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Biodegradation of xanthan by salt-tolerant aerobic microorganisms

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SUMMARY

Three salt-tolerant bacteria which degraded xanthan were isolated from various water and soil samples collected from New Jersey, Illinois, and Louisiana. The mixed culture, HD1, contained a *Bacillus* sp. which produced an inducible enzyme(s) having the highest extracellular xanthan-degrading activity found. Xanthan alone induced the observed xanthan-degrading activity. The optimum pH and temperature for cell growth were 5–7 and 30–35°C, respectively. The optimum temperature for activity of the xanthan-degrading enzyme(s) was 35–45°C, slightly higher than the optimum growth temperature. With a cell-free enzyme preparation, the optimum pH for the reduction of solution viscosity and for the release of reducing sugar groups were different (5 and 6, respectively), suggesting the involvement of more than one enzyme for these two reactions. Products of enzymatic xanthan degradation were identified as glucose, glucuronic acid, mannose, pyruvated mannose, acetylated mannose and unidentified oligo- and polysaccharides. The weight average molecular weight of xanthan samples shifted from $6.5 \cdot 10^6$ down to $6.0 \cdot 10^4$ during 18 h of incubation with the cell-free crude enzymes. The activity of the xanthan-degrading enzyme(s) was not influenced by the presence or absence of air or by the presence of $Na_2S_2O_4$ and low levels of biocides such as formaldehyde (25 ppm) and 2,2-dibromo-3-nitrilopropionamide (10 ppm). Formaldehyde at 50 ppm effectively inhibited growth of the xanthan degraders.

INTRODUCTION

Xanthan is an anionic extracellular polysaccharide produced by *Xanthomonas campestris*. The high viscosity of this polymer in solution is relatively insensitive to temperature, ionic strength, shear, and pH. For this reason, xanthan finds commercial use as a viscosity-enhancing agent for aqueous solutions. The primary structure of xanthan was established by Jansson et al. [7]. It consists of a main chain of β-1,4-linked D-glucose units, as in cellulose, but with trisaccharide side chains, composed of D-glucuronic acid and D-mannose residues, attached to alternate glucose residues. Mannosyl residues attached to the main chain bear acetyl substituents; many of the terminal, non-reducing, mannosyls are pyruvated.

Xanthan-degrading microbes are rare [9]. How-

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ever, in recent enhanced oil recovery field tests using xanthan biopolymer as the viscosity controlling agent, xanthan was found to be degraded by microbial (enzymic) activity [1]. Since then, the biodegradation of xanthan and the means for its prevention have become important research issues. Recently, Cadmus et al. [2] reported a salt-tolerant *Bacillus* that was able to degrade xanthan. They concluded that the xanthanase activity derived from a mixture of enzymes that attacked all of the side chain linkages but not the glucosidic backbone of xanthan. Sutherland [12] isolated a β-glucanohydrolase system from *Bacillus* strain R, that was capable of hydrolyzing β-1,3- and β-1,4-linked D-glucans with side-chains or other substituents.

We have been investigating microorganisms which might have been responsible for the degradation of xanthan, particularly the loss of solution viscosity under enhanced oil recovery (EOR) operation conditions. Both aerobes and anaerobes have been investigated. Results dealing with anaerobic xanthan degraders will be reported separately. In this paper, we describe the isolation of salt-tolerant, xanthan-degrading aerobic microorganisms from among two dozen soil and water samples collected from New Jersey, Illinois, and Louisiana. HD1, a mixed culture, was found to have the highest xanthan-degrading activity. It produced extracellular hydrolytic enzymes for degrading xanthan. Optimum conditions for cell growth and production of the enzymes as well as some properties of the hydrolytic enzymes are discussed.

