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Production of Extracellular Polysaccharide by *Zoogloea ramigera*

ANDERS B. NORBERG* AND SVEN-OLOF ENFORS†

Department of Technical Microbiology, Chemical Center, University of Lund, S-220 07 Lund 7, Sweden

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In batch cultures of *Zoogloea ramigera* the maximum rate of exopolysaccharide synthesis occurred in a partly growth-linked process. The exopolysaccharide was attached to the cells as a capsule. The capsules were released from the cell walls after 150 h of cultivation, which caused the fermentation broth to be highly viscous. Ultrasonication could be used to release capsular polysaccharide from the microbial cell walls. Treatment performed after 48 to 66 h of cultivation revealed exopolysaccharide concentration and apparent viscosity values in accordance with values of untreated samples withdrawn after 161 h of cultivation. The yield coefficient of exopolysaccharide on the basis of consumed glucose was in the range of 55 to 60% for batch cultivations with an initial glucose concentration of 25 g liter^{-1}. An exopolysaccharide concentration of up to 38 g liter^{-1} could be attained if glucose, nitrogen, and growth factors were fed into the batch culture. The oxygen consumption rate in batch fermentations reached 25 mmol of O_2 liter^{-1} h^{-1} during the exopolysaccharide synthesis phase and then decreased to values below 5 mmol of O_2 liter^{-1} h^{-1} during the release phase. The fermentation broth showed pseudoplastic flow behavior, and the polysaccharide was not degraded when growth had ceased.

Several bacteria are known to synthesize large amounts of extra-cellular polysaccharides, resulting in mucoid colonies on agar plates and extremely viscous broth during submerged cultivation. In many cases these polysaccharides have found technical applications, as for instance in the food and pharmaceutical industries (15). In most cases the production is oxygen dependent, and due to the high viscosity of the fermentation broth, oxygen transfer is one of the critical factors in microbial polysaccharide production (10). One well-known type of bacterium able to produce slime is *Zoogloea ramigera* (12). This bacterium is frequently found to accumulate in sewage plants (2). The exopolysaccharide formed by *Z. ramigera* strain 115 behaves like a polyelectrolyte and shows a strong affinity to metal ions (5), which makes it a potential agent for metal recovery.

This report is part of a general study on using microbial biomass as an adsorbent for heavy metal ions. Furthermore, *Z. ramigera* strain 115 has been shown to produce polysaccharide. If this property is further developed, it could be of future interest for industrial exploitation.

In this study we report on the relationship between exopolysaccharide production and growth conditions and on the rheological properties of the fermentation broth.

MATERIALS AND METHODS

Organism. *Zoogloea ramigera* strain 115 (ATCC 25935) was obtained from the American Type Culture Collection, Rockville, Md.

Media. The organism was maintained on Trypticase soy agar (BBL 11043, BBL Microbiology Systems) with weekly transfer to fresh medium. The cultivation medium had the following composition (g/liter): glu-

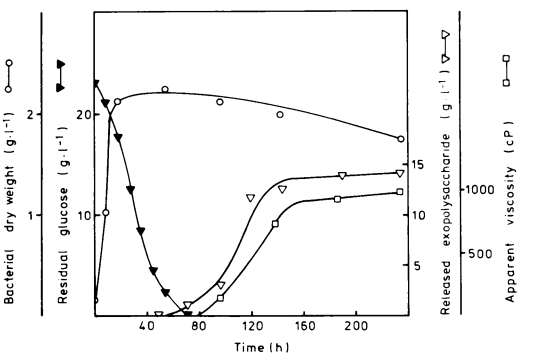

FIG. 1. Exopolysaccharide production by *Z. ramigera* in batch culture.

† Present address: Department of Biochemistry and Biotechnology, Royal Institute of Technology, S-100 44 Stockholm, Sweden.

