Journal of Industrial Microbiology, 11 (1993) 187–192

Published by Elsevier

SIM 00471

Clarification of microbial polysaccharides with enzymes secreted from *Lysobacter* species

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(Received 17 July 1992; accepted 14 December 1992)

Key words: Clarification; Lysis; Exopolysaccharide; Xanthan; *Lysobacter*

SUMMARY

Different species of Gram-negative *Lysobacter* grown in liquid culture secrete a variety of hydrolytic enzymes. The natural enzyme mixtures lyse several Gram-negative polysaccharide-producing bacteria of commercial importance, especially *Xanthomonas campestris*. We describe optimal methods for producing and using the lytic enzymes to clarify microbial polysaccharides and show that the proteases are responsible for lysing *X. campestris*.

INTRODUCTION

Many bacteria secrete useful polysaccharides which are used in cosmetics and food products to control viscosity. For aesthetic reasons, formulations often require transparent solutions. There are also utilitarian reasons to clarify microbially-produced polysaccharides. For example, in tertiary oil recovery viscous polymers push oil out of rock formations unless contaminating cells and debris plug the pores in the rock. It is mechanically difficult to separate viscous polysaccharides from cells and debris by centrifugation or filtration. Clarification of fermentation broths with alkali leads to undesirable deacetylation or degradation of the polysaccharides [8,9]. By contrast, specific enzymatic treatment offers an effective and mild way to lyse bacteria and obtain transparent polysaccharide solutions [2,6].

Gram-negative bacteria are usually not considered as a source of enzymes for the lysis of other Gram-negative microorganisms, since the enzyme-secreting bacteria might themselves be damaged by the lytic enzymes that they secrete. As a result, earlier efforts to clarify cultures of polysaccharide-producing Gram-negative bacteria such as *X. campestris* focused on the easily obtained alkaline, neutral and acidic proteases of Gram-positive bacteria, such as *Bacillus subtilis*. Based on the weight ratio of enzyme-to-polymer the alkaline proteases seem more active according to published reports, but the three types of enzymes were not tested under identical conditions and the fraction of the enzyme powder that was the active ingredient was unclear. In one study, an alkaline protease, Alcalase™ (Novo Industries), reduced the turbidity of a 1% solution of Kelzan MF™ (xanthan from Kelco Company) by about 30% in 1 h and by about 50% in 24 h [2]. The treatment at room temperature and neutral pH included 200 to 500 ppm of Alcalase, but the actual content of alkaline protease was not given. The turbidity could be reduced by 95% by using more enzyme, a different source of protease, longer treatment time or alkaline conditions. Similarly, 1700 ppm of Neutrase™ (Novo Industries), a neutral protease from *Bacillus*, reduced the absorbance (540 nm) of a fermentation broth that contained 2.7% (w/v) xanthan by about 8-fold within 2 h at 47 °C [6]. Although proteases alone can lyse *X. campestris*, an additional small increment in clarification of culture broths is achieved by adding egg white lysozyme (unpublished observation).

Here we describe the use of a naturally occurring complex of enzymes for clarifying polysaccharides, especially xanthan from *X. campestris*. The enzymes are secreted into the culture medium during growth of certain Gram-negative *Lysobacter* species [1,4]. Species of *Lysobacter* are known to lyse a variety of bacteria, fungi, algae and nematodes [1]. Among the secreted enzymes are three proteases [3,7,14], two phosphatases [13], nucleases [11,12], a lipolytic esterase [1], and a β-1,4-glucanase and chitosanase [5]. Of these activities, the proteases alone can lyse bacteria.

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MATERIALS AND METHODS

Bacterial strains and growth media

The origins for some bacterial strains are included in Table 2. *X. campestris* strain X59 (ATCC55298) is a spontaneous rifampicin-resistant derivative [10] of B1459S-4L-II from the Northern Regional Research Center (Peoria, IL), and strain X59m8 is a nonproducer of xanthan gum derived from X59 [10].