MATERIALS AND METHODS

Microorganisms. Salt-tolerant xanthan-degrading microorganisms were isolated by an enrichment technique from soil and water samples collected from Loudon, IL, Linden and Summit, NJ and Chalmette, LA. Cultures were maintained on a 2% NaCl-containing nutrient agar which contained (per liter): 2.5 g xanthan, 0.5 g $(NH_4)_2SO_4$, 0.8 g yeast extract, 0.4 g peptone, 1.5 g KH_2PO_4, 0.7 g K_2HPO_4, 2.0 g $NaNO_3$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g KCl, 0.015 g $CaCl_2$, 1.0 mg $FeSO_4 \cdot 7H_2O$, 10 ml trace metals solution, and 15 g agar. The composition of the trace metals solution was (per liter): 0.5 mg $CuSO_4 \cdot 5H_20$, 1.0 mg H_3BO_3, 0.7 mg $MnSO_4 \cdot H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, and 1 mg MoO_3. For liquid culture, agar was omitted from the medium.

Xanthan-degrading cultures were grown in Erlenmeyer flasks (300 ml) containing 50 ml of medium, or Fernbach flasks (2.8 liter) containing 1 liter of medium. These flasks were inoculated with a 3-day-old inoculum (1% v/v) that had been grown in an Erlenmeyer flask inoculated with a loopful of culture. All flasks were shaken at 200 rpm on a rotary shaker at 30°C.

Analytical methods. Viscosities were measured at 25°C with a Contraves Low Shear Model 30 viscosimeter (Middlesex, U.K.) at a shear rate of 1.3 s^{-1}. In the cell-free systems, the release of reducing sugar groups was measured by incubating 2.5 ml of xanthan stock solution and a small amount of enzyme(s) at 25°C. Xanthan stock solution was prepared by dissolving 0.188% xanthan, 0.4 mM $MgSO_4$ and 0.03 mM $NMnSO_4$ in 0.05 M sodium acetate buffer pH 5.4. At selected time intervals, samples were taken for the reducing sugar assay. Reducing sugars, calculated as glucose, were determined by the method of Somogyi [11]. One unit of enzyme activity was expressed as 1 μmol reducing sugar group released (as glucose) per min.

Thin-layer chromatography (TLC) was performed for the identification of monosaccharides. The solvent system was pyridine/ethyl acetate/acetic acid/water (5:5:1:3, v/v). Sugars on TLC plates were detected by spraying the plates with *p*-anisidine/phthalic acid reagent [10]. Acetylated hexose was determined qualitatively by spraying Hestrin reagent [5] on the plate. For determination of pyruvic acid, the area corresponding to pyruvated mannose was collected from TLC plates and was eluted with distilled water. The aqueous solution was centrifuged to remove debris and was dried by vacuum evaporation. The presence of pyruvic acid was determined by the enzymatic method of Duckworth and Yaphe [4]. Fluorescent derivatives of xanthan were prepared by isocyanide coupling [3]

of fluoresceinamine to the carboxy groups of xanthan. A method developed by Holzwarth [6] was followed for the molecular weight distribution studies.

Chemicals. Xanthan was obtained from Kelco Company (Keltrol No. 16573, Clark, NJ). Chemicals were reagent grade, used without further purification. Thin-layer precoated cellulose plates were obtained from Pierce Chemical Co., Rockford, IL. Ultrafiltration membranes (PM 10) were purchased from Amicon Corp. (Danvers, MA). 2,2-Dibromo-3-nitrilopropionamide (DBNPA) was obtained from Dow Chemical Co. (Midland, MI 48640).

RESULTS AND DISCUSSION

Screening of xanthan-degrading microrganisms

Samples of soil (1 g) or water (1 ml) collected from two dozen places in New Jersey, Illinois and Louisiana were inoculated into liquid nutrient medium containing 1560 ppm xanthan and 4% NaCl (initial solution viscosity 240 cps) to screen for xanthan-degrading aerobic microorganisms. The reduction in viscosity of culture broth was used as an indication of xanthan-degrading activity. Only three (soils from Linden and Summit, NJ, and from Chalmette, LA) showed xanthan-degradation activity in the presence of 4% NaCl. The xanthan-degrading activities were enhanced by several transfers of the cultures. The soil sample from Linden, NJ showed the highest activity. The time required to reduce the viscosity of culture broth from 400 cps to 30 cps were 1 day, 15 days, and 8 days for soil samples obtained from Linden, Summit, and Chalmette, respectively.

