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Change of the Supramolecular Structure of Cell Walls of *Saccharomyces cerevisiae* as a Result of Mechanoenzymatic Treatment

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Abstract

Changes of the supramolecular structure of cell walls of yeast under the action of mechanical activation was studied. It was established that the reactivity of polysaccharides of the cell walls towards subsequent enzymatic hydrolysis increases substantially. A new method to obtain biologically active mannanoligosaccharides was proposed. It involves mechanical activation of enzymatic hydrolysis of the components of cell wall supramolecular structure followed by extraction of the target components.

Key words: mannanoligosaccharides, supramolecular structure, cell wall, yeast biomass

INTRODUCTION

Manufacturers from the countries of the European Community have completely excluded synthetic growth stimulators and antibiotics from the fodder of agricultural animals [1, 2]. In this connection, it became urgent to make ecologically safe substituents. From the viewpoint of antibacterial activity, preparations containing mannanoligosaccharides (MOS) attract attention. It is known that the development of many intestinal diseases, for example salmonellosis, involves fixing of the pathogenic bacterium on the mucous membrane of intestines [3, 4]. Fixing occurs through binding mannan-binding lectins with mannose residues on the surface of mucous membrane. Addition of MOS blocking up lectins into forage for animals prevents the disease [5].

A promising raw material for obtaining MOS preparations is the yeast biomass, in particular the yeast cell wall which is a layered supramolecular structure containing a complex of polymers, mainly polysaccharides and glycoproteins (protein molecules bound with carbohydrates). In addition to a number of other functions, the yeast cell wall serves as the external skeleton; its strength is provided by β -glucan [6, 7]. Analysis of the published works allows us to represent the yeast cell wall as a set of interconnected structures of glucan and mannan-protein complex [8, 9].

The cell wall proteins form covalent bonds with polysaccharide molecules forming and sustaining the molecular arrangement of the cell wall. About 40 % of the total mass of cell wall is represented by proteins and mannanoproteins that penetrate the cell wall and form a mannanoprotein layer on its surface.

Mannanoproteins of the cell walls are divided into two groups according to the type of chemical bond between the protein and hydrocarbon parts of glycoproteins: O- and N-bound oligosaccharides. The mannanoligosaccharide part of O-bound mannanoproteins is easily detached under the action of a weak alkali, while the N-attached mannanoligosaccharide part may be removed only under the action of specific enzymes.

Usually the procedures necessary to extract MOS from the cell walls include induced autolysis, long-term heating in an autoclave, hydrolysis with acids and alkalis, followed by the separation of the products [10–13]. The majority of these procedures have shortcomings, such as the necessity to maintain high temperature for a long time, to use aggressive, volatile and readily inflammable reagents.

The treatment of the raw material using the 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) [14, 15]. In application to biogenic raw materials, the observed effect is explained by an increase in the specific surface and distortions of the arrangement of supramolecular structures formed by biopolymers. As a result, diffusion limitations decrease substantially. As a consequence, the rates of extraction and other processes taking place with the participation of the liquid phase increase.

The goals of the present work are to study the changes in the cell wall structure and the corresponding increase in its reactivity, search for new ways of using mechanoenzymatic treatment for obtaining the products with increased content of biologically active glycoproteins and oligosaccharides.

MATERIALS AND METHODS

Reagents and materials

We used the aqueous solution of NH_3 of the kh. ch. reagent grade (GOST 3760–79), AgNO_3 of kh. ch. grade (TU 6-09-3703–74), *D*(+)-glucose (99 %, Acros organics), *D*(+)-mannose (99 %, Acros organics), yeast *Saccharomyces cerevisiae* (GOST 171–81, Novosibirsk Yeast Plant), enzymatic complex CelloLux 2000 (Sib-

biofarm Ltd., Novosibirsk Region), H_2SO_4 of kh. ch. grade (GOST 4204–77), carbazole (95 %, Sigma Aldrich).

Mechanical and mechanoenzymatic treatment of S. cerevisiae

Weighed portion of *S. cerevisiae* containing 10 % of the enzymatic complex Cellolux 2000 was subjected to mechanical treatment in the planetary type activator AGO-2 for 2 min (20g). The resulting mechanocomposite was pressed into a tablet under the pressure of 10 kg/cm² and heated at 45 °C for 28 h.

