# Mannans of the Cell Walls of the Yeast and Mycelial Forms of *Candida tropicalis*

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Yeast-mycelium dimorphism of *Candida tropicalis* is followed with the quantitative changes in the chemical composition of the cell wall. The mycelial form contains twofold amount of the lipids and the increased amount of amino sugars and glucans in comparison with the yeast form of the microorganism. The contents of the cellular mannans and glucans are significantly different in these two morphological forms. Both mannans, however, have very similar structure with the $\alpha$-(1→6)-glycosidically linked backbone, the sugar units of which are substituted in position 2 with the oligomannosidic side chains linked through $\alpha$-(1→2)-glycosidic bonds. The mannan of the yeast form possesses relatively more longer side chains and contains unusual branching point in some side chains, *i.e.* the mannosyl unit disubstituted in positions 2 and 3.

*Candida tropicalis* belongs to the group of dimorphous fungi which produce two different morphological forms depending on the conditions of the cultivation. By the dimorphism is meant reciprocal reversibility of the yeast and mycelial forms caused by the changes of the composition and/or temperature of the cultivation medium [1].

Similar morphological changes were observed at the comparison of the growth processes of *C. tropicalis* in glucose medium and in the liquid wastes from the sulfate procedure of the cellulose production. In this paper we present the results of the investigations how the morphological changes do correlate with the changes in composition of the biomass and of the cell walls, as well as in the structure of the cellular mannans which are the main surface antigens of the microorganism.

## EXPERIMENTAL

The yeast *Candida tropicalis* CCY 29-7-6 from the Culture Collection of Yeasts and Yeast-like Microorganisms, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, was used. The yeast strain was preserved on slant wort agar under sterile paraffin oil at room temperature.

Cultivation media: semisynthetic yeast medium – 20 g glucose, 3 g ammonium sulfate, 1 g magnesium sulfate, 0.5 g potassium dihydrogenphosphate, 5 g yeast autolyzate and 1 $cm^3$ of the microelement solution [2] were dissolved in distilled water and the volume was adjusted to 1000 $cm^3$.

The prehydrolyzate obtained from Bukóza (Vranov, Slovakia) [3] was concentrated with the vacuum evaporator and subsequently hydrolyzed with 2 % sulfuric acid at 100 °C for 30 min. pH was adjusted to 5—6 with $Ca(OH)_2$ and the mixture was filtered. Charcoal was added to the filtrate and, after short boiling the mixture was filtered. Subsequently yeast autolyzate was added to the solution up to 5 g $dm^{-3}$ concentration and, upon sterilization, it was used as a cultivation medium.

Sulfate extracts were obtained from the North-Slovakian Cellulose and Paper Plants in Ružomberok. Preparation of the sulfate extracts for the cultivation purposes included removal of lignin and decreasing of the inorganic salts content to the level that could not inhibit yeast growth. Lignin was precipitated from the sulfate extracts by addition of 30 % $H_2SO_4$ until pH reached 5.5. The sediment of lignin was separated by filtration and the filtrate was diluted with water to reach 4 % content of organic substances. Afterwards, yeast autolyzate was added to the treated extract up to 5 g $dm^{-3}$ concentration.

Cultivation took place with constant stirring for 60 h at 28 °C, constant pH = 5.6 and oxygen supply. After the cultivation, the cells were separated from the medium by means of flow centrifugation, washed with ice-cold water and once more centrifuged.

Nitrogen content was determined according to the method of Dumas [4]; crude protein content was calculated by multiplication of the nitrogen content with 6.25; nucleic acids were determined according to *Spirin* [5]; amino acids were determined using an automatic amino acid analyzer, type 6020 (Developing Workshops of the Academy of Sciences of the Czech Republic, Prague); neutral sugars were determined with phenol-sulfuric acid procedure [6]; amino saccharides were determined using the modi-

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fied Morgan—Elson method [7] and the lipids according to *Suomalainen* and *Nurminen* [8]. Specific rotations of the 1 % aqueous solutions of the mannans were determined with a Perkin—Elmer 141 polarimeter at $\lambda$ = 589 nm.

