Mutagenesis of *Xanthomonas campestris* and Selection of Strains with Enhanced Xanthan Production

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ABSTRACT

Xanthan gum is a microbial polysaccharide of great commercial importance as it has unusual rheological properties in solution and consequent range of applications. In this study, a series of mutants were isolated from *Xanthomonas campestris* PTCC 1473 by ethyl methanesulfonate mutagenesis. The polysaccharide yield of one mutant, XC1473E$_2$, was 30% better than that of the parent strain. It also showed higher xanthan formation and glucose consumption rates compared to the parent strain. Xanthan produced by the mutant had enhanced viscosity, higher pseudoplasticity and larger molecular weight. Since mutant XC1473E$_2$ appeared white on agar plates, it underwent pigment extraction with methanol. Contrary to the parent strain, the mutant showed no absorption at 443nm, i.e. the wavelength related to yellow pigment. This finding suggested that yellow pigmentation and normal xanthan biosynthesis are not necessarily concurrent. In general, mutant XC1473E$_2$ seems to be a strain with interesting characteristics for use in commercial production of xanthan. *Iran. Biomed. J. 7 (3): 91-98 2003*

INTRODUCTION
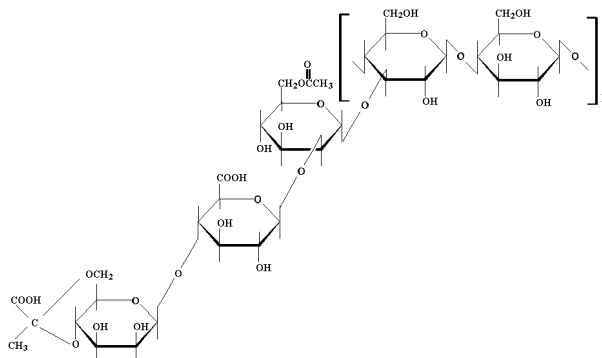
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anthan is an extracellular hetero-polysaccharide produced by the yellow-pigmented bacterium *Xanthomonas campestris* pv. campestris, a phytopathogen causing black rot in crucifers [1, 2]. The structure of xanthan consists of ß-1,4-linked D-glucose backbone carrying trisaccharide side chains composed of two mannoses and one glucoronic acid attached to alternate glucose residues in the backbone [3] (Fig. 1). The side chain is

additionally modified by acetylation of the inner and sometimes outer mannosyl residues, and pyruvylation of the outer mannosyl residue [3-5]. However, the extent of acetylation and particularly pyruvylation can vary considerably depends on fermentation conditions, as well as strain variants [6-8]. Molecular weight of xanthan ranges from 0.9×10^6 to 1.6×10^6 Daltons (Da) that is dependent on microbial source and fermentation conditions [9, 10].

Xanthan is the most commercially accepted microbial polysaccharide that is the subject of numerous studies. Worldwide consumption of xanthan is approximately 23 million kg/year estimated to grow continuously at an annual rate of 5%-10% [11, 12].

Fig. 1. Structural unit of xanthan gum.

Xanthan gum in aqueous solutions exhibits three desirable properties: (I) high viscosity at low concentrations; (II) pseudoplasticity; and (III) insensitivity to a wide range of temperature, pH,

and electrolyte concentrations. Due to its special rheological properties, the biopolymer is widely used in food, cosmetics, pharmaceuticals, paper, paint, textiles, and adhesives and otherwise in the oil and gas industry [10, 13].

However, because of its high production costs, xanthan loses some markets to polymers from algae or plants [1, 14]. In order to reduce production costs and to improve the competitive position of xanthan, its productivity should be increased [10, 14]. Various approaches have been suggested for this purpose, including (I) development of more efficient fermentation processes [15]; (II) optimization of culture media used for the gum production [15, 16]; and (III) isolation of *X. campestris* mutants with enhanced xanthan production [17-19].

The aim of the present study is to describe mutagenesis of *X. campestris* PTCC 1473 and isolation of xanthan hyperproducing strains. The mutants were further characterized for possible alterations in phenotypic properties such as extracellular enzyme production, consuming different carbon sources, and pigmentation. Pyruvyl and acetyl contents, rheological properties and average molecular weight of the different xanthan gums synthesized by the mutants were also analyzed.

