GROWTH AND EXTRACELLULAR POLYSACCHARIDE PRODUCTION BY RHIZOBIUM MELiloti IN DEFINED MEDIUM

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ABSTRACT

DUDMAN, W. F. (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia). Growth and extracellular polysaccharide production by Rhizobium meliloti in defined medium. J. Bacteriol. 88:640-645. 1964.—A defined medium was developed in which Rhizobium meliloti grew well in shaken flasks and produced large yields of extracellular polysaccharide. The medium contains 1% mannitol plus 1% sodium succinate as the carbon source, 0.2% sodium glutamate as the nitrogen source, mineral salts, thiamine, and biotin. Sucrose, glucose, and fructose were inferior to mannitol when compared as carbon sources. The presence of succinate in the medium enhanced polysaccharide production and also stimulated the appearance of an extracellular soluble pigment. In this medium, both growth and polysaccharide production were favored by low aeration.

It is desirable that bacteria grown for the purpose of immunochemical analysis should be cultured in defined liquid media to avoid contamination of the cultural products with macromolecular constituents of the medium. For this reason, before embarking on such an analysis of Rhizobium meliloti, it was necessary to find a suitable defined medium for the strains under investigation that would allow good growth of cells and large yields of extracellular polysaccharide.

Defined media suitable for good growth of R. meliloti have been reported (e.g., Ferry, Blachère, and Obaton, 1959; Bergersen, 1961; Vincent, 1962), but their suitability for the present purpose was unknown because no attempt had been made to determine polysaccharide yields in these media.

In this paper, experiments are described on the influence of a number of cultural constituents on growth and polysaccharide production in shaken cultures of two strains of R. meliloti, with a medium based on that of Bergersen (1961). An additional constituent, sodium succinate, was included in these experiments, because it was known to increase yields of cellulose in cultures of Acetobacter strains (Dudman, 1959), and it was thought possible that a similar stimulation of polysaccharide synthesis might also be obtained with R. meliloti.

MATERIALS AND METHODS

Bacteria. R. meliloti strains B and F were used. Strain B nodulates a group of 13 species of Medicago and Melilotus, but not Medicago laciniata; strain F forms nodules on a different group of 8 species of Medicago and Melilotus, including M. laciniata. The isolation and characteristics of these strains were described by Brockwell and Hely (1961), who refer to them as isolates 25 and 21, respectively. Stock cultures were maintained on yeast extract-mannitol-agar slants.

Media. The medium investigated was based on that described by Bergersen (1961). Apart from the carbohydrate, sodium glutamate, and sodium succinate, the concentrations of which varied as described below, the medium contained 5 g of KH2PO4, 0.2 g of MgSO4·7H2O, 0.5 g of NaCl, 100 µg of thiamine, 250 µg of biotin, and 1 ml of trace element solution (Dudman, 1959) in 1 liter of distilled water. The solution was adjusted to pH 6.8. After autoclaving, sterile FeCl3 (0.02 g per liter) and CaCl2 (0.05 g per liter) were added. To minimize reaction between the carbon source and other constituents, the carbon source was always separately sterilized and added to the medium later.

Cultural conditions. All small-scale experiments were carried out at 25 C with 100 ml of medium in 250-ml conical flasks mounted on a rotary shaker operated at 160 rev/min. In all flask experiments except the first, in which ordinary flasks were used, the conical flasks were baffled by
three inward indentations to increase aeration of the cultures. No antifoam was required.

A 10-liter MB fermentor, manufactured by the Marubishi Laboratory Equipment Co., Ltd., Tokyo, Japan, was used to grow cultures on the 5-liter scale after the optimal medium had been found in shake flask experiments.

**Analytical methods.** Bacterial growth was determined turbidimetrically. Polysaccharide yields were determined by an anthrone method (Fairbairn, 1953) with a glucose standard. When the medium contained mannitol, anthrone determinations were carried out directly on suitable dilutions of the culture, and a correction was applied for the polysaccharide contained in the bacterial cells. (Washed cells of strain B and strain F contained 8.5 and 5.7% of anthrone-positive polysaccharide, respectively.) In the experiment comparing glucose, fructose, sucrose, and mannitol as substrates, samples of the cultures were centrifuged to remove all the bacteria, and the supernatant fluids were dialyzed against running water for 48 hr to remove the sugars. The dialyzed cultures were diluted, and the polysaccharide was determined by the anthrone method. Where necessary, a small correction was applied for residual sugar estimated by the colorimetric Somogyi (1952) method.