The structural characteristics of the glycoproteins, polysaccharides and oligosaccharides were defined by means of the selective alkaline degradation [9], total and partial acid hydrolysis [10], Smith degradation [11], NMR spectroscopy at Bruker AM-300 spectrometer, and methylation using the modified Hakomori procedure as described by *Lindberg* [12]. Partially methylated alditol acetates were analyzed by means of gas-liquid chromatography (gas chromatograph JGC-20K) combined with mass spectrometry (mass spectrometer JMS D-100). The NMR measurements and GLC analysis of the methylated alditol acetates were carried out as described in [13].

Acetylation and acetolysis of the mannans were performed according to *Kocourek* and *Ballou* [10]. The deacetylated acetolysis products were applied to a column (2 cm × 150 cm) of Bio-Gel P-2 and eluted with distilled water at the rate of 12 $cm^3\ h^{-1}$ at room temperature. 2.5 $cm^3$ fractions were collected. The total carbohydrate content in the effluent was determined by the phenol-sulfuric acid method [6] with the absorbance having been measured at $\lambda$ = 490 nm.

### Isolation of the Cell Wall Glycoproteins and Polysaccharides from the Yeast Biomass and the Cell Walls

The isolation procedure is schematically presented in Scheme 1. The biomass was delipidized by extraction with the absolute ethanol as well as with a mixture of chloroform—methanol ($\varphi_r$ = 3 : 1). The delipidized cells were suspended in 0.2 M-NaCl in the mass ratio 1 : 20 and extracted for 20 min at the pressure of 700 kPa and temperature 145 °C three times, each time with new NaCl solution. After separation of the cells, the supernatants were pooled together and after the dialysis concentrated with vacuum evaporator. During the concentration procedure the glycoprotein was sedimented (fraction *1*), then it was separated with centrifugation and lyophilized. The concentrated supernatant was precipitated with the threefold volume of ethanol. The sediment presented mannan-protein (fraction *2*).

The cell residue was twice extracted with 2 % NaOH at 100 °C. Combined extracts were concentrated *in vacuo*, dialyzed and precipitated with the threefold volume of ethanol (fraction *3*). The rest of the cells after the alkaline extraction was washed with water until neutral reaction and lyophilized (fraction *4*). Mannans were isolated from fraction *2* according to *Gorin* and *Spencer* [14].

For quantitative determinations the substances were dried at 105 °C until the constant mass value was achieved.

## RESULTS AND DISCUSSION

The yeast *Candida tropicalis* was cultivated in the glucose cultivation medium as well as in the liquid wastes from the sulfate procedure of the cellulose production. While in the glucose medium and in the prehydrolyzate the yeast grew in normal yeast form, during the growth in the sulfate extracts filamentous cells were formed.

