

($M_r = 248.16$) $w_i(\text{calc.})$: 33.88 % C, 5.28 % H; $w_i(\text{found})$: 33.60 % C, 5.35 % H.

The same procedure, but with the addition of the precipitate II in three portions, afforded V hydrate (0.6 g, 51 %) and sodium salt of VII hydrate (0.3 g, 24 %).

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Production of Extracellular Polysaccharides by *Candida mucifera*

J. DANIŠ, A. Kardošová, and E. Sláviková

*Institute of Chemistry, Slovak Academy of Sciences,
CS-842 38 Bratislava*

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Dedicated to Dr. Ing. Š. Bauer, DrSc., in honour of his 70th birthday

Candida mucifera, CCY 29-170-1, when cultured in liquid media with various carbon sources, produced D-mannose-dominating extracellular polysaccharides; the only exception was the medium with beer wort, where the dominating sugar component was D-glucose. All the polysaccharides were heterogeneous upon electrophoresis. The protein moiety in the polysaccharides varied from 3 to 16 % and the content of phosphorus from 0.5 to 1.1 %. The lowest limiting viscosity number ($14 \text{ cm}^3 \text{ g}^{-1}$) was found with the D-glucose-dominating polysaccharide and the highest one ($67 \text{ cm}^3 \text{ g}^{-1}$) with the D-mannose-dominating polysaccharide.

In an earlier report Kocková-Kratochvílová and Sláviková [1] described a new yeast, named *Candida mucifera*. It was originally isolated from frog liver in Amazonian virgin forest, near the city Manaus (Brasil). The ability to utilize methanol as the only carbon source and produce large amounts of extracellular polysaccharides, when grown in liquid medium containing sugar, was considered an important characteristics of this species.

Therefore, it was of interest to characterize the composition and some physicochemical properties of the extracellular polysaccharides produced in liquid media containing various carbon sources, which is the subject of the present work.

EXPERIMENTAL

All evaporations were conducted under diminished pressure at 40–45 °C. Paper chromatography was performed by the descending method on Whatman No. 1 paper with the following systems: S_1 , ethyl acetate–pyridine–water ($\varphi_r = 8 : 2 : 1$); S_2 , ethyl acetate–acetic acid–formic acid–water ($\varphi_r = 18 : 3 : 1 : 4$); S_3 , ethyl acetate–pyridine–acetic acid–water ($\varphi_r = 5 : 5 : 1 : 3$). Reducing sugars were detected by spraying with anilinium hydrogen phthalate and heating the papers for 5 min at 105 °C, and alditols with alkaline silver nitrate reagent. Quantitative deter-

mination of sugar components of polysaccharides (as alditol trifluoroacetates) was carried out by GLC analysis with a Hewlett–Packard Model 5711 chromatograph using a column (200 cm x 0.3 cm) of OV-225 on 150–170 μm Chromosorb W (AW-DMCS) programmed to hold a temperature of 120 °C for 4 min, then to increase it to 170 °C at 2 °C min^{-1} .

Determination of protein content was carried out by the method of Lowry *et al.* [2] using bovine serum albumin as a standard. The amino acid composition was established with an Automatic amino analyzer, type 6020 (Mikrotechna, Prague), after hydrolysis of the polysaccharides with 6 M-HCl for 20 h at 100 °C. The methoxy groups content was determined by the method of Viebock and Brecher [3] and phosphorus content after combustion of the samples by titration with $\text{Pb}(\text{NO}_3)_2$ using dithizone as the indicator.

Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter in an 0.5 % aqueous solution at 22 °C. Free-boundary electrophoresis was effected with a Zeiss 35 apparatus on a polysaccharide ($\rho = 10 \text{ mg cm}^{-3}$) in 0.05 M sodium borate buffer at 10 V cm^{-1} and 6 μA .