Determination of the total content of glucose and mannose

To extract the components of yeast cells, we added 1.0 mL of water to the weighed portion of initial yeast or the product of treatment, then conserved the mixture with sodium azide and inactivated enzymes. Extraction was carried out at 50 °C in a thermostated shaker (rotation frequency: 750 min⁻¹) for 2 h, then the suspension was centrifuged (8000 min⁻¹, 15 min). A part of the supernatant was used for quantitative determination of carbohydrates with the help of the modified phenolsulphuric method [16]. This method is generally accepted for studies involving yeast cells and allows rapid determination of glucose and mannose content, both in the free form and in glycoproteins and oligosaccharides.

To determine glucose and mannose content, 5 mL of 85 % H_2SO_4 was added to 300 μL of the supernatant under intense mixing. After cooling the mixture to room temperature, we added 300 μL of carbazole solution in ethanol shook intensively and incubated with the boiling water bath for 10 min. After cooling to room temperature, the solutions were examined by means of photometry at λ equal to 440 and 540 nm. The extinction coefficients of glucose and mannose were determined from calibration plots. The error of measurement was less than 5 %.

Determination of the content of soluble carbohydrates not bound with proteins

Ethanol was added to the extracts of initial yeast and the products of treatment to reach the concentration of 70 %, and the mixture was

left at a temperature of 2 °C for 10–12 h. The precipitate containing proteins and glycoproteins was separated by centrifuging for 15 min at the rotation frequency of 7000 min⁻¹. The precipitate was discarded, and the supernatant was used to determine the concentrations of dissolved carbohydrates not bound with proteins. The modified phenol sulphuric method was used for this purpose. The concentration of soluble glycoproteins was determined on the basis of the difference of the total carbohydrate content and the concentration of carbohydrates not bound with proteins.

Contrasting with the ammonia complexes of silver

The reduction of complex ammonia salts of silver and the formation of metal silver was used to reveal the regions of cell walls containing the end aldehyde groups and to estimate the defect content of these regions. We added 1 mL of the working solution containing 10.0 mL of water, 1.0 mL of the solution of 1.0 mM AgNO₃, 300 μL of concentrated ammonia so-

lution to 200 mg of the weighed portion of initial yeast and yeast subjected to mechanoenzymatic treatment. Then the solutions were kept at room temperature for 24 h and used to carry out electron microscopic studies.

Preparation of ultrathin sections for transmission electron microscopy

The samples of initial yeast and yeast after mechanoenzymatic treatment were fixed with 1% solution of osmic acid [17]. Hank's solution was used as a buffer. Dehydration was carried out in the solutions with increasing ethanol concentration and in acetone; epone araldite was poured into the mixture. Ultrathin sections were prepared with an ultratome (Reichert, Austria), contrasted with uranyl acetate and lead citrate. The preparations were examined with an H-600 electron microscope (Hitachi, Japan) with the accelerating voltage of 75 kV and under the light microscope (Axio-plan-40, Zeiss, Germany).