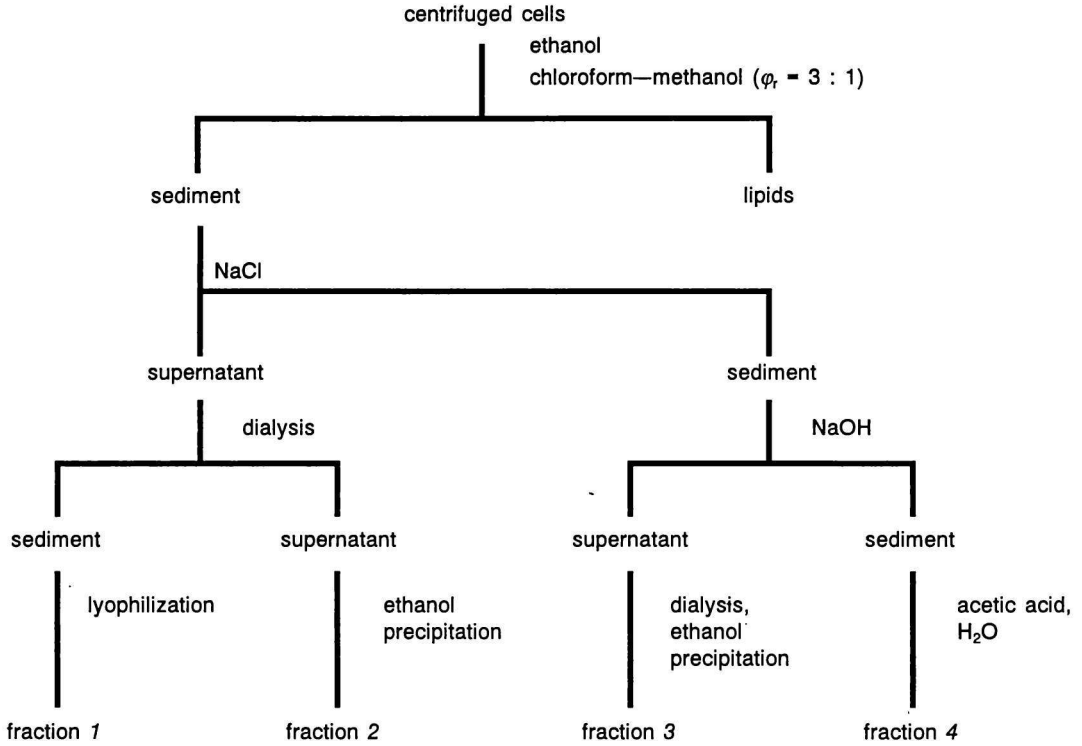
The yields of the biomass from the sulfate extracts cultivation amounted to less than 1/3 of the yields from the glucose medium, since the yeast was not able to assimilate the major component of the sulfate extract, namely glucoisosaccharinic acid and other saccharinic acids [15, 16]. The biomass isolated from the sulfate extracts (mycelial form of the yeast) differed from that isolated from the glucose and prehydrolyzate media (yeast form) in diminished content of proteins as well as in increased amount of lipids and saccharides (Table 1). Amino acid composition of the proteins was the same in both morphological forms. The ratio of glucose to mannose was 1 : 1 in the cell walls of the yeast form, while in the mycelial form it was substantially higher (1.8 : 1).

The differences observed in the chemical composition of the cell walls of both morphological forms were significant. It might be therefore suggested that definite ratio of the individual components of the cell walls, *i.e.* glucans, mannans, chitin, and proteins, is responsible for the morphology of the yeast.

**Table 1.** Yields and Composition of the Biomass of *C. tropicalis* Produced upon Cultivation in the Glucose Medium and in the Wastes of the Sulfate Procedure of the Cellulose Production

| | Glucose | Sulfate extract | Prehydrolyzate |
|---|---|---|---|
| Yield/(g $dm^{-3}$) | 7.3 | 2.2 | 6.7 |
| *w*(Crude protein)/% | 55.6 | 43.4 | 54.5 |
| *w*(Nucleic acids)/% | 5.7 | 5.3 | 6.2 |
| *w*(Saccharides)/% | 31.7 | 36.6 | 32.0 |
| *w*(Lipids)/% | 7.0 | 14.7 | 6.7 |

In order to find out whether morphological changes caused by the cultivation in glucose medium or in the sulfate extracts correlate with the structure of the yeast cell-wall antigens, we have isolated the cell-wall mannans of both the morphological forms.




*Scheme 1*

Extraction procedure for the isolation of the cell-wall glycoproteins of *C. tropicalis*.

**Table 2.** The Composition of the Glycoprotein Fractions of *C. tropicalis*

| Fraction | Yeast form | | | | Mycelial form | | | |
|---|---|---|---|---|---|---|---|---|
| | *1* | *2* | *3* | *4* | *1* | *2* | *3* | *4* |
| *w*(Protein)/% | 56.2 | 28.9 | 37.1 | 9.1 | 63.8 | 31.8 | 7.1 | 11.3 |
| *w*(Glucose)/% | 39.0 | 11.8 | 23.6 | 79.5 | 26.0 | 16.5 | 44.9 | 88.7 |
| *w*(Mannose)/% | 4.8 | 59.3 | 39.3 | 11.4 | 10.2 | 51.7 | 48.0 | – |

The cellular glycoproteins were extracted from the lipid-devoid cells with the solutions of NaCl and NaOH according to Scheme 1.

