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Polymorphism of *Sporothrix schenckii* Surface Polysaccharides as a Function of Morphological Differentiation†

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ABSTRACT: The alkali-extractable polysaccharides from different morphological types of two *Sporothrix schenckii* strains (1099.12 and 1099.18) were investigated. Dissociation of morphological phase transition and temperature effects was possible in a synthetic medium which produced cultures with 100% yeast forms either at 25 or at 37 °C. Only rhamnomannans with single-unit α-L-rhamnopyranosyl side chains were formed by the yeast forms irrespective of the incubation temperature. The higher temperature inhibited formation of 4-*O*- and 2,4-di-*O*-substituted α-D-mannopyranose units in the rhamnomannan. An apparently unsporulated mycelium culture of one *S. schenckii* strain (1099.12) synthesized a galactomannan whose structure was partially determined by methylation analysis and by proton and ^{13}C nuclear magnetic resonance spectroscopy. In another strain (1099.18), a mannan was excreted in the medium of an apparently conidia-less mycelial form at 25 °C with short incubation. Its structure was also partially determined. An apparent mixture of this mannan and a rhamnomannan rich in α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranose side chains formed in these cultures on prolonged incubation. The proportion of the excreted rhamnomannan increased as the mycelium sporulated and conidia were more numerous. Mannans or galactomannans may be transient polysaccharides in the young mycelium of *S. schenckii*. As the culture develops, rhamnomannans are formed in amounts usually masking the presence of other mannose-containing polysaccharides. It is suggested that in *S. schenckii* different polysaccharides are formed with side chains containing different proportions of rhamnose, mannose, or galactose, as a function of morphological differentiation.

Surface polysaccharides are important fungal antigens, and their fine chemical structures may differ depending on the microbial strain, species, or genus. Mannans or heteropolymers containing mannose are the usual polysaccharides present in the majority of yeasts (Gorin and Spencer, 1968; Phaff, 1971). Mannose-containing polysaccharides are also found in filamentous fungi including pathogenic organisms (Azuma et al., 1971, 1974; Kanetsuna et al., 1974). Among the latter is *Sporothrix schenckii,* which synthesizes an L-rhamno-D-mannan (Ishizaki, 1970; Lloyd and Bitoon, 1971). All studied strains of *S. schenckii* formed rhamnomannans with similar structures, as inferred by their proton nuclear magnetic resonance (^1H NMR) and ^{13}C nuclear magnetic resonance (^{13}C NMR) spectra (Travassos et al., 1974). Rhamnomannans were also found in several *Ceratocystis* and *Graphium* species and in *Taphrina deformans* (Gorin and Spencer, 1970), but were not found in pathogenic fungi besides *S. schenckii.*

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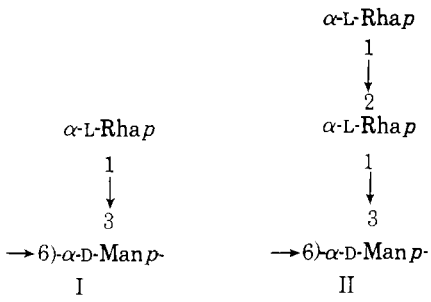

S. schenckii rhamnomannans contained varying proportions of I and II as their main structural features (Travassos et al., 1973). The rhamnomannans from *S. schenckii* differ from those from certain *Ceratocystis* species (*C. ulmi, C. stenoceras, C. minor,* and *C. pilifera*) by the presence of a higher proportion of dirhamnosyl side chains. This difference is found when the organisms are grown at 25 °C but not at 37 °C. Dirhamnosyl side chains are the main antigen determinants in *S. schenckii* rhamnomannans formed at 25 °C (Lloyd and Travassos, 1975).

S. schenckii is a dimorphic fungus displaying a yeast-like or a mycelium morphology depending on the culture conditions. Differences in the fine structures of *S. schenckii* rhamnomannans were observed in fungal cultures at two temperatures but with mixed morphology (Travassos et al., 1973, 1974). A mixture of yeasts, filaments, and conidia formed at 37 °C, with yeast forms predominating. At 25 °C cultures comprised only filaments and conidia. Variations in polysaccharide structure could have arisen because of a temperature effect, or they could be associated with the predominant fungal morphology in the particular culture.

The present paper describes attempts to correlate the structure of the polysaccharides with a specific cell morphology. In the course of these studies new polysaccharides from *S. schenckii* have been identified which were not recognized before.

Material and Methods

Microorganisms. Two strains of *S. schenckii* were investigated. Strain 1099.12 was originally regarded as a pathogenic mutant of *C. stenoceras* (Mariat, 1971). Later it was reclassified as a typical *S. schenckii* on the basis of its DNA guanine + cytosine content and DNA/DNA hybridization studies (Mendonça-Hagler et al., 1974). Strain 1099.18 was isolated from a human case of sporotrichosis. Both strains were obtained from the Mycology Section, Department of Dermatology, Columbia University, New York. Stock cultures were maintained at 4 °C in solid Sabouraud medium distributed in tubes with a layer of mineral oil.

Culture Media and Growth Conditions. The following culture media were used: (a) brain heart infusion (BHI, BBL[1]); (b) Sabouraud (glucose 2%, yeast extract (BBL) 0.5%, and peptone (Difco) 1%; (c) YCV (yeast nitrogen base (Difco), Casamino acids (BBL), and vitamins as in Travassos et al. (1973); (d) medium M (g/l.): glucose 20, Na_2HPO_4 1.6, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.25, H_3BO_3 0.0005, $CuSO_4 \cdot 5H_2O$ 0.001, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ 0.0002, $MnSO_4 \cdot H_2O$ 0.003, $Na_2MoO_4 \cdot 2H_2O$ 0.0025, $ZnSO_4 \cdot 7H_2O$ 0.003, $NaVO_3$ 0.001, $CoSO_4 \cdot 7H_2O$ 0.00005, biotin 0.00002, calcium pantothenate 0.002, thiamin-HCl 0.02, pyridoxine-HCl 0.002, nicotinic acid 0.002, inositol 0.01, choline chloride 0.01, carnitine chloride 0.01, L-glutamic acid 2.5, L-aspartic acid 1.0, L-cysteine 0.5, cytosine 0.02, orotic acid 0.02, uracil 0.01, α-glycerophosphate 10, and NH_4HCO_3 1.0; pH 7.1; glucose was autoclaved separately and then added to the medium, and NH_4HCO_3 was added after neutralization to pH 7.1 with NH_4OH or NaOH; (e) Mariat's medium B (Toriello and Mariat, 1974) with omission of gallium and lithium salts. Fresh BHI or Sabouraud slanted cultures were used as inocula. Transfers were initially made to flasks containing 200 ml of one of the media indicated above and incubation was at 25 or 37 °C with shaking (New Brunswick gyrotory shaker) for 4–5 days. Once a definite morphological type was selected the 200-ml culture was used as inoculum for 2.8-l. Fernbach flasks containing 1 l. of the same medium. To obtain cultures with 100% yeast forms, screw-capped 2-l. flasks were used to assure gas accumulation inside the flasks.

