (date not shown). The exponential cell growth rate was 0.18 h^{-1}; 1.5 g/l biomass and 5.2 g/l exopolysaccharide were produced in 60 h. The glucose to EPS conversion yield was 35%. The production of EPS was highest during the exponential cell growth phase with an overall specific productivity of 0.18 g EPS g dry weight^{-1} h^{-1}. Growth and EPS production yields and productivity on the two media were found to be similar within the error range (Table 1).

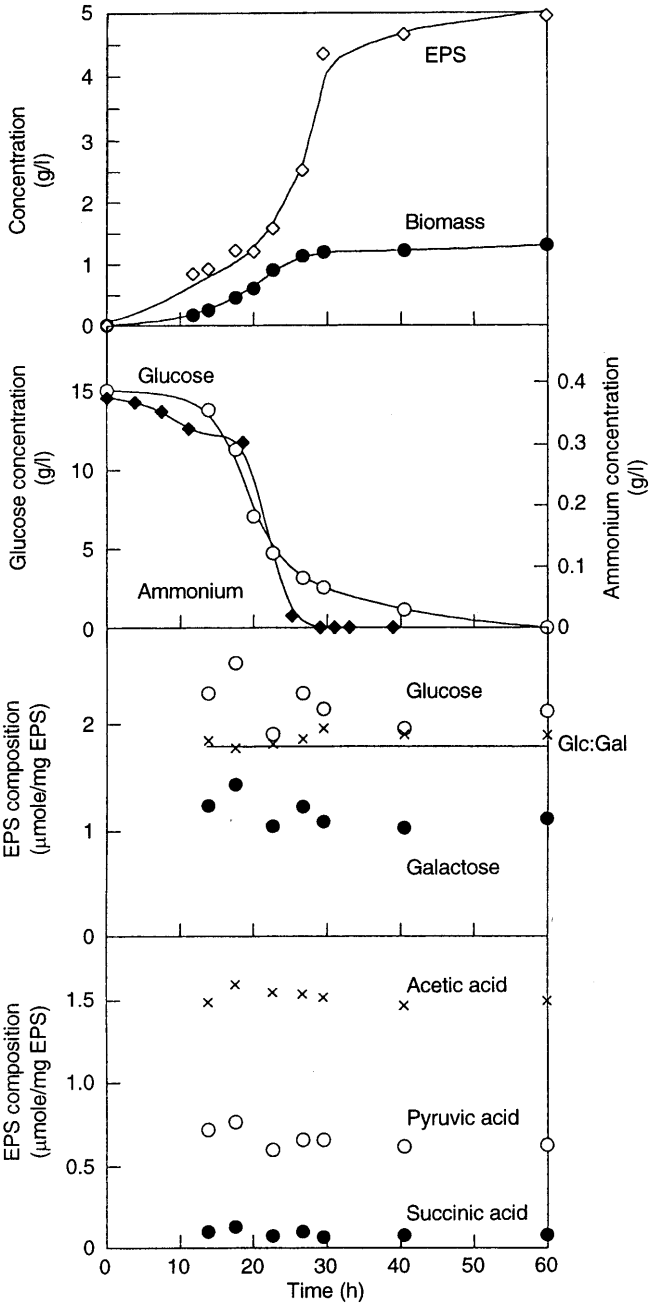

Fig. 1 Zooglan production in a batch reactor in basal medium for zooglan production (BMZP). Kinetics of growth, substrate consumption, polysaccharide production and composition. *EPS* expolysaccharide

The composition of the EPS produced in YEM was analyzed after acidic hydrolysis. It contained glucose, galactose, acetate, pyruvate and succinate. The composition of this EPS was similar to that of the EPS from BMZP (Table 2). The glucose to galactose ratios of the EPS from the two growth conditions were calculated to be 1.9 and 2. Acetic and pyruvic acid are the main organic acid constituents, comprising 1.4–1.5 and 0.66–0.69 μmol/mg EPS respectively. A low succinic acid content was also found: 0.09–0.13 μmol/mg EPS.