Fraction *1* contained mainly glucan-protein (Table 2), while fraction *2* consisted prevailingly of mannanprotein. Fraction *3* was a mixture of the peptidomannan, peptidoglucan, and the denatured proteins. Fraction *4*, the insoluble residue after the alkaline extraction and washing with acetic acid, was $\beta$-glucan [17]. By means of precipitation with copper salts, $\alpha$-mannan was isolated from fraction *2*. The composition of the glycoprotein fractions is shown in Table 2.

Mannan of the yeast form had $[\alpha]$(D, 20 °C) = + 57.5°, while that of the mycelial form had $[\alpha]$(D, 20 °C) = + 56.0°. Both mannans, thus, exhibited similar optical rotation implying predominantly $\alpha$-anomeric configuration of the glycosidic linkages. The $^{13}C$ NMR spectra of both mannans looked similar and reminded the spectra of other *Candida* mannans [13]. Fig. 1 presents $^{13}C$ NMR spectrum of the cellular mannan of the yeast form of *Candida tropicalis*. Assignment of the signals is presented in our previous paper [18].

The methylation analysis of the mannans was carried out using the modified Hakomori method [12]. The resulting mixture of the methylated alditol acetates was analyzed by means of gas-liquid chromatography and mass spectrometry. The results obtained are shown in Table 3.

The mannans were split by means of the selective acetolysis [10] and the resulting mixture of mannose and mannooligosaccharides was separated using gel filtration on Bio-Gel P-2 column (Fig. 2). The oligosaccharides obtained represented the side chains of the mannans [13] and contained only $\alpha$-(1→2) glycosidic linkages. Longer oligosaccharides obtained from the mannan of the yeast form of *C.*

**Table 3.** Methylation Analysis of the Cellular Mannans of *C. tropicalis*

| Partially methylated mannitol acetate | *x*(Yeast form) % | *x*(Mycelial form) % |
|---|---|---|
| 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol | 25.6 | 23.9 |
| 1,2,5-Tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol | 35.9 | 37.6 |
| 1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol | 12.8 | 14.7 |
| 1,2,5,6-Tetra-*O*-acetyl-3,4-di-*O*-methylmannitol | 24.4 | 23.5 |
| 1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methylmannitol | 1.3 | – |

to form a mycelial growth phase showed a decreased virulence in animal infection studies [25]. However, despite differences in the morphology and virulence, no significant differences could be found in the structure of the antigenic mannans of both forms besides somewhat lower content of $\beta$-(1→2) and $\alpha$-(1→3) linked mannosyl units in the mycelial form mannan, as well as relatively higher content of the longer oligomannosidic side chains in the yeast form mannan [21]. Thus, it seems possible that the morphological change from yeast to mycelial form is followed by the change of the composition of the cell wall, but not by the change of the structure of the carbohydrate antigens.