Microscopic examination of the salt-tolerant cultures obtained from the Linden soil sample that degraded xanthan revealed a stable mixture of two species. These two species were separated from each other on a solid medium. They are a motile, slow-growing, spore-forming short rod, forming white colonies on agar plates; and a short fat non-motile rod, forming yellow mucoid colonies on agar plates. These two species were difficult to separate from each other due to the relative slow growth rate of one of them and the apparent need for proximity to each other for growth on agar. The motile Gram-positive, catalase-positive spore-forming short rod, a *Bacillus*, was identified as the one responsible for xanthan degradation (judging from viscosity reduction: 50% viscosity reduction in 1 week). However, the presence of the other organism stimulated the xanthan-degrading activity. The stable mixed culture was designated HD1. The biodegradation of xanthan with mixed cultures to obtain the best activity was also reported previously [2,12]. The explanation and the extent to which these other species stimulate xanthan-degrading activity were not discussed. The growth of culture HD1 was not inhibited by up to 5% salt (NaCl). However, cell growth was somewhat slower in salt concentration between 6 and 8%. At 10% salt concentration, the growth of culture HD1 was completely inhibited. The salt-tolerant xanthan-degrading cultures obtained from the other two locations were also mixed cultures containing short fat and/or thin rods, both motile and non-motile, and cocci.

Time course of xanthan degradation

Fig. 1 shows the time course of xanthan degradation by culture HD1. The cell growth reached stationary phase in about 3 days. Viscosity of the culture broth decreased from 400 cps to about 30 cps within 24 h. Xanthan-degrading activity, as measured by the reducing sugar assay, continued to increase during the first 4 days of incubation.

Effect of temperature and pH on growth of HD1

The effects of temperature and pH on growth and on xanthan-degrading activity of HD1 were studied at temperatures from 4 to 60°C and pH from 3.0 to 9.0. Initial pH of the culture medium was adjusted with either NaOH or HCl. Two criteria were followed: cell growth, and the changes in viscosity of culture broth, which represented xanthan-degrading activity. Assays were conducted after 2 days of incubation. The optimum temperature for cell growth was around 30–35°C. The viscosity of the culture broth was almost completely lost when the culture was incubated for 2 days at

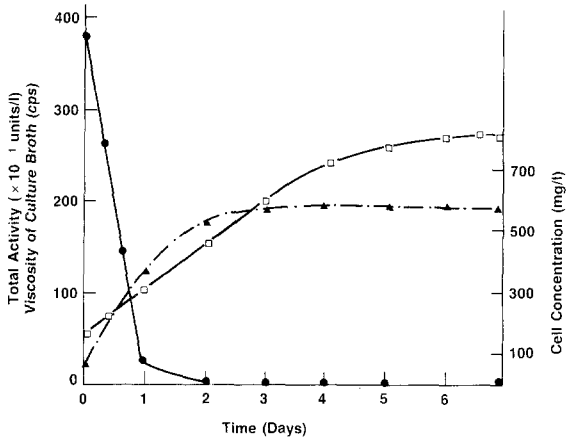

Fig. 1. Time course of degradation of xanthan by culture HD1. Culture was inoculated into nutrient medium which contained 2% NaCl and xanthan at 30°C. At each time interval, samples were taken and assayed for cell yield, viscosity, and activity. Cell yield (▲) was measured at 660 nm. Viscosity (●) was measured with a Contraves Low Shear viscosimeter at 25°C. Activity (□) was calculated by measuring reducing sugar groups released per min using fresh xanthan stock solution.

a temperature between 25 and 40°C. Culture HD1 could not grow at a temperature higher than 50°C or below 10°C, and no viscosity reduction was observed at these temperatures. Optimum pH for growth of HD1 at 30°C was found to be between 5 and 7. At a pH below 4 or above 8, there was very little growth of culture HD1, and the viscosity reduction was not significant. Based on solution viscosity measurements, pH 6 was the optimum for xanthan-degrading activity during cell growth.

