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Nutritional Studies on Xanthan Production by *Xanthomonas campestris* NRRL B1459

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The nutritional requirements of *Xanthomonas campestris* NRRL B1459 for optimal xanthan production were studied in a chemically defined medium. Of the carbon sources tested, a 4% sucrose or glucose medium yielded the highest xanthan titers. The further addition of certain organic acids, such as succinate, pyruvate, and α-ketoglutarate, stimulated xanthan production; excess concentrations of these organic acids inhibited xanthan formation. Certain amino acids (e.g., glutamate) and nitrate salts were superior to ammonium salts for xanthan production. Concentrations of these nitrogen sources higher than the optimal levels inhibited xanthan production while stimulating growth. Xanthan production was also sensitive to high concentrations of inorganic phosphate. High xanthan potencies, up to 30 g/kg of broth, were achieved in these shake-flask studies, in which completely defined media were used.

Although investigations of a biochemical engineering nature on xanthan production by *Xanthomonas campestris* NRRL B1459 have been reported (9, 11, 12), little information has appeared on the nutritional requirements of the organism for optimal heteropolysaccharide formation. Leach et al. (7) and Lilly et al. (8) studied the production of polysaccharide from various carbon sources by *Xanthomonas* species. After testing carbon sources such as maltose, glucose, sucrose, soluble starch, corn starch, and enzyme-hydrolyzed corn starch, all at 1% concentrations, they devised a medium for production of polysaccharide which consisted of glucose (or starch or sucrose), enzyme-hydrolyzed casein, and minerals. In this medium, with adequate aeration and agitation, *X. phaseoli*, *X. campestris*, *X. malvacearum*, and *X. carotae* produced 6 g of polysaccharide per liter. This paper deals with the nutritional requirements of *X. campestris* NRRL B1459 for a much higher level of xanthan formation.

MATERIALS AND METHODS

Organism. *X. campestris* NRRL B1459 was obtained from the Northern Regional Research Laboratory of the U.S. Department of Agriculture, Peoria, Ill.

Media. The initial chemically defined medium was suggested to us by G. Pace. It contained the following components (in grams per liter): glucose, 20; KH_2PO_4, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 2.0; citric acid, 2.0; H_3BO_3, 0.006; ZnO, 0.006; $FeCl_3 \cdot 6H_2O$, 0.0024; $CaCO_3$, 0.02; and HCl, 0.13 ml/liter. The pH was adjusted to 7.0 with sodium hydroxide before sterilization. Sugars were autoclaved separately. The strain was maintained in YM medium (Difco Laboratories, Detroit, Mich.).

Culture conditions. Cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium at 25°C for 96 h. The flasks were shaken at 250 rpm on a New Brunswick rotary shaker. Seed cultures were grown for 4 days in the medium defined above. After dilution with 3 volumes of sterile distilled water, the cultures were centrifuged (7,000 × *g* for 30 min). The cells were washed once with 3 volumes of sterile distilled water to remove residual polysaccharide. After centrifugation, cells were suspended in 0.4 volume of sterile distilled water and used for inoculation.

Analytical methods. Xanthan was determined by precipitation with ethanol after removing cells by the method of Jeanes et al. (6). Viscosities were measured with a Brookfield viscometer type LVT, spindle 4, 30 rpm, at 25°C. The polysaccharide was removed by centrifugation (7,000 × *g*, 20 min), and growth was measured gravimetrically and turbidimetrically (Klett-Summerson colorimeter with a red filter).

RESULTS

Xanthan production in glucose-containing medium. Glucose is the usual carbon source for the formation of xanthan. With our initial chemically defined medium containing 2% glucose, we studied the effect of additional carbon sources used at 1% on xanthan production. Among the carbon sources tested, sucrose, fructose, and xylose were the most stimulating, and all were superior to an additional 1% glucose. The other saccharides tested were starch, dex-

