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High-temperature, salt-tolerant xanthanase

Martin C. Cadmus, Morey E. Slodki and James J. Nicholson

Northern Regional Research Center, Agricultural Research Center, U.S. Department of Agriculture, Peoria, IL, U.S.A.

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SUMMARY

A new high-temperature, salt-tolerant xanthanase suitable as an enzymic viscosity breaker for xanthan-based hydraulic fracture fluids was obtained by soil enrichment growth on xanthan gum incubated at 45°C in the presence of 3% NaCl. The mixed culture produces exoenzymes functional up to 65°C in the presence of salts. Degradation products include the pyruvic acetal of mannose and branched oligosaccharides derived from cleavage of main-chain β-(1→4)-D-glucosyl linkages. Release of the terminal pyruvic acetal of D-mannose leads to oligosaccharide products that evidently contain the ene-4,5-unsaturated glucuronic acid residue.

INTRODUCTION

It is essential that an enzymic viscosity breaker for xanthan gum-based hydraulic fracture fluids, employed to enhance the recovery of natural gas and petroleum from subterranean formations of low porosity, functions under conditions of elevated temperature and salinity. Accordingly, a xanthanase was sought that would function in brine at 65°C, which is considered the prevailing *moderate* temperature in underground strata bearing gas and petroleum. Salt-tolerant bacteria that produce a xanthanase complex functional in the presence of brines have been isolated on enrichment culture with xanthan gum as the main carbon source [1]. It was further noted that the enzyme complex displayed significant resistance to thermal inactivation at 48°C in the presence of 4–10% NaCl [2]. This report describes enrichment isolation of a mixed bacterial culture that forms an extracellular xanthanase complex with the required salt and thermal tolerances. From structural characterization of degradation products, it appears that two enzymes are involved: a lyase that removes terminal pyruvated D-mannose residues and a unique β-(1→4)-D-glucanase that cleaves glycosidic linkages of backbone chain residues bearing side chains.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Correspondence: M.C. Cadmus, Northern Regional Research Center, ARS, USDA, 1815 N. University St., Peoria, IL 61604, U.S.A.

MATERIALS AND METHODS

Enrichment cultures

A heat-stable, salt-tolerant mixed culture (NRRL B-14401) was isolated by enrichment from soil. The enrichment broth consisted of xanthan, 0.25%; $(NH_4)_2SO_4$, 0.05%; yeast extract, 0.025%; tryptone, 0.025%; NaCl, 3%; and 0.03 M potassium phosphate buffer (pH 6.5). Soil samples were added to the broth in Erlenmeyer flasks (0.5 g/12 ml) and shaken at 45°C until viscosity reduction was observed (6–10 weeks). After two transfers were completed to confirm xanthan biodegradation, the positive culture was streaked on agar medium similar to the enrichment broth except that xanthan and NaCl were reduced to 0.2 and 1.0%, respectively. Neither single colony isolates nor pairs of isolates degraded the polysaccharide.

The mixed culture was stored on agar slants and in lyophilized or liquid broths. The latter method was most satisfactory for routine experiments as cell growth and viscosity reduction occurred in 1–3 days vs. 2–3 weeks when started from agar slant or lyophil cultures.

Enzyme production

Stock cultures were maintained by incubation in enrichment broth for 3–4 days at 45°C and stored in capped vials for up to 6 months. The same medium was used in the production flasks except that the NaCl level was reduced to 2% and xanthan to 0.15%. The first-stage flask was inoculated with 10% v/v from the stock broth and incubated 3–5 days until viscosity diminished and cell growth became evident. The inoculum flask (second stage) was seeded with 5% v/v from the first stage. After 72 h, 12 ml of the second stage was inoculated into Fernbach flasks containing 750 ml of medium. Maximum enzyme production was achieved in 42 h in flasks shaken (150 rpm) at 45°C. Longer incubation diminished activity.

Enzyme recovery

Fermentation broth from the mixed culture was clarified by centrifugation (20 000 × *g*, 20 min, 4°C) and then concentrated up to 20-fold in an Amicon TCF-10 membrane dialysis apparatus equipped with a PM30 membrane of nominal 30 000 molecular-weight cutoff. The concentrate was recentrifuged and dialyzed against 0.02 M potassium phosphate buffer (pH 6.0) for 48 h to remove excess salts.

