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Detection of *Xanthomonas campestris* mutants with increased xanthan production

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Several mutants of *Xanthomonas campestris* showing increased viscosity and/or gum production were detected after UV treatment. Xanthan solutions of the different mutants showed different intrinsic viscosity values, and no relationship was found between pyruvate or acetate contents and viscosifying ability of the xanthan. The best performance mutant (M-11) was obtained using halo size around colonies in starch-agar plates as the detection criterion, and proved the usefulness of this indirect screen for improved xanthan producers. A pleiotropic effect of this mutation on growth rate and total cell growth was observed.

Keywords: *Xanthomonas*; xanthan; alpha amylase; mutants

Introduction

Xanthan production by *Xanthomonas campestris* has received significant attention due to the wide range of applications of this polymer in oil, food and pharmaceutical industries, among others [4]. Genetic improvement of gum production is therefore of considerable importance for increasing the efficiency of the industrial process. However, difficulties arise due to the absence of reliable and easily measurable plate-screening criteria for xanthan production. Colony size has been used for selecting xanthan producers, but either ambiguous or contradictory results have been reported [10].

Coordinate regulation of xanthan synthesis and a series of extracellular enzymes, including alpha amylases, have been described in *Xanthomonas campestris* [15]. Based on this, colonies with larger halo size on starch-agar plates would be expected to show increased xanthan production in a mutant altered in this set of genes. This paper reports the isolation of mutants from *Xanthomonas campestris* producing increased extracellular viscosity and xanthan content by use of halo size on starch-agar plates and colony size as the screening criteria. A pleiotropic effect of one of these mutations on cell growth is discussed.

Materials and methods

Microorganism

The strain of *Xanthomonas campestris* pv *campestris* SAV [20] was used for mutagenesis.

Culture conditions and media

Growth was carried out in shaken flasks at 30°C and 150 rpm, using 500-ml Erlenmeyer flasks containing 70 ml of medium. The inoculum was prepared from 24-h slant cultures on YM medium (in g L^{-1}: yeast extract 3.0; peptone 5.0; malt extract 3.0; glucose 20; agar 15.0). Shaken cultures were grown in YM broth (YM medium without agar) and in the medium reported by Shu and Yang [12], with the composition (g L^{-1}): yeast extract 3.0; K_2HPO_4 2.0; $MgSO_4 \cdot 7H_20$ 0.1; KH_2PO_4 2.0; glucose 20. Experiments were carried out in triplicate.

Mutant isolation

A 16-h culture (A_{600} of 8, corresponding to 5.6 g L^{-1} of biomass) was centrifuged at $20\,000 \times g$ for 20 min, resuspended in the same volume of 0.9% NaCl and submitted to UV mutagenesis at 254 nm and 20 erg mm^{-2} seg^{-1} for 60 s, conditions which corresponded to a 1% survival rate. After centrifuging and washing the cells, they were incubated in shaken flasks for 16 h in YM broth and appropriate dilutions were plated on YM agar plus 1% soluble starch (BDH, Poole, Dorset, UK).

Analysis

Growth was followed by absorbance at 600 nm. Rheological properties of the fermentation broth after 72 h of cultivation were evaluated by determination of apparent viscosity in a Haake VT 181 viscosimeter (Karlsruhe, Germany) at rates of 7.3 s^{-1} and at 26°C using MK DIN axis. After appropriate dilutions of the culture broth and centrifugation at $20\,000 \times g$ for 40 min, xanthan concentration in the supernatant was determined by gravimetry. The polysaccharide was precipitated with ethanol (2:1 v/v) in the presence of 1% KCl. For physico-chemical characterization, precipitated polymer salt was submitted to successive washings with ethanol/water mixtures from 80 to 100% (v/v), suspended in deionized water and dialyzed for 48 h against deionized water. The partially purified xanthan powder was obtained by lyophilization. Xanthan gum pyruvate was determined by reaction with 2,4-dinitrophenylhydrazine according to Sloneker and Orentas [13]. Acetate content was estimated according to the method of McComb and McCready [8]. The intrinsic viscosity [η] was determined in 0.1 M NaCl in a Ubbelhode capillary viscos-

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imeter (Schott-Geräte GmbH, Hofheim, Germany) at 26°C, and calculated by graphic extrapolation as described by Torres *et al* [17]. All determinations were carried out in triplicate.

Results and discussion

Forty-two presumptive mutants were isolated from over 14 000 colonies analysed for both colony size and starch-agar halos. These cultures were evaluated in shaken tubes containing 10 ml of YM medium. Five strains showed higher viscosity production in comparison with the wild-type (data not shown). This group of mutants was further evaluated in 500 ml Erlenmeyer flasks containing 70 ml of YM broth. The results are shown in Table 1.

Four mutants produced a higher viscosity than the parent. Two strains produced a higher gum concentration, whereas one mutant displayed increased gum concentration but not broth viscosity.

The viscosity of polysaccharide solutions is a function of gum concentration and of the characteristics of the polymer. One important characteristic which reflects the polymer quality is the 'viscosifying ability', which can be defined as the viscosity generated per unit of gum concentration [10,18]. Mutant M-19 synthesized an exopolysaccharide (EPS) with a higher viscosifying ability, while *X. campestris* strain M-31 synthesized more xanthan, but with a lower viscosifying effect. Therefore, it seems that different mutations, leading to structurally different polysaccharides, could have been selected. Mutations in the different genes of a cluster produce altered xanthans with different properties [6]. Mutants that produce structurally altered xanthan have been also reported by Whitfield *et al* [21] and Tait and Sutherland [14].

