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Preparation and Preliminary Characterization of Exopolysaccharides by Yeast *Rhodotorula acheniorum* MC

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Abstract

The effects of various carbon and nitrogen sources on the synthesis of exopolysaccharides by *Rhodotorula acheniorum* MC were studied. The dynamic viscosity of cell-free culture broths during exopolysaccharide synthesis were measured. The highest values for the viscosity (10.14 MPa · s) and crude polysaccharide productivity (6.6 g/L) were obtained in a medium supplied with 5% sucrose. Ammonium sulfate was the most favorable nitrogen source for exopolysaccharide synthesis. The value of pH played a determinant role, and the obligatory condition for exopolysaccharide production was low (pH 1.7–2.0) during the fermentation. The chemical composition and sugar constituents of the crude exopolysaccharides were determined. Mannose was the main monosaccharide component, and its concentration was the highest (69.13%) in the crude exopolysaccharide synthesized in the medium that included 5% sucrose as a carbon source.

Index Entries: Polysaccharide; *Rhodotorula acheniorum*; mannose.

Introduction

Because of their diversity in composition, structure, and physical properties, microbial exopolysaccharides have found application in the food, pharmaceutical, and other industries. Among the biopolymers with industrial application are bacterial and fungal products such as xanthan, dextran, and scleroglucan *(1–5)*. Yeast belonging to different genera produce

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exopolysaccharides too. The types of polymers reported for yeast producers include mannans, glucans, glucomannans, galactomannans, and phosphomannnans *(6–12,21)*.

Microbial exopolysaccharides are related to the secondary metabolites, and their structure and physical properties depend on the composition of the fermentation medium and growth conditions. Studies on the effect of the growth-limiting substrate on the synthesis of exopolysaccharides clearly demonstrate that the composition of the growth medium can dramatically affect the specific rate of polymer synthesis *(13,14)*. Manipulation of exopolysaccharide composition by changing the growth medium is one possible mechanism to change the degree of polymerization and properties of synthesized polymer *(4,15,16)*. The amount of carbon substrate converted by the microbial cells to polymer depends on the composition of growth medium. Generally, media containing a high carbon to limiting nutrient ratio are favored for polysaccharide production *(1)*. The carbon and nitrogen sources used do not affect the qualitative monosaccharide composition of the polysaccharide, although the introduction of ammonium salts in the medium can cause a change in the monosaccharide ratio in the side chains of the polymer *(10)*. The composition of the growth medium can also indirectly affect the polymer yield by governing the charge that can occur during fermentation without pH control *(13,14)*.

In our previous investigations, the strain *Rhodotorula acheniorum* MC was chosen by multistage selection of 69 yeast strains belonging to the genera *Cryptococcus*, *Rhodotorula*, *Hansenula*, and *Sporobolomyces* according to their ability to synthesize polysaccharides on 4% glucose *(17)*.

We were interested in establishing an optimal fermentation medium and growth conditions for the synthesis of crude exopolysaccharides with high mannose concentration. This effort involved the effects of carbon and nitrogen sources on exopolysaccharide production, dynamic viscosity measurement, and their relationship to cell physiology and polymer composition.

Materials and Methods

Strain

The yeast strain *R. acheniorum* MC was selected as a suitable exopolysaccharide producer and registered in the National Bank for Industrial Microorganisms and Cell Culture, Bulgaria. It was maintained on malt slant agar and stored at 4°C.

Media and Growth Conditions

The basal medium for the testing of carbon sources contained 0.25% $(NH_4)SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% NaCI, 0.01% $CaCI_2 \cdot 2H_2O$, and 0.1% yeast extract. As carbon sources, glucose,

galactose, sucrose, sirodex (kindly supplied by Tsaremil Inc. Private Joint Stock Company, Razgrad, Bulgaria; represented hydrolyzed corn starch), and molasses were tested and supplemented to the basal medium in different concentrations.

