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Obtaining Mannanoligosaccharide Preparations by Means of the Mechanoenzymatic Hydrolysis of Yeast Biomass

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Abstract A new method of obtaining biologically active mannanoligosaccharides is proposed. It involves mechanical activation of the enzymatic hydrolysis of components forming the supramolecular structure of the cell wall. Processes that take place during mechanical treatment and enzymatic hydrolysis of yeast biomass and lead to an increase in the availability of mannanoligosaccharides of the yeast cell walls are investigated. The efficiency of the use of mechanoenzymatic approach to obtaining mannanoligosaccharide preparations is evaluated.

Keywords Mechanoenzymatic hydrolysis · Yeast biomass · Enzyme · Mannanoligosaccharide

Introduction

One of the necessary circumstances for the development of many intestinal diseases, for example salmonellosis, is fixing of a malignant bacterium on the mucous membrane of intestines [1, 2]. Fixing occurs through binding the bacterial mannan-dependent lectins to mannose residues on the surface of the mucous membrane. From this point of view, antibacterial preparations containing mannanoligosaccharides (MOS) deserve attention. Investigations show that the addition of MOS to fodder prevents the diseases [3]. The problem connected with the development of ecologically safe substitutes of antibiotics is due to the fact that starting from 2006, manufacturers from the countries of the European Union excluded synthetic growth-promoting factors and antibiotics from the fodder of agricultural animals [4, 5].

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The yeast biomass, namely the yeast cell walls, appears to be a promising raw material to obtain MOS preparations. A cell wall is a layered supramolecular structure composed mainly of polysaccharides and glycoproteins (protein molecules bound with carbohydrates). Among many other functions, the yeast cell wall plays also the part of an external skeleton; its strength is provided by β-glucan [6, 7]. Analysis of the published works allows one to represent the yeast cell wall as a set of the structures of β-glucan and mannoprotein complex bound together [8, 9].

β-Glucan of the yeast cell wall is composed of glucose residues held together by β-1,3- and β-1,6-bounds. Rigidity and strength of the cell wall and stability to the action of the environment are provided by β-1,3-glucan to a higher extent than by other components [10, 11]. The structure of yeast glucan resembles the structure of cellulose. Similar to cellulose, glucan molecules contain the crystal and amorphous parts and form micro- and macrofibrils [7, 9]. The proteins of the yeast cell wall form covalent bonds with mannanoligosaccharides [8, 9], thus giving rise to mannoproteins, and also form bonds with glucan maintaining the supramolecular arrangement of the cell wall.

Mannoproteins of the cell wall may be divided into two groups according to the type of the chemical bond between the protein and carbohydrate part of glycoproteins: *O*-bound and *N*-bound oligosaccharides. The mannanoligosaccharide part of *O*-bound mannoproteins is easily removed under the action of a diluted alkali, while the mannanoligosaccharide part attached according to the N-type is removed only under the action of specific enzymes.

The operations that are usually used to extract MOS from cell walls include induced autolysis, heating in autoclave for a long time and hydrolysis with acids and alkalis followed by separation of the products [12–15]. The majority of these procedures have disadvantages, for example the necessity to maintain high temperature for a long time and to use aggressive, volatile, and highly inflammable reagents.

The treatment of the raw material using special mechanochemical equipment, planetary and ball mills, is a recognized method to activate subsequent heterogeneous processes with the participation of liquid phases, for example extraction [16, 17]. In application to the biogenic raw material, the observed effect is explained by an increase in specific surface and distortion of the arrangement of supramolecular structures formed by biopolymers. Both the former and the latter reasons cause a noticeable decrease in diffusion limitations and therefore an increase in the rate of extraction and other processes participated in by the liquid phase. The goal of the present work is to search for the possibilities to use mechanically activated enzymatic treatment for obtaining the product with increased concentration of biologically available mannoproteins and mannanoligosaccharides.

Materials and Methods

Reagents and Materials D(+)-Glucose (99%, Acros Organics), D(+)-mannose (99%, Acros Organics), yeast *Saccharomyces cerevisiae* GOST (State Standard) 171-81 (Novosibirsk Yeast Plant, Novosibirsk, Russia), enzyme complex from *Trichoderma reesei* Celloluxe 2000 (Joint-Stock Company Sibbiofarm, Berdsk, Novosibirsk Region, Russia), H_2SO_4 (chemically pure) grade GOST 4204-77; carbazole (95%, Sigma Aldrich) were used. The enzyme complex "Celloluxe 2000" contains: xylanase (up to 8,000 U/g), cellulase (2,000 U/g), β-1,3-glucanase (1,500 U/g), and glucoamylase (up to 20 U/g).