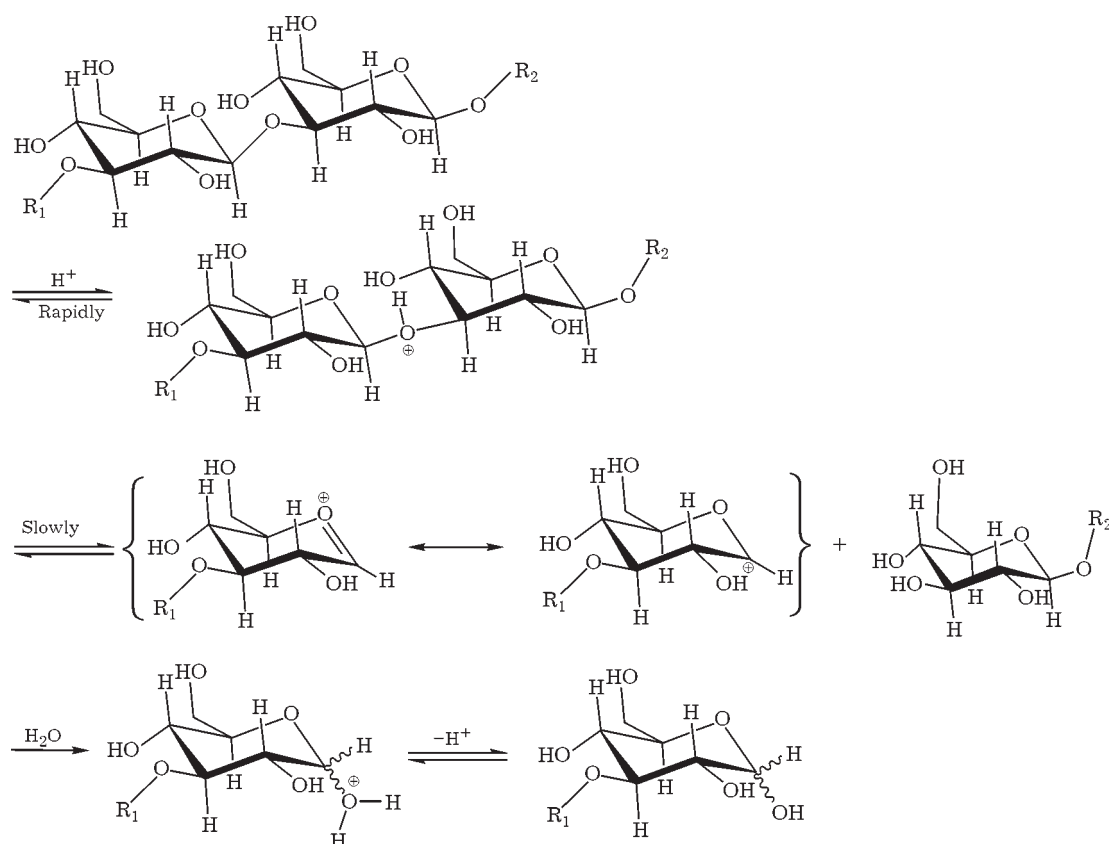


Fig. 1. Mechanism of hydrolysis of β -1,3-glucan chain.

RESULTS AND DISCUSSION

The major element of yeast cell wall responsible for sustaining its strength is β -glucan occupying the middle layer of the cell wall. The glucan layer is coated with mannanoproteins from inside and outside [6–9].

To destroy the bonds of MOS and mannanoproteins with glucan, we carried out enzymatic hydrolysis activated by the mechanical treatment (mechanoenzymatic hydrolysis). The essence of this kind of treatment is reduced to the mechanical action on the mixture of yeast biomass with the enzymatic complex, followed by the enzymatic hydrolysis of polysaccharides of the cell walls. Hydrolysis is performed in the heterogeneous mode in the presence of the limited amount of water.

At the first stage of treatment, yeast is subjected to mechanical activation with the enzymatic complex Cellolux 2000 containing 1,3- and 1,4- β -glucanases [18]. This process involves partial mechanical destruction of cells, partial damage of the supramolecular structures of cell walls causing an increase in the reactivity of glucan with respect to the enzyme, the uniform distribution of enzymes over the sample volume. Thus mechanocomposite is formed. It is a product with increased reactivity in which the diffusion routes of enzymes are shortened, and the structural layers of cell walls are disordered and have diffuse character.

The second stage is compacting of the resulting mechanocomposite. This allows us to increase the volume fraction of the enzyme, to simplify mass transfer between the composite particles and to prevent the loss of water that is necessary for enzymatic hydrolysis.

The third stage is heating the compacted composite at the optimal temperature of enzyme action (45 °C), leading to the hydrolysis of β -glucan (Fig. 1). Most probably, the cells destroyed at the first stage serve as the centres of localization of enzymatic hydrolysis (Fig. 2, b). The residual moisture promotes the propagation of reaction front, while temperature rise removes the diffusion hindrance and increases the mobility and hydrolytic activity of enzymes.

We studied the changes in yeast cell walls caused by mechanoenzymatic treatment with the help of electron transmission microscopy.

