IN SITU IMMOBILIZED CHITOSANOLYTIC ENZYMES FROM *MUCOR CIRCINELLOIDES*

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1. Introduction

Chitosan, a derivative of chitin, is a copolymer composed of 2-amino-2-deoxy-β–D-glucose and 2-acetamido-2-deoxy-β–D-glucose units. Recently, attention has been paid to chitooligosacchardies derived from this polymer, which were found to display various biological activities and are applied in medicine and in pharmaceutical, agricultural and food industries [1]. The oligomers with a relatively high degree of polymerization (DP) ranging from 4 to 7, that can be obtained by enzymatic degradation of chitosan display more diversified and stronger biological activities (immunological effects, antitumor, antiviral, antimicrobial and antioxidant activities) relative to that shown by oligomers with low DP, which are released through acid hydrolysis [1 - 3]. Enzymatic hydrolysis of chitosan is catalyzed by synergistically acting chitosanolytic system comprising endo- and exo-chitosanases.

The endo-hydrolases (chitosanases, EC 3.2.1.132 and chitinases, EC 3.2.1.14) cleave in a random manner the internal glycosidic bonds throughout the biopolymer chains thereby releasing water-soluble low molecular weight oligomers. The exo-hydrolases, comprising β-N-acetylhexosaminidase, known as chitobiase (EC 3.2.1.52) and exo-β-D-glucosaminidase attack chitosan chains and chitooligosaccharides from their non-reducing ends. The chitosan oligomers with various molecular weights were produced using the number of preparations of non-specific enzymes, such as: chitinase [4], lysozyme [5], lipase [6 - 7], tannase [7], papain [8], cellulase [9], hemicellulase [3] and pectinase [10].

Mucorales fungi contain significant amounts of chitin and chitosan in their cell walls [11 - 12] and produce enzymes involved in their degradation. Thus they can be used as a source of both the above-mentioned biopolymers and enzymes catalyzing their digestion, such as: chitosanases, chitinases, chitin deacetylases (EC 3.5.1.41), etc.

Presented studies aimed at providing evidence that the strain of *Mucor circinelloides* from our collection is an effective producer of chitosanolytic enzymes. We have developed the method of obtaining of an *in situ* immobilized preparation of intracellular chitosanolytic *M. circinelloides* enzymes and determined their substrate specificity. Presented work is a continuation of earlier researches [13].

2. Materials and methods

2.1. Chemicals

Chitosans with various molecular weights (M_v) ranging from 120.6 kDa to 421.3 kDa and deacetylation degree (DD) ranging from 66.0% to 85.8% were obtained from Vanson, Redmont (USA) and from Chemopol, Complex Pvt. Ltd, Tada (India). Chitin, glucosamine and N-acetylglucosamine were supplied by Sigma Co.

2.2. Microorganism and culture conditions

The strain of *Mucor circinelloides* from the pure culture collection of the Institute of Technical Biochemistry of TUL was cultivated for 72 h at 30 °C under agitated conditions (180 rpm) in different liquid media.

Media of series I were prepared from a basal medium containing (% w/v): glucose (0.10), $(NH_4)_2SO_4$ (0.15), KH_2PO_4 (0.80), $MgSO_4 \times 7\ H_2O$ (0.05), NaCl (0.01), $CaCl_2$ (0.01), supplemented with microelement solutions (0.1% v/v) containing (g/l): solution 1 - H_3BO_3 (500), $CuSO_4$ (40), KJ (100), $MnSO_4$ (500), Na_2MoO_4 (200), $ZnSO_4$ (400); solution 2 - $Fe_2(SO_4)_3$ (2460). This medium was alternatively supplemented with 0.5% (w/v) D-glucosamine, N-acetylglucosamine, chitosan or chitin.

Media of series II contained corn steep liquor (3.7% w/v) and olive oil (2.7% v/v) and were alternatively supplemented with 0.5% (w/v) glucose, chitin, chitosan, D-glucosamine or N-acetylglucosamine.