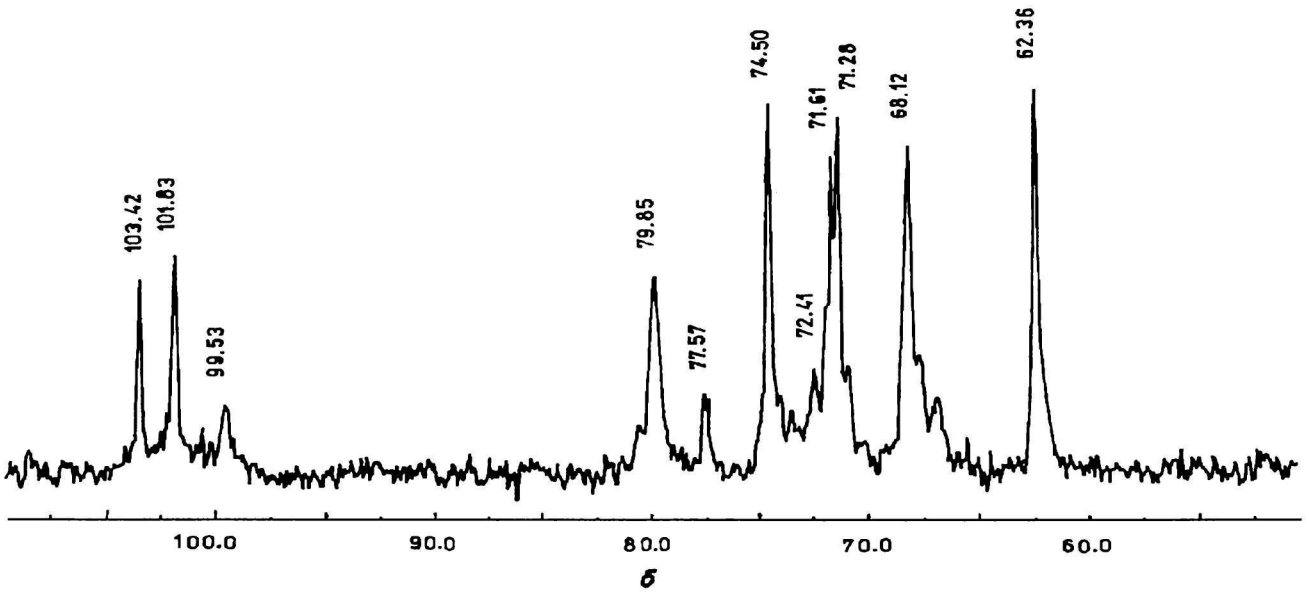



**Fig. 1.** $^{13}C$ NMR spectrum of the cellular mannan of the yeast form of *C. tropicalis*.

*tropicalis* contained an additional branching point — a mannosyl unit disubstituted in positions 2 and 3, similarly to the previously studied mannans of *C. albicans* and *C. guilliermondii* [18]. Larger nonacetolyzed fragment $V_0$ (Fig. 2) contained besides $\alpha$-(1→2) also $\alpha$-(1→6) glycosidic linkages (data not shown). This nonacetolyzed fragment might represent the inner core of the yeast mannan [19]. The reason why some $\alpha$-(1→6) glycosidic linkages were not cleaved in acetolysis conditions might be in some special secondary structure of the inner core that hindered its accessibility to the acetolysis agents.

It can be seen that despite morphological changes, the structure of the cell-wall mannan remains almost the same in both yeast and mycelial form of *C. tropicalis*.

Several papers dealt with the investigations of the yeast and mycelial forms of the pathogenic yeast *Candida albicans* [20—23]. It was observed that formation of mycelial cells was an important factor in adherence of the parasite to the host epithelium [24], while mutants of *C. albicans* that were unable

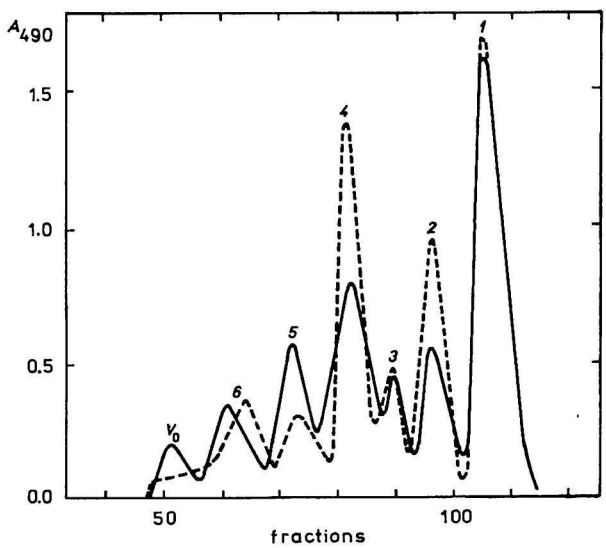



**Fig. 2.** Gel filtration profiles of the acetolysis products of the *C. tropicalis* mannans: – – – mycelial form; —— yeast form; *1.* mannose; *2—6.* mannobiose to mannohexaose; $V_0$ – larger nonacetolyzed fragment.

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