Extraction of Polysaccharides. Polysaccharides were extracted and purified via precipitation of their copper complexes with Fehling reagent as described previously (Travassos et al., 1973).

Analytical Methods. Total carbohydrate, nitrogen, and phosphate were determined by the phenol–sulfuric acid method (Dubois et al., 1956), by the ninhydrin method (Schiffman et al., 1964), and by the method of Ames (Ames, 1966), respectively. Rhamnose was determined by the cysteine–sulfuric acid method (Dische and Shettles, 1948). Quantitative determination of monosaccharides in the polysaccharides was performed by gas–liquid chromatography (GLC) of the corresponding alditol acetates (Albersheim et al., 1967; Lloyd, 1970; Sawardeker et al., 1965). Columns (6 ft × ⅛ in. diameter) containing ethylene glycol succinate–silicone 10% (EGSS-X) in 80–100 Varaport 30 or neopentyl glycol succinate (NPGS) 3% in 100–120 Gas Chrom Q at 210–215 °C were used. A detailed description of this procedure is given elsewhere (Lloyd, 1970). D-Galactose was determined by the D-galactose oxidase method (Amaral et al., 1966).[2]

Methylation–Fragmentation of Polysaccharides. Methylation of polysaccharides was carried out by the dimethylsulfinyl anion and methyl iodide method (Hakomori, 1964) as described by Sandford and Conrad (1966). Methylated polysaccharides were hydrolyzed in 90% formic acid at 100 °C and then in 0.5 N H_2SO_4 at 100 °C. *O*-Methylalditol acetates were prepared from the partially methylated sugars by reduction with $NaBH_4$ and acetylation with acetic anhydride and pyridine (Lloyd, 1970) and were separated by GLC in EGSS-X or NPGS columns. Peaks were identified by their mass spectra according to Bjorndal et al. (1967). A Varian Aerograph series 1400 gas chromatograph coupled to a Varian CH5-DF-MAT-100MS mass spectrometer data system was used with a 10% EGSS-X column (180 × 0.5 cm) at 200 °C. Comparative mass spectra of the galactomannan-derived *O*-methylalditol acetates were obtained at the Institutionen for organisk kemi, Stockholms universitet, Sweden. Peaks in the GLC were also identified by comparison of the retention times with those for the alditol acetates derived from methylation–fragmentation of baker's yeast mannan and an *S. schenckii* rhamnomannan of known structures.

Smith Degradation. A sample of polysaccharide (5 mg) was oxidized with $NaIO_4$ (20 mg) in H_2O (2 ml) for 48 h. After this period the reaction mixture was treated with a mixed-bed resin of Dowex 1 (bicarbonate form) and Dowex 50 (H^+ form)

[1] Abbreviations used are: BHI; brain heart infusion; YCV; yeast nitrogen base-Casamino acids-vitamins.

[2] The galactose oxidase reagents kit was a generous gift of Dr. D. Amaral, Department of Biochemistry, Federal University of Parana, Brazil.

TABLE I: Analyses (%) of *S. schenckii* Polysaccharides.

Polysaccharides[a]	Rhamnose[b]	Mannose GLC[c]	Galactose GLC[c]	Total Carbohydrate col.[d]	N	P	$[\alpha]^{20}D$ (deg)
12-YCV-37-C(Ymc)	47.8	56.8	tr[e]	106.9	0.2	0.2	+5
12-YCV-37-S(Ymc)	44.7	54.5	tr	108.6	0.4	0	−3
12-YCV-37-CS(Ymc)	44.2	49.2	tr	102.8	0.5	0.5	−3
12-BHI-37-C(Y)	53.4	53.6	0	97.5	0.6	0	−2
12-Sab-37-C(Y)	47.5	49.7	0	104.0	0.7	0.1	−8
12-M-37-C(Y)	48.4	47.9	0	92.9	0.5	0.2	−4
12-BHI-25-C(MCy)	42.2	51.4	5.1	102.0	0.8	0.5	−4
12-Sab-25-C(MCy)	53.2	44.2	tr	106.6	0.3	0	−6
18-M-25-C(Y)	44.4	54.5	0	101.9	0	0.5	+1
18-M-37-C(Y)	45.2	55.1	0	108.6	0.8	0.1	−4
12-BHI-25-C(M)	0	88.5	10.0	105.7	0.2	0	+69
18-B-25-C(M)	40.9	47.8	3.6	104.7	0	0.1	−22
18-B-25-S(M)	11.9	83.1	3.5	98.6	0	0.2	+40
18-B-25-S(Mc)	26.7	60.2	5.5	105.2	0.2	0.5	+22

[a] Polysaccharides are identified as follows: strain (12 or 18)/medium (YCV, BHI, Sab, M, or B)/temperature (25 or 37 C)/source (C = cells; S = supernatant liquid; CS = cells and supernatant)/predominant morphology (in parentheses: Y = yeast-forms; M = mycelium; C = conidia; capital letters indicate that one morphological type predominates over the others). [b] Rhamnose values average colorimetric and GLC determinations. [c] Gas–liquid chromatography. [d] Colorimetric. [e] Trace.

and the filtrate was partially evaporated. $NaBH_4$ (2 mg) was added and after 2 h excess acetic acid was added to destroy the reductant and the solution was shaken with Dowex 50. The filtrate was evaporated, and the residue was washed several times with methanol and then hydrolyzed in 1 N H_2SO_4 at 100 °C for 3 h. The hydrolysate was neutralized with barium carbonate and the supernatant liquid was deionized with the mixed-bed resin, evaporated, and examined chromatographically. Descending paper chromatography was carried out using Whatman No. 1 paper with the following solvents: ethyl acetate–pyridine–water (5:3:2 v/v) and 1-butanol–ethanol–water (40:11:19 v/v). Spots were developed with the alkaline silver nitrate reagent. For GLC the final deionized solution was again reduced and acetylated and the resulting alcohol acetates were separated using the NPGS column at 210 °C. Allitol hexaacetate was used as the internal standard.