Chitin and chitosan (M_v = 392.5 kDa, DD = 79.8%) were used in a powdered form.

YPG medium contained (% w/v): glucose (2.0), peptone (1.0), yeast extract (0.1), $(NH_4)_2SO_4$ (0.5), K_2HPO_4 (0.1), $MgSO_4 \times 7\ H_2O$ (0.5), NaCl (0.1) and $CaCl_2$ (0.01).

The initial pH of each medium was 4.7. Mycelia of *M. circinelloides* (suspended or pelleted) were harvested by filtration, washed with water and with acetone (three times), air-dried at room temperature and used as the in situ immobilized enzymatic preparations.

2.3. Determination of enzymatic activity

Enzymatic activity of the *in situ* immobilized enzymatic preparations were assayed by the modified method described in [13] using chitosan solution (2% chitosan in 2% acetic acid) or colloidal chitin as the substrates. The colloidal chitin was obtained as described in [14]. Chitin was dissolved in 28% HCl solution (1:14 w/w), stirred for 3 h at room temperature and precipitated by slowly adding cold (5 °C) water (1:6 v/v). Colloidal chitin was collected by filtration through coarse filter paper and washed with water until its pH was close to 4.5.

The activity was determined on the basis of a drop in an average molecular weight of chitosan (assays of activity of endo-hydrolases) and an estimation of reducing sugars content after the hydrolysis of both the substrates (measurements of activity of exo-hydrolases).

2.3.1. Reduction in average molecular weight of chitosan

Reaction mixtures containing chitosan (20 mg) and enzyme preparation (10 mg) in 1 cm^3 of 2 M CH_3COONa (pH 5.5) were incubated for 2 h at 37 °C and for 5 min in a boiling water bath, and centrifuged at 12000 rpm for 5 min. As a control, the solution of chitosan in 1 cm^3 of 2 M CH_3COONa (incubated and treated in the same manner) was used.

For optimization of conditions of chitosan biodegradation by *M. circinelloides* endo-hydrolases the reaction was performed for 1 to 6 h at different temperatures ranging from 25 °C to 55 °C, and pH ranging from 4.1 to 6.0.

2.3.2. Saccharification of chitosan or colloidal chitin

Reaction mixtures were prepared as described in section 2.3.1., but the amount of enzyme preparation was 20 mg. Processes of enzymatic degradation of chitosan or chitin were carried out for 24 h as described in section 2.3.1. The substrates and enzyme preparations were separately incubated in the solution of 2 M CH_3COONa and used as the controls.

For optimization of chitosan biodegradation by *M. circinelloides* exo-hydrolases the reaction was performed for 1 to 72 h at temperatures ranging from 25 °C to 55 °C, and pH ranging from 4.1 to 6.0.

2.4. Analytical methods

The average molecular weight (M_V) of chitosan and its digestion products was determined by the viscometric method described in [15]. The viscosity measurements were conducted at 25 °C using an Ubbelohde's viscometer (SCHOTT, K ≈ 0.01 $mm^2 \cdot s^{-2}$).

Content of reducing amino-oligomers was determined by the Somogyi-Nelson method using glucosamine or N-acetylglucosamine as standards.

HPLC analysis of low molecular weight fractions of hydrolysis products (up to 3 kDa) was carried out by using Dionex (Sunnyvale, CA, USA) chromatographic system with amperometric detection (PAD). The apparatus consisted of a GP40 pump, PAD-2 detector, CarboPac PA1 precolumn (4 × 50 mm) and CarboPac PA1 analytical column (4 × 250 mm).

3. Results and discussion

Our earlier studies revealed that chitosan-digesting enzymes from *M. circinelloides* strain display the high activity of endo-hydrolases [13]. The above-mentioned enzymes rapidly reduced the average molecular weight of chitosan and released only small amounts of reducing sugars.