Mechanical and Enzymatic (Mechanoenzymatic) Treatment of S. cerevisiae A weighed portion of *S. cerevisiae* yeast containing the "Celloluxe 2000" enzyme complex (10%) was

treated mechanically in the activator of planetary type AGO-2 for 2 min (with the centrifugal acceleration of milling bodies 200 m/s^2). The resulting mechanocomposite was pressed in a tablet at a pressure of 10 kg/cm^2 and heated at 45 °C for 28 h.

Determination of the Total Glucose and Mannose Content In order to extract the components of yeast cells, we added 1.00 ml of 0.1 M NaOH to the weighed portion of the initial yeast or the product of treatment (200 mg); then the mixture was conserved with sodium azide, and enzymes were inactivated. Extraction was carried out at 50 °C in a thermostated shaker (750 rpm) for 2 h, then the suspension was centrifuged (8,000 rpm, 15 min). A part of the supernatant was neutralized with 5% sulfuric acid and used for the quantitative determination of carbohydrates using the modified phenol–sulfuric method [18]. This method is generally accepted for manipulations with yeast cells and allows one to determine the content of mannose and glucose rapidly, either in the free form or in glycoproteins and oligosaccharides.

To determine the glucose and mannose content, we added 5 ml of 85% sulfuric acid to the 200-μl supernatant under intense mixing. After cooling the mixture to room temperature, we added 300 μl of the solution of carbazole in ethanol, under intense shaking, and kept it in a boiling water bath for 10 min. After cooling to room temperature, the solutions were examined by means of photometry at the wavelengths of 440 and 540 nm. Extinction coefficients for glucose and mannose were determined with the help of calibration plots. Measurement error is less than 5%.

Determination of the Content of Soluble Carbohydrates Non-bound with Proteins Ethanol was added up to the concentration of 70% to the extracts of initial yeast and the products of treatment; then the mixture was left at a temperature of 2 °C overnight. The precipitate of proteins and glycoproteins was separated by centrifuging for 15 min at 7,000 rpm. The precipitate was discarded, while the supernatant was used to determine the concentration of soluble carbohydrates non-bound with proteins; determination was carried out using the modified phenol–sulfuric method. The concentration of soluble glycoproteins was determined on the basis of the difference between total carbohydrate content and the content of carbohydrates non-bound with proteins.

In all the experiments, the MOS yield was calculated as a ratio of extracted MOS mass to initial biomass mass. All the results are comparable. The mass of dry biomass used for experiment was considered as 100%.

Results and Discussion

The yeast cell wall component responsible for its strength and, in particular, for the availability of mannanoligosaccharides is β-1,3-glucan. To disturb its intermolecular interactions with mannanoligosaccharides, it was proposed to use enzymatic hydrolysis, mechanical treatment in high-energy activators, and a combination of both—mechanoenzymatic hydrolysis.

To characterize the products of treatment from the viewpoint of the degree of availability of mannanoligosaccharides, it was proposed to use the total yield of mannanoligosaccharides from alkaline extraction. In the case of alkaline extraction, both the *O*-bound mannanoligosaccharides detached from the protein residue under the action of a diluted alkali and *N*-bound mannanoligosaccharides extracted into solution together with the protein component are transformed into the soluble form.

Enzymatic Treatment Treatment with cellulosolytic and proteolytic enzymes is widely used in biology. In the case under consideration, treatment of the yeast biomass with the solution of a cellulosolytic enzyme complex Celloluxe 2000 containing a set of endo- and exoglucanases was proposed for selective hydrolysis of β-1,3-glucan.

Gradual accumulation of the monomer form of glucose and a decrease in the concentration of oligomeric forms occur during hydrolysis. The curve illustrating the accumulation of the monomeric form and conversion of the oligomeric forms of glucose is shown in Fig. 1.

As a result of enzymatic hydrolysis of the part of structure-forming glucan layer of the cell wall, mannanoligosaccharides of the yeast cell wall become more available for alkaline extraction. The fraction that passes into the alkaline extract is 2.0% (1.3% passes into the extract of the initial biomass). That value of MOS yield characterizes the total yield of individual mannanoligosaccharides and protein-attached mannanoligosaccharides (mannoproteins).