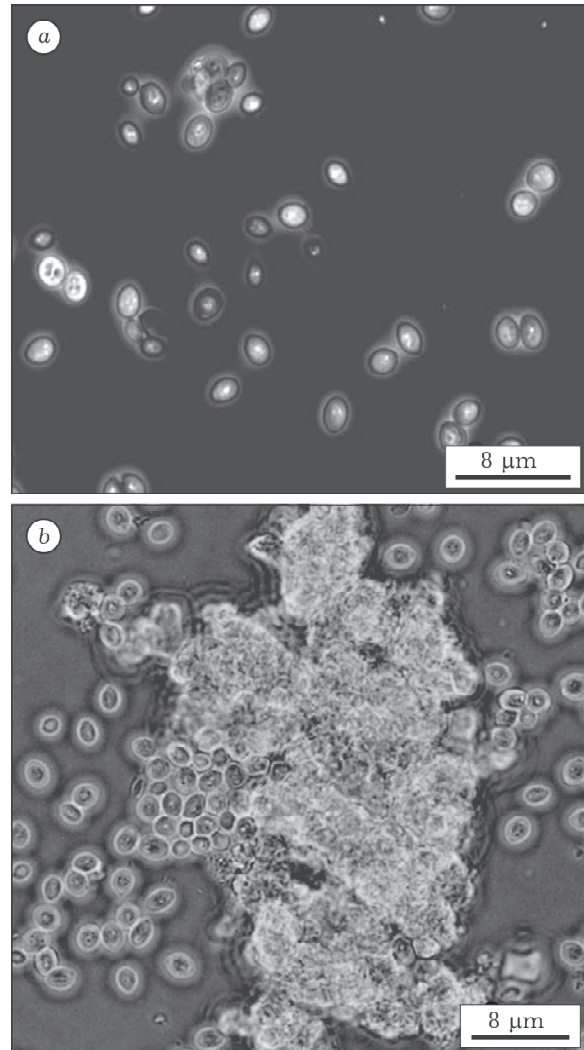


Fig. 2. Micrographs of initial yeast (a) and yeast after enzymatic hydrolysis (b).

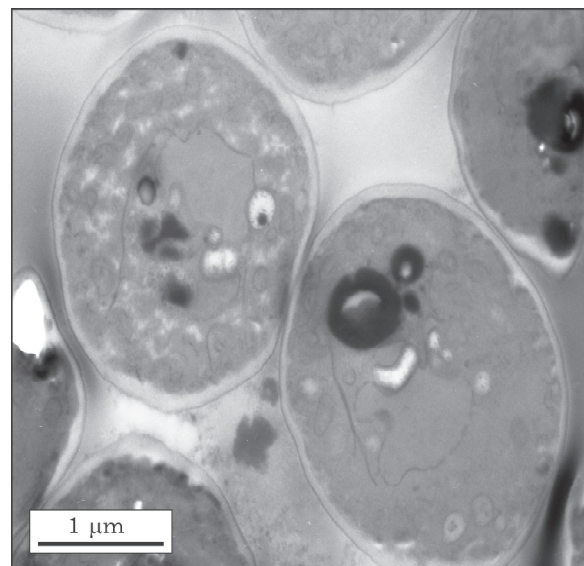


Fig. 3. Ultrathin section of initial yeast cells.

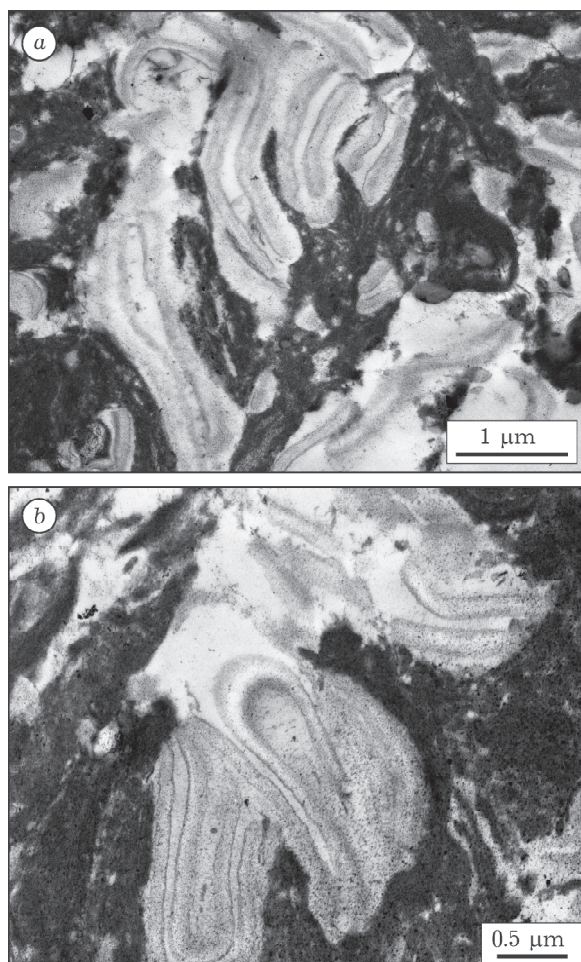


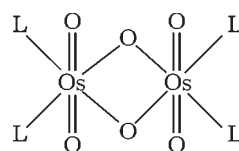
Fig. 4. Morphology of yeast cells after mechanical and enzymatic treatment. Ultrathin sections of preparations fixed with osmic acid: *a* – cell walls after mechanical activation (in the form of the strips of medium electron density) are surrounded by electron-dense cell detritus; *b* – yeast biomass after mechanoenzymatic treatment, electron-dense cell detritus and the fragments of cell walls containing osmiophil particles.