The enzyme concentrate was applied to a Sepharose 4B (Pharmacia) column (1.5 × 150 cm) equilibrated with the above buffer (15 ml/min) at room temperature. All of the xanthanase activity was recovered in the first protein peak. Specific activity increased from 195 units/mg protein (crude) to 680 units/mg protein after this partial purification. Enzyme preparations were stored as either frozen or lyophilized concentrates, or as broths at 4°C under toluene.

Xanthanase activity was measured as milligrams of mannose equivalents released [1] in 100 ml of a buffered-substrate solution containing 0.02 M potassium phosphate (pH 5.8); xanthan gum, 0.18%; $MgSO_4$, 0.01%; $MnSO_4$, 0.002%; and $CaCl_2$, 0.001%. Enzyme (0.5 ml) and buffered substrate (2.0 ml) were equilibrated at 45°C and mixed. After 60 min, the enzyme was inactivated in boiling water (10 min) prior to measurement of reducing power.

Preparation of degradation products

The enzyme concentrate was dialyzed against cold distilled water for 18 h to remove excess salts. Xanthan (1.0 g) was added to 125 ml of the enzyme concentrate and the pH adjusted to 5.6. The mixture was incubated at 45°C under toluene until significant viscosity reduction was observed (7 days). The degraded xanthan broth was centrifuged at 20 000 × *g* for 30 min.

Analytical methods

Thin-layer chromatography, determinations of *O*-acetyl, pyruvic acid and reducing power, and estimation of viscosity reduction are described elsewhere [1]. Carboxyls were converted to ethylene glycol esters for reduction with $NaBD_4$ [10].

Isolation of degradation products

As previously described [1], higher (HMW) and lower molecular-weight (LMW) products of enzy-

molysis were separated with a PM-10 membrane in the Amicon TCF-10 membrane dialysis apparatus. Lyophilized yields of LMW and HMW fractions were 620 and 266 mg, respectively. After removal of pyruvated mannose by liquid chromatography on Bio-Gel P-2, the unresolved pentasaccharide and unsaturated tetrasaccharide components of the LMW fraction were separated by HPLC. The acid forms of the oligosaccharides were chromatographed in a solvent mixture of 1:1 water/acetonitrile pumped at 6.0 ml · min^{-1} through a semi-preparative aminopropyl silica column (Dynamix-60A NH2, 10 mm i.d. × 25 cm). Only two peaks, in 45:55 ratio, were detected by refractive index. The collected peak fractions were adjusted to pH 6.8 and then concentrated in a rotary evaporator. During concentration, both oligosaccharides precipitated. Although insoluble in water, they were amenable to methylation analysis by virtue of solubility in the dimethyl sulfoxide–sodium methylsulfinylmethanide reagent.

Structure determinations

Component and methylation analyses were carried out as before [1] except for the use of capillary GC [9] for both flame ionization detection and mass spectrometry. Identification of sugar derivatives was facilitated by comparisons of GC retention times with standard compounds and by reference to the known structure of xanthan gum [5,7]:

-4Glcβ-4Glcβ-4Glcβ-4Glcβ-
3| 3|
OAc-6Manα OAc-6Manα
2| 2|
GlcAβ GlcAβ
4| 4|
PA-4,6Manβ Manβ

The structure depicted reflects the average degree of pyruvation (PA = pyruvic acetal) of the xanthan gum preparation [8] used in this work, although the sequence of pyruvation is not known.

RESULTS

Stability of substrate-free xanthanase

This enzyme was highly stable during routine handling and purification at room temperatures. About 95% of the activity remained after lyophilization and 90% remained after freezing the concentrate (–20°C). There was no loss of activity in solutions stored up to 6 months at 4°C under toluene. At 45°C and in buffer of low ionic strength, activity gradually diminished to 20% of the maximum after 7 days.

Fig. 1 depicts the results of temperature studies conducted in media of relatively low ionic strength. It can be seen that the previously reported salt-tolerant xanthanase [1] was stable only to about 40°C when heated for 20 min, with no activity remaining at 50°C. In contrast, the high-temperature xanthanase was stable to 60°C when tested under the same conditions. The enzyme(s) were inactivated at 70°C.

Salts were previously shown to increase the stability of salt-tolerant xanthanase [2]. Fig. 2 illustrates the effect of salt on the stability of high-temperature xanthanase. In the absence of salt, activity began to deteriorate above 55°C, while in the presence of 3% NaCl the xanthanase did not begin to lose activity until the temperature exceeded 60°C.