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trin, sorbose, galactose, rhamnose, mannose, maltose, trehalose, cellobiose, lactose, ribose, and arabinose. Dosage response tests revealed that the best xanthan production (measured either gravimetrically or viscometrically) occurred when 1% sucrose, 0.5% fructose, or 0.5% xylose was added to the 2% glucose medium (Fig. 1). Although the addition of excess sucrose had no detrimental effect on growth or xanthan production, the addition of excess fructose or xylose was inhibitory to both production and growth. In addition to saccharides, we tested polyols (glycerol, D-mannitol, *meso*-inositol, *meso*-erythritol, and sorbitol) and sodium salts of organic acids (lactate, succinate, oxalate, glucuronate, pyruvate, tartrate, and α-ketoglutarate). Polyols did not stimulate polysaccharide production; of the organic acids, only succinate, pyruvate, and α-ketoglutarate were stimulatory. Different concentrations of these organic acids were added to the medium containing 2% glucose (Fig. 2). The greatest amounts of xanthan were produced with concentrations of 0.3% pyruvate, 0.6% succinate, and 0.4% α-ketoglutarate; concentrations exceeding these amounts were inhibitory. For example, xanthan formation was completely checked when 2% pyruvate was added, while growth was only slightly suppressed. These three organic acids also supported growth when used as the major carbon source (i.e., in the absence of sugar), but no xanthan was produced under these conditions.

Xanthan production in sucrose-containing medium. All of the above carbon sources were tested as glucose replacements in the chemically defined medium. We found sucrose to be the best substrate for xanthan production, although it was only slightly better than glucose. Figure 3 shows the effect of different concentrations of sucrose and glucose; the highest titers of the polysaccharide were produced at a 4% concentration of each. However, sucrose showed a greater specific xanthan production (7.5 mg/mg of dry cell weight) than glucose (5.0 mg/mg of dry cell weight), so we chose sucrose as the carbon source for our remaining studies.

Figure 4 shows the marked stimulatory effect of pyruvate, succinate, and α-ketoglutarate on xanthan formation in the 4% sucrose medium. The highest potencies of xanthan were observed with concentrations of 0.3 to 1% pyruvate, 0.6% α-ketoglutarate, and 0.5 to 1% succinate. Higher

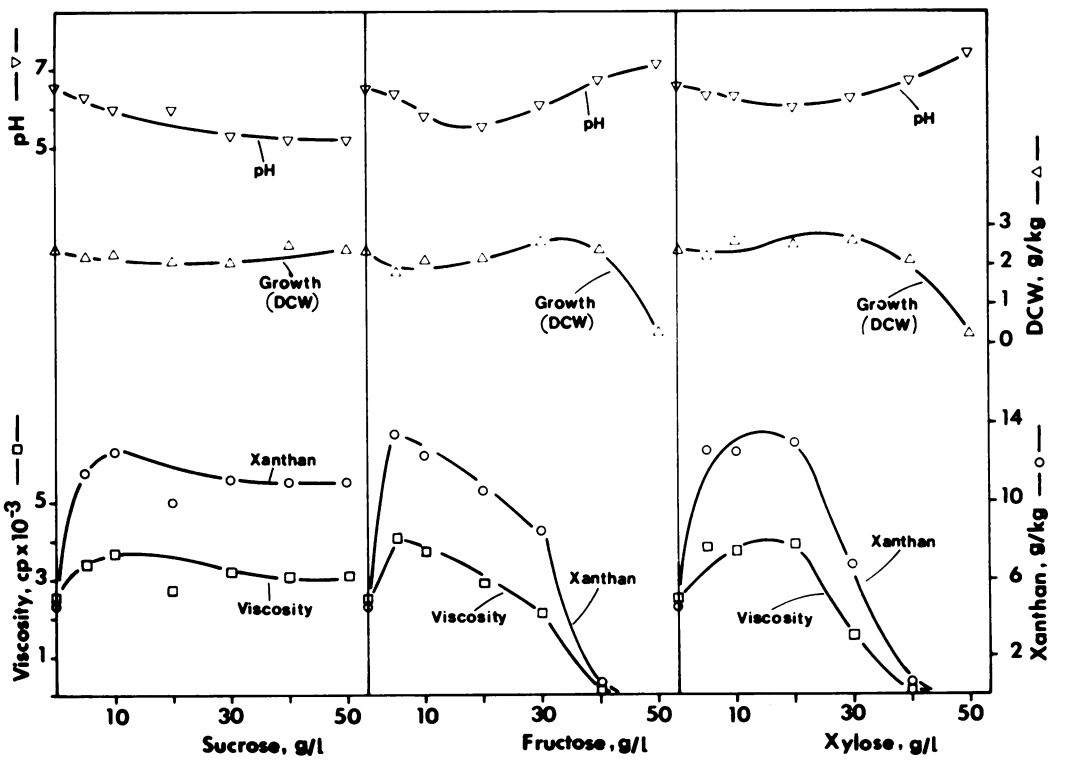

FIG. 1. *Effect of addition of increasing concentrations of carbohydrates on xanthan production and growth in the medium containing 2% glucose. Duration was for 96 h at 25°C. DCW, Dry cell weight.*