Variants in colony morphology arise spontaneously in *Lysobacter* cultures [1]. We observed colonial variants on nutrient broth plates (Difco) in samples received from the American Type Culture Collection (ATCC) and maintained them separately as follows: B23 (ATCC 55319), an opaque cream-colored colonial variant of *L. enzymogenes* ATCC 27796; B48, a yellowish variant of *L. gummosus* ATCC 29489; B41, a light brown variant of *L. gummosus* ATCC 29489; B42, a sticky-colony variant of *L. gummosus* ATCC 29489; B49, *L. brunescens* ATCC 29482; B50, *L. brunescens* ATCC 29483; B51, *L. brunescens* ATCC 29484; B52, a yellowish variant of *L. enzymogenes* subsp. *cookii* Christensen ATCC 29488; and B53, a light brown variant of *L. enzymogenes* subsp. *cookii* ATCC 29488.

LB medium contained 10 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco) and 10 g NaCl per liter. M9 minimal salts contained 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl per liter, with 10 ml 0.01 M $CaCl_2$ and 1 ml 1 M $MgSO_4 \cdot 7H2O$ added after sterilization by autoclaving. YM medium (pH 6.2 ± 0.2) contained 3 g Bacto yeast extract, 3 g Bacto malt extract (Difco), 5 g Bacto peptone (Difco) and 10 g Bacto dextrose (Difco) per liter. YPGP (pH 6.2 ± 0.2) contained 3 g Bacto yeast extract, 2 g Bacto peptone, 3 g glucose and 2 g KH_2PO_4 per liter. Agar (Difco) was at 1.5% (w/v).

Substrate cells and clarification assay

X. campestris strain X59m8 was grown at 30 °C with shaking to an absorbance at 600 nm (A600) exceeding 1 in YM medium lacking glucose. Since no xanthan gum is present the substrate cells mix efficiently with the lytic enzymes. The culture was heated at 70 °C for 90 min to kill the cells and then stored at 4 °C until use. The pH of the heat-treated culture was 8 to 9, and the starting A600 was measured before adding enzyme. Substrate (4 ml) and enzyme (1:10 to 1:100 volume) were mixed, incubated at specific temperatures without shaking, and then sampled for change in A600. Microscopically visible cell lysis corresponded with the decrease in A600. Transparency was measured at 600 nm using a 1-cm light path and with water as 100% transparent. Alkaline protease SP-4 (Nagase) was a powder containing 4% (w/w) enzyme, 20% $CaCO_3$, and 76% absorbent. The powder was suspended in deionized water at 10000 ppm, insoluble material was removed by centrifugation, and the supernatant was stored at 4 °C. The enzyme was soluble in the 10000 ppm solution, and was used at 500 ppm of powder, equal to 20 ppm of enzyme. Keltrol (xanthan) and K1A96 (welan) were from Kelco Company. Bovine serum albumin (fraction V), casein (technical grade, 90% protein), and Tween 20 were from Sigma.

TABLE 1

Absorbance (600 nm) for liquid culture of *X. campestris* after treating with *Lysobacter* cell-free supernatant

Source of supernatant		1:10 dilution[a]				1:40 dilution		
		0.5 h	1 h	2 h	3.5 h	1 h	2 h	3.5 h
None		0.91	0.91	0.91	0.91	0.96	0.96	0.96
L. brunescens	B49	0.90	0.89	0.90	0.89	0.96	0.95	0.94
	B50	0.90	0.89	0.89	0.88	0.96	0.96	0.95
	B51	0.91	0.90	0.90	0.91	0.96	0.96	0.95
L. enzymogenes	B23	0.23	0.16	0.13	0.13	0.43	0.22	0.16
	B52	0.61	0.48	0.22	0.14	0.74	0.59	0.41
	B53	0.89	0.89	0.88	0.88	0.96	0.94	0.95
L. gummosus	B48	0.79	0.73	0.64	0.52	0.89	0.85	0.76
	B41	0.91	0.91	0.91	0.90	0.95	0.95	0.95
	B42	0.90	0.87	0.84	0.82	0.96	0.93	0.93

[a] One volume of *Lysobacter* supernatant plus 9 volumes of heat-treated *X. campestris* X59m8 cells in growth medium.