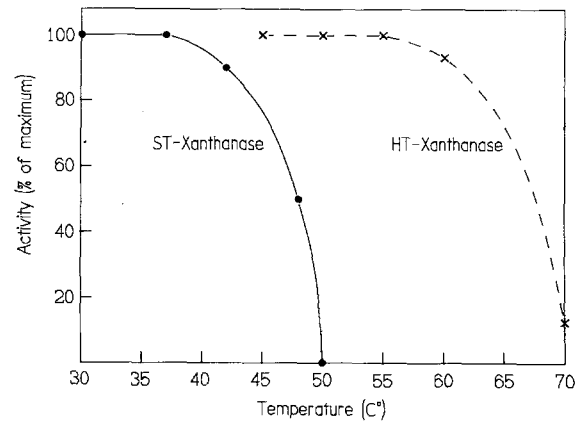

Fig. 1. Thermal stability of salt-tolerant (ST) [1] and heat-tolerant (HT) xanthanases (enzyme heated 20 min; assay 2 h, 45°C, 0.025 M buffer, pH 6.0, substrate: 1 mg/ml).

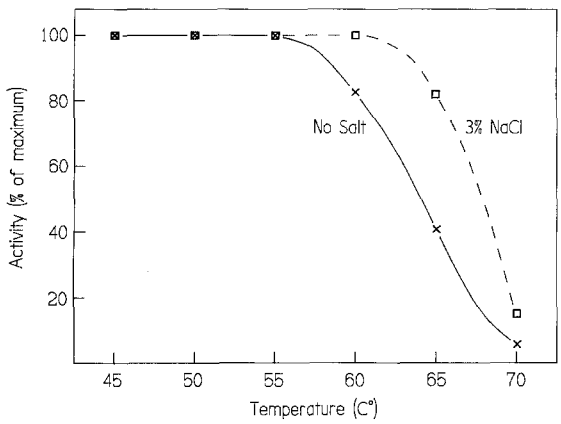

Fig. 2. Effect of added NaCl on the thermal stability of high-temperature xanthanase (conditions and assay same as in Fig. 1).

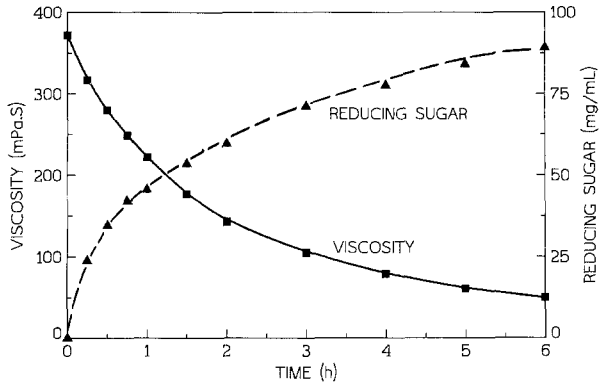

Fig. 3. Progress of high-temperature xanthanase activity as a function of incubation time (substrate: 3.5 mg/ml, 45°C, 0.025 M buffer, pH 6.0). Reducing sugar, ▲– – –▲; viscosity, ■– – –■.

At 65°C, 82% of the maximum activity remained after 20 min.

To measure stability to pH, samples were tested over the pH range of 4–9 at 25°C for 24 h. There was good stability from pH 5 to 8 with optimum stability at pH 5.8. The enzyme was more stable at the basic end of the pH range. At pH 9, 70% of the maximum activity remained, whereas only 10% remained at pH 4.5.

Catalytic properties of xanthanase

Xanthanase activity, measured as reducing power release, was determined over a temperature range of 35–70°C in the buffered-substrate solution (pH 6.0). The optimum temperature for xanthan degradation was found to be 50°C with about 10% reduction at 45°C and 58°C. For assays and substrate conversions of long duration, 45°C was used to ensure a longer period of activity. At this temperature, reaction mixtures remained active for several days.

Tests for optimum pH of activity were carried out over the range of 4–9. Xanthanase was most active at the pH of maximum stability; i.e., pH 5.8; activity dropped sharply below pH 4.8 and above pH 6.5 (data not shown).

Fig. 3 shows a typical progress curve for high-temperature xanthanase activity when measured over a period of 6 h. Loss of viscosity and release of reducing power occur inversely. Actually, product formation during the first 60 min was most rapid, after which the curve flattened out. This plateau represents about 6% conversion of substrate (in terms of total carbohydrate content). Rapid degradation by the enzyme(s) of the HMW product recovered at this stage suggests some form of inhibition by the products.