Cell wall, organelles and nucleus are clearly seen in the electron microscopic micrograph of initial yeast cells (Fig. 3).

Mechanical treatment of yeast cells under the conditions of restricted shock causes clearly exhibited damage of yeast cells; destruction of organelles, deformations of cell wall and its rupture are observed (see Figs. 3 and 4, *a*). The major part of the preparation is represented by cellular detritus appearing as electron-dense mass in which membrane-like structures of different sizes and the residues of cell wall are observed. The latter look like separate fragments and flattened walls of whole cells with ruptures. In comparison with the cells of the ini-

tial preparation, the structure of the cell wall is disordered; the wall looks homogeneous, which is the evidence of distortion of intermolecular interactions between the structural elements.

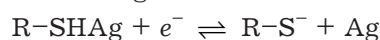
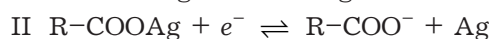
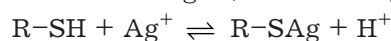
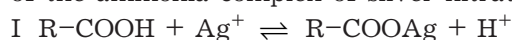
Mechanoenzymatic treatment was carried out in the presence of natural moisture of the biomass. For this purpose, yeast was subjected to mechanical activation in mixture with enzyme and subsequent heating in the form of compacted mass. One can see in the ultrathin section of the yeast biomass after mechanoenzymatic treatment (see Fig. 4, *b*) that the cell walls are injured; the cytoplasm and organelles are destroyed and form a continuous osmiophil mass localized between cell walls. Electron-dense grained structures 12–25 nm in size are detected in the cell walls. These structures are likely to be the complexes of mannanoproteins [19]:



L – lysine, methionine, tryptophan, histidine, cysteine

Enzymatic hydrolysis of glucan leads to the removal of diffusion limitations, which provides the possibility of the formation of osmiophil grained structures.

To reveal the changes in glucan-containing regions, we used the reaction of the reduction of the ammonia complex of silver nitrate [20]:



Silver salts are formed at stage I, while silver reduction proceeds at stage II. A reducing agent is necessary for the formation of the particles of elemental silver. The role of this reducing agent is played by the end aldehyde groups. One can see in the ultrathin section of the initial yeast cell treated with the ammonia complexes of silver (Fig. 5) that the electron-dense particles are localized inside the cell, which points to the intracellular reduction of silver. In the native cell wall, polysaccharides form stable structures with a small number of accessible reducing centres, so the cell wall is only weakly contrasted. In view of the insignificant content of extractive carbohydrates in the wa-

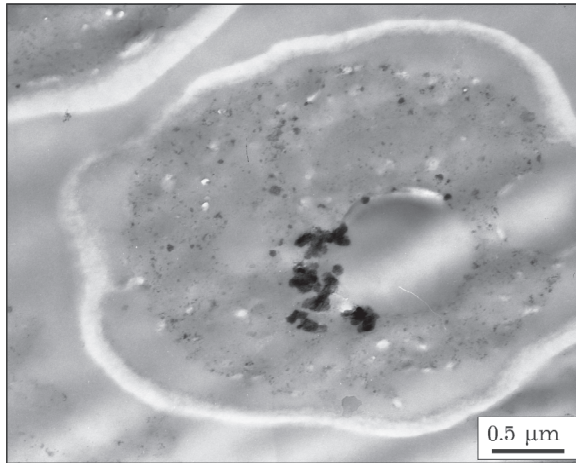


Fig. 5. Initial yeast cells, the preparation is treated with ammonia complexes of silver.

ter-soluble fraction (as confirmed by chemical analysis), the reduction of silver in the space near the cells proceeds insignificantly and no electron-dense product is formed.

Mechanical activation (Fig. 6, *a*) is accompanied by ruptures of cell walls; the structure of the fragments is diffuse in its character. The cell wall does not interact with silver complexes due to the low concentration of reducing aldehyde groups.

