ELSEVIER

Carbohydrate Polymers **25** (1994) 7–12

0144-8617/94/$07.00

Exopolysaccharide production by the *Rhizobium meliloti* M5N1 CS strain. Location and quantitation of the sites of *O*-acetylation

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(Received 29 November 1993; revised version received 20 April 1994; accepted 27 April 1994)

The mutant strain M5N1 CS of *Rhizobium meliloti* is able to produce, in a Rhizobium complete medium supplemented with fructose or sucrose (1% w/v), a partially acetylated homopolymer of glucuronic acid. During batch cultivation, the polysaccharide production is observed while the pH of the medium decreases slightly. The nature of the carbon source and the control of pH have no effect on the degree of acetylation of the polymer produced in the flasks. About 40% of glucuronic residues of the polymer are monoacetylated at C-3 or at C-2, C-3 substitution is about twice that of C-2 and remains constant during the fermentation.

INTRODUCTION

Many bacteria produce extracellular polysaccharides under specific culture conditions, resulting in the formation of a viscous broth during submerged cultivation (Sutherland, 1985). Complex exocellular polysaccharides (EPS), ranging from simple glucans to elaborate heteropolysaccharides are produced by cells belonging to the Rhizobiaceae family (Gil-Serrano *et al.*, 1990, 1992; McNeil *et al.*, 1986; Navarini *et al.*, 1992; Heyraud *et al.*, 1986).

Chemical mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was performed on the succinoglycan producing the strain *Rhizobium meliloti* M5N1. The mutant noted *R. meliloti* M5N1 CS (NCIMB 40472) was selected as it was able to induce nodule formation on roots of lucern (Courtois *et al.*, 1992). In a 20 litre fermenter containing Rhizobium complete medium supplemented with sucrose (1%), $MgSO_4$, 7 H_2O (0·6 g/litre/24 h) and stabilized at pH 7·2, the mutant produces a (1 → 4)-β-D-glucuronan partially acetylated on the 2 and/or 3 positions (Heyraud *et al.*, 1993; Courtois *et al.*, 1993).

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In this paper we studied the production of polysaccharide by the *R. meliloti* M5N1 CS strain during growth in flasks containing Rhizobium complete medium supplemented with fructose or sucrose (1% w/v). The effect of pH stabilization on the location and degree of acetylation was analysed.

MATERIALS AND METHODS

Culture conditions and polysaccharide production

For the production of EPS by the *R. meliloti* M5N1 wild strain (Courtois *et al.*, 1990), the *R. meliloti* M5N1 CS mutant strain was grown at 30°C in flasks containing 40 ml of Rhizobium complete medium (Courtois *et al.*, 1983) supplemented with fructose or sucrose (1% w/v) (termed RCF and RCS, respectively). The incubation on a rotary shaker (100 rpm) was performed to a density of 3×10^8 CFU/ml (A_{600} value = 0·8). Two series of three 2 litre Erlenmeyers flasks (termed AF, BF, CF and AF^+, BF^+, CF^+) each containing 1 litre of RCF medium were inoculated by 2 ml of the previous culture and incubated at 30°C on a rotary shaker (100 rpm).

The pH of the culture medium was controlled and maintained at 7·2 with M KOH in the flasks termed AF^+, BF^+, CF^+. The pH in the flasks termed AF, BF, CF was not controlled. The cell concentration (determined after dilution, by culture on RC agar plates), the optical density at 600 nm and the polysaccharide production were analysed during 68 h of fermentation. To determine the degree of acetylation of the polymer produced during fermentation, the EPS were extracted after 22, 46 and 68 h of incubation for flasks AF, AF^+; BF, BF^+; and CF, CF^+, respectively.

For two other series of three 2 litre Erlenmeyer flasks containing RCS medium (termed AS, BS, CS and AS^+, and BS^+ and CS^+) the procedure described previously was used; the EPS were extracted after 30, 48 and 62 h of fermentation.

The polysaccharide production was studied by high performance liquid chromatography at room temperature. The injected volumes were filtered through a 0·22 μm membrane before injection through a gel filtration column (Beckman TSK 2000 SW pre) with water as the mobile phase at a flow rate of 0·5 ml min^{-1}. The detection system was a refractive index detector (Beckman). Calibration curves were obtained using several solutions of polysaccharides, sucrose and fructose at different concentrations.

