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Exopolymer production by *Bacillus* species

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An exopolymer producing bacterium *Bacillus megaterium* was isolated from the infected plant leaf of *Aralia* species. The isolated organism produced 10–11 g/l of polymer in a synthetic medium consisting of glucose, ammonium nitrate and various salts. Optimum conditions for polymer production were determined. Amongst various carbon and nitrogen sources tested sucrose and ammonium nitrate gave maximum production. C/N ratio of the medium, temperature and pH for optimum polymer production were also investigated. Some of the physico-chemical properties, such as relative viscosity, specific viscosity and reduced viscosity of the polymer were studied at various concentrations and temperatures. Intrinsic viscosity of the polymer was also determined. © 1998 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

Microbial polysaccharides have been compared with traditional plant polysaccharides. The advantages of microbial polysaccharides are novel functionality, constant and reproducible chemical and physical property and a stable cost and supply (MacCormick *et al.*, 1996). They offer a potential new source of functional biopolymers for food, industrial and medical applications. Bacterial polysaccharides are incorporated in food as thickeners, suspending or gelling agents in order to improve food quality and texture (Stephens, 1995). They are used in metal recovery, water clarification and oil well drilling (Sutherland, 1983). They are also used as hydrophillic matrix for controlled release of drug in pharmaceutical industries (Dhami *et al.*, 1995) and some have been used for the development of bacterial vaccines (Cryz, 1990; Jennings, 1990).

Though exopolysaccharides are produced by large number of microorganisms, reports from *Bacillus* species are very few (Dedonder, 1966; Tanaka *et al.*, 1979; Han & Clarke, 1990). In the current investigation, optimum conditions for polymer production by *Bacillus megaterium* as well as some of the physico-chemical properties of the polymer are discussed.

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MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade purity.

Microorganisms

Bacillus megaterium isolated from the infected part of the *Aralia* plant leaf was grown and maintained on a medium containing glucose, yeast extract and various salts.

Polysaccharide production

Inoculum was prepared in a growth medium stated by Sutherland (1983). Medium was prepared and distributed as 100 ml in a 250 ml Erlenmeyer flask and sterilized at 15 psi/15 min. Medium was inoculated and incubated on a rotary shaker (200 rpm) for 24 h at 32±1 °C. Twenty-four-hour-old 1% inoculum was used to seed the sterile production medium with the following composition: carbon source (30 g/l), nitrogen source (0.4 g/l), potassium sulphate (1 g/l), sodium chloride (1 g/l), potassium di-hydrogen phosphate (3 g/l), di-sodium hydrogen phosphate (10 g/l), calcium chloride (0.02 g/l), magnesium sulphate (0.2 g/l), ferrous sulphate (0.001 g/l), distilled water (1000 ml) and incubated on a rotary shaker (200 rpm) at 32±1 °C. At timed intervals samples were withdrawn for analysis.

Isolation of the polysaccharide

Culture medium was diluted on the basis of its viscosity and centrifuged at 16000 rpm for 40 min. The clear supernatant thus obtained was precipitated by 3 volumes of acetone. Precipitates were washed twice with acetone under vacuum to a constant weight.

Analytical methods

Reducing sugar from the culture filtrate was determined by the DNS method (Miller, 1959). Polysaccharide estimation was carried out by gravimetric analysis. Monomers of the polysaccharide were detected by hydrolysing the polymer with 2 M TFA at 100°C for 6 h and subjecting it to paper chromatography using butanol: pyridine: water (6:4:3) as the solvent system and ammoniacal silver nitrate as the spray reagent. Glucose in the polysaccharide was estimated by the glucose oxidase kit (Sigma) using glucose as a standard. Growth was measured by resuspending the washed cell pellet in distilled water and measuring the optical density at 660 nm using a Shimadzu double beam spectrophotometer. Physical properties of the polysaccharide were studied by suspending dried polysaccharide in a known volume of distilled water and viscometric measurements were carried out using the Ubbelohde Viscometer.

RESULTS AND DISCUSSION

Bacillus megaterium produced an exopolysaccharide consisting of glucose residues in preliminary observation.