The basal medium for the testing nitrogen contained 5.0% glucose, 0.1% KH_2PO_4, 0.01% NaCI, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCI_2 \cdot 2H_2O$, 0.1% yeast extract, and different nitrogen sources. As nitrogen sources, $(NH_4)_2SO_4$, NH_4CI, $(NH_4)_2HPO_4$, NH_4NO_3, $Ca(NO_3)_2 \cdot 4H_2O$, peptone, and yeast extract were tested and were added to the basal medium in different concentrations. The initial pH was adjusted to 5.3–5.6, and the media were sterilized at 112°C for 40 min. In some experiments, $CaCO_3$ (0.5%) was added to maintain the initial pH values during the fermentations.

The inoculum from *R. acheniorum* MC was obtained on a rotary shaker (220 min^{-1}) in 500-mL Erlenmeyer flasks containing 50 mL of malt extract at 28°C for 48 h.

The fermentation media were inoculated with 1.0% w/v inoculum. The cultivation was carried out in 500-mL Erlenmeyer flasks containing 50 mL of the tested medium on a rotary shaker (220 min^{-1}) at 26°C for 144 h.

Isolation of Crude Exopolysaccharides

Whole-cell cultures were centrifuged at 6000*g* for 30 min to separate cells from the supernatant. The exopolysaccharides in the culture supernatants were precipitated with 2 vol of 96% ethanol at 4°C for 18–24 h. The resultant precipitate was recovered by centrifugation at 5000*g* for 10 min. The supernatant was discarded, and the pellet was washed twice with ethanol, dried, and weighed.

Analytical Methods

Dry weight of the yeast biomass was determined at 105°C until constant weight. The residual sugar concentration was measured by the 3-5 dinitrosalicylic acid method *(18)*.

The total amount of carbohydrates in the crude exopolysaccharides was determined using the phenol–sulfuric acid method *(19)*. The amount of total protein was determined on the solution of nonhydrolyzed polysaccharides according to the method of Lowry. The ash content was estimated after calcination for 2 h and glowing the polymer at 550°C for 3 h.

The carbohydrate composition was determined by gas chromatography using Fractovap 2407 (Carbo Erba) after hydrolysis of crude exopolysaccharides with 4 *N* H_2SO_4 at 105°C for 8 h, neutralization with barium hydroxide, followed by centrifugation and using cation exchanger Wofatit KPS for elimination of Ba^+ ions.

Dynamic viscosity of the cell-free culture broth was measured using Rheo-Viscometer type Hoppler at t^o = 25°C, K = 0.000644, P = 10 g/cm^2. The data were statistically analyzed using Sigma Plot (Version 100) and the standard deviations (SDs) were determined.

Table 1
Effect of Several Sugars on Exopolysaccharide Production

Sugar	Concentration (%) Initial	Residual[a]	Final pH	Biomass dw (g/L)[a]	Exopolysaccharide (g/L)[a]
Glucose	2.0	0.11 ± 0.02	1.84	5.80 ± 0.27	1.25 ± 0.08
	3.0	0.18 ± 0.02	1.87	6.80 ± 0.08	2.85 ± 0.10
	4.0	0.26 ± 0.01	1.84	8.60 ± 0.09	4.50 ± 0.09
	5.0	0.35 ± 0.02	1.90	9.90 ± 0.15	5.00 ± 0.13
Galactose	2.0	0.15 ± 0.03	2.19	6.57 ± 0.16	0.63 ± 0.05
	3.0	0.15 ± 0.03	1.99	8.80 ± 0.21	2.19 ± 0.05
	4.0	0.18 ± 0.02	1.85	9.63 ± 0.06	4.23 ± 0.10
	5.0	0.26 ± 0.03	1.80	10.76 ± 0.15	4.86 ± 0.12
Sucrose	2.0	0.35 ± 0.03	1.96 ± 0.17	6.60 ± 0.17	2.11 ± 0.06
	3.0	0.35 ± 0.02	1.87 ± 0.09	8.05 ± 0.09	3.52 ± 0.06
	4.0	0.37 ± 0.04	1.85 ± 0.10	9.75 ± 0.10	4.90 ± 0.08
	5.0	0.37 ± 0.04	1.87 ± 0.07	10.60 ± 0.07	6.15 ± 0.20
Sirodex	2.0	0.33 ± 0.03	1.86	5.30 ± 0.10	2.86 ± 0.06
	3.0	0.42 ± 0.03	1.84	7.48 ± 0.13	3.20 ± 0.05
	4.0	0.45 ± 0.02	1.92	10.05 ± 0.09	4.80 ± 0.14
	5.0	0.64 ± 0.04	1.92	15.11 ± 0.10	6.10 ± 0.06
Molasses	3.0	0.42 ± 0.02	5.83	13.63 ± 0.09	2.37 ± 0.06
	5.0	0.61 ± 0.07	5.10	15.42 ± 0.15	2.64 ± 0.07