Viscosity measurements of aqueous solutions of polysaccharides ($(0.1\text{--}0.5) \times 10^{-2} \text{ g cm}^{-3}$) were carried out with an Ubbelohde viscometer at a temperature of 25 °C. The relative, specific, and reduced viscosities were calculated from the following equations

$$\text{relative viscosity } \eta_r = \frac{\rho_2 t_2}{\rho_1 t_1}$$

$$\text{specific viscosity } \eta_{sp} = \eta_r - 1$$

$$\text{reduced viscosity } \eta_{red} = \frac{\eta_{sp}}{c}$$

Here ρ_1 and ρ_2 are the densities (in g cm^{-3}) and t_1 and t_2 the times of efflux (in seconds) for the solvent and solution, respectively, and c is the concentration of the solution (in $\text{g}/(100 \text{ cm}^{-3})$). Since in the range of the dilute solutions ($< 0.5 \%$) the difference in densities between the solution and solvent was insignificant, the density corrections were neglected.

Cultivation Conditions

The type strain *Candida mucifera*, CCY 29-170-1, was maintained on malt agar in the Czechoslovak Collection of Yeasts at the Institute of Chemistry, Slovak Academy of Sciences, Bratislava. *C. mucifera* was cultured on six media containing different carbon sources. The basal medium consisted of 0.1 % KH_2PO_4 , 1 % $(\text{NH}_4)_2\text{SO}_4$, 0.05 %

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.035 % CaCl_2 , and 0.3 % yeast extract. Composition of the media: 1. basal medium, 2. basal medium and 1 % methanol, 3. basal medium and 2 % methanol, 4. basal medium and 3 % methanol, 5. basal medium and 2 % D-glucose, 6. beer wort diluted to 7 mass % extract. Methanol was added into the medium after autoclaving at 120 °C and 0.19 MPa for 20 min and cooling.

A loopful of cells from malt agar was used to inoculate 25 cm^3 (in 100 cm^3 Erlenmeyer flasks) of sterile media. Incubation proceeded on a rotary shaker at the frequency of revolution 160 min^{-1} and 28 °C for 24 h.

Samples from these cultures were then transferred into 1 dm^3 growth flasks containing 0.5 dm^3 of the medium and incubation was allowed to proceed at the same cultivation conditions for 7 d. The optimum time for cultivation was found in separate experiments from the mass of the dry mycelium determined gravimetrically at time intervals during incubation.

Isolation of Polysaccharides from the Culture Media

Cells from 0.5 dm^3 of batch cultures were centrifuged at 1500 g for 10 min. The supernatant was reduced in volume thrice by evaporation and precipitated with 96 % ethanol (containing 1 % acetic acid) in the volume ratio of 1 : 3. The precipitate was separated by centrifugation, dissolved in distilled water and reprecipitated in the same manner twice. The obtained crude polysaccharide was dissolved in distilled water, the solution was dialyzed against distilled water for 48 h and lyophilized. The polysaccharides obtained from the culture media 1–6 were designated as I–VI, respectively.

Hydrolysis of Polysaccharides

The polysaccharide (5 mg) was hydrolyzed with 2 M trifluoroacetic acid (5 cm^3) in a sealed tube at 120 °C for 1 h. The products of hydrolysis were identified by paper chromatography, and their quantitative composition was determined by gas-liquid chromatography.

RESULTS AND DISCUSSION

Production of extracellular polysaccharides by microorganisms depends, beside other conditions, also on nutritional factors. The yeast *Candida mucifera* was cultured in six liquid me-

Table 1. Characterization of Extracellular Polysaccharides

Polysaccharide	Yield ^a	Content/%			[α]/ ^a _b [η]/(cm ³ g ⁻¹)	
		Protein	P	OCH ₃		
I	0.40	13.5	0.49	0.24	+ 47.7	36
II	0.40	13.8	0.52	0.25	+ 51.0	46
III	0.19	14.7	0.68	0.39	+ 41.5	51
IV	0.30	16.0	0.49	0.23	+ 45.5	40
V	0.75	10.5	1.09	0.29	+ 37.5	67
VI	0.30	3.0	0.47	0.31	+ 65.4	14
D-Mannan						8

a) in g per 500 cm³ of culture medium; b) (D, 22 °C, $\rho = 5$ g cm⁻³, water).

