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Production of xanthan gum and ice-nucleating material from whey by *Xanthomonas campestris* pv. *translucens*

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Abstract *Xanthomonas campestris* pv. *translucens* IFO13599 could produce xanthan gum (18.5 mg/100 mg, lactose) with lactose as the growth substrate in spite of a low level of β-galactosidase. This productivity corresponded to one-fifth that with glucose. This strain could also produce ice-nucleating material having an ice-nucleating temperature, T_{50}, of −2.8 °C with xanthan gum in the culture broth. We found that this strain produced both materials in whey medium from which the insoluble components had been removed. The production of xanthan with ice-nucleating material reached a maximum after cultivation for 168 h under optimum conditions. Furthermore, the xanthan obtained had a low viscosity because of its variant structure revealed, by TLC and HPLC analyses, to be lacking pyruvic acid. Furthermore, we concluded that this mixture had considerable potential as a regeneratic agent, when compared to other regeneratic agents such as carboxymethylcellulose.

Introduction

Xanthomonas campestris pv. *translucens* is an agriculturally harmful bacterium. It is known to be a bacterium that increases frost injury to plants at a temperature of −4 °C (Kim et al. 1987). On the other hand, *Xanthomonas campestris* pv. *campestris* produces xanthan gum, which has many applications in the food, cosmetics, and oil industries because of its high viscosity (Brandford and Baird 1983; Sandvik and Maerker 1977). This polymer is a substituted cellulose composed of a pentasaccharide repeating unit with alternating glucose residues in the backbone. The side-chains are composed of two mannose and one glucuronic acid molecules, mannose–glucuronic-acid–mannose, which are generally modified by the acetylation of the inner mannose and the pyruvylation of the outer mannose (Jansson et al. 1975; Melton et al. 1976). For xanthan production, various modified media with glucose, sucrose or starch as the sole carbon source are normally used but, since *X. campestris* pv. *campestris* cannot use lactose as a carbon source because of the low level of β-galactosidase, Fu and Tseng (1990) constructed a mutant strain having a mobilizable β-galactosidase expression plasmid, which was capable of producing xanthan gum from lactose. This strain produced xanthan gum from whey medium, which is a nutrient-rich dairy byproduct (4%–5% lactose) and a major problem as industrial waste. However, little is known about the simultaneous production of xanthan and ice-nucleating material by the ice-nucleating bacterium, *X. campestris* pv. *translucens*.

In this study, we report the production of xanthan and ice-nucleating material by *X. campestris* pv. *translucens* IFO13559 with whey as the growth substrate. We determined that the xanthan was a variant having a low viscosity due to a defective pyruvate. We also attempted to use the solution of xanthan and ice-nucleating material as a regeneratic agent.

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Materials and methods

Culture conditions

An ice-nucleating bacterium, *Xanthomonas campestris* pv. *translucens* IFO13559, which is capable of producing xanthan gum, was used throughout this work. This bacterium was aerobically grown in Trypticase soy medium (pH 7.0) with the following composition: Trypticase soy broth (BBL Microbiology system), 12 g/deionized water. Cells were preincubated in shaking flasks (500 ml) containing 50 ml culture medium at 18 °C for 48 h. After cultivation, the cells were harvested and washed twice with sterilized 50 mM potassium phosphate buffer (pH 7.0) by centrifugation (15 000 *g*, 10 min). These inoculum cells (1 ml) for xanthan production were adjusted with the same buffer to an approximate absorbance at

660 nm of 1.0. The whey medium was prepared by diluting the solutions of water-soluble components after removing the water-insoluble components from a 10% (w/v) whey solution by filtration (0.45-μm cellulose nitrate filters, Adventec Co.). A 50-ml sample of whey medium (pH 7.0) was used to produce the xanthan gum and ice-nucleating material. Also, the effects of various carbon sources on the xanthan production were examined by the addition of various saccharides to the medium after sucrose had been removed from the *Pseudomonas* medium (pH 7.0), which was composed of 2.0% Bacto-peptone, 1.0% sucrose, 0.86% K_2SO_4, 0.14% KCl and 0.14% $MgSO_4 \cdot 7H_2O$.