Proton NMR Spectroscopy. Proton NMR spectra of polysaccharides were obtained by using a 100-MHz Varian NMR spectrometer from 20% solutions in D_2O at 70 °C with tetramethylsilane (τ = 10) as the external standard in a coaxial capillary.

Carbon-13 NMR Spectroscopy. ^{13}C NMR spectra were obtained on samples containing a natural abundance of ^{13}C by using a Varian XL-100-15 spectrometer with Fourier transform having an 8 or 16K memory. The polysaccharides (200 mg)) were dissolved in D_2O (2.5 ml), the solution was centrifuged to remove insoluble material, introduced into a 12 mm diameter × 20 cm NMR tube, and degassed by heating, and a Teflon vortex plug was introduced. The spectral examinations were carried out at 70 °C and chemical shifts are expressed in δ_c relative to external Me_4Si whose shift relative to D_2O was obtained in a separate experiment. It followed that these spectra contain signals 0.6 ppm lower field than those obtained at 32 °C. Usually the spectral width used was 5000 Hz, the acquisition time 0.4 s, the pulse width 50 μs, and the number of transients 20 000–100 000 depending on the spectral resolution and line width. In some cases when better resolution was required a 1000-Hz sweep width was used with values of 2 s, 90 μs, and 20 000, respectively. Also, under these conditions relaxation time effects tended to be smaller and a more quantitative comparison could be made between peak magnitudes.

Results

Polysaccharides Isolated from Whole Cells and from the Culture Supernatant Liquid. In a previous publication (Travassos et al., 1973) we described strain 1099.12 polysaccharides from cells + supernatant of a 37 °C culture in the YCV medium. The polysaccharides present in the supernatant liquid were precipitated with ethanol then admixed with the cell mass, and the mixture boiled in 2% KOH at 100 °C. After neutralization of the alkali extract with acetic acid the polysaccharides were isolated by ethanol precipitation and were purified via precipitation with Fehling solution.

We observed in the present study that, if the polysaccharides were isolated independently from the cells and from the culture supernatant liquid, differences in structure emerged. Although the sugar ratios in the polysaccharides were not significantly altered (Table I), their ^{13}C NMR spectra were distinctive (Figure 1).

Spectra of polysaccharides isolated from the supernatant (Sp) of *S. schenckii* culture in the YCV medium showed signals at δ_c 103.8, 96.8, and 80.5 (Figure 1) which were missing in the spectrum of the polysaccharides isolated from the cells (Cp). The signal at δ_c 96.8–97.0 has been assigned (Table II) to the C-1 of 2-*O*-substituted L-rhamnopyranosyl units (Travassos et al., 1974). It was usually very minor in the spectra of polysaccharides isolated from cultures grown at 37 °C. Such cultures in the YCV medium consisted of a mixture of morphological types with yeasts being present in high but varying percentages depending on the culture. The above results suggest some differences in the polysaccharide preparations depending on whether they were isolated from the cells or from the culture filtrates.

Influence of Growth Media on Cell Polysaccharides. To study the influence of the growth medium on cell-bound polysaccharides two situations were examined: (a) different media in which only one morphological type (e.g., yeast) thrived; (b) different media in which a mixture of mycelium, conidia, and yeasts formed in about the same proportions.

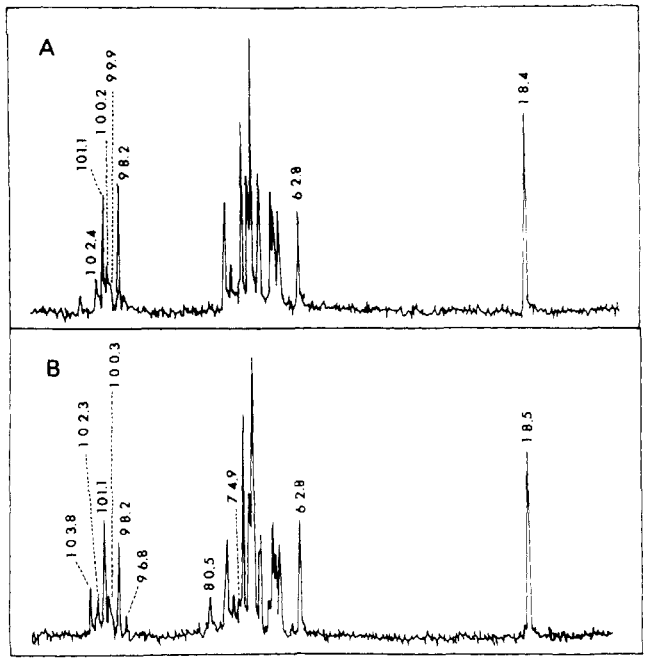

FIGURE 1: ^{13}C NMR spectra of *S. schenckii* (strain 1099.12) polysaccharides isolated from the cells (A) and from the culture supernatant liquid (B). Growth conditions: YCV medium at 37 °C.

TABLE II: Summary of the Assignments of Signals in ^{13}C NMR Spectra of Polysaccharides from *S. schenckii*.

Signal, δ_c ±0.3 (ppm)	Assignment
109.3	C-1 of β-D-galactofuranose nonreducing end units
103.7	C-1 of 3-*O*-substituted α-D-mannopyranose units
	C-1 of α-D-mannopyranose nonreducing end units
	C-1 of α-L-rhamnopyranose nonreducing end unit of *O*-α-L-Rha*p*-(1→2)-*O*-α-L-Rhap-(1→3)-D-Man
102.3	C-1 of 2-*O*-substituted α-D-mannopyranose units
101.0	C-1 of 6-*O*-substituted α-D-mannopyranose units
	C-1 of 3,6-di-*O*-substituted α-D-mannopyranose units
99.9	C-1 of 2,6-di-*O*-substituted α-D-mannopyranose units
98.2	C-1 of α-L-rhamnopyranose nonreducing end unit of *O*-α-L-Rha*p*-(1→3)-D-Man*p*
96.8	C-1 of 2-*O*-substituted α-L-rhamnopyranose units
84.8	C-4 of β-D-galactofuranose nonreducing end units
82.5	C-2 of β-D-galactofuranose nonreducing end units
80.2	C-2 of 2,6-di-*O*-substituted α-D-mannopyranose units
79.7	C-2 of 2-*O*-substituted α-D-mannopyranose units
78.5	C-3 of β-D-galactofuranose nonreducing end units
64.5	C-6 of β-D-galactofuranose nonreducing end units
62.7	C-6 of *O*-6 unsubstituted α-D-mannopyranose units
18.4	C of methyl group of α-L-rhamnopyranose units

Cultures with 100% yeast forms were obtained by growing strain 1099.12 at 37 °C in a synthetic medium (M) or in BHI. About 90% yeast forms were obtained in a Sabouraud culture at 37 °C. The component-sugar ratio of the polysaccharides isolated from these three media was about the same, with rhamnose and mannose being present, but no traces of galactose (Table I). The ^{13}C NMR spectra of the cell polysaccharides from cultures containing yeast forms were indistinguishable. These spectra were similar to those reported earlier for type III *S. schenckii* polysaccharides (Travassos et al., 1974). The ^{13}C NMR spectra of polysaccharides obtained from cultures at 25 °C with mixed morphology in BHI or Sabouraud media were quite similar, with some differences being observed in the sizes of the peaks. Both spectra contained a signal at δ_c 97.2. A small peak at δ_c 103.3 appeared in the spectrum of the Sabouraud polysaccharide but was barely identifiable in the BHI-polysaccharide spectrum. These results indicate that, provided the fungal morphology is the same, different culture media have little or no influence on the polysaccharide structure.

