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β-glucan extraction from *Saccharomyces cerevisiae* yeast using *Actinomyces rutgersensis* 88 yeast lyzing enzymatic complex

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β-glucan is a polymer of glucose which is produced by many different organisms – bacteria, fungi, plants. In this natural polymer glucose monomers are linked by β glycosidic bonds.

Nowadays non-cellulosic, fungal β-glucans are being broadly investigated. The main reason being that these biological polymers are recognized as potent immunological stimulators for human immune system. Experimental data show that β-glucans protect mammals from different infections and increase immune system cells cytotoxicity against cancer.

One of these immunomodulating substances is yeast β-glucan extracted from *Saccharomyces cerevisiae*. A large part of fungal cells mass consists of β-glucans. β-glucan is a major structural component of various yeast cells and is vital for cell functions.

In the present paper we describe a simple β-glucan extraction procedure using *Actinomyces rutgersensis* 88 yeast lysing enzymatic complex for yeast cell lysis and soft alkaline β-glucan extraction in further steps.

Key words: yeast, β-glucan, enzymatic lysis, extraction

INTRODUCTION

β-glucan is a polymer of glucose which is produced by many different organisms – bacteria, fungi, plants (Petravic-Tominac et al., 2010; Bacic et al., 2009). In this natural polymer glucose monomers are linked by β glycosidic bonds. There are a lot of β-glucan modifications that differ-glycosidic linkage position, for example β-glucan named curdlan is 1→3 β glucan and cellulose is 1→4 β glucan (Bacic et al., 2009; Novak et al., 2008).

Nowadays non-cellulosic, fungal β-glucans are broadly investigated. The main reason being that these biological polymers are recognized as

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potent immunological stimulators for human immune system (Chen et al., 2007). Experimental data show that β-glucans protect mammals from different infections and increase immune system cells cytotoxicity against cancer (Vetvicka, 2011; Chan et al., 2009).

One of these immunomodulating substances is yeast β-glucan extracted from *Saccharomyces cerevisiae*. A large part of fungal cells mass consists of β-glucans (Chen et al., 2007) which is a major structural component of various yeast cells and is vital for cell functions (Bacic et al., 2009).

Yeast cell consists of two layers – first is closer to membrane, second – to cell surface. The major polysaccharide of inner layer of yeast cell wall is branched 1→3 β-glucan, which forms continuous three-dimensional network. This layer is highly elastic and keeps cell form and saves cells from osmotic pressure changes (Klis et al., 2009).

β-glucan extraction from *Saccharomyces cerevisiae* yeast cell consists of two stages:

1. Yeast cell lysis: β-glucan is localized in cell wall, thus it is necessary to lyse cells and separate insoluble cell wall from cytoplasm;

2. β-glucan extraction from insoluble cell wall.

There are several groups of yeast lysation methods – chemical, physical and enzymatic. Chemically, yeast cells are lysed by NaOH, HCl, acetic acid, citric acid and using other chemically aggressive solutions. In most cases chemical lysis is held at high temperatures (boiling) (Pelizon et al., 2005; Hunter et al., 2005; Jamas et al., 1989; Lee et al., 2001). Physically yeasts are disrupted using sonication, homogenizers with high pressure, etc (Shokri et al., 2008; Boonraeng et al., 2000; Wenger et al., 2008). The last group of methods used for yeast degradation, is enzymatic cell lysis using natural enzymes. Enzymes partially destroy yeast cell wall, so that soluble cytoplasm leaks to the surface. This group of cell degradation can be divided into two subgroups:

1. Autolysis is self-digestion of a cell through the action of its own enzymes; the process has been defined as the hydrolysis of intracellular biopolymers under the effect of hydrolytic enzymes associated with cell death (Martinez-Rodriguez et al., 2001). Autolysis is initiated by the cell lysosomes which release digestive enzymes into the cytoplasm. The process is quite long and can proceed for a few days (Vosti et al., 1954; Lowry et al., 1951).