Ice-nucleating activity measurement

The ice-nucleating activity was measured by a previously described method (Vali 1971). For this, the cells after culturing were harvested by centrifugation at 20 000 *g* for 10 min and washed once in 10 ml 50 mM potassium phosphate buffer (pH 7.0). The cell concentration was then adjusted with the same buffer to an absorbance at 660 nm of 0.1. The ice-nucleating spectra of the cell were obtained by the modified droplet-freezing method (Lindow et al. 1982; Obata et al. 1987).

Preparation of xanthan gum from culture broth

After the strain had been cultured under various conditions, the cultures were centrifuged at 20 000 *g* for 10 min to remove the cells. The resulting supernatant was mixed with deionized water in a volume of 1:1, and the xanthan was then precipitated from this solution by the addition of the same volume of acetone. The precipitated xanthan was filtered with suction through a 9-cm filter-paper (no. 2, Advantec Co.) and the filter, containing adsorbed xanthan, was then dried at 120 °C for 30 min. The xanthan was then resuspended in deionized water, and was precipitated by the addition of the same volume of ethanol. The precipitated xanthan was collected by centrifugation at 20 000 *g* for 10 min and used as the purified xanthan.

Characterization of xanthan gum from this strain

For measurements of viscosities, the purified xanthan solutions were diluted with distilled water. For each concentration, the viscosities of all samples were measured with a B-type viscometer equipped with a no. 18 spindle at a shear rate of 8 s^{-1}. The sample temperature was measured at 25 °C. The components of the purified xanthan were analyzed as follows. Purified xanthan (10 mg/ml) was hydrolyzed in 2 M trifluoroacetic acid at 120 °C for 2.5 h. After removal of the acid from the solution by KOH, a portion of the hydrolysate was extracted with ether for determining the presence of acetate and pyruvate. The water solution obtained was neutralized with $BaCO_3$ before the hexose and uronic acid determinations.

The sugars and organic acids were analyzed by thin-layer chromatography and HPLC. Sugars were analyzed on Kieselgehr 60F254 with the solvent system ethyl acetate/acetic acid/ether/water (12:3:3:2, by vol.) and anisaldehyde reagent for detection, and the organic acids with the solvent system ethanol/ammonium/water (70:4:16, by vol.) and 2,6-dichloroindophenol reagent for detection. The sugar contents were also measured on a Tosoh sugar analysis system with a column of TSKgel AXG at 60 °C. Sugar elution was monitored with a spectrofluorometer (excitation at 288 nm, emission at 470 nm) after reaction with benzamidine at 110 °C. The contents of organic acids were also measured by HPLC using a Shimpack CLC-ODS column. The eluant, 0.1% H_3PO_4, was used at a flow rate of 1.0 ml/min and the detection wavelength was 210 nm.

Measurement of the freezing curve

The strain was cultured under optimum conditions and the culture broth obtained (30 ml) was centrifuged at 12 000 *g* for 10 min to remove the cells; it was then dialyzed against distilled water at 4 °C for 4 days. The freezing curve of the prepared culture broth containing both materials was obtained by placing samples into silicone-coated Pyrex tubes (18 × 130 mm), which were cooled in a methanol bath at −10 °C using a Program culture freezer MPF-1000 (EYELA Co.). A temperature sensor was inserted into the sample and changes in the sample temperature were measured for each 30-s period.