Anthrone determinations on samples of purified polysaccharide from strains B and F indicated 64 and 79% "glucose," respectively (Dudman, 1964). The polysaccharide yields estimated in the cultures in terms of glucose were adjusted, therefore, by multiplying the results for the two strains by the factors 1.56 and 1.27, respectively.

The pigment produced by the organism was estimated in samples of supernatant liquid from centrifuged cultures by measurement of optical density at 405 mμ.

**Results**

**Comparison of mannitol, glucose, fructose, and sucrose.** A suitable carbon source was selected by comparing the performance of the strains in cultures grown in media with mannitol, glucose, fructose, and sucrose, both alone and in the presence of sodium succinate. One set of media contained 1% carbohydrate and another set, 1% carbohydrate plus 1% sodium succinate as carbon sources. Medium containing succinate without carbohydrate was also prepared. The concentration of sodium glutamate was 0.2% and the other constituents were used as shown above. The cultures were all grown in un baffled flasks.

The organisms were adapted to the various carbon sources by 48 hr of growth in each medium before inoculating 2-ml volumes into the test cultures. The cultures were examined for growth after 5, 10, and 15 days, but for polysaccharide production only after 15 days, by which time the cultures were well into the stationary phase. Preliminary experiments established that the concentration of polysaccharide in cultures of these *Rhizobium* strains did not decrease with age, even after 6 weeks at 25 C on the shaker.

The results obtained with the 15-day cultures are shown in Fig. 1. Growth of strain B was best on mannitol, and was progressively less on sucrose, fructose, and glucose. Succinate made little difference to these results. Growth of strain...
mannitol, and containing 1% (○), 2% (△), 5% (▽), and 10% (□) mannitol, and 1% mannitol plus 0.8% sodium succinate (●) as carbon sources, all grown in 100-ml cultures in shaken baffled flasks. Results for 1% mannitol medium in shaken unbaffled flasks are shown (×).

(a) Polysaccharide production, and (b) growth of strain B. (c) Polysaccharide production, and (d) growth of strain F.

F was approximately equal with all the four carbohydrates in the absence of succinate, but was best on mannitol when succinate was present.

The general level of polysaccharide synthesis was different in the two strains. Strain B produced small amounts of polysaccharide on all the substrates; the addition of succinate to the medium gave equal increased yields from mannitol, sucrose, and fructose. Polysaccharide yields were much higher in cultures of strain F. Mannitol was the best substrate, and gave increased yields in the presence of succinate.

The terminal pH values of the cultures were as expected. The succinate cultures were distinctly alkaline, as the result of removal of the oxidizable anions, whereas the succinate-free cultures were close to neutrality except in the case of the glucose cultures, which were acidic. The fructose cultures were not alkaline, despite the presence of succinate.

The cultures containing succinate alone or succinate plus carbohydrate were distinctly colored after a few days of growth. Succinate appeared to stimulate the production of a yellow-brown pigment. When the supernatant liquids from centrifuged cultures were dialyzed, some 30% of the pigment remained inside the sacs.

It was concluded that mannitol, supplemented by succinate, was the best of the carbon substrates examined.

Effect of mannitol concentration. The optimal mannitol concentration in the medium was sought by examining growth and polysaccharide production in media containing 1, 2, 5, and 10% mannitol and 0.25% sodium glutamate. For comparison, medium containing 1% mannitol and 0.5% sodium succinate as the carbon substrates was also used. These cultures were grown in indented flasks in the belief that the increased aeration would assist higher yields. To find whether aeration at this level was really necessary, the medium containing 1% mannitol was prepared also in unbaffled flasks. The flasks were inoculated with 1-ml volumes of heavy suspensions of 5-day cultures grown on Bergersen's mannitol medium in agar. The cultures were sampled at intervals.

Growth of strain F (Fig. 2d) reached essentially the same level at the end of 14 days at all mannitol concentrations in the succinate-free cultures, whereas strain B (Fig. 2b) gave reduced growth in cultures containing 5 and 10% mannitol. Comparison of the growth patterns in cultures grown in unbaffled flasks showed that aeration differences of this level made no difference to growth. The presence of succinate in the medium had little effect until the later stages of growth, when the cultures of strain B were partially inhibited by succinate and the cultures of strain F were stimulated to greater growth.