The amount of NaCl that high-temperature xanthanase tolerated is depicted in Fig. 4. Enzymic analyses were performed at salt concentrations up to 10%. Activity gradually diminished over that range with 40% of the maximum activity still remaining in 10% salt. Magnesium and calcium chlo-

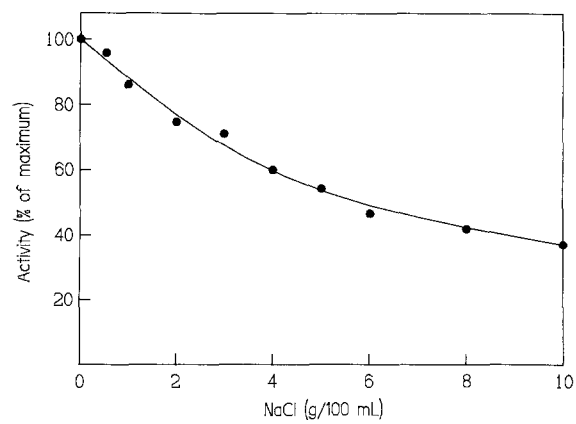

Fig. 4. Effect of added NaCl on activity of high-temperature xanthanase. Activity measured as reducing power (6 h, 45°C, 0.025 M buffer, pH 6.0, substrate: 1 mg/ml).

rides were also tested at 1% concentrations; neither of these salts affected enzyme activity (data not shown).

Characterization of LMW products

Thin-layer chromatography of the LMW fraction revealed three spots: pyruvated mannose [4,6-*O*-(1-carboxyethylidene)-D-mannose] and two oligosaccharides. Both oligosaccharides were *O*-acetylated, but one displayed an intense short-wave UV absorbance characteristic of ene-4,5-uronic acids. A positive thiobarbituric acid test and UV absorption spectra identical to those published [6] accorded with this conclusion. When a portion of the fraction was reduced with $NaBH_4$, hydrolyzed, and then examined by GC as a mixture of per-*O*-acetylated aldononitrile (PAAN) and alditol acetate derivatives, a 1:1 ratio of glucose/mannose was obtained. Half of the glucose and a quarter of the mannose were reduced. Because the original xanthan gum substrate bears pyruvic acetal substituents on about half of the side chains, this result suggested an equimolar mixture of pyruvated mannose, pentasaccharide and unsaturated tetrasaccharide. This mixture probably was derived through a lyase that removed terminal pyruvated mannosyl residues and a hydrolase that cleaved one of the backbone β-(1→4)-D-glucosyl linkages. Uronic acid residues would not be detected by the procedure unless the carboxyls were reduced.

Methylation analysis (Table 1) of the fraction, following reduction of carboxyls with ethylene oxide and $NaBD_4$ [10], revealed that both oligosaccharides were branched. The glucose residue representing the glucuronic acid of the pentasaccharide was identified by the presence of two deuterium atoms on C-6 of a PAAN derivative. The branch-point glucose residues were identified by the presence of a single deuterium atom on C-1 of 3,4-di-*O*-acetyl-tetra-*O*-methyl-D-glucitol, which also represents the reducing end groups. Pyruvated mannose was represented by the 4,6-di-*O*-acetyl-tetra-*O*-methyl-D-mannitol-1-*d* derivative. Molar ratios of the various methylated components agreed closely

Table 1

Methylation analyses of deuterio-reduced oligosaccharide products of high-temperature xanthanase

Methylated component[a]	Mol ratios in oligosaccharides					
	Carboxy-reduced mixture[b]		Mixture[c]		Isolated	
	Found	Calc.[d]	Found	Calc.[d]	A	B
2,3,4,6-Glc	2.0	2	2.0	2	1.0	1.0
2,3,4,6-Man	0.9	1	0.9	1	1.0[e]	1.0
2,3,6-Glc(6-d_2)	1.1	1				
3,4,6-Man	1.8	2	1.8	2		0.9
1,2,5,6-Glc OH(1-*d*)	2.3	2	2.2	2	1.0	1.1
1,2,3,5-Man OH(1-*d*)[f]	1.2	1				

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. After hydrolysis of per-*O*-methylated products, free sugars were analyzed by GC-MS as per-*O*-acetylated aldononitriles, and alditols as di-*O*-acetates.

[b] Glucuronic acid carboxyls reduced with ethylene oxide-$NaBD_4$.