Enzymatic treatment of mechanically activated biomass leads to a noticeable increase in the reactivity of cell wall towards the ammonia complexes of silver nitrate. Previously [21] studying yeast cells in the electron microscope on grids we established that the cells are electron-impermeable structures. Hydrolysis of polysaccharides is likely to be accompanied by substantial accumulation of reducing aldehyde groups in the wall; these groups are potential centres of nucleation for metal silver particles during reactions in solution.

In addition to endoglucanase activity, the enzymatic complex involved in the work exhibits exoglucanase activity, so substantial amounts of reducing carbohydrates providing the growth of silver phase nuclei are accumulated in the system. Due to these factors, a dense, almost continuous layer of elemental silver is formed on the surface of cells.

Ultrathin sections (see Fig. 6, *b*) exhibit accumulation of silver nanoparticles inside and on the surface of cell walls, which is due to

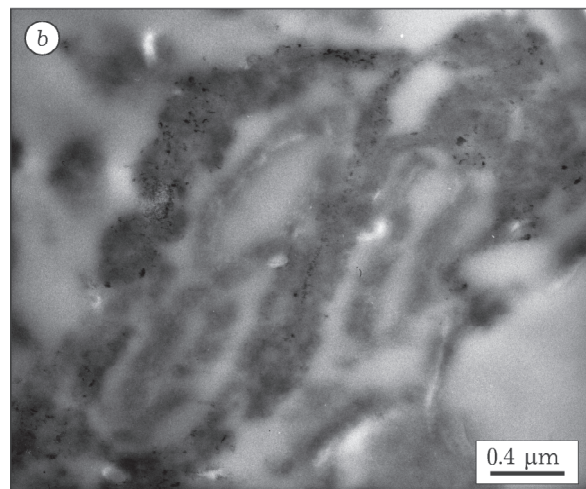
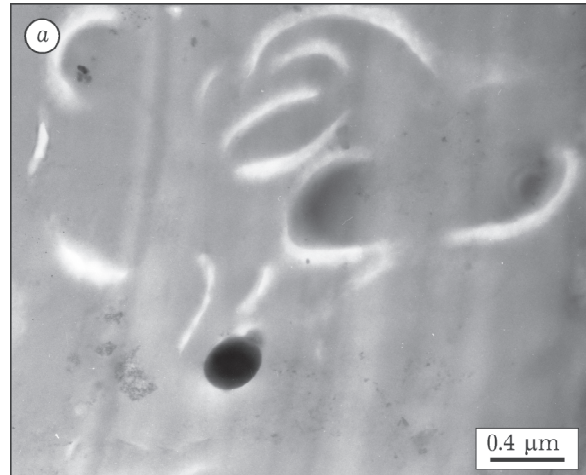


Fig. 6. Preparation of yeast subjected to mechanoenzymatic treatment: *a* – cell walls after mechanical activation; *b* – fragments of membrane-like structures after mechanical activation and enzymatic hydrolysis.

the presence of isolated immobile nucleation centres. According to the data of chemical analysis, as a result of mechanoenzymatic treatment, the amount of water-soluble carbohydrates accumulating in the system is sufficient for the growth of nuclei.

The data obtained are confirmed by the results of chemical analysis. Due to mechanical activation, the yield of the sum of free MOS and mannanoproteins increases during extraction by a factor of 2.8 – from 1.3 to 3.8% (Fig. 7). After enzymatic treatment, the yield of MOS increases substantially; the fraction of glucosaccharides bound with protein decreases. The latter effect is connected with hydrolysis resulting in the detachment of glucosaccharides from glucoproteins.

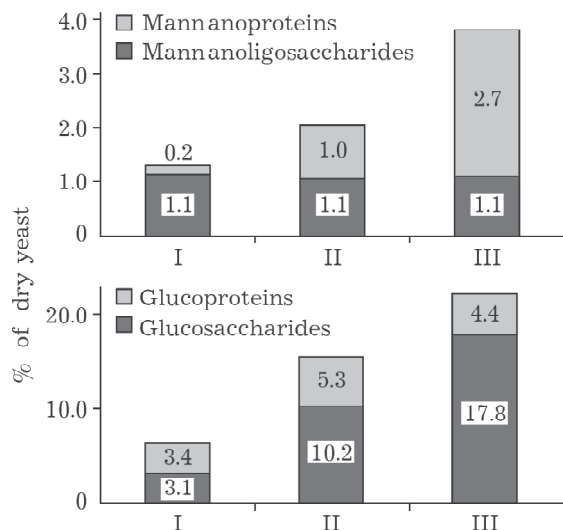


Fig. 7. Yield of oligosaccharides depending on treatment conditions: I–III – extracts of initial yeast (I), yeast after mechanical activation (II), and after mechanical activation and enzymatic hydrolysis (III).

