

ISOLATION AND SOME APPLICATIONS OF FUNGAL CHITIN - GLUCAN COMPLEX AND CHITOSAN

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INTRODUCTION

Fungal mycelial wastes from biotechnological plants can become free and rich alternative sources of chitin - chitosan materials, beside the traditional industrial source - shellfish waste materials. Moreover, the fungal chitosans can have unique properties compared with those derived from *Crustacea*.

We have tested possibilities of production and some applications of chitin-glucan complex and chitosan from industrial waste mycelia of *Aspergillus niger* from a citric acid production plant and *Penicillium oxalicum* from production of a red anthraquinone dye in Czech Republic.

The alkali-insoluble cell-wall residue of the mycelia consists mainly of chitosan, chitin and β -glucans, with a significant preponderance of (1-3)- β -D-glucan. Chitin is thought to be present as microfibrils physically embedded in the β -glucan matrix.

EXPERIMENTAL

Hypal wall isolation and chitosan extraction

Aspergillus niger mycelium was obtained as a by-product of submerge production of citric acid from AKTIVA (Kaznějov, Czech Republic). *Penicillium oxalicum* mycelium was obtained from ASCOLOR - BIOTEC (Uničov, Czech Republic) as a by-product of production of a red anthraquinone dye.

Mycelia were washed exhaustively with water and dried by freeze-drying. Chitosan and chitin-glucan complex were extracted from the mycelial wastes with alkali hydroxide and hydrochloric acid using a method developed by combination and modification of methods suggested by Muzzarelli [1] and White et al. [2]. Mycelia were treated with 0.5M-10M aqueous KOH or NaOH solutions (10 ml per 1 g of dry mycelium) at 95 or 120°C for 1-3 h to remove alkali-soluble biopolymers and deacetylate chitin to chitosan. The insoluble material was washed with DEMI water to obtain neutral pH and concentrated ethanol and freeze-dried. On applying this procedure, an alkali-insoluble fraction of cell walls consisting of chitosan and chitin-glucan complex is left as a white powder.

For chitosan isolation this freeze-dried alkali insoluble fraction was treated with diluted 2% HCl solution (50 ml per 1g of dry matter) for 1-12 h at 95°C. Value of pH of the acidic supernatant was increased to 9.5 with 2M NaOH. The alkali-insoluble precipitate (presumable chitosan) obtained was repeatedly centrifuged and washed with distilled water, freeze dried and weighed.

Measurement of IR spectrum of the isolated chitosan fraction

The IR spectra of the KBr discs containing the isolated alkali-insoluble precipitate after the acidic extraction (presumable chitosan), and a commercial chitosan, medium molecular weight (Sigma-Aldrich) were measured from 4 000 cm^{-1} to 450 cm^{-1} with a Nicolet-Impact 410 FT-IR spectrophotometer.

Measurement of ^1H NMR spectrum of the isolated chitosan fraction

10 mg of the alkali-insoluble precipitate (presumable chitosan) and a commercial chitosan, medium molecular weight (Sigma-Aldrich) were dissolved in D_2O (pH=3.1). Chemical shifts in ppm units are related to DSS standard (sodium dimethylsilylsulphonate). From the ratio of acetyl CH_3 group (2.0 ppm) and H2 group (deacetylated form, 3.13 - 3.10 ppm) signal intensities degree of acetylation of the isolated chitosan was estimated.

Preparation of carboxymethyl derivatives of chitosan and chitin-glucan complex

CM-derivatives of the isolated chitosan and chitin-glucan complex were prepared by a modified procedure suggested according to US4304905 and US4619995 patents. Principle of the method is treating the samples with monochloroacetic acid in hot concentrated solutions of alkali hydroxides.

Preparation and AFM analysis of membranes

Membranes were prepared by a direct solvent evaporation in Petri dishes. Solutions containing 1 - 2% (w/v) of chitosan in 2% (w/v) acetic acid or CM-derivatives in water were used in the experiments. Surfaces of thin films of the samples on microscope cover slips were analysed using commercial atomic force microscope TOPOMETRIX Explorer (TopoExplorer).

RESULTS and DISCUSSION

Hypal wall isolation and chitosan extraction

Yields of the alkali-insoluble material after the treatment of the fungal biomass with 0.5M - 10M aqueous KOH or NaOH solutions at 120°C were in the range 15 - 33% on a dry cell weight basis according to conditions of the procedure. There was no significant difference between both the mycelia.

The maximum yields of the chitosan fraction after the treatment with 10M aqueous KOH or NaOH solutions and the acidic extraction were 13.1% for *A. niger* and 11.3% for *P.oxalicum*.

The results are in a good agreement with results published by other authors. Miyoshi, et al. [3] obtained yields varied from 1.2 to 10.4% (of a dry fungal cell weight) of chitosan-like material extracted from 5 different fungal strains other than *Aspergillus* sp. Muzzarelli, et al., [4] found 11 - 14% of chitosan in *A. niger* biomass under comparable conditions of alkali extraction.