2. Enzymatic yeast cell lysis is digestion of the cell using special lytic enzymes. There are a lot of organisms which are using yeast cell wall biopolymers (cellulose and other β-glucans) as a nutritional source. In addition, there are microorganisms which are parasitizing on yeast cells. They produce lytic enzymes to the surface, which can digest the cell wall. Therefore the culture solution of these microorganisms is suitable to be used for lysis of yeast cells (Fleet et al., 1974; Saeki et al., 1994; Ferrer, 2006; Adamisch et al., 2003; Кислухина et al., 1990; Шкляр, 1977).

β-glucan is extracted from insoluble cell wall after yeast cell digestion. As a rule, NaOH, different acids and H_2O_2 solutions are used to extract β-glucan from the cell wall. β-glucan extraction process from yeast cell wall is based on other cell wall polymers and low molecular weight β-glucans ability to dissolve in aggressive chemical solutions (Bacic et al., 2009, Jamas et al., 1989; Lee et al., 2001; 23). On the other hand, high molecular weight β-glucan is insoluble even in high concentration of alkali (up to 10 M) (Hayen et al., 2001; Bahl et al., 2009).

The use of aggressive chemical solution for yeast cell lysis and further β-glucan extraction require a large amount of alkali and / or acids. On the other hand, to minimize usage of aggressive materials, yeast autolysis or enzymatic cell digestion may be performed. In the present paper we describe a simple β-glucan extraction procedure using *Actinomyces rutgersensis* 88 yeast degrading enzymatic complex for yeast cell lysis and soft alkaline β-glucan extraction in further steps. We do not use yeast autolysis because of its time-consuming process, which may continue for days. The method is based on *Saccharomyces cerevisiae* yeast digestion by *Actinomyces rutgersensis* 88 culture solution, which has yeast degrading enzymatic complex. In *Actinomyces rutgersensis* 88 culture solution were determined the following enzymatic activities: laminarinase (β-1,3), licheninase (β-1,3; 1,4), gentibiosinase (β-1,6), β-glucanase (β-1,3; 1,6), mannanase (α-1,6; 1,2; 1,3), galactomannanase (β-1,4), chitinase (β-1,4), proteinase (-CO-NH-) (Гуреева 1983).

MATERIALS AND METHODS

All processes and reactions were performed using reagent grade materials.

Microorganism cultivation. *Actinomyces rutgersensis* 88 microorganism was cultivated in SARTORIUS B fermenter for 48 h, 30 °C. After cultivation, culture solution with yeast lysing complex (further YLC) was separated from microorganism cells by centrifugation (4 500 rpm) and filtrated with 2 μm acetylcellulose membrane till clear solution; afterwards yeast lysis activity was determined.

YLC preparation t °C and pH optimal condition determination. The t °C optimum of YLC was determined in the following way: the reaction solutions consisted of 0.03 mg of dry *Saccharomyces cerevisiae* yeast, 1 ml (1,5 a.u.) of YLC solution, 1 ml of distiled water and 1 ml of pH 6 buffer (3M); reaction temperatures: 30, 40, 50, 60, 70 and 80 °C. Reactions had been carried out for 1 h, then solutions were centrifuged (4 500 rpm), insoluble substrate (yeast) was separated from reaction solutions. Concentration of the soluble protein in reaction solutions was determined using Lowry method and compared with concentration at the beginning of the reaction (Lowry et al., 1951). The difference between starting and post reaction concentrations is leaked cytoplasmic proteins of lysed yeast. The t °C optimum was determined when there was the largest difference between starting and post reaction concentrations.

pH optimum of YLC was determined in the same way as t °C optimum, namely measuring soluble proteins difference between starting and post reaction concentrations. Reaction solutions: 0.03 mg of dry *Saccharomyces cerevisiae* yeast, 1 ml (1,5 a. u.) of YLC solution, 1 ml of distiled water and 1 ml of different pH (1–12 pH) 3 M buffer solutions in 50 °C. As pH optimum, the pH buffer was selected where the largest difference between starting and post-reaction concentrations was determined.