[c] Carboxyls not reduced; pyruvated mannose removed by gel filtration.

[d] Mole ratios expected for unimolecular mixture of unsaturated tetrasaccharide A, pentasaccharide B and pyruvated mannose (where present).

[e] Ene-4,5-D-glucopyranosyluronic acid end group removed prior to methylation.

[f] Derived from free 4,6-*O*-(1-carboxyethylidene)-D-mannose.

with ratios calculated for oligosaccharides A and B (below) and pyruvated mannose in an equimolar mixture.

ene-4,5-GlcAβ2(OAc6)Manα3(Glcβ4)Glc (A)

Manβ4GlcAβ2(OAc6)Manα3(Glcβ4)Glc (B)

The oligosaccharides were separated from pyruvated mannose, but not from each other, by aqueous column chromatography on Bio-Gel P-2. Methylation analysis (Table 1) of the mixture, which was reduced with $NaBD_4$, accords with an equimolar ratio of A:B. This result was confirmed by analyses of the oligosaccharides separated by HPLC. It is apparent from the analysis listed in Table 1 that the acid-labile, unsaturated uronic acid residue of A had been removed to form a branched trisaccharide (C):

OAc6Manα3(Glcβ4)Glc (C)

Characterization of the HMW fraction

The fraction of the enzymic digest retained by a PM-10 membrane filter contained *O*-acetyl substituents, but no pyruvic acetal. Its UV spectrum was similar to that given by the mixture of oligosaccharides. Prior to methylation, the fraction was reduced with $NaBD_4$ in order to label the reducing end group and also to prevent alkaline elimination of a side chain. The carboxyl groups were not reduced. Table 2 lists the methylation analysis, which agrees closely with structure D (below) that consists of three trisaccharide and two unsaturated disaccharide side chains appended to a backbone chain having two additional unsubstituted β-(1→4)-linked glucosyl residues. The lower than expected value for 2,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-D-mannononitrile is attributed to resistance of the aldobiuronic acid linkage to hydrolysis.

Glcβ-4Glcβ-[4Glcβ-4Glcβ-4Glcβ-4Glcβ]$_2$-4Glcβ-4Glc

3| 3| 3|

OAc-6Manα OAc-6Manα OAc-6Manα (D)

2| 2| 2|

GlcAβ ene-4,5-GlcAβ GlcAβ

4| 4|

Manβ Manβ

Table 2

Methylation analysis of the deuterio-reduced HMW product of high-temperature xanthanase

Methylated component[a]	Mol ratios	
	Found	Calc.[b]
2,3,4,6-Glc	1.0	1
2,3,4,6-Man	3.2	3
2,3,6-Glc	6.2	6
3,4,6-Man	3.0	5
2,6-Glc	3.9	4
1,2,5,6-Glc OH(1-*d*)	0.8	1

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. After hydrolysis of the per-*O*-methylated product, free sugars were analyzed by GC-MS as per-*O*-acetylated aldononitriles, and the glucitol as the di-*O*-acetate.

[b] According to structure D (see text).

In the above structure (calculated K^+ salt MW $\approx$ 4600), the locations of additional main-chain glucosyl residues are not known. A similar excess of unsubstituted glucosyl residues was noted by Sutherland [13] in a product from another type of xanthanase.

DISCUSSION

Bacterial xanthanases described prior to 1982 [3,6,11,12] were sensitive to salt concentrations >0.3 M and temperatures above 30°C. These extracellular enzyme mixtures appear, from a consideration of the products, to consist primarily of a lyase that removes terminal pyruvated mannose residues and a β-(1→4)-D-glucanase that cleaves the backbone chain of xanthan gum to give *linear* oligosaccharides that contain either terminal mannose or ene-4,5-D-glucuronic acid. In 1982 [1] we described salt-sensitive and salt-tolerant bacilli from soil enrichment cultures that produced exoenzymes having a different action pattern in which all side-chain residues were liberated and the component resistant to further degradation was primarily (1→4)-linked D-glucan. This type of side-chain degradation with

salt-tolerant isolates was confirmed by Hou et al. [4] whose preparations also degraded the glucosidic backbone chain.