RESULTS AND DISCUSSION

Screening of Lysobacter species for lytic activity

We tested several isolates of *Lysobacter* from three species (*enzymogenes*, *gummosus* and *brunescens*) for their ability to clarify liquid cultures of *X. campestris*. *Lysobacter* cultures were grown for 42 h at 30 °C with shaking in YPGP medium and then centrifuged at 10 000 × *g* for 3 min to obtain cell-free supernatants containing the lytic enzymes. Samples of 50 or 200 μl of supernatant were added to 2 ml of heat-treated *X. campestris* X59m8 cells, and A600 was measured as a function of the time of incubation at 45 °C. As shown in Table 1, the most active supernatant was from strain *L. enzymogenes* (B23), and the second most active was from *L. enzymogenes* subsp. *cookii* (B52).

We also surveyed *Lysobacter* cultures for their ability to clarify various species of bacteria embedded in agar, including many that secrete polysaccharides. The target bacteria were grown to saturation in LB medium, killed by heating at 80 °C for 60 min, and then incorporated into agar plates containing M9 minimal salts and 0.1% glucose. The *Lysobacter* strains were grown to saturation at 30 °C in M9 minimal salts supplemented with 0.5% yeast extract and trace minerals. A drop of each culture was spotted onto the bacteria-containing plates. The digestion of cellular material caused a visible zone of clearing around each spot of *Lysobacter*. We calculated the amount of clearing as the ratio of the diameter of the zone of clearing to the diameter of the zone of growth of *Lysobacter* cells. As seen previously, *Agrobacterium radiobacter* was refractory to lysis by these enzymes [4]. However, contrary to the earlier study, a xanthan-producing species of *Xanthomonas* was readily lysed. The ratios given in Table 2 reveal differences in total clarification activity. Although the liquid assay is more discriminating, at least for the *X. campestris* target cells, the plate assay requires minimal handling and the clearing zones are visible within 16 h.

Preparation of the Lysobacter cell-free supernatant

Ensign and Wolfe [3] demonstrated the importance of yeast extract for preparing an *Arthrobacter*-specific extracellular lytic activity from *Lysobacter*. First we confirmed their results, and then we surveyed a variety of growth substrates for stimulating the secretion of the lytic enzymes. We could not stimulate the desired activity by adding whole proteins such as casein or killed *X. campestris* cells. The most active cell-free supernatants were from media derived from YM, which includes yeast extract, peptone, malt extract and glucose. However, we found that malt extract was not essential and that inclusion of potassium phosphate stimulated the growth of *Lysobacter* and moderated the final pH of the culture to about 8. YPGP (see Materials and Methods) was the most productive medium for both shake-flask and jar fermentations. An initial pH of 6 was better than either 7 or 8. With

TABLE 2

Clarification of exopolysaccharide-producing bacteria in agar

Substrate bacteria and ATCC number		Ratio of clear zone to growth zone[a]			
		L. enzymogenes		*L. gummosus*	*L. brunescens*
		B23	B52	B48	B49, B50, B51
X. campestris	55298	1.6	1.6	1.6	1.0
P. aeruginosa	27853	1.3	1.6	1.2	1.0
A. viscosus	19584	1.6	1.8	1.2	1.0
E. coli K12	53323	1.5	1.9	1.3	1.0
Pseudomonas sp.	31554	2.0	2.3	1.3	1.0
P. elodea	31461	1.3	1.8	1.3	1.0
Alcaligenes sp.	31555	1.7	2.5	1.3	1.0
K. pneumoniae	12657	1.4	1.8	1.1	1.0
B. indica	21423	2.0	2.5	1.6	1.0
A. radiobacter	31643	1.0	1.0	1.0	1.0
Alcaligenes sp.	31853	1.4	2.0	1.3	1.0
Alcaligenes sp.	31961	1.7	2.0	1.5	1.0
B. subtilis W168		1.6	1.2	1.3	1.0

[a] A ratio of 1.0 indicates no visible clearing zone. One standard deviation in the ratio from replicate measurements was less than ± 0.2.

an inoculum size of only 0.2% the peak lytic activity accumulated within 32 h. However, a 5% inoculum produced the lytic enzymes within 24 h. Organic antifoam 204 (Sigma) at less than 0.033% (v/v) can be included without a detrimental effect on the lytic activity. The accumulation of lytic activity closely coincided with the accumulation of cell mass. The A600 in a jar fermenter peaked at about 4–5, and then during stationary phase it decreased by about 50%. However, the lytic activity remained stable in the broth for at least 24 h, and remained stable at 4 °C for at least 5 months.