The pattern of polysaccharide production varies with the type of organism and medium composition. Polysaccharide production may either be growth-associated (Deavin *et al.*, 1977), non-growth-associated (Evans *et al.*, 1978) or mixed (Moraine & Rogovin, 1973). The growth pattern of our isolate was studied in batch culture. It was found that polysaccharide production increased with cell growth. Optimum polysaccharide production as well as cell growth was obtained after 96 h. (Fig. 1). With the decrease in cell growth, a decrease in polysaccharide production was observed which suggests the growth-associated production of polysaccharide. *Bacillus megaterium* has a fermentation cycle of 4 days which is much shorter than the widely reported Gram positive *Bacillus polymyxa* which is 10 days (Han & Clarke, 1990).

The type of carbon source plays an important role in growth as well as polysaccharide production. A carbon source which supports excellent growth may not always yield significant polysaccharide. A substantial change in the polysaccharide production was observed with different carbon sources. Lactose supported maximum

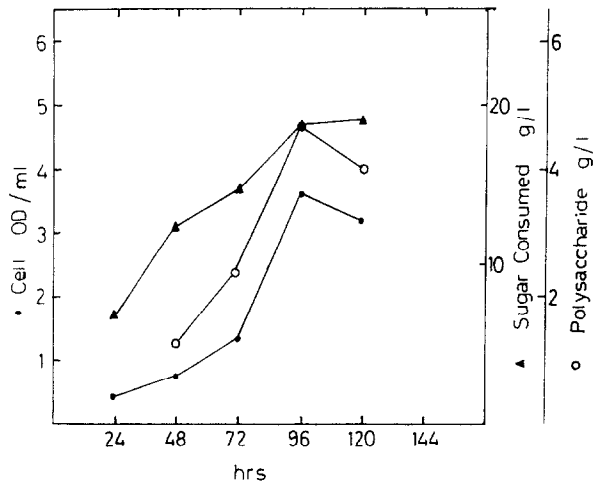

Fig. 1. Growth and polysaccharide production at different time intervals.

growth without detectable polysaccharide production. Sucrose supported maximum polysaccharide production of 9.2 g/l, whereas fructose gave optimum yield coefficient (YP/S) of 0.68 as shown in Table 1. The yield is higher than many of the polysaccharides reported in the literature under similar conditions (Scheepe-Leberkuhne & Wagner, 1986; Roberts *et al.*, 1995; Okabe *et al.*, 1981). Amongst different nitrogen sources ammonium nitrate gave maximum polysaccharide production of 11.7 g/l and YP/S of 0.48 as compared with other nitrogen sources (Table 2).

Carbon/nitrogen ratio (C/N ratio) in the growth medium plays an important role in polysaccharide production (Scheepe-Leberkuhne & Wagner, 1986). In this context we examined C/N ratios by varying carbon and nitrogen concentrations as depicted in Fig. 2. By varying carbon concentration at a fixed nitrogen (concentration) in the range of C/N 50–200, it was observed that by increasing the C/N ratio from 50 to 75, polysaccharide production increased with the reduction in growth. In the range C/N 75–150, there was no substantial effect on growth as well as polysaccharide production. Beyond the C/N of 150, polysaccharide production increased but growth decreased. At fixed carbon (concentration) and varying nitrogen concentrations, it was observed that with the increase in C/N ratio, growth increased but the polysaccharide production decreased. At a very high C/N ratio, growth as well as polysaccharide production decreased.

Temperature is often a critical factor in polysaccharide synthesis. The effect of temperature on growth and polysaccharide production was studied in the range 25–35°C. Low polysaccharide production of 3.1 g/l and YP/S of 0.17 was obtained at 25°C, whereas optimum polysaccharide production of 9.8 g/l and YP/S of 0.45 were obtained in the temperature range of 30–35°C.

Table 1. Effect of carbon sources on growth and polysaccharide production by *Bacillus* species

Carbon source	Cell O.D. (660/ml)	Sugar[a] consumed (g/l)	Polysaccharide (g/l)	YP/S
Glucose	2.20	22.40	4.00	0.18
Fructose	2.88	16.48	1.10	0.68
Sucrose	2.48	19.27	9.20	0.48
Lactose	3.41	12.77	ND	ND
Maltose	2.61	22.33	2.80	0.13

ND, not determined.
[a]Initial concentration of sugar was 30 g/l.