Table 1 Exopolysaccharide (*EPS*) and biomass production yields (*Y*) and overall specific EPS productivity of the cultures grown on defined basal medium for zooglan production, (*BMZP*) and yeast extract (*YEM*) medium. (The results are expressed as the mean ± standard error of values obtained from two independent batch cultures)

Medium	*Y*(EPS/Glc) (g/g)	*Y*(biomass/Glc) (g/g)	*Y*(EPS/biomass) (g/g)	Productivity at 24 h (g EPS g biomass^{-1} h^{-1})
YEM	0.35 ± 0.02	0.10 ± 0.02	4.10 ± 0.30	0.18 ± 0.03 g/g h
BMZP	0.32 ± 0.02	0.08 ± 0.01	3.85 ± 0.15	0.13 ± 0.04 g/g h

At the end of the cultures a sample was extracted in order to determine the mol mass and the molecular weight distribution of the polysaccharide (Fig. 2). The average mol mass of the polysaccharide obtained on the defined medium was lower ($6 \times 10^6 \pm 0.6 \times 10^6$ g/mol; the 95% confidence interval was determined from five independent determinations) than that produced on YEM ($1 \times 10^7 \pm 0.1 \times 10^7$ g/mol; the 95% confidence interval was determined from five independent determinations) while its polydispersity index was higher, respectively 1.4 and close to 1.0.

Discussion

We report here the development of a new defined medium (BMZP) for the study of exopolysaccharide production by *Z. ramigera* 115SLR. We examined growth and polysaccharide production kinetics in BMZP and in a medium containing yeast extract. The kinetics of growth and exopolysaccharide production of *Z. ramigera* 115SLR on the two media were similar, within the error range, with a growth rate of 0.18 h^{-1} and a glucose to polysaccharide conversion yield of around 35%. Production of zooglan was previously reported for growth on YEM (Norberg and Enfors 1982). The authors found, with the original strain, *Z. ramigera* 115, a glucose to polysaccharide conversion yield of 40% with a $C_{Glc}/N_{ammonium}$ ratio of 26 and showed that yield increased with an increasing ratio.

Our kinetic studies show that the EPS production occurs in two phases: the highest EPS productivity occurred during the exponential cell growth. In the stationary growth phase, a strong decrease in EPS production was observed. Nevertheless, the glucose to polysaccharide conversion yield remained constant over the two phases. A culture grown with twice the initial ammonium concentration showed similar behavior (data not shown), indicating that the decrease in growth and EPS production is not due to ammonium limitation.

The composition of the exopolysaccharides produced in the two media was found to be identical within the error range of the analytical procedures. Our data are consistent with the identification of the EPS as zooglan. The exopolysaccharide contains glucose, galactose, acetate, pyruvate and succinate. The glucose/galactose ratio is approximately 2, in agreement with previously reported values of 2.1 (Franzen and Norberg 1984), 2.3 (Troyano et al. 1996) and 1.95 (Lee et al. 1997). We also showed that this ratio remained constant over the time of the culture.

Although the composition of zooglan produced in BMZP is indistinguishable from that of zooglan produced in YEM, we found that the growth medium had a great effect on the degree of polymerization. Zooglan produced in YEM has a higher molar mass (1.10^7 compared to 6.10^6 g/mol) and a lower polydispersity index than zooglan produced in BMZP. These data show that the YEM leads to a more homogeneous polysaccharide solution. Little has been reported in the literature about the effect of culture conditions on the quality of polysaccharides. The dissolved oxygen level under no limitation of oxygen (Flores et al. 1994) and the oxygen transfer rate under oxygen-limited conditions

Table 2 Components of zooglan produced in defined (BMZP) and yeast extract (YEM) medium. (The results are expressed as the mean ± 95% confidence intervals of five independent determinations)