Fig.1 shows graphs of dependence of chitosan yields on NaOH concentration used for the alkali extraction at 120°C for 2 h period. The curves are similar for both the mycelia.

The graphs show that substantial yields of chitosan can be obtained already at lower NaOH concentrations than 10 mol.l⁻¹. Substantial chitosan yields from *P.oxalicum* biomass were achieved even after the alkali extraction at 95°C, in contrast with *A. niger* biomass (results unshown). The reason of the higher chitosan yields from *P.oxalicum* biomass at 95°C can be better availability of chitin for the alkali deacetylation because of a fiber mycelial structure in contrast to a globular structure of *A. niger*.

Measurement of IR spectrum of the isolated chitosan fraction

To prove that the acid extractable material contains chitosan, its IR spectrum was measured in comparison with IR spectrum of a commercial chitosan purchased from Sigma-Aldrich (see Fig.2). The isolated fraction gave IR spectrum similar to that of the commercial chitosan. The result indicates a strong similarity of both the compounds.

Measurement of ^1H NMR spectrum of the isolated chitosan fraction

Fig. 3 shows ^1H NMR spectrum of the isolated chitosan fraction. ^1H NMR spectrum of the commercial chitosan sample is not shown because of its low solubility at the conditions of the method. However comparisons with the published chitosan spectra proved that the isolated fraction is chitosan.

From the ratio of acetyl CH_3 group (2.0 ppm) and H2 group (deacetylated form, 3.13 - 3.10 ppm) signal intensities degree of acetylation (DA) of the isolated chitosan was calculated as cca 4%. The result is in a good correspondence with our previous result obtained with a direct spectrophotometric method.

The calculation of DA: $\text{DA} = [(0.118/3)/1] \times 100 \approx 4$

Kobayashi, et al. [5] prepared a chitosan product from mycelia of *Absidia* strains with a degree of acetylation of 8-21%. Degree of acetylation of different fungal chitosans ranged from 6 to 15%, according to Rane and Hoover [6].

Preparation and AFM analysis of membranes

A photograph of a membrane prepared from the isolated chitosan from *Aspergillus niger* is shown at Fig. 4. Similar membranes from the commercial chitosan PRIMEX and the CM-derivative of the chitin-glucan complex from *Aspergillus niger* were also prepared. The prepared membranes are fragile.

Fig. 4 also shows AFM images of thin films prepared from the chitosan and CM-derivative samples. The globules perceptible on the AFM images probably correspond to single chitosan molecules. Sizes of the molecules of the isolated chitosan and the CM-derivative of the chitin-glucan complex are between 80-100 nm.

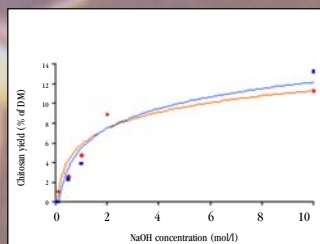


Fig.1 Graph of dependence of chitosan yields on concentration of NaOH solution used for the alkali extraction at 120°C for 2 h period

Blue line: *Aspergillus niger*. Red line: *Penicillium oxalicum*.

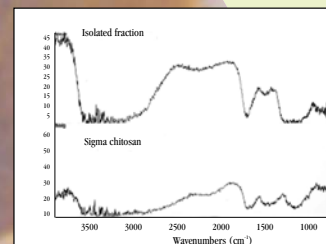


Fig.2 Comparison of IR spectra of the isolated chitosan fraction and a commercial chitosan sample from Sigma-Aldrich

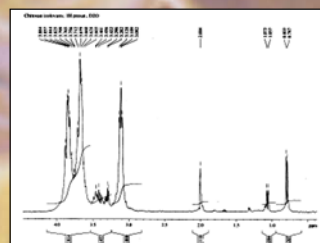


Fig.3. ^1H NMR spectrum of the isolated chitosan fraction



Fig.4. Membrane sample and AFM images of thin film surfaces

CONCLUSIONS

The industrial waste mycelia of *Aspergillus niger* and *Penicillium oxalicum* fungi are good alternative source for chitosan production. The maximum chitosan yields were 13.1% for *A. niger* and 11.3% for *P.oxalicum*. Substantial chitosan yields can be obtained after a treatment of the fungal biomass with diluted solutions of alkali hydroxides, in contrast with the traditional industrial source-shellfish waste materials. The isolated chitosans were characterised by IR and NMR spectroscopy.

Water soluble carboxymethyl derivatives of the isolated chitosan and the chitin-glucan complex from *Aspergillus niger* were also prepared. The isolated chitosans and the carboxymethyl derivatives were suitable for preparation of membranes.

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