Results

Effects of carbon source on the production of xanthan gum and ice-nucleating material

Xanthan production using *X. campestris* pv. *campestris* has been investigated by many researchers, but few studies have considered the production of both xanthan and ice-nucleating material by *X. campestris*. Obata et al. (1995) reported that, when *X. campestris* pv. *translucens* IFO13559 was cultured in medium with citric acid as a carbon source, the cells were lyzed and this strain could produce ice-nucleating material and xanthan in the culture broth. As shown in Table 1, when this strain was cultured in various saccharides other than xylose, it could produce both materials in the culture broth. The highest production of xanthan (76.6 mg/ 100 mg, sucrose) occurred in the culture with sucrose as the growth

Table 1 Effects of carbon sources on the ice-nucleating activity and production of xanthan gum. Growth was monitored by measuring the absorbance (A) at 660 nm. T_{50} the temperature required to freeze 50% of the cell suspension. The viscosity of the sample was measured by using a B-type viscometer. The preparation of xanthan gum from the culture broth was as described in Materials and methods

Carbon source	Growth (A_{660})	pH	T_{50} (°C)	Viscosity (cP)	Xanthan gum (mg 100 mg, sugar)
Glucose	5.3	7.4	−2.8	38.4	40.2
Sucrose	6.0	7.4	−2.9	79.5	76.6
Galactose	4.2	7.3	−3.0	2.2	1.5
Xylose	0.0	5.9	–	2.2	1.5
Fructose	5.8	7.2	−2.8	32.1	32.3
Mannose	7.8	7.1	−2.9	2.3	1.8
Arabinose	0.1	6.7	−2.8	2.1	1.2
Maltose	3.0	7.3	−2.9	2.1	1.3
Lactose	3.5	7.3	−2.8	2.9	8.5

substrate, which is approximately the combined yield from the cultures with glucose and fructose. When this strain was cultured in medium with lactose, xanthan production was one-fifth that with glucose. We found that this production from lactose was higher than the amounts obtained from other *X. campestris* strains under the same conditions. Next, we attempted culture in medium containing only whey, in which the components of the water-soluble fraction were lactose (56.1%, w/v), protein (18.4%, w/v), lipid (12.0%, w/v) and ash (8.5%, w/v).

Culture conditions in whey medium for the production of both materials

Figure 1 shows the results of culturing this strain in medium prepared from whey at various concentrations (2%–10%, w/v). The results show the optimum whey concentration for xanthan production to be 6% (w/v). In 6% whey medium, this strain had maximal growth, but the whey concentration for ice-nucleating activity was 5% (w/v). Also, the optimum temperature and pH for xanthan production were 18 °C and 6.0 respectively (Figs. 2, 3).

The effects of culture time on the production of both materials, when this strain was cultured in 6% whey medium (pH 6.0) at 18 °C, are shown in Fig. 4. There was exponential growth for 72 h after a lag phase of 48 h. After the mid-exponential phase (absorbance at 660 nm = 3.0), the ice-nucleating temperature was rapidly lowered from −2.8 °C to −4.2 °C. On the other hand, xanthan production rapidly increased with increasing viscosity of the culture broth after a 72-h cultivation in which it was the stationary phase; the maximal production of xanthan was 28 mg/100 mg whey. From these results, we concluded that this strain should be cultured in the whey medium (6% w/v, pH