3.1. Stimulation of biosynthesis of chitosan-degrading enzymes by *M. circinelloides* and optimization of the culture medium composition

Biosynthesis of the majority of chitosanases is known to be activated by glucose (Glc), glucosamine (GlcN), N-acetyglucosamine (GlcNAc), chitosan or chitin, that are used as a sole C-source in a culture medium [16]. The effect of these compounds on biosynthesis of chitosan-degrading enzymes by *M. circinelloides* strain and yield of biomass was evaluated and the results are shown in Figures 1 and 2.

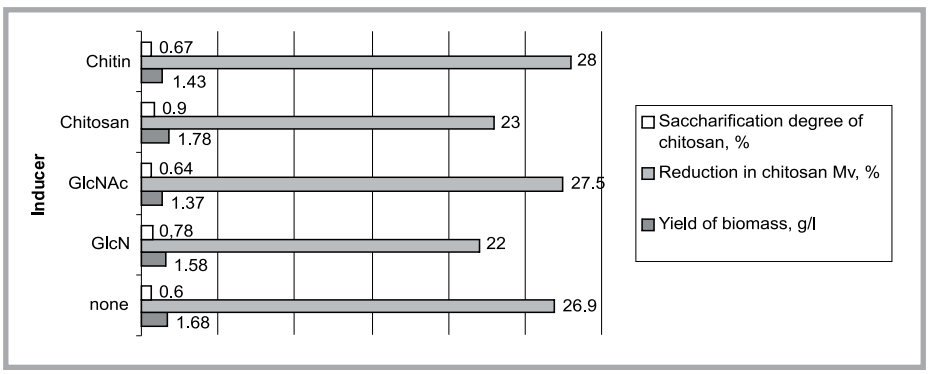

Figure 1. *The effect of different inducers on biosynthesis of chitosan–degrading enzymes by M. circinelloides (media of series I, section 2.2.). The activity of chitosanases was assayed using chitosan solution as described in section 2.3.*

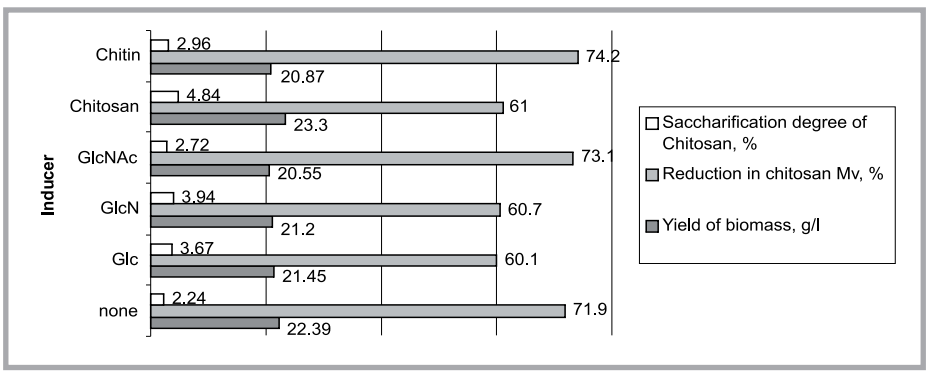

Figure 2. *The effect of different inducers on biosynthesis of chitosan–degrading enzymes by M. circinelloides (media of series II, section 2.2.). The activity of chitosanases was assayed using chitosan solution as described in section 2.3.*

It was revealed that chitin (0.5% w/v) and GlcNAc (0.5% w/v) present in the media of series I and II, insignificantly stimulated the biosynthesis of endo-chitosanolytic enzymes by *M. circinelloides*. By contrast, supplementation of the culture medium with chitosan (0.5% w/v) and GlcN (0.5% w/v) lowered the yield of their production (Figures 1 and 2).

On the other hand, the exo-chitosanase activity and biomass yield were increased (in low degree) when chitosan (0.5% w/v) was added to the media of series I and II (Figures 1 and 2). It is to note that the *M. circinelloides* strain did not grow in the media, which contained either chitosan or chitin in concentrations above 1.0% (w/v) or GlcN or GlcNAc in concentrations above 2.0% (w/v).