MATERIALS AND METHODS

Materials. Ethyl methanesulfonate (EMS) was purchased from Sigma Chemical Co. (USA) and antifoam A emulsion from Fluka (Switzerland). Rifampicin and all media bases were supplied by Merck Co. (Germany). All other chemicals were of analytical grade.

Microorganism. *X. campestris* PTCC 1473 (NRRL B-1459) was obtained from Persian Type Culture Collection, Iranian Research Organization for Science and Technology (Tehran, Iran) and used as the parent strain. The stock cultures were maintained on yeast malt (YM) agar slants containing (g/l) glucose, 10; peptone, 5; yeast extract, 3; malt extract, 3; and agar, 20, and stored at 4^oC. They were transferred every 14 days to maintain good viability and stability for xanthan production.

Batch culture conditions. The culture medium used for xanthan production was the one optimized elsewhere [16]. This culture medium had the following composition (g/l): glucose, 30; $MgSO_4.7H_2O$, 0.24; $(NH_4)_2SO_4$, 3.33; H_3BO_3, 0.0072; $FeCl_3.6H_2O$, 0.0042; KH_2PO_4, 7.2; $CaCO_3$, 0.029; citric acid, 2; ZnO, 0.006; yeast extract, 0.75; peptone, 0.34; antifoam, 0.06; and HCl, 0.16 ml/l. The initial pH of the medium was adjusted to 7.0 ± 0.1. Batch fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of culture medium inoculated with 10% (v/v) of a 24-h preculture. The cultures were then incubated at 30^oC on a rotary shaker at 200 rpm for 96 h. Samples were withdrawn at regular intervals and analyzed for concentrations of biomass, xanthan, and residual glucose.

Mutagenesis. The technique for EMS treatment was based on that described by Miller [20] with some modifications. *X. campestris* PTCC 1473 was grown at 30^oC in 100 ml YM broth to ca. 2×10^8 cells/ml. Portions (5 ml) of the culture were centrifuged at 5,000 $\times$g for 15 min, washed twice in minimal A buffer (g/l: K_2HPO_4, 10.5; KH_2PO_4, 4.5; $(NH_4)SO_4$, 1 and sodium citrate.$2H_2O$, 0.5; pH 7.0 ± 0.1) [20], and resuspended in 2.5 ml of the same buffer. The cell suspensions were pooled in a flask and subsequently dispensed as 2-ml aliquots into screw-capped tubes. Then 40 µl EMS was added to each tube, and the tubes were incubated at 30^oC with mild shaking for 5 to 60 min. To quench the mutagen, 4 ml of a freshly prepared

10% (w/v) sodium thiosulfate sterile solution was added to each tube [21]. The treated cells were then centrifuged (5,000 ×g for 15 min), washed twice in minimal A buffer and finally resuspended in 2 ml of the same buffer. A 0.5-ml aliquot of each suspension was transferred into 10 ml YM broth and incubated at 30°C overnight to allow mutations to segregate. The day after, suitable dilutions of the cultures were made in sterile 0.9% (w/v) NaCl and bacteria were spread over the surface of YM agar containing rifampicin (50 µg/ml) and incubated at 25-30°C for 72 h [19]. The isolated mutants were further characterized by transferring onto plates of (I) minimal medium (g/l: glucose, 5; K_2HPO_4, 10.5; KH_2PO_4, 4.5; $(NH_4)SO_4$, 1; sodium citrate.$2H_2O$, 0.5; $MgSO_4.7H_2O$, 0.246; thiamine hydrochloride, 0.005 and agar, 15; pH 7.0 ± 0.1) [20] ; (II) minimal medium supplemented with casein hydrolysate (acid hydrolyzed) [22]; (III) M9 medium plus glucose, mannose, fructose or lactose at 1% (w/v) as the carbon source [19] ; and (IV) YM agar containing 1% (w/v) soluble starch, skim milk or sodium carboxymethyl cellulose. The use of these media permitted detection of (i) auxotrophs in general; (ii) amino acid-requiring auxotrophs; (iii) ability of mutants in utilizing different carbon sources; and (iv) extracellular enzymatic activities of α-amylase, protease and cellulase, respectively. The plates were then incubated at 25-30°C for appropriate periods of time. Cleared zones appearing around colonies with protease activity were readily observed on plates containing skim milk. Starch plates were fumigated with iodine to develop halos. To visualize cleared zones produced by cellulase activity, the plates were treated using Congo red as previously described [23].