Table 2. Effect of nitrogen sources on growth and polysaccharide production by *Bacillus* species

Nitrogensource	Cell O.D. (660/ml)	Sugar[a] consumed (g/l)	Polysaccharide(g/l)	YP/S
Ammonium sulphate	2.50	23.12	10.04	0.45
Ammonium chloride	1.94	21.47	9.10	0.37
Ammonium oxalate	2.88	21.12	7.86	0.37
Ammonium nitrate	3.60	24.00	11.70	0.49
Triammonium citrate	3.66	23.42	9.20	0.39
Diammonium phosphate	3.20	21.47	9.90	0.46
Sodium nitrate	3.44	25.63	7.60	0.30
Potassium nitrate	3.64	23.07	11.10	0.48
Urea	2.94	22.60	0.10	0.01

[a]Initial concentration of sugar was 30 g/l.

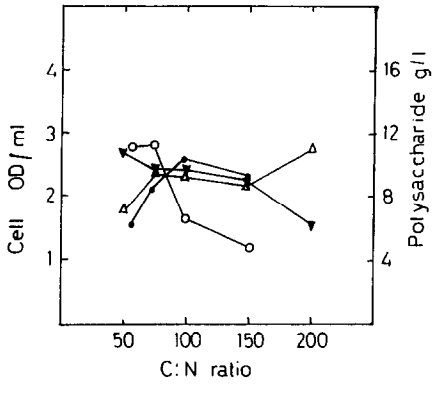

Fig. 2. Effect of C/N ratio on growth and polysaccharide production. Growth (▲) and polysaccharide production (△) at a fixed carbon concentration. Growth (•) and polysaccharide production (○) at a fixed nitrogen concentration.

Optimum pH for growth and polysaccharide production for bacteria ranges from 6.0 to 7.5 (Margaritis & Pace, 1986). *Bacillus megaterium* was grown at a different pH for polysaccharide production and the results are depicted in Table 3. Optimum growth, polysaccharide production and YP/S were obtained in the neutral pH range.

Successful application of polysaccharide largely depends on their physico-chemical properties rather than yield alone. To gain insight about properties and behaviour, relative viscosity (nr), specific viscosity (nsp) and reduced viscosity (nsp/c) were studied at different concentrations, temperature and pH values (Figs 3-5). It was observed that with the increasing concentration of the polysaccharide there was an increase in viscosity functions (nr, nsp, nsp/c) (Fig. 3). Decreases in viscosity functions were observed when the polysaccharide solution was subjected to the temperature range of 35–70°C (Fig. 4). Similarly, by

Table 3. Effect of initial pH of medium on growth and yield of polysaccharide in *Bacillus* species

Initial pH	Cell O.D. (660/ml)	Sugar[a] consumed (g/l)	Polysaccharide(g/l)	YP/S	Final pH
5.00	1.53	16.43	2.80	0.17	5.25
6.00	2.80	22.00	9.80	0.45	6.00
7.00	3.53	23.27	10.25	0.44	6.50
8.00	3.11	21.36	8.90	0.42	7.80
9.00	2.30	19.17	8.10	0.42	8.90

[a]Initial concentration of sugar was 30 g/l.

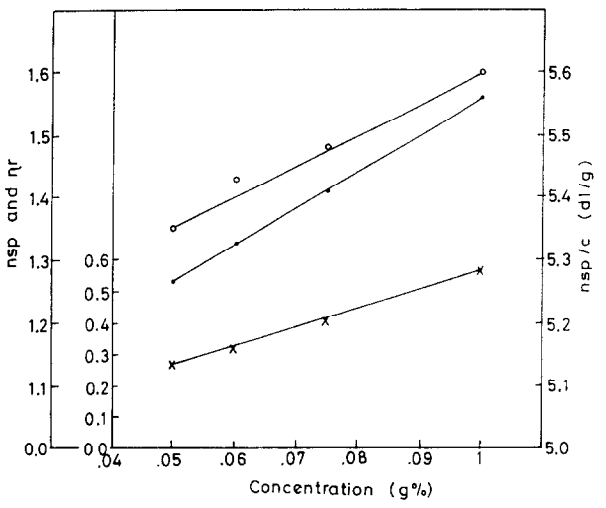

Fig. 3. Effect of concentration of polysaccharide on viscosity functions of *Bacillus* polysaccharide. (•) Relative viscosity (nr). (×) Specific viscosity (nsp). (○) Reduced viscosity (nsp/c).