However, with all the revealed advantages (selectivity of the hydrolysis of β-1,3-glucan, increase in the availability of mannanoligosaccharides), enzymatic hydrolysis of the yeast biomass possesses a number of technological disadvantages; the major one among them is the necessity to perform the reaction in aqueous solution, which is connected with evaporation of a large amount of water to obtain the final forms of the product.

Mechanoenzymatic Hydrolysis To increase the efficiency of enzymatic hydrolysis of β-glucan, we propose a hydrolysis scheme in which the stages of heterogeneous enzymatic hydrolysis in a limited amount of water are preceded by the stage of mechanical activation of the powdered mixture of the substrate and the enzyme. In order to increase the reactivity of the cell wall biopolymers, the dry mixture of yeast biomass *S. cerevisiae* with the enzymatic preparation Celloluxe 2000 is subjected to mechanical activation.

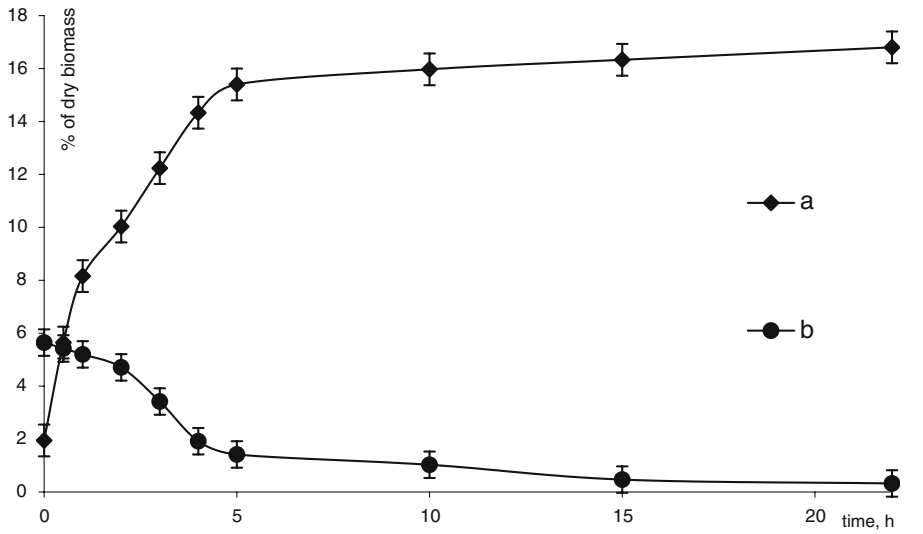

Fig. 1 Accumulation of the monomeric form and conversion of oligomeric forms of glucose as a result of enzymatic hydrolysis of yeast biomass: *a* yield of the monomeric form of glucose, *b* yield of oligomeric forms of glucose

Ultrastructural investigations [19] showed that the treatment of the yeast biomass causes mechanical destruction of a part of cells as well as distortion of intermolecular interactions between the structural elements of the cell wall. It was assumed that the observed distortion of the structure of cell wall, in particular accumulation of defects in the structure of β-1,3-glucan, may accelerate subsequent enzymatic hydrolysis. It is also shown that mechanical treatment causes an increase in the availability of mannanoligosaccharides for subsequent extraction. The fraction that passes into the alkaline extract is 2.1%.

Changes of the structure and physical and chemical properties as a result of mechanical activation allows one to speak of the formation of a powdered composite, which is a product with increased reactivity; it also exhibits structure and morphology differing from those of the initial mixture [19]. During mechanical activation, a uniform distribution of the enzymes over the sample volume is achieved. As a consequence of water deficit in the system, the reaction of enzymatic hydrolysis cannot proceed to a substantial extent at the moment of activation. Complete mechanical destruction of all the cells of the sample does not occur due to the insufficient activation time.

The second stage includes compacting of the powdered mechanocomposite. As a result, the volume concentration of the enzyme increases, mass transfer between the particles of the composite is simplified, and the loss of water necessary for hydrolysis is prevented.

The third stage is heating of the compact at the temperature that is optimal for the action of enzymes (45 °C); this is necessary for the heterogeneous enzymatic hydrolysis of β-glucan to proceed. Destroyed cells obtained at the first stage serve as the centers where subsequent hydrolysis is localized. Water (humidity of the yeast biomass is about 5%), released from the destroyed cells, dissolves the enzyme. Enzymes get sorbed on the substrate and catalyze the reaction of hydrolysis. In turn, water released from the next hydrolyzed cells promotes the propagation of reaction front.

Enzymatic hydrolysis proceeds most efficiently when the first two stages are involved: at the first stage, the reactivity of biopolymers of the cell wall increases, enzymes get dispersed, and localization centers are formed; at the second stage, the necessary concentrations of enzymes in the transformation region are provided.