As it was described in [17 - 18], the YPG medium (section 2.2.) was found to be optimal for biosynthesis of chitosanases by several microorganisms. Preparations of *M. circinelloides* mycelium harvested from this medium displayed the highest exo-chitosanase activity (the degree of chitosan saccharification approached 9.63%), and the relatively low endo-chitosanase activity (the drop in chitosan M_v reached 49%). Surprisingly, the last activity was higher (the decrease in chitosan M_v was 73.6%) when the medium contained corn steep liquor and olive oil (Figure 3) – the known inducer of intracellular lipases [19].

3.2. Specificity of Mucor enzymes in chitosan biodegradation

The optimum conditions for chitosan hydrolysis by *M. circinelloides* enzymes were the temperature of 37 °C and pH 5.5. The highest drop in the average molecular weight of the biopolymer (of circa 60%) was observed within the 1st h of polymer degradation. After 6 h of the process, the M_v of chitosan dropped by more than 80% while the saccharification degree reached only 1 - 2%. Even the significantly longer time of digestion (72 h) resulted in a saccharification degree of only 7.5%. It implies that the activity of exo-hydrolases in the *Mucor* preparation and their contribution to chitosan depolymerization were insignificant.

Chitosanases from various sources display different hydrolytic action patterns, which depend on the substrate deacetylation degree (DD) and/or molecular weight (M_v) of chitosan [16]. Effects of DD and M_v of chitosan on activity of *M. circinelloides* chitosanolytic enzymes are shown in Table 1 and in Figure 4.

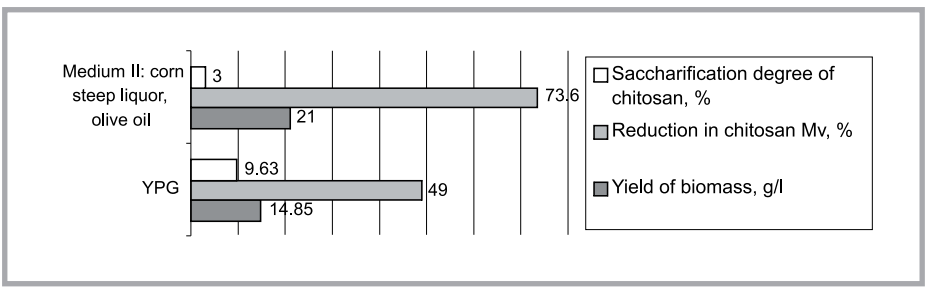

Figure 3. *The effect of medium composition on the yield of M. circinelloides mycelium and activity of intracellular chitosanolytic enzymes. Compositions of the applied culture media are shown in section 2.2. Conditions of chitosan hydrolysis are described in section 2.3.*

Table 1. *Substrate specificity of chitosanases from M. circinelloides.*

Type of substrate	Reduction in substrate M_v, %	Saccharification degree, %
Colloidal chitin	-	1.29
Chitosan (DD = 66.0 %; M_v = 421.3 kDa)	72.7	1.43
Chitosan (DD = 78.9 %; M_v = 120.6 kDa)	60.0	2.50
Chitosan (DD = 79.8 %; M_v = 392.5 kDa)	62.0	2.56
Chitosan (DD = 85.8 %; M_v = 309.0 kDa)	50.5	3.12