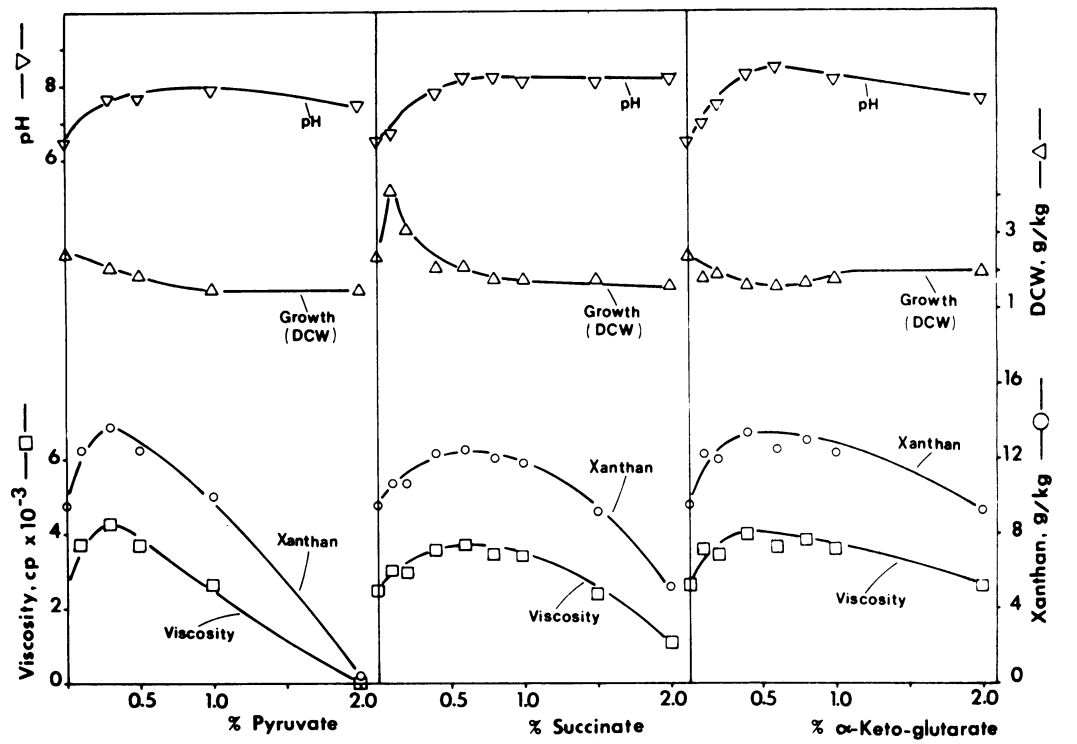

FIG. 2. *Effect of addition of increasing concentrations of organic acids on xanthan production and growth in the medium containing 2% glucose. Incubation was at 25°C for 96 h. DCW, Dry cell weight.*