Dissociation of Morphological Differentiation and Temperature Effects. Since strain 1099.18 tended to form yeasts more easily than strain 1099.12, it was used in these experiments. It was grown in medium M, distributed in flasks with tightened screw caps, and incubated at 25 or 37 °C with shaking. Depending on the source of the inoculum, medium, and morphology, pure yeast cultures were obtained after different incubation periods. Usually longer incubation was necessary to obtain pure yeast cultures at 25 °C. Yeasts obtained at 25 or 37 °C had the same morphology and resembled the cigar-shaped bodies found in infected tissues in sporotrichosis. The component-sugar ratios of the polysaccharides isolated at 37 (M-37) or 25 °C (M-25) from strain 1099.18 were very close, with rhamnose and mannose being present but no galactose (Table I). Methylation–fragmentation of these polysaccharides and GLC of the resulting partially methylated alditol acetates showed two main peaks corresponding to the acetates of 2,3,4-tri-*O*-methyl-L-rhamnitol and 2,4-di-*O*-methyl-D-mannitol. A very small peak appeared in both chromatograms corresponding to the acetate of 3,4-di-*O*-methyl-L-rhamnitol (Figure 2). Minor peaks corresponding to the acetates of 3,6-di-*O*- and 2,3,6-tri-*O*-methyl-D-mannitol were also identified in both cases, but they were more prominent in the M-25 spectrum. In the ^{13}C NMR spectrum of M-37 the C-1 region showed only two signals (δ_c 101.1 and 98.3); that for the M-25 polysaccharide also had these signals but also showed minor signals at δ_c 102.4, 100.3, and 99.9. Although the assignments of signals at δ_c 102.4 and 100.3 are still under study, we suggest that they correspond to the C-1's of 4-*O*- and 2,4-di-*O*-substituted α-D-mannopyranose units. A signal at δ_c 100.1 previously observed in the ^{13}C NMR spectra of *S. schenckii* and *C. stenoceras* rhamnomannans was tentatively assigned to the C-1 of 2,4-di-*O*-substituted α-D-mannopyranose units (Travassos et al., 1974). The spectrum of M-25 had also more prominent signals at δ_c 76.0 and 62.8 which were very minor in the M-37 spectrum. No traces of signals at δ_c 96.8–97.0 were observed, thus excluding the presence of 2-*O*-substituted L-rhamnose units (Figure 2). By combining the methylation data with the ^{13}C NMR spectra of both M-25 and M-37 we suggest that the rise in temperature inhibited formation of 4-*O*- and 2,4-di-*O*-substituted α-D-mannopyranose units in the rhamnomannan. It was concluded that rhamnomannans rich in dirhamnosyl side chains are not formed by yeast forms even when cells are grown at 25 °C.

New S. schenckii Polysaccharides Synthesized at 25 °C. When inoculated in BHI at 25 °C from agar-slanted cultures in the mycelium phase, strain 1099.12 grew as a mycelium which did not sporulate for several days. From this mycelium which lacked visible conidia a polysaccharide was extracted with hot aqueous alkali and purified via precipitation with Fehling reagent. Acid hydrolysis provided mannose and a small proportion of galactose which were identified by GLC of the derived alditol acetates. This technique showed that 7–10% of

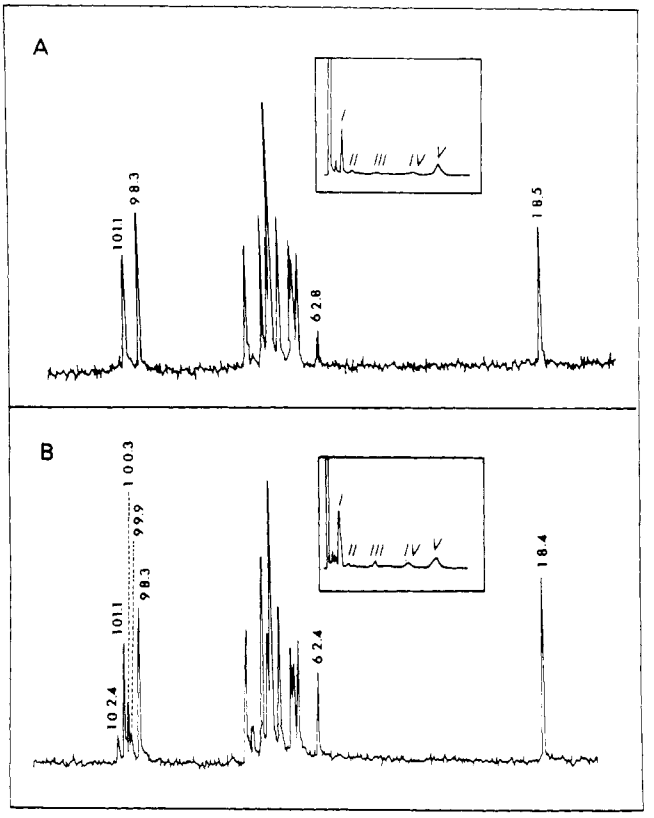

FIGURE 2: ^{13}C NMR spectra and GLC (insets) of the partially methylated alditol acetates from methylation analysis of cell-bound polysaccharides from *S. schenckii* (strain 1099.18) growing in the yeast form (medium M) at 37 °C (A) or at 25 °C (B). Acetate derivatives: I, 2,3,4-tri-*O*-methylrhamnitol; II, 3,4-di-*O*-methylrhamnitol; III, 2,3,6-tri-*O*-methylmannitol; IV, 3,6-di-*O*-methylmannitol; V, 2,4-di-*O*-methylmannitol.