Polysaccharide production (Fig. 2a and c) by both strains was markedly influenced by the mannitol concentration in the medium. The highest yields were obtained with the lower mannitol concentrations, 2% for strain B and 1% for strain F. Polysaccharide synthesis at these concentrations appeared to proceed almost linearly over the 14-day period. Succinate stimulated polysaccharide synthesis by both strains, but only after 6 days of growth.
Mannitol at a 1% concentration, supplemented with succinate, appeared to be the optimal concentration of carbon source in the medium. 

Effect of sodium succinate concentration. The optimal concentration of succinate to be used in conjunction with 1% mannitol was investigated by examining cultures grown in a medium containing 1% mannitol and 0.2% sodium glutamate and to which four different levels (0.25, 0.5, 0.75, and 1.0%) of sodium succinate were added. The flasks were inoculated with 2-ml volumes of a dense suspension of the bacteria grown for 48 hr on yeast extract-mannitol-agar. The results after 14 days of growth (Fig. 3) suggest that 0.25% sodium succinate was the optimal concentration for maximal growth, but that polysaccharide synthesis was increasingly stimulated by the presence of increasing amounts of succinate over the concentration range examined, the highest yields being obtained with 1% sodium succinate in the medium.

Effect of sodium glutamate concentration. The optimal nitrogen concentration to be used in the medium was found by comparing cultures grown in 1% mannitol medium containing 0.05, 0.1, 0.2, and 0.4% sodium glutamate. Succinate was omitted in this experiment. The flasks were inoculated with 1-ml volumes of a 48-hr culture grown in the medium containing 0.2% sodium glutamate. Growth was examined at intervals, but polysaccharide production was determined only after 9 days. The level of bacterial growth at all stages was found to increase with increasing glutamate concentration in the medium (Fig. 4), but polysaccharide yields were highest in the cultures containing intermediate glutamate concentrations, 0.1% in the case of strain B and 0.2% with strain F.

Pattern of growth and polysaccharide synthesis in the optimal medium. It was concluded from the above experiments that a defined medium containing 1% mannitol, 1% sodium succinate, and 0.2% sodium glutamate, plus the other constituents already described, would be suitable for good growth and polysaccharide yields with the two strains of *Rhizobium meliloti* under investigation. This was tested more closely by growing cultures in this medium and sampling at more frequent intervals to establish the growth pattern. To ensure that growth would proceed at the maximal rate, the flasks were given a heavy inoculum in the form of 5 ml of a 60-hr culture grown in the same medium.

The growth curves (Fig. 5) for both strains showed a short exponential phase, complete within 24 hr from inoculation, followed by a slower rate of increase which continued for 3 to 4 days. A bacterial count on the fourth day indicated $6 \times 10^9$ to $10 \times 10^9$ cells per ml. Growth was virtually complete after 7 to 8 days, by which time a considerable degree of clumping caused the turbidity of the cultures to decrease. Polysaccharide production was insignificant for the first 2 days, after which synthesis appeared to parallel the growth rate until both processes ceased after 8 to 9 days.

The retention of the symbiotic characteristics of the organisms when grown in this medium was tested with 3-day cultures by inoculating test plants [*Medicago truncatula* Desr. and *M. lacini-
TABLE 1. Growth and polysaccharide production by *Rhizobium meliloti* strain *F* in 7 days on the defined medium in a 10-liter fermentor, under conditions of low and high aeration.

<table>
<thead>
<tr>
<th>Air flow</th>
<th>Stirrer</th>
<th>OAR*</th>
<th>Cell dry wt</th>
<th>Polysaccharide</th>
<th>Terminal pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>liter/min</td>
<td>rev/min</td>
<td>mg/ml</td>
<td>mg/ml</td>
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</tr>
<tr>
<td>10.0</td>
<td>750</td>
<td>400</td>
<td>0.95</td>
<td>0.25</td>
<td>10.5</td>
</tr>
<tr>
<td>0.8</td>
<td>250</td>
<td>5</td>
<td>2.90</td>
<td>2.95</td>
<td>7.3</td>
</tr>
</tbody>
</table>

*Oxygen absorption rate, expressed in millimoles of O₂ per liter per hour, determined by sulfite oxidation (Cooper, Fernstrom, and Miller, 1944).

*niiata* (L) Mill.] grown under bacteriologically controlled conditions (Thornton, 1930). The nodulating and nitrogen-fixing behavior of each strain was similar to that demonstrated by Brockwell and Hely (1961). Therefore, growth in this medium did not cause the bacteria to lose their symbiotic properties.