Temperature and pH stability of the lytic preparation and optimal conditions for use

When the lytic enzymes were incubated for 3 h at various temperatures before adding substrate cells, the lytic activity was insensitive to temperatures at or below 37 °C, but was inactivated at 55 °C or higher. Treatment at 65 °C for 3 h eliminated all of the activity. There was no effect on enzyme stability of pH in the range of 7 to 9 at either room temperature or 45 °C. However, the combination of pH 10 and 45 °C inactivated the lytic enzymes.

Panels A and B in Fig. 1 show the optimum pH and temperature for using the *Lysobacter* enzymes to clarify *X. campestris* cultures. The optimum pH was between 8 and 9, as seen for the 15-min reactions. The optimum temperature was 55 to 60 °C, also indicated by the 15-min reactions. The optimum temperature for the 15-min reactions was about 15 °C higher than the optimum indicated by the 180-min reactions. The longer times at high temperature cause enzyme inactivation. The extent of clarification is probably more important for processing large quantities of fermentation broth.

Inhibition of cell lysis by soluble proteins

Which hydrolytic activities are responsible for lysis of the *X. campestris* cells? Of the hydrolytic activities secreted by *Lysobacter* only the proteases have been shown to cause lysis by themselves [3,5,14]. The β-1,4-glucanase/chitosanase, phosphatases, and nucleases are unlikely to cause lysis of bacterial cells. We confirmed that the proteases were mainly responsible for lysing *X. campestris* by adding bovine serum albumin (BSA), a substrate for each of the three proteases, to the lytic reactions and observing a reduced rate (inhibition) of lysis (Fig. 2). Casein was also an effective inhibitor of lysis, but there was no inhibition by added Tween 20. Addition of Tween 20 alone to 10, 20 and 50 mg/ml caused reductions in A600 of 10, 17 and 28%, respectively.

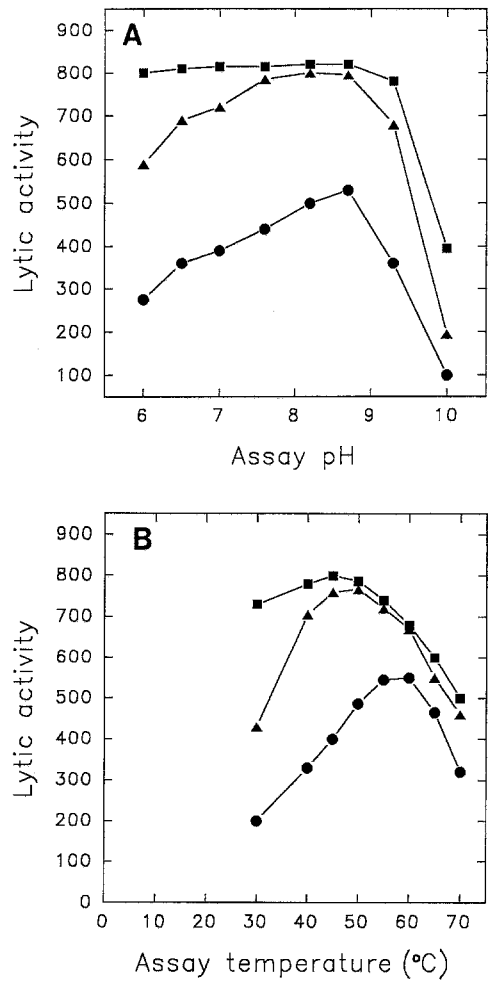

Fig. 1. Optimization of conditions for treating cultures of *X. campestris* with *Lysobacter* enzymes. Panels A (pH) and B (temperature) show lytic activities calculated as 1000 × (initial A600 − final A600). The initial A600 was 0.865. Symbols: ■, 180 min assay, ▲, 60 min assay; ●; 15 min assay.