Absorption spectra of xanthomonadin pigments. Extraction of xanthomonadin pigments was carried out as previously described with some modifications [24]. Overnight cultures of the parent strain and the mutants grown in YM broth were harvested by centrifugation (10,000 ×g for 20 min) in 80-ml polypropylene centrifuge tubes and washed twice in distilled water. After adding methanol at a ratio of 80 ml to 1 g of wet biomass, the tubes were immersed in a boiling water bath for 5 min. After removing cells by centrifugation, the supernatants were concentrated by evaporation in a hot water bath (75°C) to give appropriate concentrations for scanning the absorption spectra. The extracts were scanned using a CECIL CE 9260 spectrophotometer (Cecil Instruments Ltd., England).

Analytical methods. For biomass estimation, cells were pelleted by centrifugation (20,000 ×g for 1 h), washed twice and resuspended in distilled water and absorbance was measured at 650 nm. The corresponding dry weight of the cells was obtained from the established standard curve in which absorbance was plotted against dry biomass [14]. Xanthan was removed from the fermentation medium by precipitation with three volumes ethanol in the presence of 1% (w/v) KCl as the electrolyte [25]. The precipitate was then dried to constant weight at 50°C. Glucose concentration in the cell free supernatants was determined using the glucose oxidase method [26]. The pyruvate content of the polymer was estimated by reaction with 2,4-dinitrophenylhydrazine as described elsewhere [27]. The acetyl content was determined by hydroxamic acid reaction using glucose pentaacetate as the standard [28].

Rheological studies were carried out using a Brookfield DV-II cone and plate viscometer, equipped with a CP-40 spindle. Viscosities of 3 g/l xanthan solutions in 0.01 M KCl were measured at 25°C between shear rates of 2.25 and 225 s^{-1}. The viscosity coefficient (η′) and flow behavior index (N) were derived by linear regression using the following power law model:

$$F^N = η'G \qquad (1)$$

where F is the shear stress and G is the shear rate [29, 30].

The average molecular weight of xanthan was obtained by measuring the viscosities of xanthan solutions in 0.1 M KCl at concentrations between 1 and 1.5 g/l. The temperature was maintained at 25°C using a circulatory water bath. The value of the intrinsic viscosity ([η]) was determined and related to the polymer average molecular weight (M_W) by means of a Mark-Houwink type equation proposed by Milas *et al.* [31]:

$$[η] = 1.7×10^{-7}M_W^{1.14} \qquad (2)$$

Statistical analysis. Comparisons between the groups were made with the Dunnett t-test (2-sided) following one-way analysis of variance (ANOVA) treating the parent strain as the control group. In terms of glucose consumption rate, Mann-Whitney U test was used to compare each mutant against the parent strain. Differences between experimental groups were considered significant at the 0.05 level. All analyses were performed using the statistical software SPSS$^®$ 10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Isolation and characterization of mutants. Exposing *X. campestris* PTCC 1473 to EMS generated a collection of mutants. Of 4400 colonies appeared on the rifampicin-containing media, 22 colonies were selected according to their size, color or morphology. These isolates were then checked for their xanthan producing ability in the fermentation medium. They showed xanthan yields between 8.07 and 17.30 g/l compared to the 13.31 g/l obtained from the parent strain (data not shown). Four mutants designated as $XC1473E_1$ to $XC1473E_4$ were observed to accumulate higher amounts of xanthan with respect to the parent strain. Experimentation with these mutant strains was repeated for a one-year period, revealing no

Table 1. Biochemical characteristics of *X. campestris* PTCC 1473 and the mutants [a].