[a]Values represent the mean ± SD from assays of three samples; dw = dry weight.

Results and Discussion

The effects of several sugars in different concentrations on exopolysaccharide synthesis were examined. The results, summarized in Table 1, show that high polysaccharide production was obtained when the medium was supplied with 5% sucrose as well as sirodex (6.15 and 6.10 g/L, respectively). In all cases of cultivation, the final pH values declined below 2.0 with the exception of molasses. There was no acidification in the medium supplemented with molasses, which resulted in poor polysaccharide production although the biomass concentration reached 15.42 g/L. Of the sugars tested, sucrose was the most suitable for both cell growth (biomass concentration) and polysaccharide production. The time course of polysaccharide synthesis, biomass concentration, sugar consumption, and pH values in a medium containing 5% sucrose are shown in Fig. 1. Note that the pH value dropped rapidly to 1.7–2.0 within 24 h, remaining constant during 144 h of incubation. Polysaccharide concentration reached the maximum when sugars were almost exhausted by the late stationary phase (96–120 h), and it was not identical to the maximum of biomass (72 h).

Dynamic viscosities of the cell-free culture broths during exopolysaccharide synthesis by *R. acheniorum* MC on sucrose is shown in Fig. 2. As can be seen in Fig. 2, the culture broths were with low viscosity when the strain was cultivated in media with 2 or 3% sugars. Dynamic viscosity

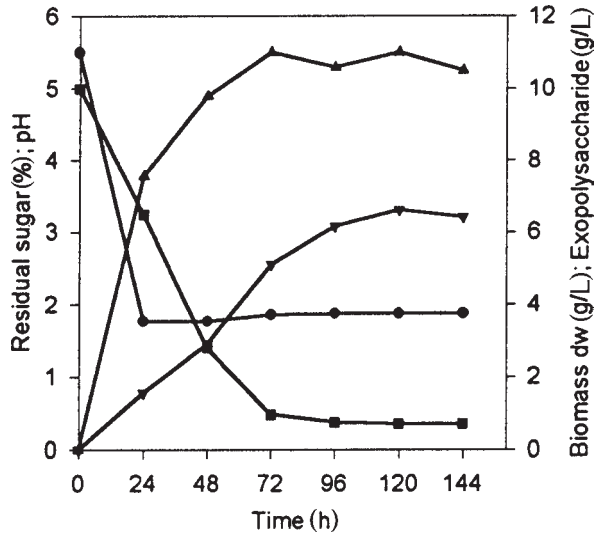

Fig. 1. Time course of polysaccharide synthesis, cell growth, pH, and sucrose consumption. (—●—), pH; (—■—), residual sugar; (—▲—), biomass; (—▼—), exopolysaccharide. dw = dry weight.