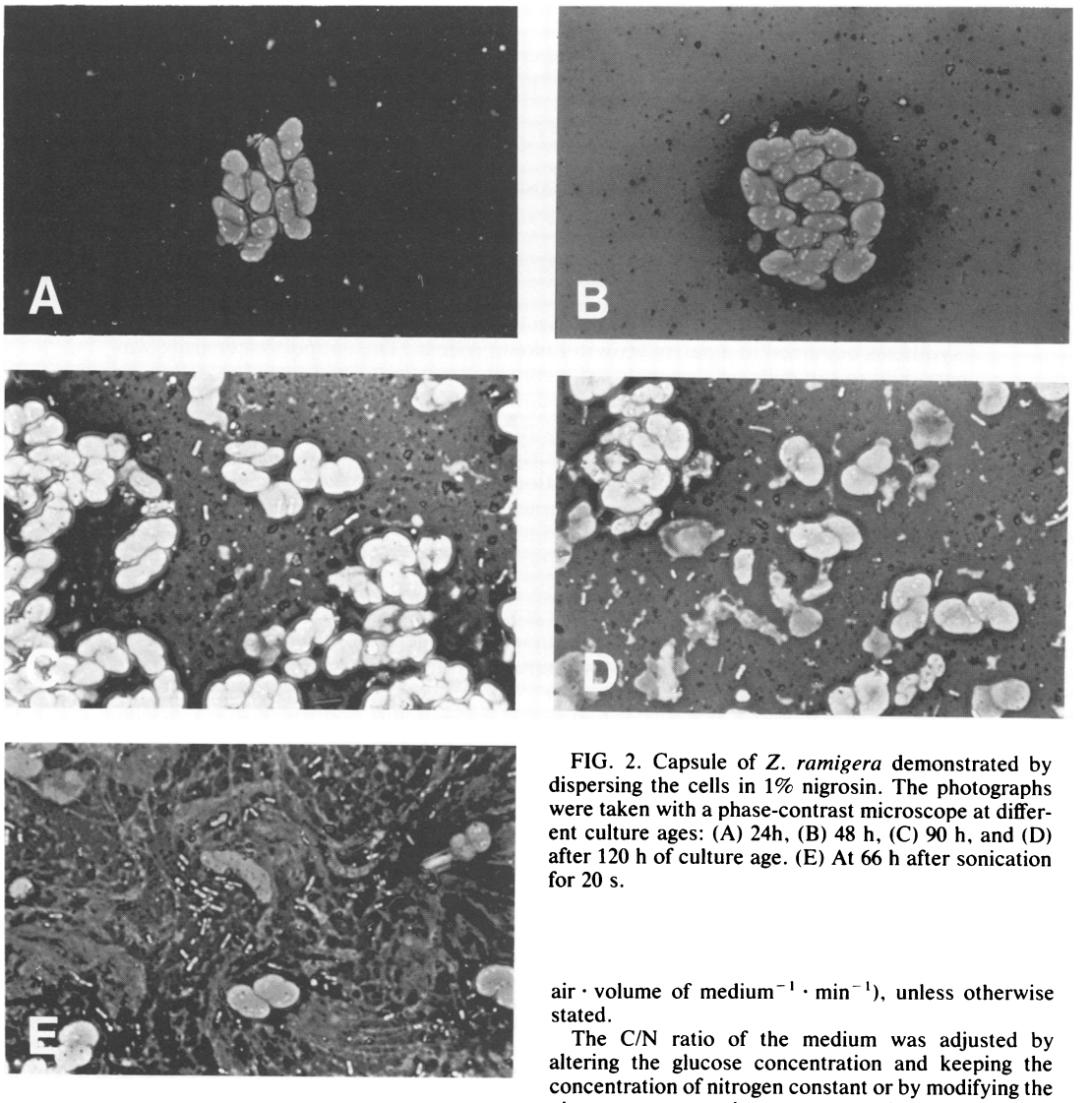

FIG. 2. Capsule of *Z. ramigera* demonstrated by dispersing the cells in 1% nigrosin. The photographs were taken with a phase-contrast microscope at different culture ages: (A) 24h, (B) 48 h, (C) 90 h, and (D) after 120 h of culture age. (E) At 66 h after sonication for 20 s.

cose, 25; K_2HPO_4, 2; KH_2PO_4, 1; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.2; yeast extract (Difco Laboratories), 0.01. Glucose and $MgSO_4 \cdot 7H_2O$ were autoclaved separately. The solution used for feeding contained (g/liter): glucose, 400; NH_4Cl, 16; yeast extract, 0.16. The components were autoclaved separately.