Clarification of commercial Keltrol (xanthan) and K1A96 (welan)

The *Lysobacter* enzymes are also useful for clarifying aqueous suspensions of commercial polysaccharides such as Keltrol from *X. campestris* (Kelco Company) and K1A96 from *Alcaligenes* sp. (Kelco). Both products contain residual amounts of isopropyl alcohol which is used to concentrate the polysaccharides from the fermentation broths. Keltrol powder was dissolved in deionized water at 0.5% (w/v) and K1A96 at 1%, and then divided into samples of 20 ml that received either 0.5 ml of *Lysobacter* enzymes or water. All the samples were incubated at 55 °C without constant mixing, but were inverted several times before removing samples for measuring transparency. The

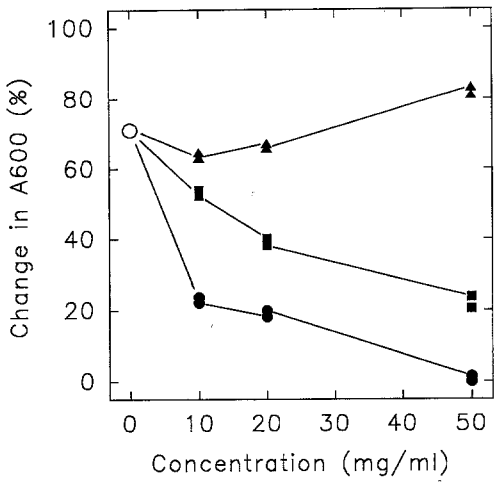

Fig. 2. Inhibition of lysis of *X. campestris* by added protein. Substrate X59m8 cells (1 ml) were mixed with different amounts of Tween 20 (▲), bovine serum albumin (■) or casein (●), and then a rate-limiting amount (12.5 μl) of lytic enzymes from strain B23 was added. The percent change in A600 was calculated from the A600 measured before adding the lytic enzymes and after 1 h of enzyme digestion at 45 °C. The open circle (○) indicates the 71% change in A600 observed for the control digestion. The initial absolute A600 values were different and depended on the amount of each added substance.

results are given in Fig. 3. An additional 0.5 ml of enzyme was added to both samples at 3.25 h. For both polysaccharides the enzyme treatment caused about a 3–4-four fold increase in transparency, reaching a maximum clarity within 3 h. Addition of more enzyme at this time did not further increase the transparency.

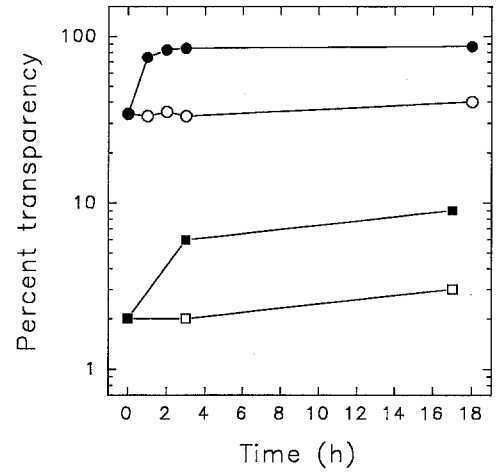

Fig. 3. Clarification of commercial polysaccharides by *Lysobacter* enzymes. Symbols: enzyme-treated (●) and untreated (○) Keltrol (xanthan); enzyme-treated (■) and untreated (□) K1A96 (welan).

Enzymes from Lysobacter do not degrade xanthan

We tested enzyme-treated and untreated xanthan samples for degradation by measuring viscosity. A 2% solution of Keltrol was incubated with or without *Lysobacter* enzymes at about 23 °C for 67 h with constant mixing by inversion on a rotating wheel. The enzymes caused the transparency to increase about 7-fold from 3 to 20. After dilution to 0.5% the transparencies were 40 and 82%, respectively. The polymers were diluted to 1% with deionized water and viscosities were measured at various shear rates with a Brookfield LVT-DVII rotating spindle viscometer. For shear rates of 12.54, 2.51 and 0.63 s^{-1} the enzyme-treated xanthan had viscosities of 1040, 3900 and 13600 cp, respectively, while the untreated control had 972, 3540 and 12500 cp. Therefore, the xanthan was not degraded by the *Lysobacter* enzymes. In addition we demonstrated that a 3–4-h treatment of a mixture of xanthan and *X. campestris* cells at 50 °C with the *Lysobacter* enzymes did not release hydrolytic activities from the lysing *X. campestris* cells (results not shown). However, there was a slight viscosity decrease for xanthan caused by longer enzyme treatments at higher temperatures (55 °C).

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