Strain	MM	MM+CH	M9 medium				Extracellular enzymatic activity		
			+G	+F	+M	+L	A	P	C
Parent	+	+	+	+	+	+/–	+	+	+
$XC1473E_1$	+	+	+	+	+	+/–	+	+	+
$XC1473E_2$	+	+	+	+	+	+	+	+	+
$XC1473E_3$	+	+	+	+	+	+	+	+	+
$XC1473E_4$	+	+	+	+	+	+	+	+	+

[a]Symbols: MM (Minimal medium); CH (Casein hydrolysate); G (Glucose); F (Fructose); M (Mannose); L (Lactose); A (α-Amylase); P (Protease); C (Cellulase); + (Growth); +/– (Faint growth).

alterations in stability or performance. Mutant $XC1473E_1$ appeared yellow on YM agar, the same as the parent strain, but mutants $XC1473E_2$, $XC1473E_3$ and $XC1473E_4$ gave rise to white colonies on the medium. Characterization studies of the strains demonstrated that the mutants were similar to the parent strain except for lactose exploitation as the only carbon source (Table 1). In lactose-containing medium, the parent strain and $XC1473E_1$ grew weakly opposing to the three other mutants. Of these three mutants, $XC1473E_2$ gave a distinctively larger colony in this medium.

Batch fermentation kinetics. Time course fermentation studies were performed for the strains with glucose (30 g/l) as the carbon source to compare the xanthan production and other fermentation parameters of the mutants with the parent strain. The specific growth rate (μ) was determined from the slope of the semi logarithmic plot of cell density versus fermentation time. After 48 h, cultures entered the stationary phase and the rate was calculated in the exponential growth phase. As shown in Table 2, the specific growth rate of $XC1473E_1$ (0.059 h^{-1}) was significantly larger than that of the parent strain (0.054 h^{-1}), resulting in a markedly higher average cell density (reached at the stationary phase) for $XC1473E_1$ compared to the parent strain (4.46 vs. 3.44 g/l). The xanthan formation rate was estimated from the slope of the xanthan production curve of the batch culture. Since the curve almost leveled off after 72 h, the xanthan formation rate was only calculated up to the time. Mutants $XC1473E_2$ and $XC1473E_3$ had higher xanthan formation rates (0.23 and 0.20 g/l.h, respectively) with respect to the parent strain (0.16 g/l.h) (Table 2). The glucose consumption rate was determined from the slope of the glucose consumption curve of the batch culture. As shown in Table 2, mutants $XC1473E_2$ and $XC1473E_3$ exhibited considerably higher glucose consumption rates (0.39 and 0.38 g/l.h, respectively) than the parent strain (0.35 g/l.h), which may be attributed to better xanthan formation rates of the two strains. Mutant $XC1473E_2$ was the only strain to use up glucose within 72 h.

Mutants $XC1473E_2$ and $XC1473E_3$ showed the highest xanthan yield (17.30 g/l) and (16.18 g/l), respectively when compared to the parent strain (13.31 g/l) (Table 2). The yield increases observed for $XC1473E_2$ and $XC1473E_3$ were better than those previously reported by Harding *et al.* [32] and Thorne *et al.* [33] that were 10% and 20%, respectively. However, in more suitable fermentation conditions (e.g. in a

fermentor) the yields may be better, because broth viscosity arising from the xanthan production significantly reduces the mass transfer rate for oxygen and other nutrients [34]. Since synthesis of xanthan by *X. campestris* is a very efficient process [34] only relatively small

Table 2. Fermentation kinetic parameters of *X. campestris* PTCC 1473 and the mutants [a,b].

Strain	μ (h^{-1})	R_P (g/l.h)	R_S (g/l.h)	X_S (g/l)	Xanthan[c] (g/l)
Parent	0.054 ± 0.001	0.16 ± 0.01	0.35 ± 0.00	3.44 ± 0.82	13.31 ± 0.46
XC1473E$_1$	0.059 ± 0.002^d	0.17 ± 0.03	0.35 ± 0.03	4.46 ± 0.49^d	14.08 ± 1.37
XC1473E$_2$	0.054 ± 0.002	0.23 ± 0.01^d	0.39 ± 0.03^e	3.23 ± 0.28	17.30 ± 0.81^d
XC1473E$_3$	0.055 ± 0.002	0.20 ± 0.01^d	0.38 ± 0.01^e	3.54 ± 0.87	16.18 ± 1.12^d
XC1473E$_4$	0.057 ± 0.001	0.18 ± 0.01	0.36 ± 0.01	3.69 ± 0.68	14.49 ± 1.61

[a]Symbols: μ (Specific growth rare during 0-48 h); R_P (Xanthan formation rate during 0-72 h); R_S (Glucose consumption rate during 0-72 h); X_S (Average dry biomass in the stationary phase). [b]Data are the means of three independent experiments $\pm$ standard deviation. [c]Maximal xanthan is given here. [d]Significant difference from the parent strain (Dunnett t-test, $P<0.05$). [e]Significant difference from the parent strain (Mann-Whitney U test, $P<0.05$).