Culture conditions. Seed cultures were derived from a single colony isolate and grown for 24 h on a rotary shaker at 26°C in the medium defined above. These cultures were used for inoculation of a 3-liter working volume fermentation vessel (Chemoferm AB, Hägersten, Sweden) fitted with a six-blade open turbine and a condenser for the outlet air to reduce the evaporation of water during long-term experiments. The fermentation pH was maintained at 7.0 by titration with 1 M NaOH. The initial foaming was controlled by a mechanical foam breaker. Agitation was at 800 rpm, and the aeration was controlled at 0.5 VVM (volume of air · volume of medium^{-1} · min^{-1}), unless otherwise stated.

The C/N ratio of the medium was adjusted by altering the glucose concentration and keeping the concentration of nitrogen constant or by modifying the nitrogen concentration at constant glucose concentration.

Viscosity. Viscosity was measured with an STV Epprecht Rheometer (Contraves AG, Zürich) at 25°C. The apparent viscosities were determined at a fixed shear rate of 77.9 s^{-1}, unless otherwise stated. The consistency index (apparent viscosity at a shear rate of 1 s^{-1}) was determined by using a Rheomat-30 viscometer (Contraves AG).

Glucose determination. Glucose was determined by the hexokinase method using a Glucoquant kit (Boehringer Mannheim Corp.).

Oxygen measurement. Oxygen in the outlet gas was measured continuously with a paramagnetic oxygen analyzer (Leeds & Northrup, England). Dissolved oxygen tension of the broth was measured with a measured continuously with a paramagnetic oxygen analyzer (Leeds & Northrup Ltd., Birmingham, England). Dissolved oxygen tension of the broth was measured with a galvanic oxygen electrode according to Johnson et al. (7).

TABLE 1. Influence of the C/N ratio on cell and polysaccharide production at a glucose concentration of 25 g liter^{-1}

C/N ratio	Polysaccharide concn (g/liter)	Bacterial dry wt (g/liter)	Yield coefficients (g/g)	
			Y_{PS}	Y_{XN}
38	15.5	2.4	0.62	9.2
26	10.0	3.7	0.40	9.7
19	5.1	5.0	0.20	9.4

Cell-bound bacterial polysaccharide. Bacterial cells and capsular polysaccharide could not be thoroughly separated by centrifugation. Samples were first subjected to ultrasonication three times for 20 s each time at an amplitude of 10 μm in an MSE Soniprep 150 (MSE Scientific Instruments, Crawley, United Kingdom). Centrifugation was then performed at 31,300 × *g* for 40 min to remove cells and cellular debris in a Beckman J2-21 centrifuge. When the sonicated sample became viscous, it was diluted with 3 volumes of distilled water before centrifugation to achieve good separation of the cells and polymer. KCl was added to the supernatant to a 1% (wt/vol) concentration. Precipitation of the polysaccharide was performed by adding 2 volumes of propanol. The resultant precipitate was filtered off by using a preweighed GF/A filter dish (Whatman Ltd., Maidenstone, England). The filter was dried at 105°C to a constant weight. The dry weight was determined gravimetrically.

Released exopolysaccharide. To determine the released portion of the exopolysaccharide, the withdrawn sample was diluted with 3 volumes of distilled water. Centrifugation was performed at 31,300 × *g* for 40 min. This sedimented the cells with and without capsules. The supernatant was decanted, and KCl was added to a 1% (wt/vol) concentration. Precipitation was performed by adding 2 volumes of propanol. The amount of released exopolysaccharide was then determined gravimetrically as described above.

Cell dry weight. The cell-bound polysaccharide was removed as described above, and the cells were resuspended in water to the initial volume. The dry weight was then measured gravimetrically using Sartorius membrane filters with a pore size of 0.2 μm after desiccation at 105°C for 2 h.