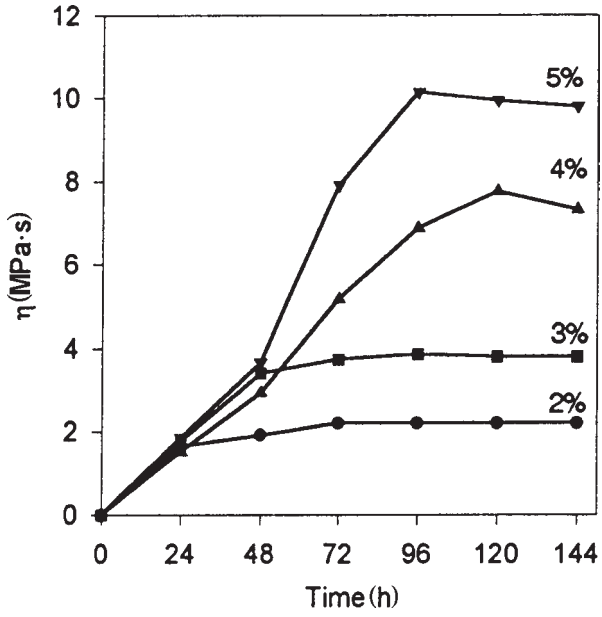

Fig. 2. Dynamic viscosity of the cell-free culture broth during polysaccharide synthesis on different percentages of sucrose.

appeared to be associated with cell physiology and exopolysaccharide production. Carbon sources in low concentrations were not suitable for exopolysaccharide synthesis by *R. acheniorum* MC because the carbon was almost used up for biomass production.

The highest value of the viscosity showed the cell-free culture broth during exopolysaccharide synthesis in the medium with 5% sucrose. The viscosity reached a maximum of 10.14 MPa · s at 96 h of yeast incubation on this substrate. Note that this maximum was not identical to that of exopolysaccharide production (Fig. 1). Probably polysaccharide macromolecules undergo depolymerization, and the values of viscosity therefore decline.

The chemical composition of the crude exopolysaccharides synthesized on different carbon sources is shown in Table 2. Total carbohydrates ranged from about 68 to 76%. Protein and ash were present in all products. The constituent monosaccharides were determined using gas chromatographic analysis after hydrolysis of the polymers. As can be seen in Table 2, mannose was the main component of the yeast polymers, and its concentration was the highest (69.13%) for the crude exopolysaccharide synthesized on sucrose. On the basis of these results, sucrose could be selected as the most suitable carbon source for synthesis of exopolysaccharide by *R. acheniorum* MC and used for pure mannan production.

The effects of several nitrogen sources on the production of exopolysaccharides by *R. acheniorum* MC were examined (Table 3). The yeast was incubated at 26°C for 96 h in the media mentioned previously. Comparing the influence of different ammonium salts on the yeast growth and metabolic production, it was found that the NH_4^+ ion played a central role in nitrogen metabolism as the form in which nitrogen incorporated into organic cell components (biomass). All ammonium salts tested ensured active cell growth, but for exopolysaccharide synthesis only, $(NH_4)_2HPO_4$ was not suitable. As can be seen from the polysaccharide yields, the low concentration of ammonium salts ensured the highest biomass production, but exopolysaccharide extraction was impossible. The growth with NH_4^+ was accompanied by a rapid decrease in pH values when NH_4^+ components were utilized. The highest exopolysaccharide production and viscous culture broth were obtained with a concentration of ammonium salts of 0.25–0.30%, but there was no correspondence with the yield of biomass. Nitrate can be used by some yeasts for their growth. In the medium supplemented with $Ca(NO_3)_2 \cdot 4H_2O$, cell growth of *R. acheniorum* MC was poor and polysaccharides were barely produced.

Many organic nitrogen sources can be utilized if the yeasts are capable of breaking down these components into smaller units that can be transported into the cells. As Table 3 shows, *R. acheniorum* MC utilized peptone and yeast extract. Higher concentrations promoted the cell growth but not polysaccharide production. The pH value did not change remarkably during cultivation on these substrates, and culture broths did not become viscous. This was probably one of the reasons for poor biopolymer production.

The viscosity was measured on the cell-free culture broths after 96 h of cultivation of *R. acheniorum* MC in media supplemented with 0.25% $(NH_4)_2SO_4$, NH_4Cl, or NH_4NO_3, and the values of viscosity were 5.16, 4.36, and 4.53 MPa · s, respectively. The chemical composition of the crude

Table 2
Chemical Composition of Crude Exopolysaccharides Synthesized in Media with Different Carbon Sources

Carbon source	Carbohydrate (%)[a]	Protein (%)[a]	Ash (%)[a]	Monosaccharide (% of carbohydrate content)				
				Arabinose	Fucose	Mannose	Galactose	Glucose
Glucose	76.40 ± 1.6	6.30 ± 0.20	15.4 ± 0.9	—	4.00	64.40	8.20	23.40
Galactose	68.52 ± 1.5	8.30 ± 0.30	23.1 ± 0.8	4.38	7.28	64.85	9.49	14.00
Sucrose	75.30 ± 1.7	7.25 ± 0.20	17.8 ± 1.2	3.07	5.47	69.13	8.30	13.73
Sirodex	75.15 ± 1.5	7.16 ± 0.30	17.6 ± 1.2	—	3.35	64.05	8.25	24.35

[a]Values represent the mean ± SD from assays of three samples.