Chemical analysis of the alkaline extract of the product of mechanoenzymatic hydrolysis showed that the yield of mannanoligosaccharides into the alkaline extract is 3.8% of the mass of initial *S. cerevisiae*, which exceeds the yield of mannanoligosaccharides as a result of liquid-phase enzymatic hydrolysis (2.0%).

Chemical analysis of the alkaline extracts of the initial yeast biomass, the mechano-composite after activation, and the final product of mechanoenzymatic hydrolysis was carried out. The results of the investigation are presented in Fig. 2.

One can see in the diagram that a part of glucosaccharides extracted from the initial biomass is bound to proteins. After mechanical activation, the fraction of extracted glucosaccharides bound to proteins increases, which is an evidence of disordering of the supramolecular structure of biopolymers. After enzymatic hydrolysis, the fraction of extracted glucosaccharides bound to proteins decreases due to hydrolysis of the carbohydrate chain.

Chemical analysis showed (Fig. 3) that mainly *O*-bound mannanoligosaccharides, able to get detached from the protein molecule under the action of alkali, pass into the alkaline extract of the yeast biomass. These values characterize separate yields of mannanoligo-saccharides and mannoproteins. The sum of these separate yields is equal to the total yields given earlier. The fraction of *O*-bound mannanoligosaccharides remains constant in the entire set of experiments, which is an evidence of their complete extraction into solution due to weak binding with proteins.

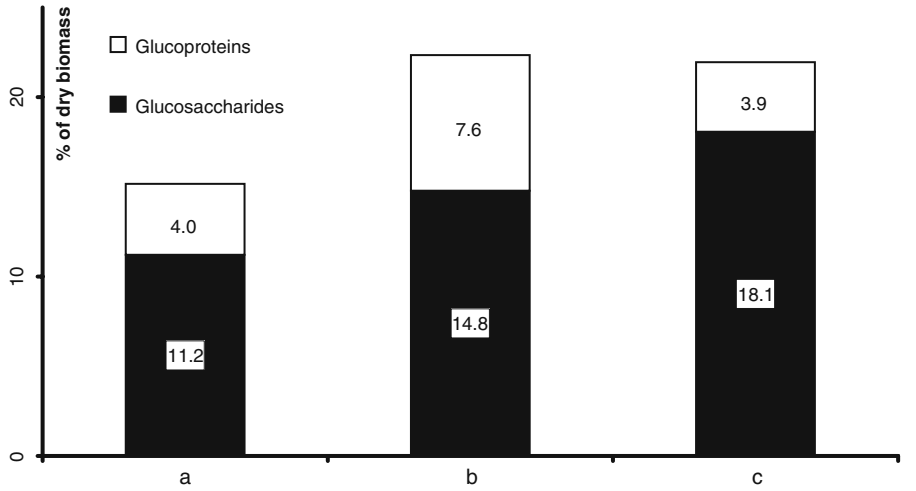

Fig. 2 Chemical analysis of alkaline extracts of the initial biomass and the products of mechanoenzymatic hydrolysis: *a* extract of the initial biomass, *b* extract of mechanically activated biomass, *c* extract of the product of mechanoenzymatic hydrolysis

After mechanical activation (Fig. 3), due to distortions of the supramolecular structure of the cell wall, a part of *N*-bound mannanoligosaccharides becomes available for alkaline extraction. They pass into the alkaline solution in the form of mannoproteins. The fraction of *N*-bound mannanoligosaccharides increases substantially after heterogeneous enzymatic hydrolysis in a limited amount of water, which is an evidence of the fact that hydrolysis of structure-forming β-glucan allows one to increase the availability of mannoproteins.