DNase treatment. DNase treatment was performed to ensure that the increase in viscosity, obtained after sonication, was not due to liberation of DNA. Samples (40 ml), withdrawn from the fermentor at different culture ages, were sonicated three times for 20 s each time. The enzyme solution, prepared by dissolving 5,400 Kunitz units in 2 ml of 0.15 M NaCl, was added to the sonicated sample. The temperature was maintained at 25°C, and the pH was 7.0. Apparent viscosity was recorded continuously for 6 h after the addition of DNase (Sigma Chemical Co.). To demonstrate that hydrolysis of DNA occurred under these conditions, the increase in absorbancy at 260 nm was measured.

A separate sample was supplied with magnesium (final concentration, 1 mM) to determine if sufficient magnesium was present to allow hydrolysis of DNA.

RESULTS

Production of exopolysaccharide in batch culture. The cell growth phase was finished within 20 h. However, the glucose consumption continued far beyond this time, as shown in Fig. 1. During the first 70 h of cultivation, microscopical analysis of the culture showed that the polysaccharide was attached to the microbial cells as capsules (Fig. 2). After about 90 h of culture, polysaccharide also occurred in the extracellular fluid, as measured by viscosity (Fig. 1). The final apparent viscosity value was in the range 800 to 1,000 cP. The consistency index of the broth was around 17,500 cP. The viscosity remained constant when the culture was held at 26°C for 100 h after the viscosity had reached its maximum value, thus indicating that no degradation of polysaccharide occurred. The yield of polysaccharide from glucose was around 60% on a weight basis.

The C/N ratio had a pronounced effect on the polysaccharide production. The final amount of exopolysaccharide was negatively affected if the C/N ratio was decreased below 38 at a fixed glucose concentration of 25 g/liter. Instead, the culture responded by producing more cells. The yield coefficient $Y_{X/N}$ was constantly around 9 irrespective of the C/N ratio (Table 1).

Results from gas analysis during batch fermentations show that the oxygen absorption rate reached a peak value of 25 mmol of O_2 liter^{-1} h^{-1} at 40 h of growth. During the viscosity-increasing phase the oxygen uptake never exceeded 5 mmol of O_2 liter^{-1} h^{-1}. Krul (8), who studied the activity of *Z. ramigera* in flocs and suspension, showed that the potential oxygen demand of the cells occurred in the early growth phase of the cultures.

To verify that the microscopically observed capsules contained polysaccharide during the period when growth had ceased but glucose was still being consumed, samples were subjected to ultrasonication. This treatment resulted in the release of polysaccharide from the cells as could

TABLE 2. Effect of sonication on the apparent viscosity of the culture broth at different ages

Culture age (h)	Treatment	Resulting viscosity (cP)	Bacterial dry wt (g liter^{-1})	Released polymer concn (g liter^{-1})
48	None	5	2.0	0.5
48	Sonication	940	2.2	12.9
66	None	10	ND[a]	0.4
66	Sonication	2,670	2.1	13.2
161	None	880	2.3	14.0
161	Sonication	2,900	ND	ND

[a] ND, Not determined.

TABLE 3. Polysaccharide production by *Z. ramigera* in fed batch system

Nutrients fed	Amt of glucose added (g liter^{-1})	Residual glucose concn (g liter^{-1})	Polysaccharide concn (g liter^{-1})	Polysaccharide yield from glucose used (%)	Bacterial dry wt (g liter^{-1})	Apparent viscosity (cP)
Glucose	42.6	16	12.5	47	2.5	520
Glucose plus NH_4Cl plus yeast extract[a]	53	0	32.8	62	5.0	2,340
Glucose plus NH_4Cl plus yeast extract	59.2	0	38	64	6.0	3,100

[a] Concentration of dissolved oxygen above 15% of saturation throughout the cultivation.