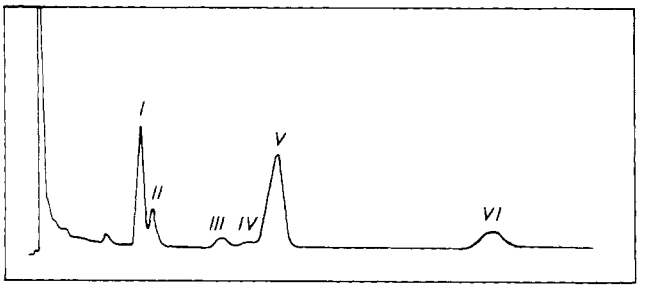

FIGURE 3: Gas chromatogram of the partially methylated alditol acetates from methylation analysis of the *S. schenckii* (strain 1099.12) galactomannan. Growth conditions: BHI medium at 25 °C. Acetate derivatives: I, 2,3,4,6-tetra-*O*-methylmannitol; II, 2,3,5,6-tetra-*O*-methylgalactitol; III, 3,4,6-tri-*O*-methylmannitol; IV, 2,4,6-tri-*O*-methylmannitol; V, 2,3,4-tri-*O*-methylmannitol; VI, 3,4-di-*O*-methylmannitol.

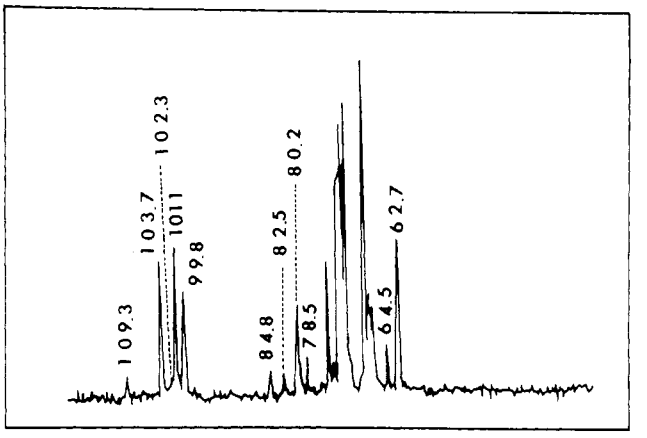

FIGURE 4: ^{13}C NMR spectrum of *S. schenckii* (strain 1099.12) galactomannan.

the total consisted of galactose which was characterized as the D isomer by oxidation with D-galactose oxidase (Amaral et al., 1966). The specific rotation of the galactomannan was +69°, indicative of predominant α-D-mannose units, but lower than +88° of completely α-D-linked baker's yeast mannan (Haworth et al., 1941), thus suggesting the presence of some β-D-galactosyl units. By methylation–fragmentation of this polysaccharide several partially methylated alditol acetates were identified by GLC on EGSS-X. The retention times of the peaks corresponded to 2,3,4,6-tetra-*O*-, 3,4,6-tri-*O*-, 2,4,6-tri-*O*-, 2,3,4-tri-*O*-, and 3,4-di-*O*-methyl derivatives of mannitol and to 2,3,5,6-tetra-*O*-methylgalactitol (Figure 3). The fragmentation patterns obtained on mass spectrometry of the *O*-methylalditols agreed with these assignments. The polysaccharide thus contains mannopyranose and galactofuranose nonreducing end units, 6-*O*- and 2,6-di-*O*-substituted mannopyranose units, and small proportion of 2-*O*-linked mannopyranose units. The presence of a minor proportion of 3-*O*-linked mannopyranose units was confirmed by Smith degradation which provided glycerol and some unoxidized mannose. The configuration of the galactofuranose units in the galactomannan was deduced from its ^{13}C NMR spectrum. The three most prominent peaks in the C-1 region at low field correspond in their chemical shifts to structures typical to α-D-mannans (Figure 4). These are from nonreducing end units and/or 3-*O*-substituted (δ_c 103.7) units, 6-*O*-substituted units (δ_c 101.1), and 2,6-di-*O*-substituted α-D-mannopyranose units (δ_c 99.8). Prominent signals were present at δ_c 80.2 and 62.7 arising from C-2 of 2,6-di-*O*-substituted α-D-mannopyranose units and C-6 of α-D-mannopyranose nonreducing end units, respectively (Gorin, 1973), thus agreeing with the chemical structures indicated by the methylation experiment. Five of the minor signals in the ^{13}C NMR spectrum have chemical shifts that are very close to those of methyl β-D-galactofuranoside (Gorin and Mazurek, 1975). These are at δ_c 109.3, 84.8, 82.5, 78.5, and 64.5 and correspond in their chemical shifts to the C-1, C-4, C-2, C-3, and C-6 signals, respectively, of the glycoside, and in the case of a furanoside 1,5-linked β-D-galactotetraose (Gorin and Spencer, 1959) obtained from the galactomannan of *Penicillium charlesii* (Table III) to the C-1, C-4, and C-6 signals at δ_c 109.3, 84.8, and 64.5. The ^{13}C signals of methyl α- and methyl β-D-galactopyranose and methyl α-D-galactofuranoside have different chemical shifts (Gorin and Mazurek, 1975).

Other signals corresponding to *O*-substituted galactofuranose units could not be detected, and it appears that all the galactofuranose occurs as nonreducing units attached to α-D-mannopyranose units. The position of the linkage is not yet clear. It is possible that a minor signal at δ_c 102.3 arises from C-1 of a 2-*O*-substituted α-D-mannopyranose unit. Such a substitution would displace the C-1 signal upfield from that (δ_c 103.7) of an unsubstituted unit (Gorin, 1973). *O*-Mannosylation, rather than *O*-galactosylation, would give a signal with the same shift. However, if this occurred a signal should also be present at δ_c 80.0 corresponding to that of C-2 of a 2-*O*-substituted α-D-mannopyranose. The latter signal, however, was not detected. β-Galactofuranose units could also be linked through 1→3 linkages to α-D-mannopyranose units. Smith degradation of the galactomannan confirmed the presence of

TABLE III: Comparison of Chemical Shifts of ^{13}C Signals in the Galactomannan with those of Methyl β-D-Galactofuranoside and the Nonreducing End Unit of a 5-*O*-β-Linked Galactotetraose.

Sugar	Chemical shifts as δ_c (ppm[a])				
	C1	C2	C3	C4	C6
Methyl β-D-galactofuranoside	109.9	82.5	78.4	84.6	64.5
Galactomannan (at 70 °C)	109.3	82.5	78.5	84.8	64.5
Nonreducing end unit of 5-*O*-β-linked galactotetraose from *P. charlesii*	108.0	—	—	84.3	64.4

[a] A correction factor of +0.6 ppm from the reported values for methyl β-D-galactofuranoside (Gorin and Mazurek, 1975) and the 5-*O*-β-linked galactotetraose (Gorin and Mazurek, submitted for publication) was required since the spectra were obtained at 32 °C rather than 70 °C.