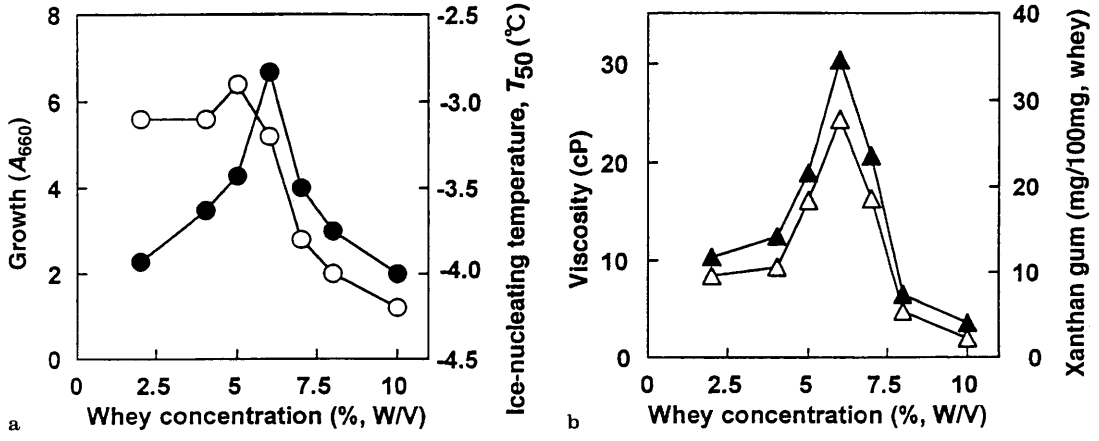

Fig. 1 Effects of whey concentration on the production of ice-nucleating matter and xanthan. ● Growth, ○ ice-nucleating temperature T_{50} (°C), ▲ viscosity, △ amount of xanthan

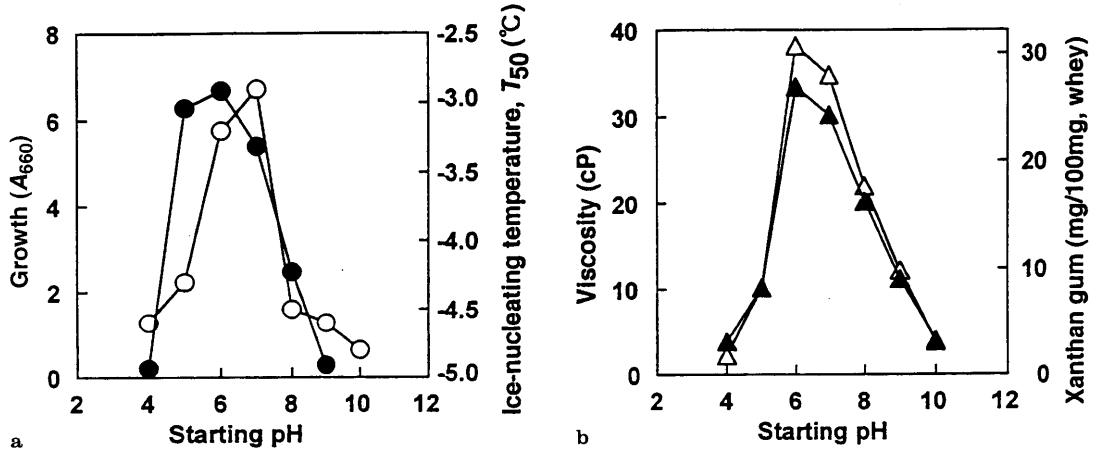

Fig. 2 Effects of pH on the production of ice-nucleating matter and xanthan. ● Growth, ○ ice-nucleating temperature T_{50} (°C), ▲ viscosity, △ amount of xanthan

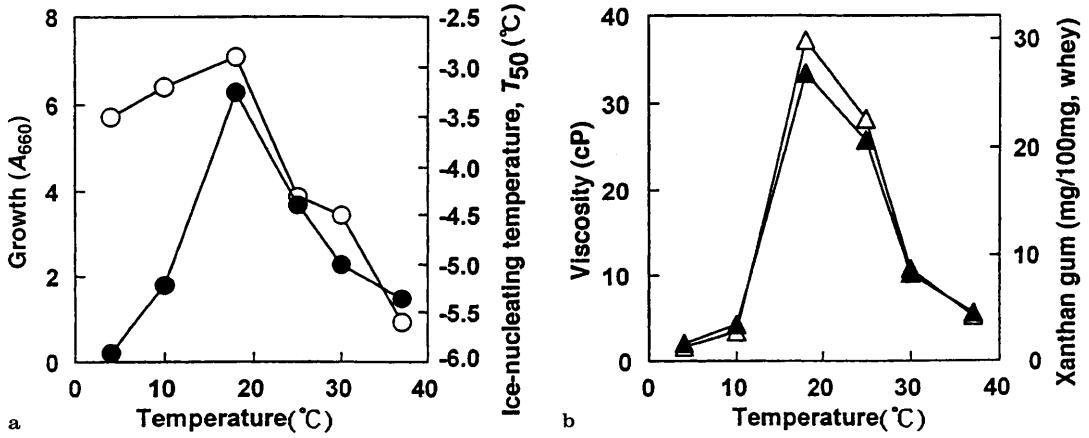

Fig. 3 Effects of temperature on the production of ice-nucleating matter and xanthan. ● Growth, ○ ice-nucleating temperature T_{50} (°C), ▲ viscosity, △ amount of xanthan

6.0) at 18 °C for 168 h in order to produce both materials in the culture broth.