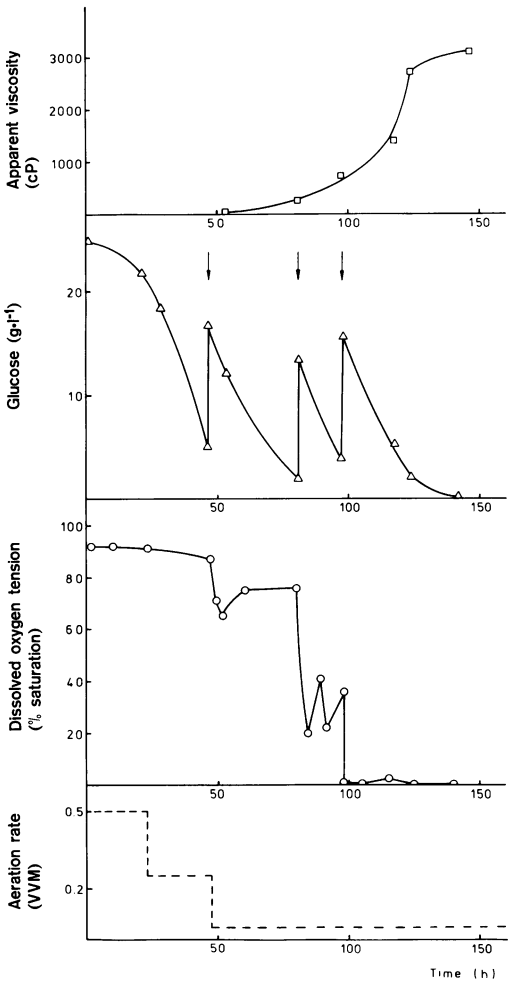

FIG. 3. Exopolysaccharide production by *Z. ramigera* in fed batch culture. Arrows indicate addition of feeding solution.

be observed microscopically (Fig. 2). The sonicated samples were also stained for light microscopy by using two dyes, namely, alcian blue 8GX and toluidine blue. Toluidine blue, which is a metachromatic dye, produced a red color when exposed to the sonicated sample. Samples stained with alcian blue turned dark blue. Both reactions confirm that the sample contains polysaccharide. Furthermore, a steep increase in viscosity was obtained after sonication (Table 2). Cell mass and exopolysaccharide determinations performed on the sonicated samples were comparable to the results obtained on untreated samples after 161 h of culture. To check the presence of DNA in the medium after sonication, DNase was added to the sonicated sample. This treatment did not influence the viscosity. However, the presence of DNA was demonstrated, as we recorded an increased absorbance at 260 nm upon the addition of the enzyme. Addition of magnesium did not affect the hydrolysis.

Production of exopolysaccharide in fed batch culture. The first attempt to increase final polysaccharide concentration was performed by the feeding of glucose. Glucose was fed in two portions, the first after 34 h and the second after 71 h of cultivation. In both cases, glucose concentration in the fermentor increased by 9 g/liter. The results showed that no increase in final polysaccharide concentration was gained compared with batch cultivation. The final apparent viscosity was 500 cP (Table 3). In the following experiment the feeding solution also contained NH_4Cl and yeast extract. The C/N ratio of the feeding solution was 38. The solution was fed in two equal portions, each increasing the glucose concentration by 13 g/liter. Feeding was performed at 44 and 72 h of cultivation. The dissolved oxygen tension never decreased below 15% of air saturation during the polysaccharide production phase. The maximum polysaccharide concentration was reached within 100 h of growth, with a 63% yield on a glucose basis. The apparent viscosity was 2,340 cP, and the consistency index of the broth was 72,000 cP.

To evaluate the effects of oxygen limitation, another cultivation was performed in which the aeration rate was lowered from 0.5 VVM to 0.25 VVM at 23 h of growth and further decreased to 0.05 VVM at 47 h of growth. The feeding solution contained glucose, NH_4Cl, and yeast ex-

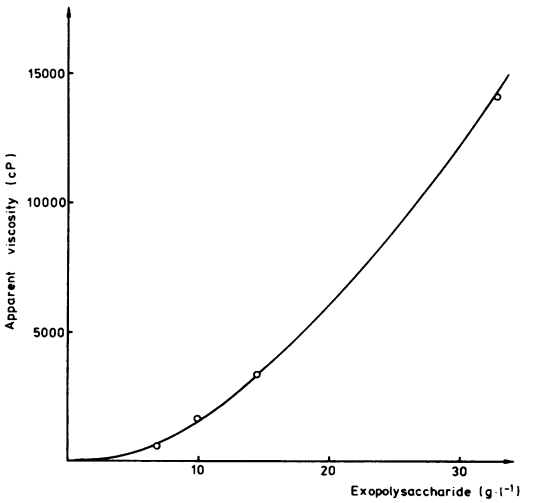

FIG. 4. Concentration of exopolysaccharide in fermentation broth versus apparent viscosity at a shear rate of 8 s^{-1}.