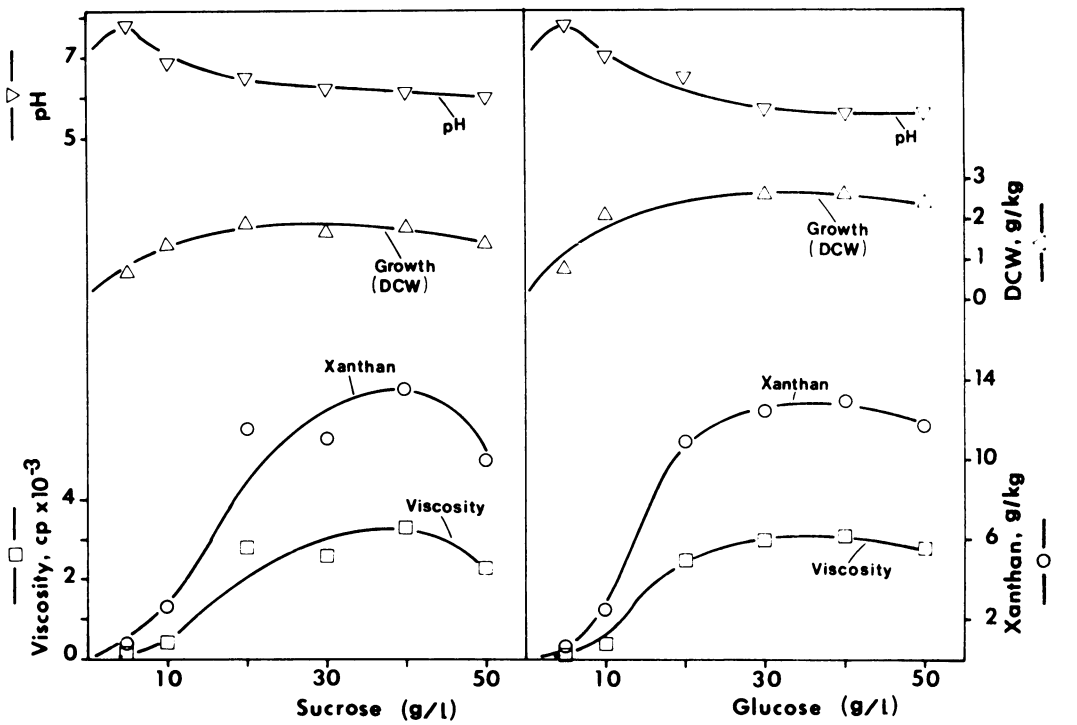

FIG. 3. *Xanthan production and growth in increasing concentrations of sucrose or glucose as carbon sources. Incubation was at 25°C for 96 h. DCW, Dry cell weight.*

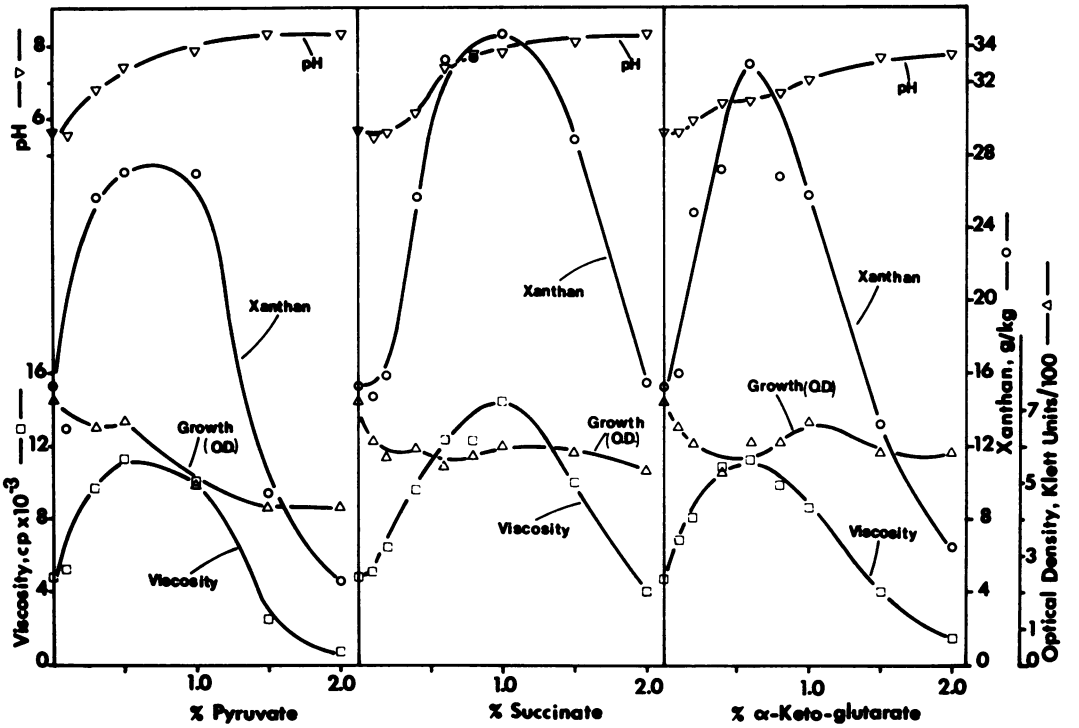

FIG. 4. *Effect of addition of increasing concentrations of organic acids on xanthan production and growth in the medium containing 4% sucrose as the carbon source. Incubation was at 25°C for 96 h.*

concentrations of organic acids inhibited xanthan production, although growth was suppressed only slightly, at most.