Table 3
Effect of Several Nitrogen Sources of Polysaccharide Production

Nitrogen source	Concentration (%)	Final pH	Biomass dw (g/L)[a]	Polysaccharide (g/L)[a]
$(NH_4)_2SO_4$	0.100	2.40	11.83 ± 0.31	0.00
	0.150	2.32	10.52 ± 0.10	2.00 ± 0.12
	0.200	2.16	9.75 ± 0.07	3.30 ± 0.09
	0.250	2.00	8.40 ± 0.14	4.92 ± 0.07
	0.300	1.81	7.55 ± 0.11	3.80 ± 0.10
	0.350	1.75	7.26 ± 0.12	3.40 ± 0.12
NH_4Cl	0.100	2.14	14.20 ± 0.10	0.00
	0.150	1.98	13.30 ± 0.13	1.90 ± 0.04
	0.200	1.70	10.40 ± 0.11	3.20 ± 0.14
	0.250	1.60	9.20 ± 0.13	3.92 ± 0.12
	0.300	1.56	8.75 ± 0.12	4.14 ± 0.10
	0.350	1.45	8.32 ± 0.09	3.80 ± 0.08
$(NH_4)_2HPO_4$	0.100	2.67	13.20 ± 0.13	0.60 ± 0.06
	0.150	2.34	11.30 ± 0.12	1.50 ± 0.08
	0.200	2.22	10.70 ± 0.15	1.80 ± 0.11
	0.250	2.14	9.80 ± 0.13	1.75 ± 0.09
	0.300	2.05	9.50 ± 0.22	1.55 ± 0.19
NH_4NO_3	0.100	2.16	11.70 ± 0.16	0.30 ± 0.06
	0.150	2.03	11.50 ± 0.13	2.40 ± 0.13
	0.200	1.86	10.80 ± 0.22	3.50 ± 0.16
	0.250	1.78	9.30 ± 0.12	4.22 ± 0.11
	0.300	1.60	8.85 ± 0.14	4.40 ± 0.18
	0.350	1.55	8.50 ± 0.15	4.45 ± 0.07
$Ca(NO_3)_2 \cdot 4H_2O$	0.050	4.60	5.80 ± 0.08	0.80 ± 0.07
	0.100	4.45	5.60 ± 0.18	1.70 ± 0.20
	0.150	4.38	5.60 ± 0.11	2.05 ± 0.10
	0.200	4.23	5.00 ± 0.18	0.00
Peptone	0.100	4.50	9.40 ± 0.19	1.29 ± 0.15
	0.200	4.38	10.60 ± 0.18	1.54 ± 0.17
	0.300	4.15	12.80 ± 0.18	1.38 ± 0.10
Yeast extract	0.150	4.58	7.70 ± 0.16	1.24 ± 0.07
	0.300	4.20	13.50 ± 0.16	1.87 ± 0.15
	0.450	4.02	17.90 ± 0.11	0.16 ± 0.07

[a]Values represent the mean ± SD from assays of three samples; dw = dry weight.

exopolysaccharides isolated by alcohol precipitation of these cell-free culture broths is shown in Table 4. The total carbohydrate content ranged from about 72 to 76%. Protein and ash were present in all products. The data in Table 4 also illustrate that constituent monosaccharides were glucose, mannose, galactose, and fucose. The main component was mannose, and its concentration was the highest (67.50% of carbohydrate content) in the crude exopolysaccharide synthesized in the medium with NH_4NO_3. Ammonium sulfate was the most favorable nitrogen source for exopolysaccharide synthesis by *R. acheniorum* MC because of the highest viscosity of culture broth.