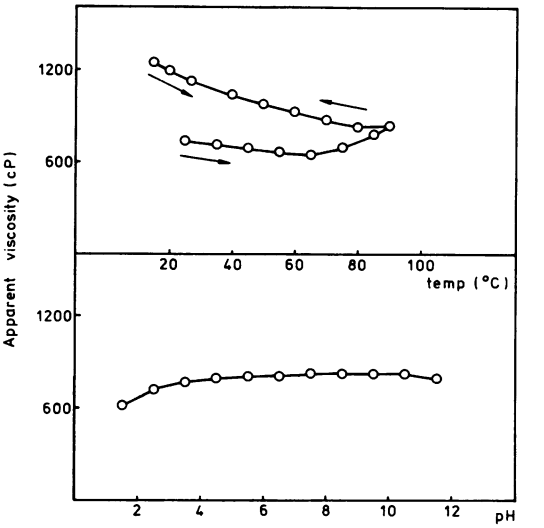

FIG. 5. Effects of temperature and pH on apparent viscosity of fermentation broth. Concentration of exopolysaccharide is 1.5%.

tract as described above. The solution was fed in three equal portions, each increasing the glucose concentration by 11 g/liter. Feeding was performed at 46, 81, and 97 h of growth. During the feeding experiment, glucose concentration in the fermentor never fell below 2 g/liter. After 145 h of cultivation, the apparent viscosity of the broth was 3,100 cP, and the consistency index of the broth reached 94,000 cP. Of the glucose supplied to the fermentor, 64% was converted into polysaccharide. The signal of the dissolved oxygen probe declined to values close to zero after the third addition of nutrients and remained at this low level throughout fermentation. Two factors caused this: first, increased oxygen consumption, and second, the fact that the probe is sensitive to viscosity (1) which reduces the signal of the electrode at increasing viscosity (Fig. 3 and Table 3).

Rheological properties of fermentation broth. Figure 4 shows the apparent viscosity at 7.99 s^{-1} versus the concentration of exopolysaccharide in fermentation broth. The effect of temperature on the viscosity of fermentation broth containing 1.5% polysaccharide indicated an irreversible behavior when the temperature was successively raised from 25 to 90°C and back again. This phenomenon occurred only once and might indicate a thermal rearrangement of the polysaccharide during the first heat treatment that resulted in a higher viscosity at low temperature (Fig. 5). The viscosity of the cultivation broth, containing 1.5% polysaccharide, is essentially independent of pH between pH 4 and 11. Decreasing the pH below 4 results in a decrease of viscosity (Fig. 5). If pH is increased above 11, the polysaccharide forms a brittle gel which makes viscosity measurements impossible. Figure 6 shows the change in rheological behavior of the growing culture. As shown by the almost straight line corresponding to 81 h of cultivation, the broth was close to Newtonian at the beginning of the fermentation. The broth became pseudoplastic in its flow behavior at the beginning of the release period.

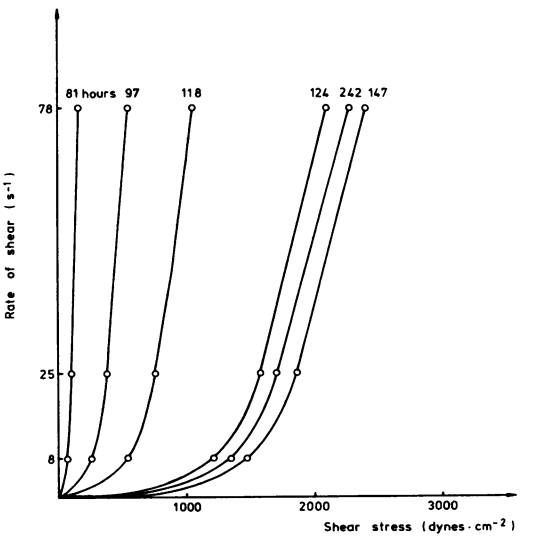

FIG. 6. Rheograms of the broth from a batch culture of *Z. ramigera* taken at different cultivation times.