Characterization of xanthan from this strain

We first examined the relationship between the viscosity and the concentration of the purified xanthan. The xanthan had a viscosity lower than that of the commercial xanthan. The viscosities of the 0.1% and 0.2% solutions were 5 cP (5 mPa s) and 9 cP respectively. The pH and thermal stabilities of this solution were also the same as those of the commercial xanthan. We postulated that the low viscosity of the xanthan was caused by the lack of acetate and/or pyruvate in the side-chain. On the basis of TLC (Fig. 5) and HPLC analyses, we found that the xanthan from this strain had glucose (R_F 0.41), mannose (R_F 0.33), glucuronic acid (R_F 0.18) and acetic acid (R_F 0.33) as components, and lacked pyruvic acid (R_F 0.43) in the side-chain. This absence also resulted in different IR spectrum for each xanthan (data not shown). Furthermore, from the results of the HPLC analysis, we found that one repeating structural unit had two moles of acetic acid in the side-chain. These properties were the same as those of mutant xanthan (X1397) produced by the mutant strain lacking the *L* gene, the ketalase gene, which pyruvylates the outer mannose (Hassler and Doherty 1990).

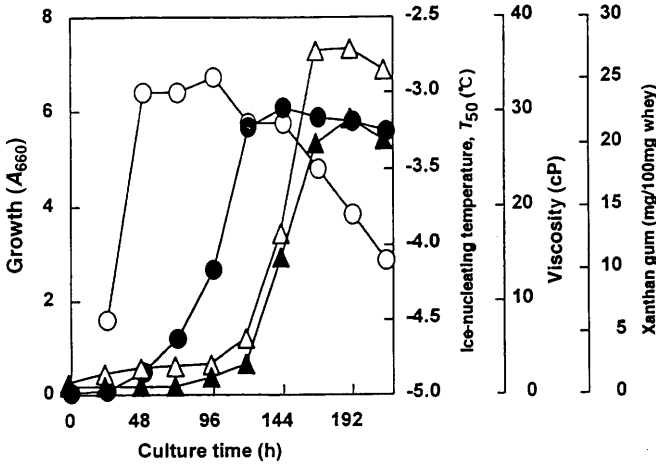

Fig. 4 Effects of culture time on the production of ice-nucleating matter and xanthan. ● Growth, ○ ice-nucleating temperature T_{50} (°C), ▲ viscosity, △ amount of xanthan