Exopolysaccharide isolation and purification

The *Rhizobium* suspensions were centrifuged at 13 870 *g* for 30 min and the cell free supernatants separated from the pellets. The cell free broths were concentrated by ultrafiltration (with a 100 000 NMWCO membrane from Sartorius). The concentrate diluted by 1 volume of distilled water was purified by ultrafiltration with the same membrane as previously, this step was repeated three times. The product was desiccated under vacuum at room temperature.

Reduction and hydrolysis

The glucuronic residues were reduced by the action of *N*-cyclohexyl-*N'*[*β*-(*N*-methyl morpholino)ethyl] carbodiimide-*p*-toluene sulfonate at pH 4·75 and sodium borohydride at pH 7 (to prevent alkaline hydrolysis) according to a method described previously (Bouffar & Heyraud, 1987); the reduction was repeated twice. The reduced EPS was hydrolysed in 70% sulfuric acid (2 ml) for 30 min at room temperature, then diluted with water (10 ml) and kept overnight at 100°C. The mixture was neutralised with $BaCO_3$ before concentration and filtration.

Separation of neutral monosaccharides was achieved by high performance liquid chromatography (HPLC) on a Waters instrument using a CHO-682 column from Interchim (France) with water as eluent as 85°C. The detection system was an analytical IOTA refractive index detector (Jobin-Yvon, France) in series with a Perkin-Elmer model 241 polarimeter equipped with a 80 ml microflow cell operating at 365 nm with 0·05 degree full scale (Heyraud & Rinaudo, 1990).

Methylation analysis

Methylation of the reduced EPS was carried out by the Kakomori method (Hakomori, 1964). The methylated polysaccharide was recovered by dialysis. A CF_3COOH hydrolysis was performed, the resulting sugars were reduced with $NaBH_4$ and acetylated with acetic anhydride-pyridine. The *O*-acetylated–*O*-methylated alditols were analysed by gas liquid chromatography (GLC) on a Hewlett instrument equipped with a Macrobore SP-5380 column (25 × 0·53 mm).

NMR measurements

All NMR measurements were carried out on a Bruker AM-300 spectrometer, operating at 300·13 MHz and equipped with an aspect 3000 computer. Samples containing 10 g $litre^{-1}$ of native exopolysaccharide in D_2O were used. ^1H NMR spectra were obtained using a spectral width of 2000 kHz and 16 K data block. The probe temperature was kept at 85°C to reduce the solution viscosity and thereby the signal line width.

The solvent peak (HDO) was partially suppressed under a 180°, *t*, 90° pulse sequence (t = 3 s) with a 7 s recycle time (Varum *et al.*, 1991). Chemical shifts were referenced to external sodium 3 (trimethylsilyl) propionate d_4.

The proton homonuclear chemical shift correlation experiment (COSY) and COSY with one, two and three steps relayed coherence transfer were recorded using Bruker software. The acquisition involved 32 scans over 1 K data points for 256 experiments. The transformed data were displayed as a magnitude spectrum after multiplying the data with a sine-bell squared function with no phase shift. A 90° mixing pulse was used in the COSY experiments.

RESULTS AND DISCUSSION

Production of exocellular polysaccharides by *R. meliloti* M5N1 CS

During batch cultivation of the *R. meliloti* M5N1 CS strain in RCF medium, the pH decreases slightly (Fig. 1), the pH variation between the beginning and the end of fermentation was 0·8. The growth was not affected by the slight pH decrease in the medium (data not shown), the cell concentration during the stationary growth phase in the medium with and without controlled pH correspond to $6·5 \times 10^8$ CFU ml^{-1}. We determined that the production of EPS in RCF medium maintained at pH 7·2 was slightly superior to the production in a medium where the pH value decreased (Fig. 1) though