DISCUSSION

When growth ceased in the batch process (after 20 h), only 5 g of glucose per liter had been consumed, and about 2 g of biomass per liter had been formed. This gives a yield coefficient of biomass ($Y_{X/S}$) of about 0.4 (grams of cells per grams of glucose) which is in accordance with typical yield coefficients for aerobic growth on carbohydrates (9). In spite of the growth cessation, glucose continued to disappear from the broth and reached zero after 70 h without any concomitant increase of biomass or appearance of polysaccharide in the broth (Fig. 1). A possible explanation of this phenomenon would be that glucose is used in the stationary phase for synthesis of polysaccharides which are not excreted into the medium. The microphotos (Fig. 2) demonstrate the presence of large quantities of capsular material around the cells during the glucose consumption phase (0 to 70 h), and a further support for this hypothesis is the observation that sonication of these cells increases the viscosity of the medium (Table 2) and detaches the capsular material from the cells (Fig. 2).

The unexpected high viscosity of samples sonicated after 66 and 161 h raised the question whether cell disintegration and DNA release might have caused the increased viscosity during the treatment. However, treatment of the sonicated samples with DNase did not reduce the viscosity of the medium. Furthermore, the untreated 48-h sample contained no free polysaccharide, while the same sample after sonication showed a polysaccharide content of 12.9 g/liter (Table 2). These observations further support the hypothesis that polysaccharides are formed during the glucose consumption phase but stick to the cells as capsules till a later phase when they are at least partly released to the medium. To achieve a total release of polysaccharides from the cells, thorough sonication of the sample has to be performed (Fig. 2).

The data from the batch culture (Fig. 1) give the impression that it is glucose starvation that induces the release of polysaccharides to the medium. However, the results from the batch culture contradict this since the release of polysaccharides started at about the same time (60 to 70 h) in both types of cultures while in the fed batch culture the glucose concentration was high throughout the process.

The importance of nitrogen limitation for the polysaccharide production is shown in Table 1. The more nitrogen source in the initial medium, the less polysaccharides were produced. However, the cell amount produced was proportional to the initial concentration of nitrogen source as visualized by the constant yield coefficient $Y_{X/N}$. This is a common characteristic of exopolysaccharide production both by yeasts (4) and bacteria (6).

It was therefore expected that more polysaccharides could be produced by increasing the glucose supply by fed batch cultivation. However, more feeding with glucose did not improve the polysaccharide production. To increase the final product yield, the nitrogen source and yeast extract had to be supplemented (Table 3). With this technique, the culture reached viscosities so high that adequate mixing could not be achieved in an ordinary laboratory fermentor. Cadmus et al. (3) reported that 65% of the glucose consumed by *Xanthomonas campestris* was converted into xanthan gum. Parsons and Dugan (12) estimated that *Z. ramigera* converted approximately 34% of the carbon source supplied into exopolysaccharide. In our experiments, 60 to 64% of the glucose used was converted into exopolysaccharide.

Microbial synthesis of exopolysaccharides is dependent on phosphorylating reactions (14) and must therefore be dependent on energy metabolism which involves respiration in the case of aerobic organisms. Oxygenation of polysaccharide processes as in xanthan fermentation is a major technical problem because of the severe reduction of the oxygen transfer coefficient (K_La) caused by the highly viscous broth. However, unlike *X. campestris* (13), *Pseudomonas aeruginosa* (11), and *Pseudomonas* NCIB 11264 (16) which formed and secreted polymer throughout the fermentation, *Z. ramigera* in our study produced polysaccharides which attached to the cell walls. This tendency of the polysaccharide to attach to the cells and not make the broth viscous during the synthesis phase allows high oxygen transfer rates to be maintained during the high oxygen demand period. Furthermore, very low oxygen consumption was observed during the period of polysaccharide release when the oxygen transfer rate cannot be raised to reasonable values without very high energy inputs.

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