Xanthan degradation by culture supernatant

Experiments were conducted to determine whether xanthan-degrading enzyme(s) are located extracellularly. 3-day-old culture broth of culture HD1 grown on xanthan in the presence of 2% salt was centrifuged to remove cells. The supernatant was concentrated and washed three times with 0.05 M phosphate buffer (pH 7.0) in an Amicon ultrafiltration unit with a PM 10 membrane. The cells were washed twice with a 10 times volume of 0.05 M phosphate buffer (pH 7.0), centrifuged, and were resuspended in a small amount of the same buffer. A portion of this washed cell suspension was disintegrated by two passages through a French pressure cell (American Instruments Co., Silver Springs, MD) at 20 000 lb/in^2. The disintegrated cell suspension and the washed cell suspension were also tested for their xanthan-degrading ability; neither of these two samples could degrade xanthan. Only the concentrated culture supernatant showed xanthan-degrading activity (Fig. 2). The sharpest reduction in solution viscosity was found during the initial 5 min of incubation. After that, the decrease in solution viscosity became slower. However, the production of reducing sugar groups showed increases during the entire incubation period, indicating further cleavage of fragmented xanthan molecules.

Effect of growth substrate on production of xanthan-degrading enzyme(s)

In order to determine whether the xanthan-degrading enzyme(s) is constitutive or inducible, culture HD1 was grown on nutrient medium containing different carbon sources, i.e, glucose, mannose, glucose + mannose, xanthan, cellulose, carboxymethyl cellulose, DEAE-cellulose, succinate, pyruvate, starch, cellulose acetate, or without addition. Cell growth was observed in all of the flasks (*A* at 660 nm ranging from 0.7 to 2.17, grown for 2 days at 30°C). However, only the xanthan-containing flask produced xanthan-degrading enzyme activity

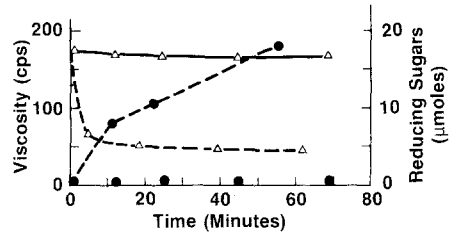

Fig. 2. Degradation of xanthan by a cell-free system of culture HD1. 3-day-old culture broth of HD1 was centrifuged and the cell-free supernatant was concentrated and washed three times with 0.05 M sodium acetate buffer (pH 5.4) in an Amicon ultrafiltration unit with PM 10 membrane. A 1.24 mg protein sample of the cell-free concentrate was incubated with xanthan in 14 ml 0.015 M sodium acetate buffer (pH 5.4) at 40°C. At each time interval, samples were taken for reducing sugar (●--●) and viscosity (△--△) assays. Solid line control (with washed cells suspension, with disintegrated cells suspension, or without addition).

in the cell-free culture broth. Therefore, it is clear that the xanthan-degrading enzyme(s) was induced only in the presence of xanthan.

Effect of temperature on activity of xanthan-degrading enzyme(s)

The effect of temperature on the activity of xanthan-degrading enzyme(s) was studied at temperatures ranging from 4 to 55°C by measuring the changes in solution viscosity. A crude enzyme fraction obtained from cell-free culture broth of HD1 grown at 30°C for 4 days was used for this study. The viscosity of the reaction mixture was recorded at both 5 min and 5 h of incubation at the indicated temperature. Results are shown in Fig. 3. It is clear that the optimum temperature for the activity of xanthan-degrading enzyme(s) is between 35 and 45°C. The enzyme(s) degraded xanthan slowly at 4°C and was not active at a temperature higher than 50°C.

On storage, the crude enzymes were stable at 4°C for at least 6 months.

Effect on pH on the activity of xanthan-degrading enzyme(s)

Xanthan was dissolved in different buffer solutions (0.05 M) at various pH values: sodium acetate buffer for pH 3.5–6.0; sodium phosphate buffer for pH 6.0–7.5; and Tris (hydroxymethyl)aminomethane buffer for pH 8.0–9.0. The same enzyme fraction used for temperature studies was used. Enzyme activity was followed by both the decrease in solution viscosity and the production of reducing sugar groups. Results are shown in Fig. 4. In the cell-free system, the optimum pH for reducing solution viscosity and for producing reducing sugar groups appears to be about 5.0 and 6.0, respectively. This suggests that these two reactions are carried out by different enzymes. In the absence of added enzyme preparation, there was no change in viscosity and no release of reducing sugar at the pH values tested.