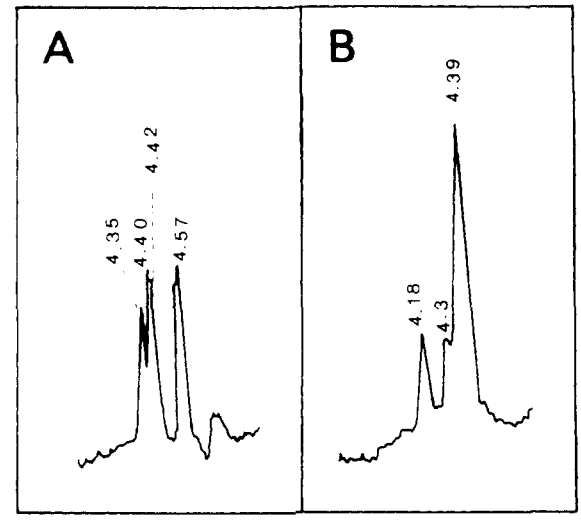

FIGURE 5: Proton NMR spectra (H-1 region) of *S. schenckii* (strain 1099.12) galactomannan (A) and baker's yeast mannan (B).

3-*O*-substituted mannopyranose units, but the methylation experiment showed that the peak in the GLC corresponding to the 2,4,6-tri-*O*-methylmannitol acetate was very small when compared with the peak corresponding to 2,3,5,6-tetra-*O*-methylgalactitol acetate (Figure 3).

The proton NMR spectra of the galactomannan and of a baker's yeast mannan were compared. Both contained a signal at τ 4.39–4.40 corresponding to the H-1 of 2,6-di-*O*-substituted α-D-mannopyranose units (Gorin et al., 1968). A signal at τ 4.18 corresponding to the H-1 of repeated 2-*O*-substituted α-D-mannopyranose units did not appear in the proton NMR spectrum of the galactomannan (Figure 5). There were additional signals in the latter spectrum at τ 4.42 and 4.57 which could correspond to the H-1's of nonreducing α-D-mannopyranose end units and 6-*O*-substituted α-D-mannopyranose, respectively (Gorin et al., 1969).

The position of the 1→2 and 1→6 links in the galactomannan was determined as follows: the α-D-mannopyranose nonreducing end units were removed with an exo-α-D-mannosidase (Jones and Ballou, 1969). Examination of the precipitated degraded polysaccharide in D_2O at 70 °C by proton NMR spectroscopy showed only one signal at τ 4.57 in the H-1 region, corresponding to a (1→6)-linked α-D-mannopyranose main chain (Gorin et al., 1969).

The above data suggest that the galactomannan from *S. schenckii* has the predominant structure III.

α-D-Man*p*
1
↓
2
·α-D-Man*p*(1→6)-α-D-Man*p*(1→6)·

III

The same procedure was used to isolate a similar polysaccharide from *S. schenckii* strain 1099.18. This strain, however, did not form an unsporulated mycelium in BHI at 25 °C at least under the conditions used in the experiment. Attempts were then made to obtain pure mycelium cultures of this strain in Mariat's medium (medium B). In this medium, sporulation was evident after 2 days of incubation at 25 °C. In contrast to strain 1099.12, the polysaccharide isolated from the apparently unsporulated mycelium of strain 1099.18 was a typical *S. schenckii* rhamnomannan with a high proportion of dirhamnosyl side chains as deduced by the methylation–fragmentation technique and since a very prominent peak appeared in the gas chromatogram corresponding to 3,4-di-*O*-methyl-1,2,5-tri-*O*-acetyl-L-rhamnitol. Polysaccharides in the supernatant fluid of this culture apparently consisted of a mixture of a mannan and small amounts of the rhamnomannan. By prolonging incubation, increasing amounts of the rhamnomannan were excreted in the medium in parallel with the increasing proportion of conidia in the culture. This was followed by methylation of the polysaccharides isolated from the culture filtrates after 2, 3, 4, and 5 days of incubation. By methylation analysis and separation of the resulting alditol acetates of polysaccharide mixtures isolated after 3 and 4 days of incubation the following acetyl derivatives were identified: 2,3,4-tri-*O*- and 3,4-di-*O*-methyl-L-rhamnitol; 2,3,4,6-tetra-*O*-, 3,4,6-tri-*O*-, 2,3,6-tri-*O*-, 2,3,4-tri-*O*-, 3,6-di-*O*-, 3,4-di-*O*-, and 2,4-di-*O*-methyl-D-mannitol. Assuming a rhamnomannan structure similar to the one previously described in this strain in another culture medium at 25 °C (Travassos et al., 1973) we suggest that peaks corresponding to 2,3,4,6-tetra-*O*-, 3,4,6-tri-*O*-, 2,3,4-tri-*O*-, and 3,4-di-*O*-methyl-D-mannitol represent the alditols which arose from the mannan in the presumed polysaccharide mixture. The mannan contains α-D-mannopyranose nonreducing end units, 2-*O*-, 6-*O*-, and 2,6-di-*O*-substituted α-D-mannopyranose units. There was no evidence for the presence of β-D-galactofuranose units as in the polysaccharide preparation from strain 1099.12. Methylation data were confirmed by the ^{13}C NMR spectrum of the presumed polysaccharide mixture from a culture filtrate of strain 1099.18 in which the mannan largely predominated over the rhamnomannan (Figure 6). Signals at δ_c 103.5, 102.0, 101.0, and 99.9 were assigned to the C-1's of the nonreducing α-D-mannopyranose end units, 2-*O*-, 6-*O*-, and 2,6-di-*O*-substituted α-D-mannopyranose units, respectively. The small peak at δ_c 18.4 and the very minor peaks at δ_c 98.2 and 97.1 denoted the presence of small numbers of rhamnose units in the sample. The peaks at δ_c 80.0 and 79.7 possibly corresponded to the C-2's of 2,6-di-*O*-, and 2-*O*-substituted α-D-mannopyranose units, respectively, and the peak at δ_c 62.7 to the C-6 of the nonreducing end unit. Studies are in progress to obtain this polysaccharide free from the rhamnomannan for further investigation of its structure.

TABLE IV: Summary of Structures of Mannans Isolated from *S. schenckii* under Different Conditions.