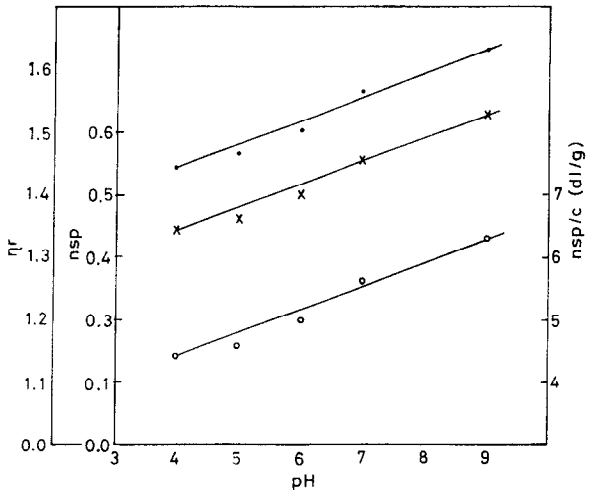

Fig. 5. Effect of pH on viscosity functions of *Bacillus* polysaccharide. (•) Relative viscosity (nr). (×) Specific viscosity (nsp). (○) Reduced viscosity (nsp/c).

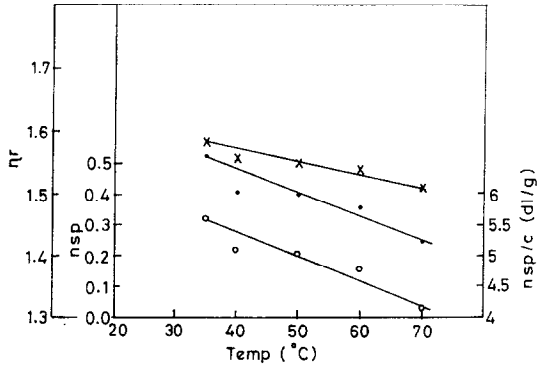

Fig. 4. Effect of temperature on viscosity functions of *Bacillus* polysaccharide. (•) Relative viscosity (nr). (×) Specific viscosity (nsp). (○) Reduced viscosity (nsp/c).

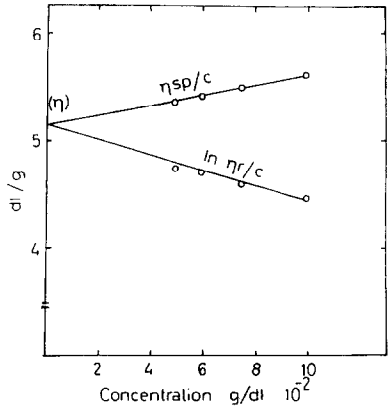

Fig. 6. Determination of the intrinsic viscosity of the polysaccharide.

increasing the pH of the polysaccharide solution, increases in the viscosity functions were observed (Fig. 5). Intrinsic viscosity is considered as a characteristic parameter which governs many physico-chemical aspects of the polymers. It also explains the hydrodynamic property of colloidal or macromolecular solutes. Intrinsic viscosity was determined from the intercept of the plot of ln nr/c vs concentration and nsp/c vs concentration. At 35°C intrinsic viscosity of the polysaccharide was 5.2 dl/g (Fig. 6). This will enable us to predict the molecular weight (DP) of the polysaccharide upon further characterization.

CONCLUSION

Optimum production of the polysaccharide is obtained in a medium containing sucrose and ammonium nitrate. The optimum C/N ratio for polymer production is found to be 75. A temperature range of 30–35°C and pH of 7 is found to be optimum. Temperature and pH have pronounced effects on the polysaccharide solution. The polysaccharide has an intrinsic viscosity of approx. 5.2 dl/g.

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