Compound	EPS (μmol/mg)	
	YEM	BMZP
Glucose	2.50 ± 0.20	2.20 ± 0.20
Galactose	1.30 ± 0.20	1.10 ± 0.13
Acetic acid	1.40 ± 0.04	1.52 ± 0.04
Pyruvic acid	0.69 ± 0.03	0.66 ± 0.05
Succinic acid	0.13 ± 0.02	0.09 ± 0.02

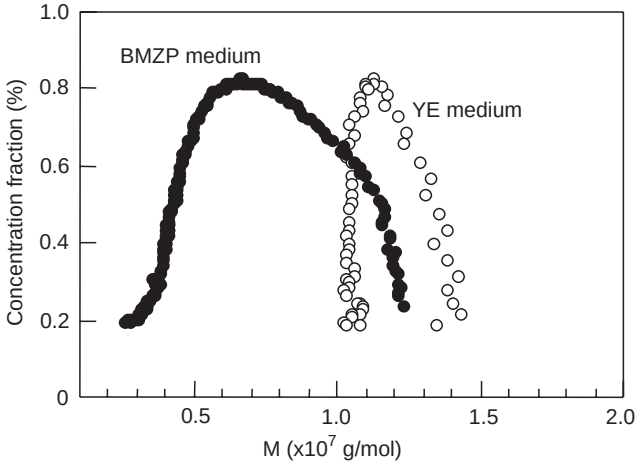

Fig. 2 Molar mass distribution of zooglan obtained at the end of the cultures in yeast extract medium (*YEM*) and BMZP

(Suh et al. 1990) were reported to affect the average molecular weight of xanthan significantly. In both fermentations, agitation and aeration settings were chosen in order to obtain identical oxygenation profiles. It is reasonable to assume that the micromixing phenomena were identical in the two fermentations since the EPS production profiles were similar. Therefore the differences observed in the homogeneity of the polysaccharides can not be related to oxygen or micromixing problems.

The osmolarity of the growth media can also affect the molecular weight distribution of succinoglycan, a polysaccharide produced by *Rhizobium meliloti* (Breedveld et al. 1990). The osmolalities of both of our media were measured and were found to be equal at 265 mosmol/kg.

Our results show that one or more component(s) of yeast extract affect the polydispersity of the polysaccharide. It is known that the molecular weight distribution of the polyhydroxybutyrate polymer can be controlled by the concentration of polyhydroxyalkanoate synthase, the enzyme responsible for the polymerization of the polyhydroxybutyrate (Sim et al. 1997). Depolymerization is a second mechanism that may affect the molecular weight distribution of a polysaccharide. York and Walker (1998) have shown that the expression of glycanases by *R. meliloti* controls the production of low- or high-molecular-mass succinoglycan. In our case, no depolymerase activity was detected in supernatant or crude extract of *Z. ramigera* 115SLR for either native or deacylated zooglan. It is reasonable to assume that yeast extract contains a range of components that are missing in the defined medium and could have an effect on polymerase or depolymerase activity or synthesis. It should be possible to fractionate yeast extract and add the fractions to BMZP in order to find the components involved. Although it has been reported that the degree of polymerization of polysaccharide during a culture changes (Herbst et al. 1988) with the oxygenation conditions (Suh et al. 1990; Flores et al. 1994) and with the genetic background of strains (Reuber and Walker 1993; Becker et al. 1995), this is the first report of the influence of nutrients, and particularly yeast extract, on the molecular weight and polydispersity of a polysaccharide.

A completely defined and synthetic medium has been developed that enables production of the exopolysaccharide, zooglan, from *Z. ramigera* 115SLR with comparable growth and production performances and zooglan composition comparable to those obtained in yeast extract medium. While the factors governing the degree of polymerization in polysaccharides are still largely unknown (Ielpi et al. 1993), this medium will be a very useful tool for further investigation of the effects of nutrients on polysaccharide quality. Additional studies are under way to determine the parameters that affect the molecular weight of the polysaccharide and its distribution.

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