Table 3. Characteristics of xanthan gums produced by *X. campestris* PTCC 1473 and the mutants [a,b].

Strain	η' (mPa.s)	N	Pyruvyl (%)	Acetyl (%)	M_W (Da)
Parent	16.87 ± 3.62	1.22 ± 0.02	7.17 ± 0.21	3.58 ± 0.05	946908 ± 11862
XC1473E$_1$	15.44 ± 1.70	1.22 ± 0.01	6.82 ± 0.55	3.56 ± 0.08	988771 ± 57090
XC1473E$_2$	26.47 ± 4.89^c	1.27 ± 0.02^c	7.85 ± 0.29	3.51 ± 0.03	1182634 ± 61627^c
XC1473E$_3$	24.08 ± 4.36	1.25 ± 0.03	7.92 ± 0.42	3.38 ± 0.26	1078048 ± 47827^c
XC1473E$_4$	18.94 ± 1.37	1.22 ± 0.01	6.62 ± 0.51	3.56 ± 0.01	1057787 ± 40802

[a]Symbols: η' (Viscosity coefficient); N (Flow behavior index); M_W (Average molecular weight). [b]Data are the means of three independent experiments $\pm$ standard deviation. [c]Significant difference from the parent strain (Dunnett t-test, $P<0.05$).

increases in yield are to be expected by strain development. Thus, an enhancement of the productivity by 30% (as shown in this study)
is significant for the commercial production
of xanthan.

Rheological properties. After resuspending dried potassium salt of xanthan (3 g/l) in 10 mM KCl, the effect of the shear rate on the viscosity of each product was determined and the viscosity coefficient and flow behavior index were derived by linear regression of the observed results. The regression coefficients were all greater than 0.999, indicating a very good correlation. According to equation (1), the higher the flow behavior index the greater the pseudoplasticity of system [30].
As shown in Table 3, maximum values of viscosity coefficient and flow behavior index were observed for mutant XC1473E$_2$ (26.47 mPa.s and 1.27, respectively), which were significantly greater than the parent strain. Even though the values obtained with mutant XC1473E$_3$ were reasonably high, they were not significantly different from the parent strain. Xanthan solutions show pseudoplastic behavior, i.e. apparent viscosity decreases as shear rate increases; no hysteresis loop is evident and shear thinning and structural recovery are instantaneous. Due to these features, xanthan is extensively used as thickener and stabilizer in emulsions as well as in paints [13, 15]. In fact, a higher pseudoplasticity index, as observed with XC1473E$_2$, is counted as an advantage for xanthan solutions. The other important characteristic, which reflects on the polymer quality, is the viscosity coefficient. Xanthan produced by XC1473E$_2$ gives a more viscous solution than that of the parent strain.
Rheological properties of xanthan solutions change with polymer nature, i.e. pyruvate [25] and acetate contents as well as average molecular weight [10, 35]. Some researchers have considered the pyruvate content as the best indicator of the polysaccharide quality; a polymer with a pyruvic acid content of 4% or more is defined as a quality gum [25]. This effect appears to be primarily associated with a promotion of the

aggregated form of xanthan by pyruvyl residues, and thereby enhances pseudoplasticity and viscosity of the gum solutions [10]. Inversely, acetate has a stabilizing effect upon the xanthan ordered conformation and leads to a decreased gum viscosity [35, 36]. As shown in Table 3, the pyruvate and acetate contents of the xanthan gums produced by the mutants were not significantly different from that obtained from the parent strain, suggesting that the changed rheological characteristics were attributable to the third factor. Actually, the higher molecular weight of xanthans produced by $XC1473E_2$ (1,183 kDa) and $XC1473E_3$ (1,078 kDa) compared to that of the parent strain (947 kDa) (Table 3) seems to account for its increased viscosity coefficient and flow behavior index, since molecular weight of the polymer has a direct influence on its rheological behaviors [6, 10].