Nitrogen nutrition. Ammonium sulfate (0.2%) was the nitrogen source in the basic chemically defined medium. Twenty-one protein amino acids, six ammonium salts (acetate, oxalate, lactate, chloride, phosphate, and nitrate), sodium nitrate, and urea were tested as sole nitrogen sources in the medium containing 4% sucrose. The nitrogen sources were added at a nitrogen level equivalent to 2 g of ammonium sulfate per liter. Table 1 presents the results for those compounds which supported better xanthan production than did $(NH_4)_2SO_4$. No growth occurred when the sole nitrogen source was serine, arginine, tryptophan, or cysteine. L-Glutamic acid was chosen to represent the active amino acids in further experiments.

Increasing concentrations of glutamate, ammonium nitrate, and sodium nitrate were tested in xanthan fermentations (Fig. 5). Glutamate at its optimal concentration (15 mM) yielded the highest xanthan titer (33 g/kg of broth). Sodium nitrate (15 mM) and ammonium nitrate (7.5 mM) were not as effective as glutamate, although they were decidedly superior to $(NH_4)_2SO_4$ at its optimal concentration of 7.5

TABLE 1. *Effect of sole nitrogen sources on xanthan production and growth*[a]

Nitrogen source	Viscosity (cP)	Xanthan (g/kg)	pH	Growth (Klett units)	Specific xanthan production (cP/Klett units)
L-Threonine	12,200	28	6.3	775	15.8
Sodium nitrate	12,200	33	8.5	780	15.7
L-Hydroxyproline	13,040	31	6.1	1,000	13.0
L-Glutamic acid	15,610	33	6.7	1,300	12.0
L-Alanine	13,600	35	6.9	1,260	10.8
L-Asparagine	13,920	30	6.9	1,300	10.7
Ammonium nitrate	13,800	30	6.9	1,380	10.0
L-Proline	12,800	37	6.4	1,340	9.5
L-Aspartic acid	13,350	32	6.7	1,720	7.8
Ammonium sulfate	3,840	15	5.4	960	4.0

[a] Sucrose (4%) used as carbon source. Nitrogen sources added at a nitrogen level equivalent to 2 g of $(NH_4)_2SO_4$ per liter. Incubation was at 25°C for 96 h.

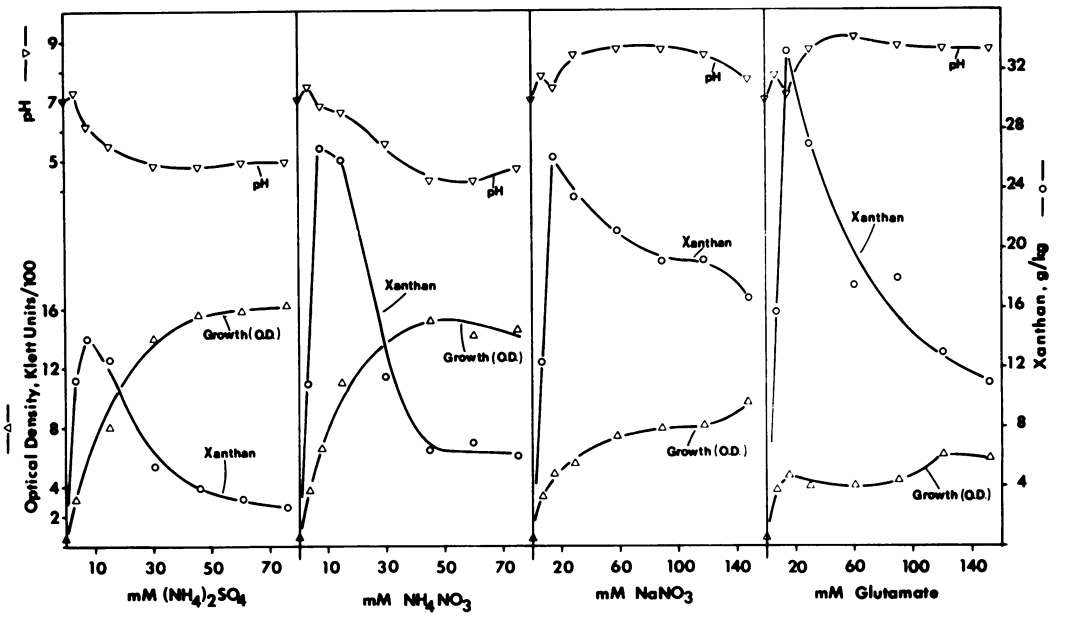

FIG. 5. *Influence of increasing concentrations of sole nitrogen sources on xanthan production and growth in the medium containing 4% sucrose as the carbon source. Incubation was at 25°C for 96 h.*

mM. The specific xanthan production with glutamate was much greater than with the other nitrogen sources. Quite striking was the vigorous inhibitory effect of all four nitrogen sources at concentrations greater than their optima, despite the fact that growth was stimulated. Thus, it is evident that nitrogen sources strongly influence xanthan production.