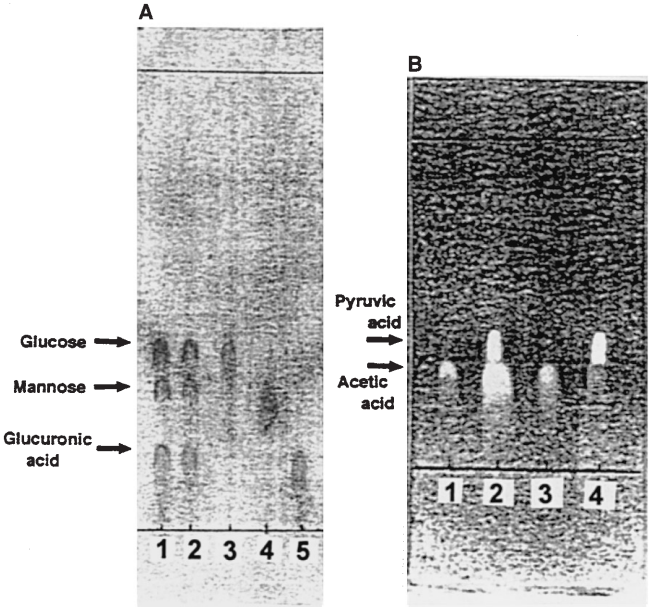

Fig. 5A, B Thin-layer chromatography of the components of extrapolysaccharide. **A** Sugar: TLC, Kieselgehr 60. Solvent: ethyl acetate/acetic acid/ether/water (12:3:3:2, v/v); detection: anisaldehyde reagent. *1* Purified xanthan, *2* commercialized xanthan, *3* glucose *4* mannose, *5* glucuronic acid. **B** Organic acid: TLC, Kieselgehr 60. Solvent: ethanol/ammonium/water (70:4:16, v/v); detection: 2,6-dichloroindophenol reagent. *1* Purified xanthan, *2* commercialized xanthan, *3* acetic acid, *4* pyruvic acid

Freezing curve of the solution containing xanthan and ice-nucleating material

The culture broth containing both materials, after culture under optimum conditions as previously described, was examined and the results are shown in Fig. 6. The ice-nucleating temperature (T_{50} = −3.6 °C) was lower than that (T_{50} = −2.9 °C) of the culture broth after culturing in *Pseudomonas* sucrose medium. However, this culture broth had a normal ice-nucleating spectrum. We therefore compared the freezing abilities of the mixture, having a viscosity of 28.3 cP and T_{50} = −3.6 °C, with those of carboxymethylcellulose solution (CMC, 0.3%) as a regeneratic agent (Fig. 7). The CMC solution only froze at −4 °C after 15 min, but the mixture started to freeze after 3 min and freezing was complete at 10 min. Also, if the CMC solution (0.15%) was added to the mixture (0.15%) the resulting solution froze in 10 min in the same manner. This result suggests that the mixture could be used as a regeneratic agent with the ability to freeze at high temperature (−1 °C).

Discussion

The production of xanthan gum by *X. campestris* in a culture broth has been extensively studied (Slodki and Cadmus 1978) and most commercial xanthan is currently produced in this way. However, most of these studies have been confined to growth in complex media and the effects of growth conditions on the composition and properties of the polymer (Tait et al. 1986). Such studies used *X. campestris* pv. *campestris* as the producing strain, and the study of xanthan production by the ice-nucleating bacterium, *X. campestris* pv. *translucens* has received little attention. We reported that the strain IFO13559 could produce ice-nucleating material and xanthan gum simultaneously in a particular medium (Obata et al. 1995).

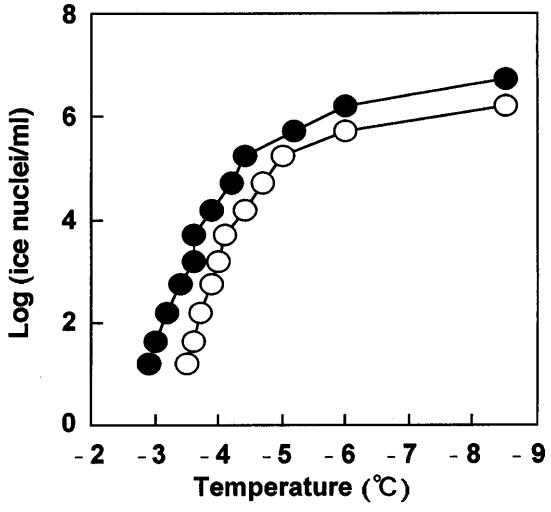

Fig. 6 Ice-nucleation spectra of ice-nucleating matter in whey medium. ● *Pseudomonas* sucrose medium, ○ whey medium