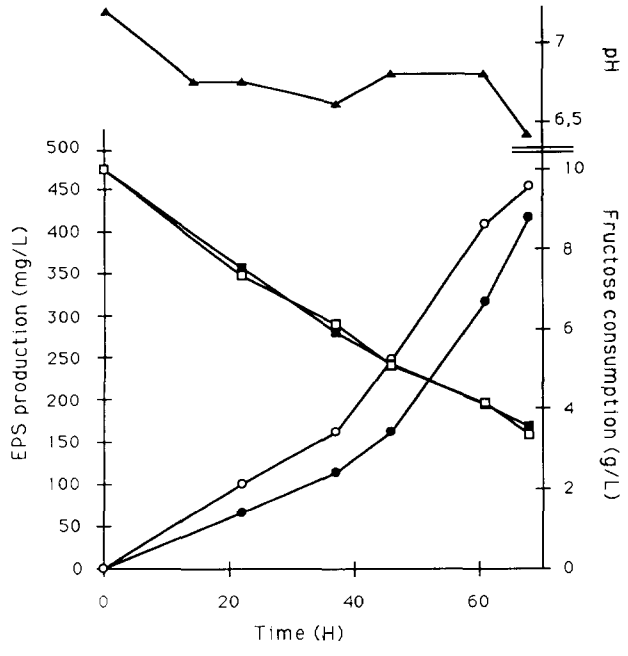

Fig. 1. Polysaccharide production (○) and fructose consumption (□) by the *R. meliloti* M5N1 CS strain in a RC medium supplemented with fructose (1%) maintained a pH 7.2 and without controlled pH (●; ■). pH evolution in the medium without addition of KOH (▲).

the consumption of fructose was not affected by the pH decrease. The optimal production period corresponds to the stationary growth phase. Similar results were obtained during batch cultivation of the *R. meliloti* M5N1 CS strain in RCS medium.

Chemical analysis

EPS extracted from CF, CF^+, CS and CS^+ flasks were analysed. As the EPS were not soluble in a 1 M sulfuric acid solution, and due to their strong resistance towards acid hydrolysis, they were compared to uronic acid-rich polysaccharides, so the polymers were reduced before being submitted to an acidic hydrolysis. Glucose was the only component detected after analysis of the hydrolysates by HPLC. A positive value for the signal obtained from this component using a polarimetric detector on line with a refractometric detector, indicated the component was D glucose.

After *O*-methylation and CF_3COOH hydrolysis of the reduced EPS, the resulting sugars were reduced and acetylated. GLC analysis revealed the presence of a single component having the same retention time as that of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol. This result suggested that in the four samples tested, the glucuronic acid (GlcA) residues are 1 → 4 linked.

NMR studies

In order to determine the polymer structure, we first determined the ^1H and ^{13}C chemical shifts of glucuronic acid monomer and of *O*-deacetylated polymer (deacetylation was carried out by NaOH treatment [pH 11·5] at 50°C during 15 h).

The ^1H and ^{13}C spectra of *O*-deacetylated polymer revealed the presence of only five protons and five carbons corresponding to β-glucuronic acid residues. The β anomeric assignment was confirmed by the $^3J_{1,2} = 7{\cdot}7$ Hz and the acidic function by the chemical shift value for C-6 (175·67 ppm). The ^1H NMR data of the *O*-deacetylated polymer (Table 1) are in good agreement with the values determined in the literature (De Ruiter *et al.*, 1992) for the (1 → 4)-β-linked glucuronic acid polymer derived from EPS of *Mucor hiemalis*.

The twelve samples obtained after cultivation of the *R. meliloti* M5N1 CS strain in RCF and RCS medium, free of protein (from microanalysis data) were analysed by ^1H NMR spectroscopy. As identical spectra were obtained with the different samples, we concluded that the EPS produced in the different conditions were the same. The ^1H NMR spectrum of a native polymer solution (Fig. 2) presents a complex system in the ring proton region and signals in the 2 ppm region characteristic of *O*-acetyl groups. These results clearly indicate that the polysaccharide is partly acetyled. Integration of the resonances in the ^1H NMR spectrum of the native EPS in the downfield region (4·3–5·1 ppm) in the upfield region (3·1 to 4·0 ppm) and in the acetyl region (1·9–2·2 ppm) provided the degree of substitution per residue (ds). The ds values of the samples obtained from RCF and RCS medium (Table 2) were not significantly modified by the duration of fermentation nor by pH stabilization.