As reported earlier in this paper, organic acids such as succinate noticeably improved xanthan production when they were added to the medium containing ammonium sulfate as nitrogen source (Fig. 4). We next studied the effect of 1% succinate in media containing ammonium nitrate (0.6 g/liter), sodium nitrate (1.25 g/liter), and glutamic acid (2.25 g/liter) as nitrogen sources. As expected, succinate stimulated polysaccharide production in the presence of $(NH_4)_2SO_4$ (Table 2). However, in each case where nitrogen sources more favorable for xanthan formation were used, succinate inhibited both growth and xanthan production.

Phosphorus nutrition. Potassium phosphate at a concentration of 37 mM was used as a buffer in the initial chemically defined medium. Figure 6 summarizes the effect of phosphate concentration on maximum xanthan titer and maximum growth; 50 mM supported the best xanthan production (25 g/kg of broth). Above this phosphate concentration, formation of the polysaccharide was inhibited, though no growth inhibition occurred. There was, however, a curious growth depression between 5 and 50 mM which was not investigated further.

TABLE 2. *Effect of succinate on xanthan production and growth with different nitrogen sources*[a]

Additives	Viscosity (cP)	Xanthan (g/kg)	pH	Growth (Klett units)	Specific xanthan production (cP/Klett units)
Ammonium nitrate	6,380	16	7.1	610	10.5
Ammonium nitrate + succinate	3,000	14	8.4	420	7.1
Sodium nitrate	7,400	21	7.5	590	12.5
Sodium nitrate + succinate	2,360	13	8.4	420	5.6
L-Glutamic acid	10,400	28	7.6	530	19.6
L-Glutamic acid + succinate	3,800	15	8.5	450	8.6
Ammonium sulfate	2,700	13	5.8	780	3.5
Ammonium sulfate + succinate	8,400	25	7.9	705	12.0

[a] Nitrogen sources were used at the following concentrations (grams per liter): NH_4NO_3, 0.6; $(NH_4)_2SO_4$, 2; $NaNO_3$, 1.25; L-glutamate, 2.25. Incubation was 25°C for 96 h.

DISCUSSION

Our data show that glucose and sucrose are the best carbon sources for producing xanthan.