dia containing various carbon sources (see Experimental). Optimum growth of the yeast, monitored by gravimetric measurement of the dry biomass, was observed on the 7th day of cultivation. The highest amount of the biomass (11 g per 500 cm³ of culture medium) was produced in the medium with beer wort and the lowest one (2 g per 500 cm³ of culture medium) in the medium containing 1 % methanol as the carbon source. The seven days old culture media were centrifuged and the supernatants were treated with acidified ethanol to give the extracellular polysaccharides I–VI. The amount of the obtained polysaccharides varied between 0.19 g and 0.75 g per 500 cm³ of culture medium (Table 1). The lowest amount was obtained from the medium with 2 % methanol as the carbon source and the highest one from the medium containing D-glucose.

All polysaccharides were heterogeneous upon free-boundary electrophoresis. Acid hydrolysis of polysaccharides I–V revealed that their constituent sugars were similar (Table 2). D-Mannose was the dominating sugar in all cases, the amount of D-glucose was by 3 to 10 times lower, while D-galactose and L-arabinose were present in low and trace amounts, respectively. The sugar composition of the polysaccharide VI differed from the foregoing ones in presence of D-xylose and

Table 2. Sugar Composition of Extracellular Polysaccharides

Polysaccharide	Mole ratio of sugar components				
	D-Glc	D-Gal	D-Man	L-Ara	D-Xyl
I	0.32	Traces	1.00	Traces	–
II	0.17	Traces	1.00	Traces	–
III	0.18	0.05	1.00	Traces	–
IV	0.24	Traces	1.00	Traces	–
V	0.10	0.08	1.00	Traces	–
VI	5.14	–	1.00	2.88	3.99

Traces – mole ratio below 0.05.

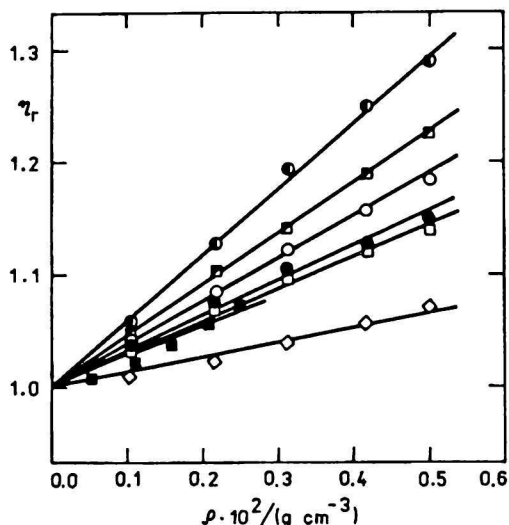


Fig. 1. Concentration dependence of relative viscosity of aqueous solutions of polysaccharides I–VI and D-mannan (M). □ – I; ○ – II; ■ – III; ● – IV; ▲ – V; ◇ – M.

absence of D-galactose. Moreover, the dominating sugar was D-glucose, while D-mannose was present in the least amount.

As seen from Table 1, all polysaccharides contained proteins, phosphorus, and methoxy groups. The lowest amount of proteins (3 %) was determined in VI, isolated from the medium not containing yeast extract, while phosphorus dominated in V (1 %), obtained from the medium with D-glucose as the carbon source. The content of methoxy groups was very low (≈ 0.25 %) in all polymers. The values of optical rotation, ranging from +37° to +65° may indicate the presence of both α - and β -anomeric bonds in the polysaccharides. The amino acids composition of the protein moieties of the polysaccharides was found to be similar (Table 3).