Table 4
Chemical Composition of Crude Exopolysaccharides Synthesized in Media with Different Nitrogen Sources

Nitrogen source	Carbohydrate (%)[a]	Protein (%)[a]	Ash (%)[a]	Monosaccharide (% of carbohydrate content)				
				Arabinose	Fucose	Mannose	Galactose	Glucose
$(NH_4)_2SO_4$	76.40 ± 1.8	6.30 ± 0.4	15.4 ± 0.9	—	4.00	64.40	8.20	23.40
NH_4Cl	72.80 ± 1.7	8.50 ± 0.3	17.6 ± 1.2	—	4.28	62.87	6.37	26.48
NH_4NO_3	74.60 ± 1.7	7.20 ± 0.3	18.2 ± 1.1	—	3.02	67.50	5.10	24.38

[a]Values represent the mean ± SD from assays of three samples.

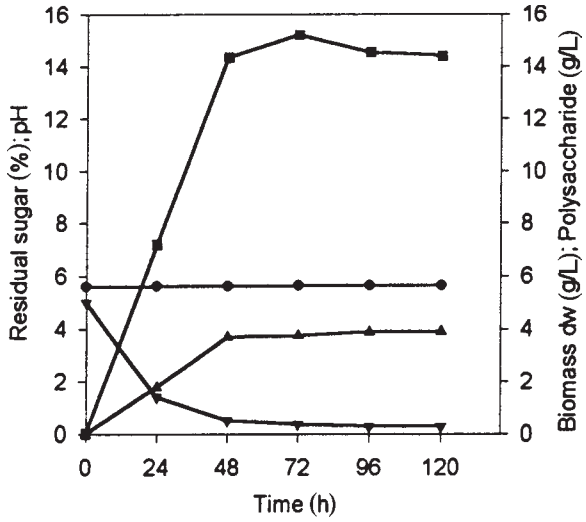

Fig. 3. Time course of polysaccharide synthesis, cell growth, pH, and glucose consumption in a media with $CaCO_3$. (—●—), pH; (—■—), biomass; (—▲—), polysaccharide; (—▼—), residual sugar.

To confirm our standpoint for the determinant role of pH value in exomannan synthesis, the yeast strain was grown in media containing 5.0% glucose, 0.10% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.01% $CaCl_2$, 0.1% yeast extract, and 0.5% different ammonium salts and $CaCO_3$ to maintain initial pH value during fermentation. The time course of polysaccharide synthesis, biomass production, pH, and glucose consumption in the medium supplemented with 0.25% $(NH_4)_2SO_4$ are shown in Fig. 3. In this medium, pH value was maintained constant during 120 h of incubation. Under these conditions, 15.2 g/L of biomass were produced at 72 h of cultivation and 3.8 g/L of exopolysaccharides were synthesized. Note that the cell-free culture broth had low viscosity (2.43 MPa · s) and that the resultant exopolysaccharide precipitate was different compared with that of the culture broth without $CaCO_3$. The total carbohydrate content in this product was very low (32.80%), since salts were coprecipitated. The constituent monosaccharides were 27.14% mannose, 13.25% galactose, 44.50% glucose, 10.58% xylose, and 4.00% arabinose (percentage of carbohydrate content). Their ratio was different compared with the monomer compositions of exopolysaccharides synthesized in media without $CaCO_3$ (Table 4). The concentration of mannose in this product was low, but it was high for the glucose. These results showed that maintaining the initial pH value during fermentation was not suitable for the synthesis of exomannan by *R. acheniorum* MC. These findings revealed additional information that correlates quite well with the results of other investigators *(20)*. When NH_4Cl or NH_4NO_3 were added as nitrogen sources, high biomass productions (17.7 or 15.10 g/L) were obtained, but under these conditions exopolysaccharides were not synthesized.

A medium containing 5.0% sucrose, 0.25% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% NaCl, 0.01% $CaCl_2 \cdot 2H_2O$, and 0.1% yeast extract was selected on the basis of these results.

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