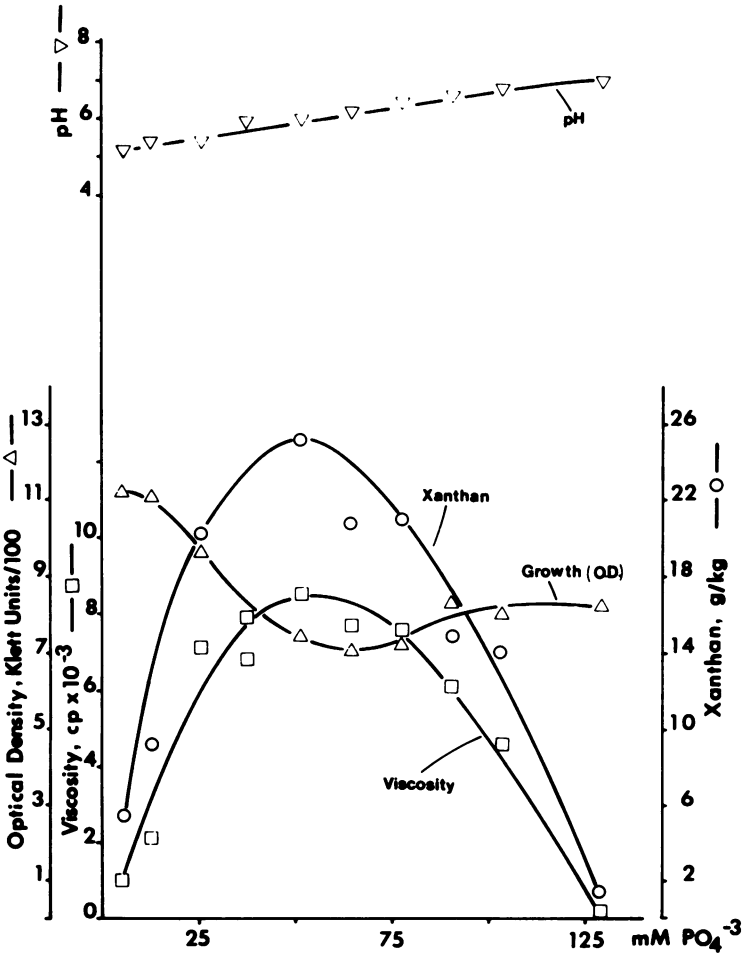

FIG. 6. *Effect of phosphate concentration on xanthan production and growth in the medium containing 4% sucrose as the carbon source. Incubation was at 25°C for 96 h.*

We preferred sucrose because it supported a somewhat higher specific xanthan production than did glucose. Leach et al. (7) found that sucrose was the most favorable carbon source for polysaccharide formation in *X. phaseoli*. Although we found low concentrations of fructose to support satisfactory xanthan production, higher fructose concentrations were severely inhibitory. For example, although 50 g of sucrose per liter could be added to a 2% glucose medium without any decrease in xanthan production (Fig. 1), fructose additions greater than 5 g/liter inhibited xanthan formation, without affecting growth. Experiments with xylose showed similar inhibitory effects at concentrations higher than 15 g/liter. Before we can conclude that carbon catabolite regulation is responsible for these inhibitory effects on polysaccharide production, enzyme studies must be done. It should be noted that the addition of very high levels of fructose or xylose (50 g/liter) drastically suppressed growth.

Several organic acids, such as pyruvate, succinate, and α-ketoglutarate, markedly stimulated xanthan production when added to glucose or sucrose media. Concentrations of these organic acids higher than their optima inhibited xanthan formation without significantly affecting growth. It is doubtful that pH increases, which were observed in the presence of organic salts, caused the inhibition of polymer formation, but this will have to be examined in fermentors utilizing pH control.

Nitrogen nutrition is another important factor in the xanthan fermentation. Amino acids (alanine, threonine, aspartate, asparagine, glutamate, proline, and hydroxyproline) and nitrates (ammonium nitrate and sodium nitrate) were better nitrogen sources for xanthan production than ammonium sulfate (Table 1). Goto et al.

(5) found that amino acids (glutamic acid, proline, histidine, and leucine) were suitable for polysaccharide production by *Pseudomonas aeruginosa*. In our experiments, excess concentrations of effective nitrogen compounds strongly inhibited xanthan production while stimulating growth (Fig. 5). From this observation, we conclude that xanthan production is best under nitrogen limitation. Similar effects have been observed with other polysaccharide-producing organisms, e.g., *Aerobacter aerogenes* (4), *Coccidioides immitis* (10), *Chromobacterium violaceum* (3), *Pullularia pullulans* (2), and *Pseudomonas* NCIB 11264 (13).

Phosphorus nutrition also appears to be important in the xanthan fermentation. The effect of inorganic phosphate in our studies indicated that xanthan production is controlled by phosphate; phosphate concentrations higher than the optimal level (50 mM) suppressed xanthan production (Fig. 6). Again, fermentor studies with constant pH will have to be done to eliminate pH as a factor.

It is interesting to compare our data with those of others, e.g., Cadmus et al. (1). Surprisingly high xanthan yields were observed in our experiments. Upon organic acid (Fig. 4) or glutamate (Fig. 5) supplementation of the 4% sucrose medium, conversions of over 70% (grams of xanthan per 100 g of sucrose plus additive charged) were achieved in a chemically defined medium. Cadmus et al. (1) indicated that the highest yield obtained in other defined media was 56%, whereas complex media containing distiller's dried solubles supported yields of about 65%. The highest viscosities reported by Cadmus et al. (1) were on the order of 7,000 cP for defined media and 11,000 cP for complex media using 2.5% glucose as carbon source. In our experiments, a viscosity of 15,000 cP was obtained from 4% sucrose plus 1% succinate. Cadmus et al. (1) reported xanthan titers as high as 1.4% in defined media and 1.7% in complex media. Our potencies reached 3.3 to 3.5% when the 4% sucrose medium was supplemented with succinate, α-ketoglutarate (Fig. 4), or glutamate (Fig. 5).

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