Products of xanthan degradation

The degradation of xanthan by concentrated cell-free supernatant was conducted at 40°C, pH 6 for 18 h. The reaction mixture was filtered through an ultrafiltration unit using a PM 10 membrane to separate a low molecular weight fraction (LMWF, less than 10 000 molecular weight) and a high molecular weight fraction (HMWF). The LMWF was

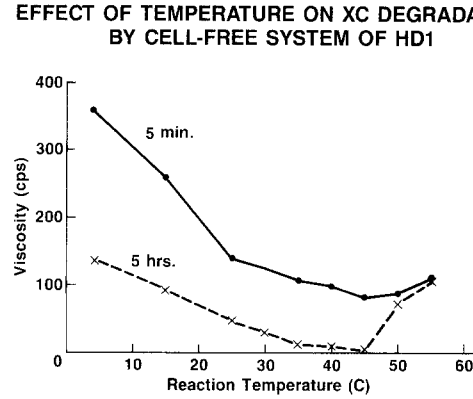

Fig. 3. Effect of temperature on the activity of xanthan-degrading enzyme(s). Concentrated crude enzyme fraction obtained from cell-free culture broth of HD1 grown at 30°C for 3 days was used. A 100 μl sample of crude enzyme fraction was added into 6 ml of reaction mixture which had been preincubated at the set temperature for 15 min. The initial viscosity of the reaction mixture was 410 cps. The reaction was conducted at the temperature indicated for both 5 min (solid line) and 5 h (broken line). The viscosity of the reaction mixture was recorded.

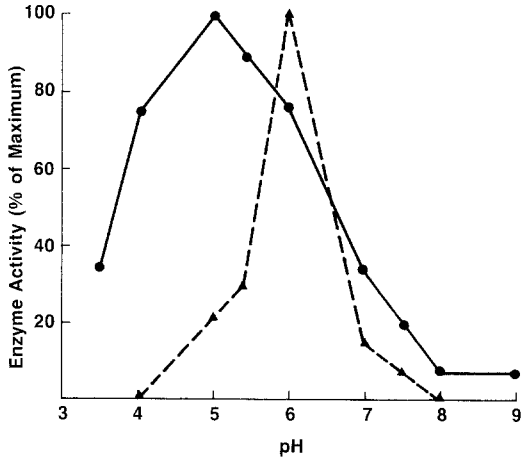

Fig. 4. Effect of pH on the activity of xanthan-degrading enzyme(s). The same crude enzyme fraction as for Fig. 3 was used. Various buffer solutions (0.05 M) are described in the text. Enzyme activities are expressed as % of maximum for viscosity reduction (solid line) and for release of reducing sugar groups (broken line).

Table 1

Distribution of total carbohydrate and reducing sugar (r. sugar) in different molecular weight fractions produced from xanthan by concentrated cell-free culture supernatant of HD1

Time (h)	LMWF		HMWF	
	r. sugar (μg/5 ml)	total CH_2O (%)	r. sugar (μg/5 ml)	total CH_2O (%)
0	0.330	30	0.090	70
18	106.13	87	0.124	13

concentrated with a rotary evaporator and was de-salted with a Bio-Gel P-2 column. The de-salted LMWF was again concentrated and analyzed on thin-layer chromatography plates for monosaccharides. All of the components of xanthan, i.e., glucuronic acid, pyruvated mannose, glucose, mannose and acetylated mannose were detected in the LMWF. This pattern was different from that exhibited by xanthanase [2] which attacked all of the side-chain linkages without breaking the glucosidic backbone of xanthan.

The distribution of total carbohydrate and reducing sugar groups produced in LMWF and HMWF were studied. Results are listed in Table 1. After 18 h incubation in the presence of the crude enzyme(s), almost all of the reducing sugar groups and total carbohydrate were found in the LMWF. The remaining carbohydrate in the HMWF was only 13%.

The HMWF was assayed for its molecular weight distribution by using the fluorescent derivative method [6]. The weight average molecular weight of xanthan decreased from $6.5 \cdot 10^6$ to $6.0 \cdot 10^4$ in 18 h of incubation in the presence of the cell-free crude enzyme preparation (Fig. 5). This is the first time that the molecular weight shift of xanthan samples by the action of enzyme(s) has been reported.