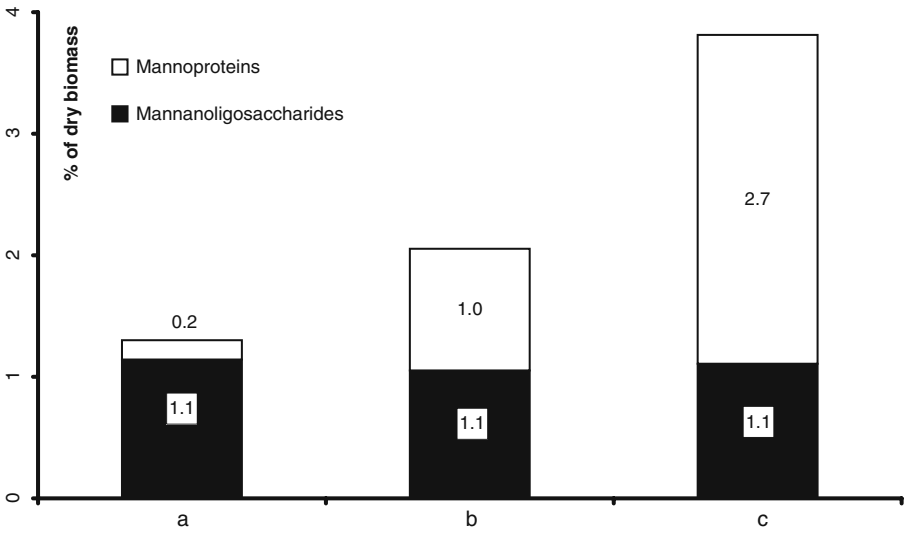

Fig. 3 Chemical analysis of alkaline extracts of the initial biomass and the products of mechanoenzymatic hydrolysis: *a* extract of the initial biomass, *b* extract of mechanically activated biomass, *c* extract of the product of mechanoenzymatic hydrolysis

Conclusions

Combination of mechanical activation and subsequent enzymatic hydrolysis allows one to achieve a 2.9-fold increase in the yield of available mannanoligosaccharides from the yeast biomass of *S. cerevisiae*. As a result of mechanical activation of the yeast biomass, the reactivity of the biopolymers of cell wall with respect to enzymatic hydrolysis increases. The effect is based on disordering of the supramolecular structure of the cell wall, which causes a decrease in diffusion limitations and increase in the efficiency of enzymatic hydrolysis of the structure-forming component—β-glucan.

References

1. Wold, A. E., Mestecky, J., & Tomana, M. (1990). *Infection and Immunity, 58*(9), 3073–3077.
2. Emmerik, L. C. V., Kuijper, E. J., & Fijen, C. A. P. (1994). *Chin. Exp. Immunol., 97*, 411–416.
3. Dawson, K.A., Sefton, A.E. Methods and compositions for control of coccidiosis. Pat. USA No. 7048937 B2. (23.05.2006).
4. Commission proposes new safety rules for feed additives and to prohibit antibiotics as growth promoters, Scientific Steering Committee of the EU, Brussels, 25 March 2002
5. Future of growth-promoting factors. European Lecture Tour, February 13 – March 5, 2006 Available from: www.alltech.com. Accessed January 15, 2010
6. Bjorndal, H., & Lindberg, B. (1973). *The Biochemical Journal, 135*, 31–36.
7. Manners, D. J., Masson, A. J., & Patterson, J. C. (1973). *The Biochemical Journal, 135*, 19–30.
8. Biryuzova, V. I. (1993). Ultrastructural arrangement of yeast cell, Moscow, Nauka. (in Russian).
9. Kalebina, T. S., & Kulaev, I. S. (2001). Biochemistry (Moscow). Special issue. *Biological Chemistry Reviews, 41*, 105–130 (in Russian).
10. Harthmann, C., & Delgado, A. (2004). *Journal of Biomechanics, 37*, 977–987.
11. Pereira, R. S. (2000). *FEBS Letters, 475*(1), 43–46.
12. Belousova, N. I., Gordienko, S. V., & Eroshin, V. K. (1990). *Biotekhnologiya, 3*, 6–9 (in Russian).
13. Kanegae, Y., Minami, K. Method for producing yeast extract. Pat. USA № 4810509. (07.05.1989).
14. Lazzari, F. Product based on polysaccharides from bakers' yeast and its use as a technological coadjuvant for bakery products. Pat. USA No. 6060089. (09.05.2000).
15. Kitamura, K., Matsuki, S., Tanabe, K. Physiologically active polysaccharides, production and uses thereof. Pat. USA No. 4313934. (02.02.1982).
16. Shakhtshneider, T. P., & Boldyrev, V. V. (1999). *Reactivity of molecular solids*. England: Wiley.
17. Lomovsky, O.I., Boldyrev, V.V. (2006). Mechanochemistry in solving ecological problems, Novosibirsk, SPSTL SB RAS (in Russian).
18. McMurrought, A., & Rose, A. H. (1967). *The Biochemical Journal, 105*, 189–203.
19. Bychkov, A. L., Ryabchikova, E. I., Korolev, K. G., & Lomovsky, O. I. (2009). *Chemistry for Sustainable Development., 5*, 479–486.

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