Strain	Culture Medium	Temp. (°C)	Morphology	Source: Cells (Cp) or Supernatant (Sp)	Major Structure
1099.12	YCV	37	Mixed (yeasts predominating)	Cp	Monorhamnosylmannan
1099.12	YCV	37	Mixed (yeasts predominating)	Sp	Monorhamnosylmannan with small proportion of dirhamnosyl side chains
1099.12	M, BHI	37	100% yeasts	Cp	Monorhamnosylmannan
1099.12	Sab	37	90% yeasts	Cp	Monorhamnosylmannan
1099.12	BHI, Sab	25	Mixed (Mycelium plus conidia predominating)	Cp	Mainly dirhamnosylmannan
1099.18	M	37	100% yeasts	Cp	Monorhamnosylmannan
1099.18	M	25	100% yeasts	Cp	Monorhamnosylmannan with minor amounts of 4-*O*- and 2,4-di-*O*-substituted α-D-Man*p* units
1099.12	BHI	25	Unsporulated mycelium	Cp	Galactomannan
1099.18	B	25	Unsporulated mycelium	Cp	Dirhamnosylmannan
1099.18	B(2 d)	25	Unsporulated mycelium	Sp	Mannan and trace of rhamnomannan
1099.18	B (4 d)	25	Mycelium plus few conidia	Sp	Mannan and small amount of rhamnomannan

A summary of structures of mannans from *S. schenckii* isolated under different conditions is given in Table IV.

Discussion

Previous studies on the L-rhamno-D-mannans from *S. schenckii* and *Ceratocystis* species (Travassos et al., 1973, 1974) established the predominant chemical structures of these polymers. Generally, the polysaccharides had a (1→6)-linked α-D-mannopyranosyl main chain substituted in the 3-positions by α-L-rhamnopyranosyl and in many cases by α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl side chains. Differences among polysaccharides resided in the proportions of the dirhamnosyl side chains and of 4-*O*- and 2,4-di-*O*-substituted mannose units. The NMR spectra of these polysaccharides revealed differences which were tentatively used to classify the polysaccharides from *S. schenckii* and *C. stenoceras* in different structural types (Travassos et al., 1974).

Studies on *S. schenckii* rhamnomannans referred to above were performed on mixtures of polysaccharides isolated from the cells and excreted in the medium. Toriello and Mariat (1974) showed that differences could arise in the polysaccharide preparations from *S. schenckii* if they were isolated independently from the cells or from the culture supernatant fluids. For instance, a polysaccharide isolated from the yeast forms of an *S. schenckii* culture at 38 °C contained 15.3% galactose along with mannose and rhamnose. The polysaccharide isolated from the culture filtrate had twice as much rhamnose and only 1.8% galactose. The proton NMR spectra of *S. schenckii* polysaccharides isolated from the supernatant liquid of cultures at 35 °C with prolonged incubation showed a peak at τ 4.40 (δ 5.60) which was not observed in the spectra of polysaccharides extracted from the cells (Toriello et al., 1973). These differences suggest the heterogeneity of the polysaccharide preparations. Using the same semisynthetic medium (YCV) as before (Travassos et al., 1973) we determined the ^{13}C NMR spectra of *S. schenckii* polysaccharides isolated at 37 °C from the cells (Cp) and from the culture supernatant liquid (Sp). The differences observed in these spectra also suggested some heterogeneity in the polysaccharide preparations. Minor peaks at δ_c 103.8, 96.8, and 80.5 were observed in the Sp ^{13}C NMR spectrum but were absent in the Cp spectrum. Such signals are consistent with the presence of a small proportion of dirhamnosyl side chains in the rhamnomannan excreted in the medium in contrast with the rhamnomannan isolated from the cells which contained only single-unit α-L-rhamnopyranosyl side chains. Previous studies on the ^{13}C NMR spectra of polysaccharides obtained at 37 °C had not disclosed the presence of these signals (Travassos et al., 1974). Such discrepancies probably arise due to the variability of fungal morphology in the cultures. In fact, varying proportions of yeasts, filaments, and conidia formed in the YCV medium at 37 °C depending on factors such as the morphology of the inoculum and the incubation time. In the earlier experiments neither the inoculum nor the culture conditions (except for culture medium and temperature) were standardized. The above results show that rhamnomannans with different structures are synthesized in cultures of *S. schenckii* of mixed morphology.

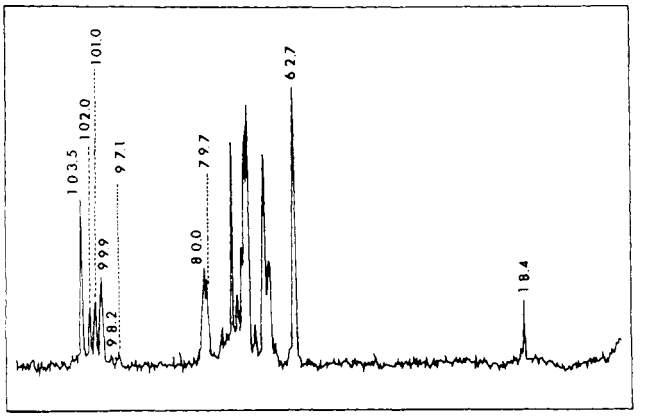

FIGURE 6: ^{13}C NMR spectrum of a *S. schenckii* (1099.18) polysaccharide preparation apparently consisting of a mannan and small amounts of a rhamnomannan. Growth conditions: medium B at 25 °C with short incubation.

To determine the structure of the polysaccharides specifically synthesized by the yeast forms a medium was devised

yielding cultures with 100% yeast forms either at 37 or at 25 °C. The rhamnomannans formed by *S. schenckii* yeast forms, irrespective of the incubation temperature, contained α-L-rhamnopyranosyl rather than *O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl side chains. Minor differences in the polysaccharides from the yeast forms could be attributed to a temperature effect: apparently, the rise in temperature lowered the ability of *S. schenckii* yeast forms to synthesize 4-*O*- and 2,4-di-*O*-substituted α-D-mannopyranose units. The temperature effect was determined by examining the ^{13}C NMR spectra of polysaccharides isolated from the yeast forms grown at both temperatures. Data obtained with the yeast forms of *S. schenckii* suggested also that polysaccharides containing dirhamnosyl side chains were more likely synthesized by the fungal mycelium or by the conidia or both.