The pyruvic and acetic acid contents of xanthan have been reported by some authors to be important factors in the viscosifying effect of the solutions [10,11]. The contents of pyruvic and acetic acid from the wild-type and mutants xanthan are shown in Table 2.

The acetate content of the polysaccharide was similar for all mutants and the parent strain. Lower pyruvate content appeared in the M-11 mutant in comparison with the wild-type. However, this mutant, although showing increased xanthan and broth viscosity, had similar viscosifying ability

Table 1 Growth and xanthan production by the mutants and wild-type strain in flask cultures and YM medium

Strain	Growth ($A_{600\,nm}$)	Viscosity (mPa.s)	Xanthan (g L^{-1})	Viscosifying ability (mPa.s/g L^{-1})
SAV (WT)	8.1	1360	7.8	174
M-11[a]	6.1*	1890*	11.4*	166
M-18[b]	7.5	1490*	8.2	178
M-19[b]	8.3	1550*	7.4	210*
M-20[b]	7.8	1410	9.2*	166
M-31[b]	8.1	1510*	10.2*	149*

[a]Selected by larger halo in starch-agar plates.
[b]Selected by colony size.
*Significant difference with the wild-type strain (Student's *t*-test, 95% reliability).

Table 2 Pyruvate and acetate content and intrinsic viscosity [η] of the xanthan gum produced by the mutants and wild-type strain

Strain	Pyruvate content (%)	Acetate content (%)	η (ml g^{-1})
SAV (WT)	5.9	3.7	1.66
M-11	4.4*	3.9	2.65*
M-19	6.2	3.7	1.38*
M-31	6.7*	3.4	1.85*

*Significant difference with the wild-type strain (Student's *t*-test, 95% confidence interval).

(Table 1). By contrast, *X. campestris* M-31, which showed a decreased viscosifying ability, seems to produce a gum with higher pyruvate content, while mutant M-19, with increased viscosifying ability, did not show any significant difference from its parent. Therefore our results do not support the idea of a relationship between contents of these residues and xanthan viscosifying ability. This conclusion coincides with the observations of Torrestriana *et al* [18] and Torres *et al* [17], who did not find any correlation between these parameters and the rheological properties of the polymer solutions. Furthermore, culture conditions like oxygen concentration (not controlled during shake-flask cultures) can influence the pyruvate content of xanthan gum in *Xanthomonas campestris* [9].

Considering the usefulness of measurements in the dilute regime for showing differences in biopolymers at the molecular level [17], values of intrinsic viscosity [η] were determined for polymers of the wild-type and mutant strains (Table 2).

The values of η for all the mutants were significantly different from those of the wild-type strain. The highest value corresponded to the polymer produced by mutant M-11. The factor which mainly influences η is the effective hydrodynamic volume in solution [22]. This could mean that the xanthan produced by mutant M-11 has a higher molecular mass or assumes a different spatial conformation than polysaccharides from the other mutants and from the wild-type. No correlation was found between η values and pyruvate or acetate contents. This result coincides with the reports of Callet *et al* [1], who found no influence of the substituent contents on the intrinsic viscosities of xanthan solutions.

Mutant M-11 showed the highest increase in viscosity (39%) and xanthan concentration (46%) with respect to the parent strain (Table 1). These increments are higher than those obtained by mutagenesis by Marquet *et al* [7] or by genetic engineering techniques [16,19], and comparable to the results of Destefano and Rosato [2] by transposon mutagenesis. Even higher viscosity and gum production was achieved by mutant M-11 when evaluated in the production medium (Table 3). These results show the potential of this strain for xanthan production. This mutant was selected by halo size on starch-agar plates, and thus shows the feasibility of using this criterion for the selection of improved xanthan producers.

Mutant M-11 showed a lower total cell growth and a lower growth rate than the parent strain (Tables 1 and 3). This suggests that the mutation(s) led to metabolic changes

Table 3 Growth and xanthan production of mutant M-11 and the parent *X. campestris* in the production medium

Strain	Growth ($A_{600\ nm}$)	μ (h^{-1})	Viscosity (mPa.s)	Xanthan ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Viscosifying ability ($mPa.s/g\ L^{-1}$)
SAV	4.9	0.23	1530	10.0	0.21	153
M-11	3.9*	0.15*	2150*	13.5*	0.28*	159

*Significant difference with the wild-type strain (Student's *t*-test, 95% of reliability).

which decreased growth but favored xanthan synthesis. Since mutant M-11 was selected by its larger halo on starch-agar plates, it seems that multiple mutations or a pleiotropic mutation took place which affected at the same time cell growth, xanthan production and alpha amylase expression. *Xanthomonas campestris* [5], and *Pseudomonas solanacearum* [3], have gene clusters involved in the biosynthesis of both EPS and LPS. Considering that an effect on LPS biosynthesis could be related to cell growth, a mutation in one of these genes could account for the observed behaviour of the M-11 mutant.

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