The concentration dependences of relative and reduced viscosities, respectively, of aqueous solutions of polysaccharides in the concentration range of $(0.1\text{--}0.5) \times 10^{-2}$ g cm⁻³ are illustrated in Figs. 1 and 2. The upper concentration was limited by solubility of polysaccharides. For comparison also the viscosity of a reference D-mannan ($\bar{M}_N = 28\,000$), originating from *Candida albicans*, has been measured in the same concentration range. Extrapolation of reduced viscosities to zero concentrations showed that the limiting viscosity number [η] was in all cases higher ($14\text{--}67$ cm³ g⁻¹) than that of the D-mannan (8 cm³ g⁻¹) (Table 1). As seen from the lines in Fig. 2, the reduced viscosities increased in the whole concentration range with dilution of the solution, except in the cases of VI and D-mannan. We cannot give any exact explanation for this phenomenon yet. How-

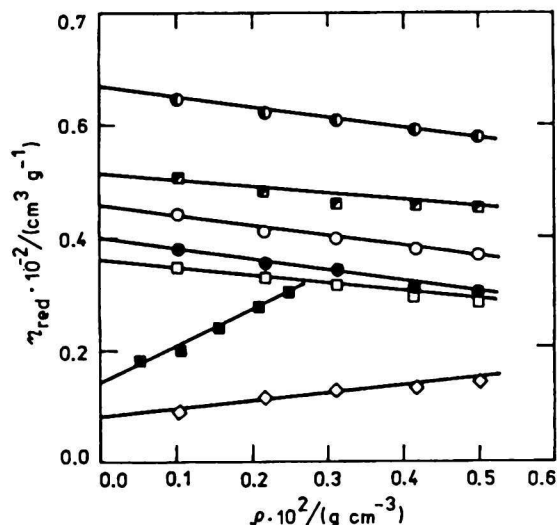


Fig. 2. Concentration dependence of reduced viscosity of aqueous solutions of polysaccharides I–VI and D-mannan (M). □ – I; ○ – II; ■ – III; ● – IV; ◐ – V; ◇ – M.

ever, a similar phenomenon was observed in the case of a salt-free polyelectrolyte (pectin) solution, where it was attributed to the decreased screening of the fixed charges by the counterions and the consequent coil expansion due to the mutual repulsion between the increasing number of like charges attached to the coil [4].

It is also known that some yeasts of the *Candida* genus produce phosphomannans or phosphomannan–protein complexes with phosphoric diester linkages [5, 6]. When considering the above-mentioned facts and the fairly common feature of extracellular polysaccharides to possess a net negative charge, which is conferred not only by uronic acids, but also by nonsugar substituents, e.g. phosphorus [7], then phosphorus found in all polysaccharides studied herein might have affected the viscosity properties of aqueous solutions of polysaccharides. However, explanation of this phenomenon will require deeper investigation into the primary structure and properties

Table 3. Amino Acids Composition of Extracellular Polysaccharides

Amino acid	x/%					
	I	II	III	IV	V	VI
Lysine	4.85	4.52	5.62	4.80	4.43	3.86
Histidine	1.52	1.78	1.75	1.55	2.84	2.81
Arginine	2.33	2.43	2.25	0.91	1.94	2.51
Aspartic acid	10.45	10.51	10.76	11.90	9.10	10.06
Threonine	13.30	13.58	13.37	13.45	14.40	8.26
Serine	13.45	13.58	13.61	13.47	13.88	9.34
Glutamic acid	10.45	10.70	10.45	10.80	9.26	14.72
Proline	6.23	5.46	4.98	5.90	9.11	6.92
Glycine	8.23	8.95	8.05	8.30	7.54	8.86
Alanine	10.01	10.20	10.19	10.10	11.18	8.58
Valine	5.28	5.45	5.48	5.53	4.46	5.39
Methionine	0.51	–	–	0.63	–	1.79
Isoleucine	3.45	3.78	3.27	3.25	3.14	4.43
Leucine	4.70	3.90	4.07	4.22	3.43	5.76
Tyrosine	2.90	2.51	3.80	2.62	2.60	2.72
Phenylalanine	2.25	2.62	2.30	2.49	2.71	3.98

of the individual homogeneous polysaccharides, which will be the subject of our future work.

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