Fortuitously, one strain of *S. schenckii* grew for a while as an unsporulated mycelium in a complex medium (BHI) at 25 °C provided the inoculum was in the mycelium phase. The polysaccharide isolated from these cells contained no rhamnose and was characterized as a galactomannan with the predominant structure consisting of 6-*O*- and 2,6-di-*O*-substituted α-D-mannopyranose units. A small proportion of the mannose end units were substituted, possibly in their 2 or 3 positions by β-D-galactofuranose units which accounted for 7–10% of the total carbohydrate content. The amount of galactomannan isolated from the cells was very small when compared with the usual production of rhamnomannans by sporulated mycelia. Since *S. schenckii* (strain 1099.12) cultures in other media at 25 °C consisted of mycelium and conidia (with no yeast forms) and the polysaccharides extracted from the cells were characterized as rhamnomannans (Travassos et al., 1973, 1974), we suggest that the latter polysaccharides were synthesized mainly by the conidia or by conidia-forming well-developed mycelium. Rhamnomannans from conidia or conidia-forming mycelia are rich in dirhamnosyl side chains. The situation of an unsporulated young mycelium forming a galactomannan may be quite transient. For some reason the BHI medium at 25 °C favors the stabilization of a morphological type with these characteristics.

The correlation of specific polysaccharide formation and cell morphology was not as clear in another strain of *S. schenckii* (1099.18). In contrast to strain 1099.12, an unsporulated mycelium culture of this strain could not be obtained in the BHI medium. In a synthetic medium introduced by Mariat (Toriello and Mariat, 1974) this strain formed an apparent conidia-less mycelium culture with short incubation (2 days at 25 °C). After this period sporulation definitely increased. The polysaccharide isolated from the mycelium with apparently no conidia attached was a typical *S. schenckii* rhamnomannan with a high proportion of dirhamnosyl side chains. Only a very small amount of a second polysaccharide was suggested by the presence in the methylation–fragmentation technique of minor peaks in the GLC run corresponding to 3,4,6-tri-*O*- and 2,3,4-tri-*O*-methyl-D-mannitol, and traces of 2,3,4,6-tetra-*O*- and 3,4-di-*O*-methyl-D-mannitol derivatives. However, in the supernatant liquid of this culture an apparent mixture of a mannan and a rhamnomannan was obtained. These polysaccharides can be separated on the basis of their reactivity with concanavalin A (Lloyd, unpublished data) into mannan-rich (38%) and rhamnomannan-rich (53%) fractions. Methylation data and ^{13}C NMR spectra were obtained on the unfractionated mixture. The mannan differed from the galactomannan isolated from the other *S. schenckii* strain by its high proportion of 2-*O*-substituted D-mannopyranose units. It seems that in the case of strain 1099.18 the mannan was excreted in the medium by the mycelium. As conidia became more numerous the amount of rhamnomannan excreted in the medium surpassed that of the mannan. This result suggests that the rhamnomannan was predominantly synthesized by the conidia or was associated with conidia formation. Since, however, the latter polysaccharide was also found in the apparently unsporulated mycelium, a clear correlation is not evident. It is possible that strain 1099.18 mycelium growing in Mariat's medium for a short incubation already had several precursors of conidia which synthesized rhamnomannans. Mannans could be formed in much lower concentrations and be more readily excreted in the medium. The new mannose-containing polysaccharides from *S. schenckii*, suggested in the present paper, was generally characteristic of mycelium cultures. When cultures consisted entirely of yeasts there was no evidence for the occurrence of other cell-bound mannose-containing polysaccharide in addition to the rhamnomannan.

Thus, the present investigation suggests that there is a preferential synthesis of different polysaccharides by specific cell types: yeasts synthesize rhamnomannans with monorhamnosyl side chains; conidia or the conidia-forming mycelia synthesize rhamnomannans with dirhamnosyl side chains; the unsporulated mycelium synthesizes galactomannans or mannans which are readily excreted in the medium. It cannot be excluded that in some strains the transient unsporulated mycelium also synthesizes a rhamnomannan rich in dirhamnosyl side chains. Similar findings have not been reported in other studies on the biochemistry of differentiation in fungi although Wang and Bartnicki-Garcia (1973) reported the appearance of a specific phosphoglucan in the sexual phases of *Phytophthora palmivora*.

In view of the results of Toriello and Mariat (1974) who found variations in the polysaccharide constitutions depending on the carbon source used to grow *S. schenckii*, we also investigated the influence of the medium on polysaccharide formation. To discount morphological transition as a differential factor, three media, one synthetic (medium M) and the other complex (BHI, Sabouraud), were used; these yielded cultures at 37 °C which were 90–100% in the yeast form. Under these conditions the polysaccharides synthesized by *S. schenckii* were indistinguishable on the basis of their ^{13}C NMR spectra. This result seemed to exclude the influence of the medium on polysaccharide formation provided the fungal morphology in the cultures was the same. As expected, some variation occurred in the polysaccharides formed at 25 °C in two different media. Neither medium (Sabouraud or BHI) promoted cultures at this temperature with one morphological type only. The ^{13}C NMR spectra of the latter polysaccharides were, however, similar, and both contained signals at δ_c 97.0 which are consistent with the presence of 2-*O*-substituted α-L-rhamnopyranose units in the corresponding rhamnomannans. The peak at δ_c 103.5 which appeared in the ^{13}C NMR spectrum of the Sabouraud-derived polysaccharide could be assigned to C-1 of L-rhamnopyranose nonreducing end units in the *O*-α-L-Rha*p*-(1→2)-α-L-Rha*p* sequence. This assignment, which was not made in our previous study (Travassos et al., 1974), was made by comparison with the ^{13}C NMR spectrum of a trisaccharide α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)-D-mannopyranose prepared by partial acetolysis of an *S. schenckii* rhamnomannan (Lloyd and Travassos, 1975).

Galactose-containing heteropolymers, as the one isolated from the mycelium of *S. schenckii*, are common among pathogenic fungi. Galactomannans isolated from *Histoplasma*

capsulatum, Paracoccidioides brasiliensis, and *Blastomyces dermatitidis* and type I galactomannans from dermatophytes have (1→6)-linked D-mannopyranose units as the main chain (Azuma et al., 1974; Grappel et al., 1974). Galactomannans I from dermatophytes are branched polysaccharides with branch points through the C-2 and C-6 hydroxyl groups of D-mannopyranose. D-Mannopyranose and D-galactofuranose are the nonreducing end units. In *Cladosporium werneckii* mannose is mainly (1→2)-linked. Branch points are through 2,6-di-*O*-substituted D-mannopyranose units. Galactose in the latter polysaccharide is present in both D-pyranosyl and D-furanosyl forms (Lloyd, 1970). In dimorphic fungi it was generally observed that the mycelial rather than the yeast-like forms synthesized galactomannans (Azuma et al., 1974; Kanetsuna et al., 1974). Our results with *S. schenckii* are in agreement with this generalization.

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