The spectrum obtained with a solution of native polymer reveals an envelope of overlapping multiplets in the region from 3·1 to 4·0 ppm (Fig. 2). Downfield from 4.3 ppm several broad signals labelled A to E can be assigned to protons linked to either C-1 or to C-*O*-Ac groups. The peaks were assigned using a combination of COSY and relayed COSY experiments and by

Table 1. Experimental and calculated[a] ^1H chemical shifts of the ring protons in deacetylated and native polysaccharide at 85°C

	H-1	H-2	H-3	H-4	H-5
Deacetylated EPS	4·53	3·36	3·61	3·69	3·85
Native EPS residue:					
β-D-Glc*p*A (1)[b]	4·39	3·22	3·53	3·66	3·84
β-D-Glc*p*A (1)[b]	4·44	3·25	3·56	3·73	3·84
β-D-Glc*p*A (2)[b]	4·53	3·35	3·60	3·71	3·86
β-D-2-*O*-Ac-Glc*p*A	4·69	4·69	3·78	3·89	3·92
	(4·71)	(4·77)	(3·80)	(3·77)	(3·86)
β-D-*O*-Ac-Glc*p*A	4·61	3·50	4·98	3·92	3·89
	(4·63)	(3·54)	(5·10)	(3·88)	(3·94)

[a] Calculated chemical shifts are in parentheses.
[b] (1) The β-D-Glc*p*A residue is situated behind or after acetylated residues. (2) The β-D-Glc*p* residue belongs to a set of unacetylated residues.

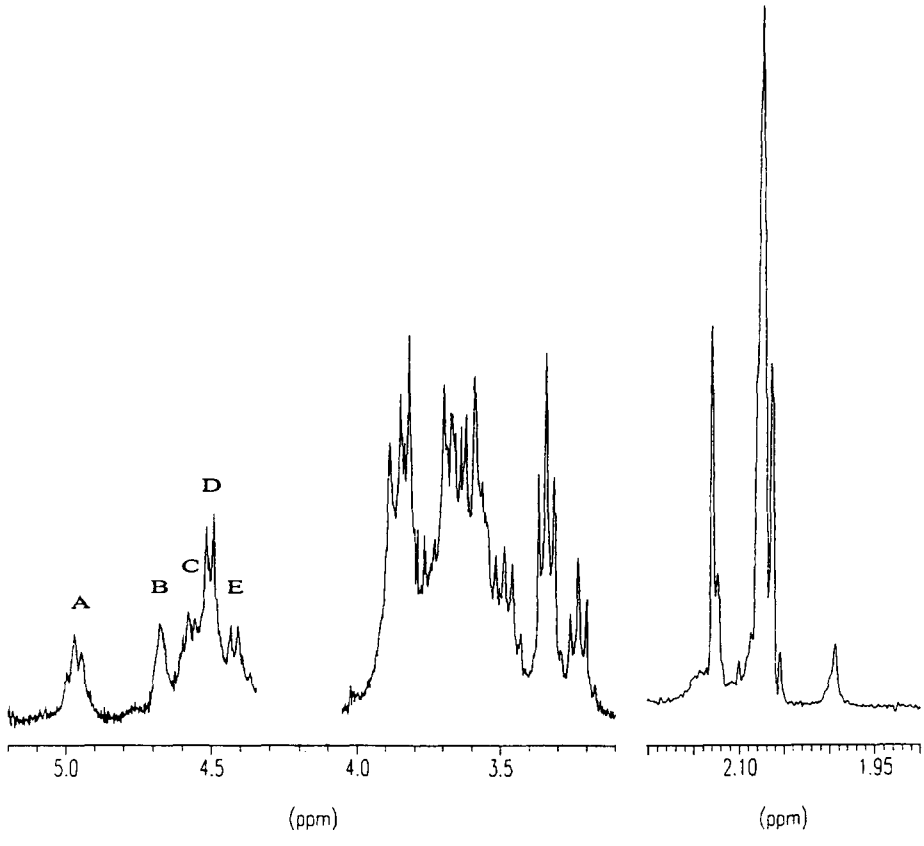

Fig. 2. ^{1}H NMR spectrum of the native exopolysaccharide (at 85°C) obtained after 68 h fermentation of the *R. meliloti* M5N1 CS strain in a RCF medium, maintained at pH 7.2. A: H_3 of 3-*O*-acetylated residues, B: $H_1 + H_2$ of 2-*O*-acetylated residues, C: H_1 of 3-*O*-acetylated residues, D: H_1 of residues unacetylated in a set of unacetylated residues, and E: H_1 of residues unacetylated behind or after acetylated residues.

observing the chemical shift variations induced by *O*-acetylation (Haverkamp *et al.*, 1982; Jansson *et al.*, 1987).