Only a small amount of MOS passes into the extract of the initial yeast (see Fig. 7), mannanoproteins are almost non-extractable after mechanical activation, the yield of mannanoproteins increases. They get extracted to a higher extent from the product obtained by the combined mechanical activation and enzymatic hydrolysis.

Thus, it was demonstrated that mechanical activation of the yeast biomass followed by enzymatic hydrolysis allows one to achieve a substantial increase in the content of mannanooligosaccharides and mannanoproteins available for extraction by solutions.

CONCLUSION

As a result of mechanical activation of the yeast biomass, the reactivity of polysaccharides of cell wall towards enzymatic hydrolysis increases. This effect is based on disordering of the supramolecular structure of cell walls. Due to this, the diffusion limitations are removed and the efficiency of enzymatic hydrolysis of the structure-forming component – glucan –

increases substantially. A combination of mechanical treatment and subsequent enzymatic hydrolysis allows one to increase by a factor of 2.9 the overall content of mannanooligosaccharides and mannanoproteins available for extraction in the yeast preparations.

REFERENCES

- 1 Commission Proposes New Safety Rules for Feed Additives and to Prohibit Antibiotics as Growth Promoters, Scientific Steering Committee of the EU, Brussels, March 25, 2002.
- 2 Budushcheye Stimulyatorov Rosta (European Lecture Tour), February 16–March 5, 2006.
URL: <http://www.alltech.com>
- 3 A. E. Wold, J. Mestecky, M. Tomana *et al.*, *Infect. Immun.*, 9, 58 (1990) 3073.
- 4 L. C. V. Emmerik, E. J. Kuijper, C. A. P. Fijen *et al.*, *Chin. Exp. Immunol.*, 97 (1994) 411.
- 5 US Pat. No. 7048937 B2, 2006.
- 6 H. Bjorndal, B. Lindberg, *Biochem. J.*, 135 (1973) 31.
- 7 D. J. Manners, A. J. Masson, J. C. Patterson, *Biochem. J.*, 135 (1973) 19.
- 8 V. I. Biryuzova, *Ultrastrukturnaya Organizatsiya Drozhzhevoy Kletki*, Nauka, Moscow, 1993.
- 9 T. S. Kalebina, I. S. Kulaev, *Usp. Biol. Khim.*, 41 (2001) 105.
- 10 N. I. Belousova, S. V. Gordienko, V. K. Eroshin *et al.*, *Biotekhnol.*, 3 (1990) 6.
- 11 US Pat. No. 4810509, 1989.
- 12 US Pat. No. 6060089, 2000.
- 13 US Pat. No. 4313934, 1982.
- 14 T. P. Shakhtshneider, V. V. Boldyrev, *Reactivity of Molecular Solids*, John Wiley&Sons, Chichester, 1999, pp. 271–311.
- 15 O. I. Lomovsky, V. V. Boldyrev, *Mekhanokhimiya v Reshenii Ekologicheskikh Problem*, Izd-vo GPNTB SO RAN, Novosibirsk, 2006.
- 16 B. Kh. Shklyar, *Fermentativny Lzis Drozhzhey*, Nauka i Tekhnika, Minsk, 1977, pp. 166–167.
- 17 R. P. Barykina, *Spravochnik po Botanicheskoy Mikrotekhnike. Osnovy i Metody (Handbook)*, Izd-vo MGU, Moscow, 2004.
- 18 A. P. Sinitsyn, A. V. Gusakov, V. M. Chernoglazov, *Biokonversiya Lignotsellyuloznykh Materialov*, Izd-vo MGU, Moscow, 1995.
- 19 J. A. Nielson, W. P. Griffith, *J. Histochem. Cytochem.*, 27, 5 (1979) 997.
- 20 L. S. Strizhko, *Tsv. Met.*, 2 (2003) 40.
- 21 A. L. Bychkov, E. I. Ryabchikova, K. G. Korolev, O. I. Lomovsky, *Nauch.-Prakt. Konf. "Nanotekhnologii i Nanomaterialy dlya Biologii i Meditsiny" (Proceedings)*, vol. 1, Novosibirsk, 2007.