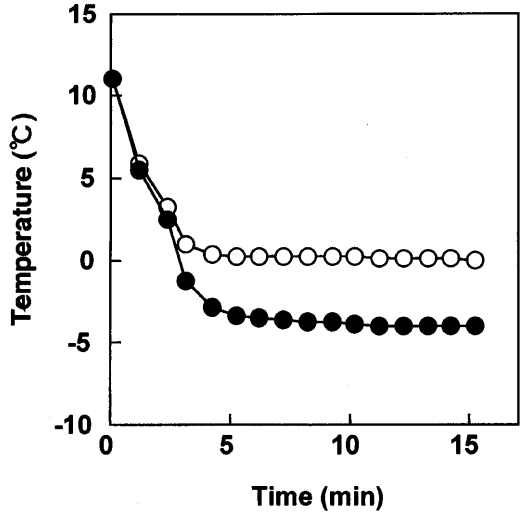

Fig. 7 Freezing curves of the mixture solution (○) and carboxymethylcellulose solution (●)

This study reconfirmed these observations in *Pseudomonas* sucrose medium. The strain produced more xanthan gum with lactose as the growth substrate than has been achieved with other xanthan-gum-producing strains (Table 1). Previously, attempts have been made by several groups to construct lactose-utilizing *X. campestris* strains for xanthan production, as *X. campestris* possesses a low level of β-galactosidase (Frank and Somkuti 1979). These lactose-utilizing strains contain the β-galactosidase expression plasmid (Fu and Tseng 1990). Starting from the wild strain, Shwartz and Bodie (1985) were able to select a strain that could utilize lactose for xanthan production, but the strain was not stable. Furthermore, the production of xanthan with whey as the growth substrate has been attempted with these strains (Fu and Tseng 1990) and with *X. cucurbitae* (Baig et al. 1990).

The strain used in this work could produce xanthan with ice-nucleating material by utilizing lactose under stable conditions. By examining the effects of the growth conditions (Figs. 1–3), this strain produced maximal amounts of both substances under optimum conditions (Fig. 4). To our surprise, we found that this production (50 mg/100 mg, lactose in whey medium) was higher than that obtained with *X. campestris*17 containing the β-galactosidase expression pKMΦLT (Fu and Tseng 1990). However, the reasons for the high production in whey medium by this strain are unknown. Furthermore, we found that the xanthan produced has a lower viscosity than that of commercial xanthan, because the structure differed from that of xanthan gum, lacking the pyruvic acid residues. The viscosity of this xanthan was the same as that of type X1397, which lacks the *L* gene encoding ketalase (Hassler and Doherty 1990).

This work was the first to report the structure of the xanthan produced by the ice-nucleating bacterium, *Xanthomonas* strain. We have previously reported that a

marine ice-nucleating bacterium, *Pseudomonas* sp. KUIN-5, could produce ice-nucleating material and cellulose at the same time (Kawahara et al. 1996). Also, because of the low viscosity of the xanthan, the cell could be readily removed from the culture broth, and the two materials could be separated from the solution by centrifugation. We predict, in the light of our previous report, that this ice-nucleating material was produced by cell lysis after the stationary phase (Obata et al. 1995). The organic acids, including phenyl acetic acid, propionic acid and isovaleric acid, originate mainly from the breakdown of amino acids liberated during autolysis of cells induced by oxygen starvation caused by xanthan gum accumulation around the *Xanthomonas* cells (Van et al. 1987).

This ice-nucleating material corresponded to the ice-nucleating material from the cells in having similar ice-nucleating spectra (Fig. 7). The optimum activity at pH 7.0 was greatly decreased by treatment at 40 °C for 30 min and was completely lost by treatment at 90 °C. Furthermore, as we had previously reported that the aluminum alginate capsule, with immobilized ice-nucleating material, was successful in freeze-concentrating various solutions, such as sea water, dimethylsulfoxide and ethanol solutions (Obata et al. 1991), we attempted to use this mixture as a generatic agent. The mixture was able to freeze quickly at a higher temperature (Fig. 6). We confirmed that this mixture was gelled by the addition of cations such as Ca^{2+} (data not shown). We expect that this mixture could be utilized for freeze-drying various foods or freeze-concentrating foods by using this gelling ability of the ice-nucleating material (Watanabe et al. 1989). It is hoped that continuing studies will yield further insight into the xanthan metabolism of *X. campestris* pv. *translucens* and an increased production of xanthan and ice-nucleating material.

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