Two cross peaks at 3·35 and 3·60 ppm are observed for signal D (4·53 ppm) in the one-step relayed-COSY spectrum (Fig. 3). Comparison of the 3·35 ppm value with the H-2 proton chemical shift of deacetylated EPS (Table 1) led us to assign the signal at 3·35 ppm to the proton linked to unacetylated C-2. The resonances at 4·53 and 3·60 ppm were assigned respectively to H-1 and H-3 of an unacetylated residue. A similar observation was made for signals E at about 4·40 ppm; further studies of this resonance (Fig. 2) and cross peaks to this signal in the 2D spectrum (Fig. 3) suggested an overlapping of two doublets, attributed to two species of unacetylated residues.

Signal A at 4·98 ppm connected to peaks at 3·50 and 3·92 ppm as determined by a COSY contour plot experiment and to four peaks at 3·50, 3·89, 3·92 and 4·61 ppm, as determined from a one-step relayed-COSY experiment (Fig. 3), was assigned to the H-3 linked to an acetylated carbon. The four resonances at 3·50, 3·89, 3·92 and 4·61 ppm, were attributed respectively to H-2, H-5, H-4, and H-1 of residues acetylated at C-3. These assignments were confirmed by the COSY correlation at

Table 2. Molar proportions of β-D-glucuronic acid residues in native exopolysaccharides for different incubation times in RCF and RCS medium

A- in RCF medium Residue	22 h	22 h +[a]	46 h	46 h +[a]	68 h	68 h +[a]
ds[b]	42%	42%	40%	46%	45%	50%
3-*O*Ac-β-D-Glc*p*A	28	27	28	27	25	34
2-*O*Ac-β-D-Glc*p*A	14	14	12	17	12	16
β-D-Glc*p*A	56	56	55	54	59	46
B- in RCS medium Residue	30 h	30 h +[a]	48 h	48 h +[a]	62 h	62 h +[a]
ds[b]	42%	40%	36%	32%	40%	38%
3-*O*Ac-β-D-Glc*p*A	20	19	20	18	19	21
2-*O*Ac-β-D-Glc*p*A	10	10	11	9	12	13
β-D-Glc*p*A	59	58	61	64	59	57

Molar proportions of 2,3-di-*O*-Ac-β-D-Glc*p*A residues are estimated at about 4% in RCF medium (A) and 5% in RCS medium (B).

[a] The EPS were produced in a medium maintained at pH 7·2 with M KOH.

[b] Degree of substitution per residue determined from integration of the resonances in the downfield, upfield and acetyl region.