The salt-tolerant xanthanase complex had greater thermal stability than those previously reported. Salts enhanced stability; e.g., up to 50°C for 30 min in >0.15 M NaCl [2]. Use as a viscosity breaker for hydraulic fracture fluids based on xanthan gum, however, requires activity in brines at 60–70°C. Accordingly, soil samples were screened for both salt- and thermal-tolerant xanthanase producers. Even though this type of screen was successful at 45°C, a mixed culture was obtained that displayed salt tolerance and activity in the desired temperature range. The culture required the presence of 0.5% salt for growth on xanthan gum as the sole source of carbon. Individual isolates alone or combined failed to produce xanthanase activity. Although slow-growing and difficult to cultivate on agar media, the mixed culture was easily propagated in shaken flasks.

It is clear from an analysis of the enzymic products that high-temperature xanthanase comprises two activities. One is a lyase that catalyzes the elimination of pyruvated mannose residues with formation of an ene-4,5-D-glucopyranosyl uronic acid. The other activity is a hydrolase that cleaves the β-D-glucosyl linkages of backbone residues bearing side chains to yield *branched* oligosaccharides. With this finding, enzymic cleavages of all glycosidic linkages in xanthan gum have been demonstrated. High-temperature xanthanase is active on carboxymethyl cellulose as are the xanthanases that attack the other backbone linkage. The gradual decrease in viscosity that parallels release of reducing power suggests an exo-type of backbone cleavage. At present, it is not known whether removal of pyruvated mannosyl residues occurs after release of oligosaccharides. Isolation of a completely depyruvated HMW fragment, however, indicates that the lyase can attack the intact polysaccharide.

This work has shown the potential for use of the enrichment culture technique to obtain depolymerases active on microbial polysaccharides in brines at elevated temperatures. In retrospect, the various enzymes obtained by this technique have given linear and branched oligosaccharides that confirm the chemically determined structure of xanthan gum. Excess backbone glucosyl residues present in HMW enzymolysis products, however, suggest a biosynthetic irregularity not apparent from chemical studies.

REFERENCES

1 Cadmus, M.C., L.K. Jackson, K.A. Burton, R.D. Plattner and M.E. Slodki. 1982. Biodegradation of xanthan gum by *Bacillus* sp. Appl. Environ. Microbiol. 44: 5–11.

2 Cadmus, M.C. and M.E. Slodki. 1985. Enzymic breakage of xanthan gum solution viscosity. Dev. Ind. Microbiol. 26: 281–289.

3 Cripps, R.E., H.J. Sommerville and M.S. Holt. 1981. Xanthanase enzyme. Eur. Pat. Appl. 30,393.

4 Hou, C.T., N. Barnabe and K. Greaney. 1986. Biodegradation of xanthan by salt-tolerant aerobic microorganisms. J. Ind. Microbiol. 1: 31–37.

5 Jansson, P.-E., L. Kenne and B. Lindberg. 1975. Structure of the extracellular polysaccharide from *Xanthomonas campestris*. Carbohydr. Res. 45: 275–282.

6 Lesley, S.M. 1961. Degradation of the polysaccharide of *Xanthomonas phaseoli* by an extracellular bacterial enzyme. Can. J. Microbiol. 7: 815–825.

7 Melton, L.D., L. Mindt, D.A. Rees and G.R. Sanderson. 1976. Covalent structure of the extracellular polysaccharide from *Xanthomonas campestris*: evidence from partial hydrolysis studies. Carbohydr. Res. 46: 245–257.

8 Sandford, P.A., J.E. Pittsley, C.A. Knutson, P.R. Watson, M.C. Cadmus and A. Jeanes. 1977. Variation in *Xanthomonas campestris* NRRL B-1459: characterization of xanthan products of differing pyruvic acid content. In: Extracellular Microbial Polysaccharides (Sandford, P.A. and A. Laskin, eds.), pp. 192–210, American Chemical Society, Washington, DC.

9 Slodki, M.E., R.E. England, R.D. Plattner and W.E. Dick, Jr. 1986. Methylation analyses of NRRL dextrans by capillary gas-liquid chromatography. Carbohydr. Res. 156: 199–206.

10 Sutherland, I.W. 1970. Structure of *Klebsiella aerogenes* type 8 polysaccharide. Biochemistry 9: 2180–2185.

11 Sutherland, I.W. 1981. *Xanthomonas* polysaccharides – improved methods for their comparison. Carbohydr. Polym. 1: 107–115.

12 Sutherland, I.W. 1982. An enzyme system hydrolysing the polysaccharides of *Xanthomonas* species. J. Appl. Bacteriol. 53: 385–393.

13 Sutherland, I.W. 1984. Hydrolysis of unordered xanthan in solution by fungal cellulases. Carbohydr. Res. 131: 93–104.