Effect of chemicals on the growth of HD1 and on the activity of xanthan-degrading enzyme(s)

Chemicals and biocides, including those used in enhanced oil recovery operatins (EOR), were studied for their effect on the growth of culture HD1 and on the activity of xanthan-degrading enzyme(s). Both aerobic and anaerobic conditions were tested. In order to simulate the conditions of an EOR field operation, xanthan was produced in the laboratory by growing *Xanthomonas compestris* [8]. The centrifuged xanthan culture broth was autoclaved and was diluted with brine to a viscosity about 300 cps. A cell suspension of culture HD1 obtained from colonies grown on agar plates was used as the inocolum. Growth of HD1 was followed by monitoring decreases of viscosity of xanthan and by microscopic observation. Results are listed in Table 2. The presence of 10 ppm of a biocide, DBNPA, was not effective in preventing the activity of HD1. The solution viscosity was lost within 5 days of incubation. 25 ppm of formaldehyde was somewhat more effective; it took 9 days for HD1 to degrade the xanthan solution. In the presence of 50 ppm formaldehyde, no changes in solution viscosity or cell growth were observed during 30 days of incubation. A combination of sodium dithionite, formaldehyde, and DBNPA at concentrations sim-

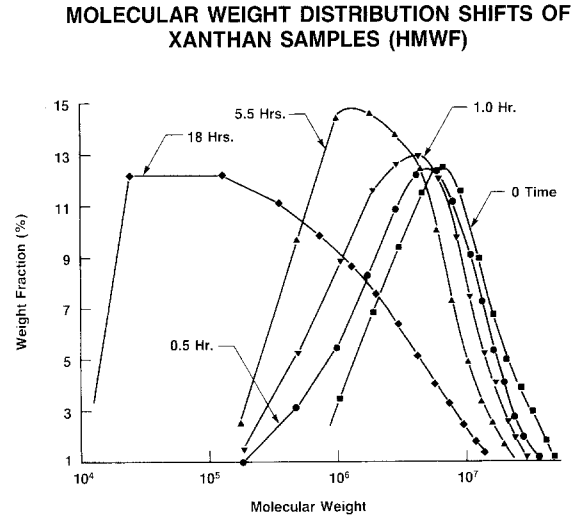

Fig. 5. Molecular weight distribution shifts of xanthan samples (HMWF). The same crude enzyme fraction (10 mg protein) as for Fig. 3 was incubated with 144 mg xanthan in 80 ml 0.05 M sodium acetate buffer (pH 6.0) at 40°C. Samples were taken at indicated times and were boiled for 5 min to deactivate the enzymes. The HMWF of the reaction products was subjected to molecular weight distribution studies by using the fluorescent derivative method [6].

Table 2

Effect of chemicals and biocide on growth of culture HD1

Compounds	Concentration (PPM)	Viscosity (cps after 3 days)
Control	—	0.9
$Na_2S_2O_4$	75	1.0
HCHO	25	1.5 (9 days)
	50	300
DBNPA	10	1.5 (5 days)
$Na_2S_2O_4$ + HCHO + DBNPA	(75 + 25 + 10)	1.5 (9 days)
Triton X-100	500	90
Polypropylene glycol	500	120

ilar to those used in an EOR operation slowed cell growth and solution viscosity loss over 9 days. Under anaerobic conditions, there was no growth of HD1 and no loss in solution viscosity either in the presence or absence of these chemicals.

The above experiments were repeated using cell-free enzyme preparations obtained from culture broth of HD1 grown on xanthan for 3 days. A concentrated crude enzyme fraction was incubated with the individual chemicals or a combination of these chemicals either in the presence or absence of air. None of these chemicals at the concentrations indicated (same as Table 2), tested individually or in combination, in either anaerobic or aerobic conditions, inhibited the activity of the xanthan-degrading enzyme(s). The solution viscosities in all cases (except those control tests which contained no enzyme(s)) were lowered markedly within the first hours of incubation. These data indicate that the presence of a small quantity of this enzyme(s) could cause problems in an EOR operation that uses xanthan as a solution viscosity-enhancing agent.

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