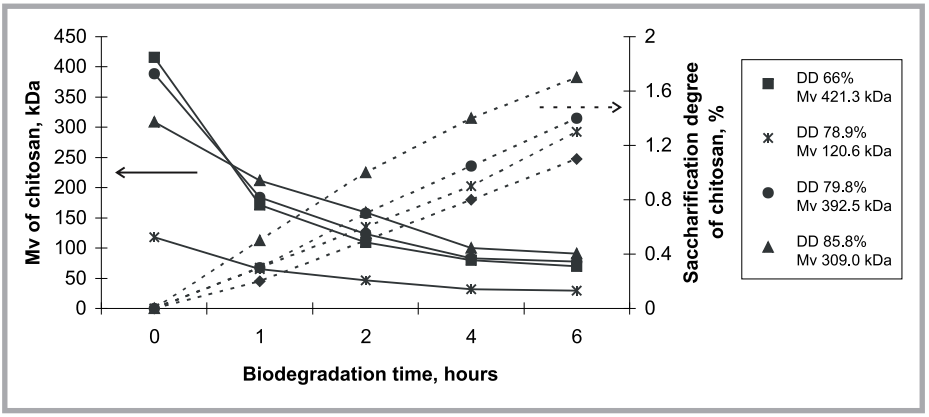

Figure 4. *Effects of DD and M_v of chitosan on activity of M. circinelloides chitosanases. Chitosan hydrolysis conditions: the ratio of enzymatic preparation (air-dried mycelium) : chitosan - 2:1(w/w), pH 5.5, 37 °C.*

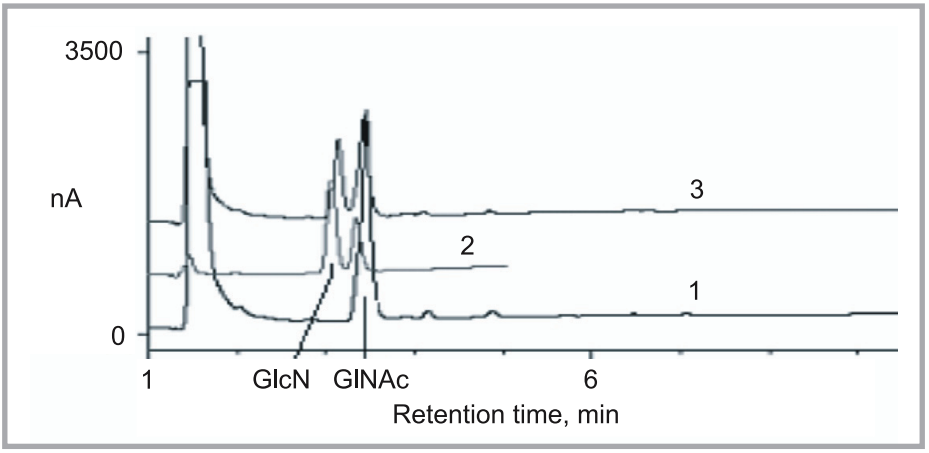

Figure 5. *HPAEC profile of low molecular weight fractions of chitosan biodegradation products. Chitosan hydrolysis conditions: the ratio of enzymatic preparation (air-dried mycelium):chitosan - 1:1 (w/w), pH 5.5, 37 °C, 24 h; 1) Mixture of digestion products released from chitosan (DD 78.9%, M_v 120.6kDa), 2) Mixture of GlcN and GlcNAc standards, 3) Mixture of digestion products released from chitosan (DD 78.9%, M_v 120.6 kDa) + GlcN standard*

Chitosan and colloidal chitin hydrolysis conditions were described in section 2.3.

The results shown in Table 1 and Figure 4 indicate that the endo-hydrolases produced by *M. circinelloides* strain display the high specificity for chitosan with the low DD, whereas the polymer with high DD favored the function of exo-hydrolases. The results shown in Table 1 revealed that these enzymes can also hydrolyze glycosidic bonds in a colloidal chitin.

The anion-exchange high performance liquid chromatography (HPAEC) was used for analysis of the low molecular weight fractions released by chitosan degradation. An example of HPAEC-profile of these fractions is shown in Figure 5.

The occurrence of GlcNAc in the mixture of products of chitosan digestion suggests that also the exo-chitinase contributes to the biodegradation of chitosan by the M. circinelloides enzymes.

4. Conclusion

The results of research provide evidence that the enzymatic complex from *M. circinelloides* can be used for production of oligomers from chitosan or chitin. It was found that Mucor mycelium displayed mainly the activity of endo-hydrolases capable of cleaving β-1,4-glycosidic bonds in chitosan. Their preferred substrates was chitosan with relatively low DD. These enzymes reduce its average molecular weight by circa 80%.

5. References

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