**Effect of aeration on fermentor cultures.** Cultures of strain *F* were grown on a 5-liter scale in the optimal medium in a 10-liter stirred, baffled, aerated fermentor to provide material for antigenic analysis. Comparison of the results (Table 1) obtained under conditions of high and low aeration show that aeration is an important cultural factor. Low aeration gave good growth and polysaccharide synthesis, comparable with that obtained in the shaken flasks, but vigorous aeration gave meager results.

**DISCUSSION**

The present results show that a defined medium containing 1% mannitol plus 1% sodium succinate as the carbon source and 0.2% sodium glutamate as the nitrogen source gave satisfactory levels of growth and extracellular polysaccharide, and is thus suitable for the growth of this organism for immunochromatographic analysis.

The observation that mannitol is the preferred carbon source for growth by *R. meliloti* conflicts with the results of Ferry et al. (1959), who reported that glucose gave better growth than mannitol with their strain of the organism. However, apart from strain differences and the difference in the length of the cultural period used, the results for the two media are not comparable because they differ in a number of ways, including an essential feature, namely, the nature and concentration of the nitrogen source. The present medium contains 0.02% nitrogen in the form of glutamate, whereas that of Ferry et al. (1959) contained 0.14% nitrogen as ammonium sulfate.

The nitrogen concentration in the present defined medium affected growth and polysaccharide yields differently (Fig. 4). Growth levels increased with increased concentrations of glutamate over the range examined (0.05 to 0.4%), whereas polysaccharide yields did not, being highest with intermediate glutamate concentrations. This response of polysaccharide production by *R. meliloti* to nitrogen concentration in the medium, found also in complex media with other strains of *Rhizobium* by Davis and Clapp (1961), resembles that of *Aerobacter aerogenes*, *A. cloacae*, and *Escherichia coli* (Duguid and Wilkinson, 1953; Wilkinson, Duguid, and Edmunds, 1954). With bacteria such as these, which are capable of synthesizing polysaccharides without simultaneous growth, polysaccharide yields appear to be highest in media in which growth is limited by nitrogen and excess carbon is available for continued polysaccharide synthesis.

A corollary of this is that the level of polysaccharide synthesis of *Rhizobium* strains cannot be assessed validly without reference to the medium used. Thus, with the present two strains, growth in the optimal medium leads to higher polysaccharide yields from strain *F* than from strain *B* (Fig. 1). However, in medium containing 0.1% sodium glutamate (Fig. 4b), strain *B* produced more polysaccharide than strain *F*. The concentration of 0.2% glutamate chosen for the optimal medium is not as favorable to cultures of strain *B* as to strain *F*, but represents a compromise choice between maximal growth and polysaccharide yields from both strains. When information is available about the behavior of other strains in
this medium, it may be found necessary to vary the nitrogen level in the medium to obtain maximal growth and polysaccharide yields from bacteria with widely different cultural characters in this respect.

The possibility of increasing polysaccharide yields in bacterial cultures by using components of the tricarboxylic acid cycle in addition to a carbohydrate seems to have been neglected in studies with most bacteria. Acetate, citrate, and succinate are known to stimulate synthesis of cellulose by Acetobacter strains (Greathouse, Shirk, and Minor, 1954; Dudman, 1959). The present experiments show that succinate is effective with \textit{R. meliloti}. Although little increase in polysaccharide production was found with strain B, with strain F the yield from mannitol plus succinate was greater than the sum of the yields obtained when these components were used separately. With \textit{Acetobacter} strains, which are unable to use succinate as the sole carbon source, the mechanism by which increased yields are obtained appears to be a sparing action, but in the case of \textit{R. meliloti}, which is able to grow in the presence of succinate as sole carbon source, the mechanism by which yields are increased is not so clear. The difference in cultural pH between cultures in mannitol with and without succinate may be a factor, because cultures with succinate always have a higher terminal pH level than do those in the same medium without succinate. No differences were detected in the composition of the polysaccharides produced in mannitol cultures with and without succinate. The results of the analysis will be reported separately.

Although no differences were found between cultures grown in smooth-walled and baffled flasks on the rotary shaker, showing that aeration differences at this level were unimportant, larger differences in aeration rates, in a fermentor, led to important differences in growth and polysaccharide yields. These differences were subsequently found to be reflected in differences in the precipitin patterns obtained in immune diffusion analysis of the extracellular soluble antigens of strain F (Dudman, 1964). It is not known whether these cultural and antigenic differences arise directly from the difference in aeration or indirectly from the effect of aeration on the pH level of the cultures. This will be investigated further in cultures grown under conditions of controlled pH.

Acknowledgments

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Literature Cited