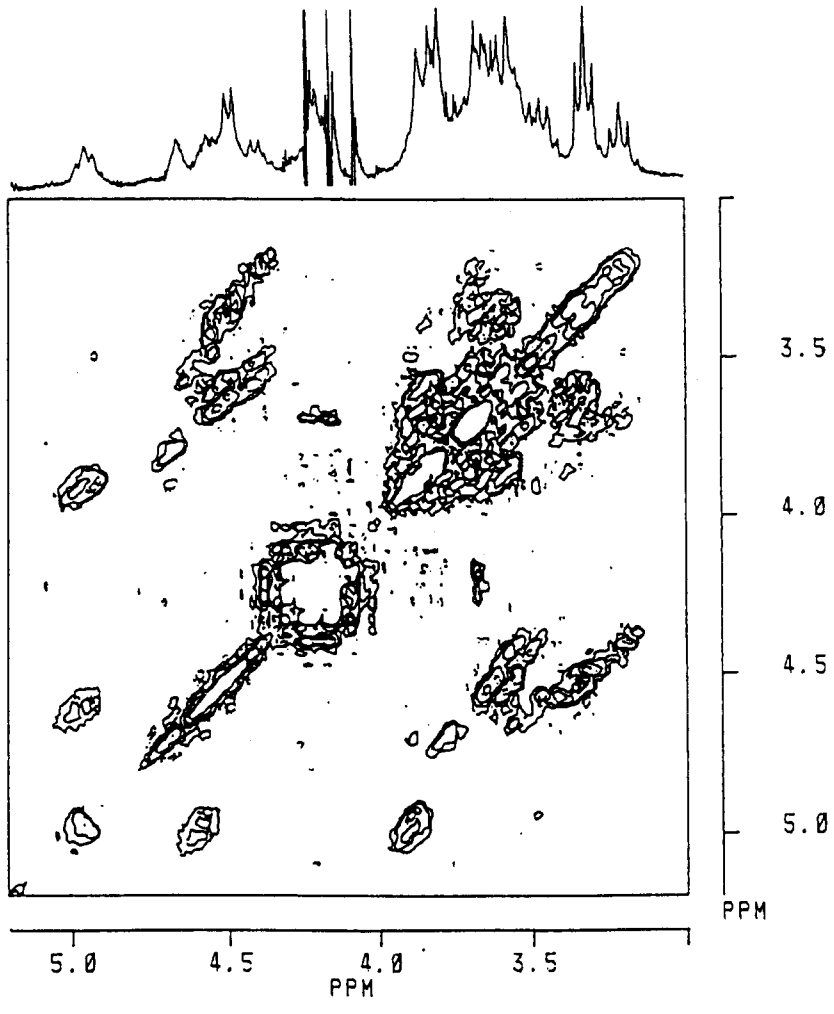

Fig. 3. 300 MHz homonuclear ^{1}H relayed COSY plot at 85°C of native exopolysaccharide extracted from Cl^- flask.

4·61 ppm with the H-2 resonance. Proton chemical shifts for the 3-*O*-acetyl residue agree with those reported by Canter-Cremers *et al.* (1991).

Signal B (4·69 ppm) shows only one connectivity at 3·78 ppm on COSY spectrum and two cross peaks at 3·78 and 3·89 ppm on the relayed COSY spectrum. As no cross peak was observed in the region of H-2 (3·20–3·40 ppm), we propose that the residues studied are acetylated on C-2 and that the H-2 and H-1 chemical shifts are equivalent and appear at 4·69 ppm.

Table 1 lists calculated chemical shifts for the various protons alongside their experimental values. The calculated values were determined using ^{1}H chemical shift variations (Jansson *et al.*, 1987) induced by acetylation of β-D-glucopyranoside at ambient temperature. The calculated values agree with the experimental values obtained from both 1- 2-D spectra. The overlapping of the H-4 and H-5 signals made assignments and measurements of the chemical shifts difficult. Attribution was made from one and two-step relayed-COSY spectrum. The small signals at 5·10 and 4·80 ppm could be attributed to H-3, H-1 and H-2 resonances of 2,3-di-*O*-Ac-Glc*p*A, no signals from the end residues have been detected.

From these results, it is now possible to determine the proportions of the different species of residues (Table 2) in the native glucuronan by integration of the downfield resonances (A to E). Signal areas were determined by integration or by peak deconvolution.

By comparison of peak intensity at 2·15 and 2·09 ppm with proportions of 2-*O*-acetyl and 3-*O*-acetyl residues, the resonances at 2·15 and 2·09 ppm were assigned to the methyl of acetyl group at C-2 in the 2-*O*-acetyl residue and at C-3 in the 3-*O*-acetyl residue, respectively. The small signal at 2·01 ppm and shoulder at central peak indicated that a small fraction of residues were also diacetylated. The chemical shifts of unacetylated residue labeled (2) in Table 1 are very close to chemical shifts of deacetylated EPS, so we propose that this residue belongs to a set of (1–4)-β-linked glucuronic acid units.

In previous studies, we have determined that the pH in RCF medium used for production of succinoglycan by the wild type strain *R. meliloti* M5N1 remained

constant at 7·2, a pH decrease (from 7·2 to 4·5) has been detected only when RC medium was supplemented with glucose (Courtois *et al.*, 1983). In this study, we have determined that the behaviour of the mutant strain *R. meliloti* M5N1 CS is different from the wild type strain, a pH decrease (from 7·2 to 6·4) is observed both in RCF and RCS medium. The polysaccharide production in flasks by the *R. meliloti* M5N1 CS strain, the degree of acetylation and the distribution of acetyl group in the polymer are not affected by the nature of the carbon source and the pH decrease in the media. The duration of fermentation has no effect on the degree of acetylation of the polymer produced in RCS and RCF medium by the *R. meliloti* M5N1 CS strain during batch cultivation in flasks with and without pH control and the distribution of *O*-acetyl groups in the EPS produced remains constant.

As in the conditions of production described, only a few residues are diacetylated in contrast to the polymer obtained under the fermenting conditions described previously (Heyraud *et al.*, 1993; Courtois *et al.*, 1993). we conclude that the pO_2 parameter or the $MgSO_4$ concentration may influence the degree of acetylation of the polymer. Studies are actually in progress to confirm this.

ACKNOWLEDGEMENTS

This work was supported by ANVAR Picardie and by Biopole (Conseil Regional de Picardie).

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