YLC activity units. It was decided to define 1 unit of YLC activity, thus, when in a yeast lysis reaction (0.03 g of dry *Saccharomyces cerevisiae* yeast, 1 ml of YLC solution, 1 ml of dist. water, 1 ml of buffer (1 M); reaction time – 1 h; pH and t °C – optimal) the soluble protein concentration rises by 0.1 mg/ml during reaction.

Unknown solution's YLC activity determination. YLC activity of a solution is determined in the following way: the reaction solution consists of 0.03 mg of dry *Saccharomyces cerevisiae* yeast, 1 ml of unknown activity YLC solution, 1 ml of distiled water and 1 ml of buffer solution (3 M), t °C and pH are optimal. Reaction is done for 1 h, then solutions are centrifuged, and soluble protein concentration in reaction solution is measured. YLC activity (a. u.) is the difference between starting and post-reaction concentrations (mg/ml).

Yeast enzymatic lysis and autolysis; lysis efficiency determination. *Saccharomyces cerevisiae* yeast lysis reaction: 40 ml of YLC solution (1,5 a. u.), 20 ml of buffer (3 M), 3 g of dry yeast, t °C and pH are optimal. Yeast autolysis reaction: 40 ml of distiled water, 20 ml of buffer (3 M), 3 g of dry yeast, t °C and pH are optimal. Lysis efficiency was determined with microbiological (cell viability) and microscopic (cell shape) methods. After lysation yeast cell walls were separated from soluble supernatant, washed for 3 times and stored at –20 °C until further usage.

Yeast cell viability determination after lysis. *Saccharomyces cerevisiae* cells viability after lysis was determined cultivating yeast on *Petri* plates. Yeast was inoculated at the plates after the lysis process. At the same time, as control, the plates with normal dry yeast were inoculated (the same dry material concentration). Yeast was cultivated for 24 h. Afterward the plates with intact yeast were compared with plates inoculated with post lytic cells.

Optimization of alkaline β-glucan extraction

Effect of NaOH concentration on β-glucan extraction efficiency. 5 g of the yeast cell walls were suspended in 25 ml with various concentrations: 0,5; 1; 2; 3; 4; 5 M of NaOH. The mixture was heated at 100 °C for 2 h with stirring. Then β-glucan solution was centrifuged, supernatant was poured off, β-glucan was washed with distiled water till neutral pH; carbohydrate and protein concentration in β-glucan residue were determined.

Effect of reaction time on β-glucan extraction efficiency. Optimization of the reaction length was performed using the same method as the optimization of NaOH concentration. Concentration of NaOH – optimal; reactions were done for 30 min, 1 h, 2 h, 3 h, 4 h and 5 h.

β-glucan assay

β-glucan hydrolysis. β-glucan was added to aqueous 50% H_2SO_4 solution so that β-glucan concentration was 2 mg/ml. Hydrolysis was held for 24 h. Then solution was neutralized with 2 M NaOH aqueous solution (5.1 ml 2 M NaOH for 1 ml β-glucan hydrolysis solution with 50% H_2SO_4), and glucose concentration assay was done.

Glucose concentration analysis. Glucose monomers concentration in solutions was determined using reducing sugars assay with the dinitrosalicilic acid (Miller et al., 1959).

Protein concentration analysis. Proteins concentration in solutions was determined using Lowry protein assay (Lowry et al., 1951).

RESULTS AND DISCUSSION

***Saccharomyces cerevisiae* yeast lysis**

In our experiments yeast was lysed by *Actinomyces rutgersensis* 88 culture solution which had yeast lytic activity (Гуреева 1983). Firstly, we decided to determine optimal yeast lysis conditions (t °C, pH).

Our data (Figs. 1 and 2) show that optimal external conditions for *Actinomyces rutgersensis* 88 YLC action are:

1) t = 50 °C;

2) pH = 10.

The next step after determination of the optimal activity conditions for YLC was full *Saccha-*

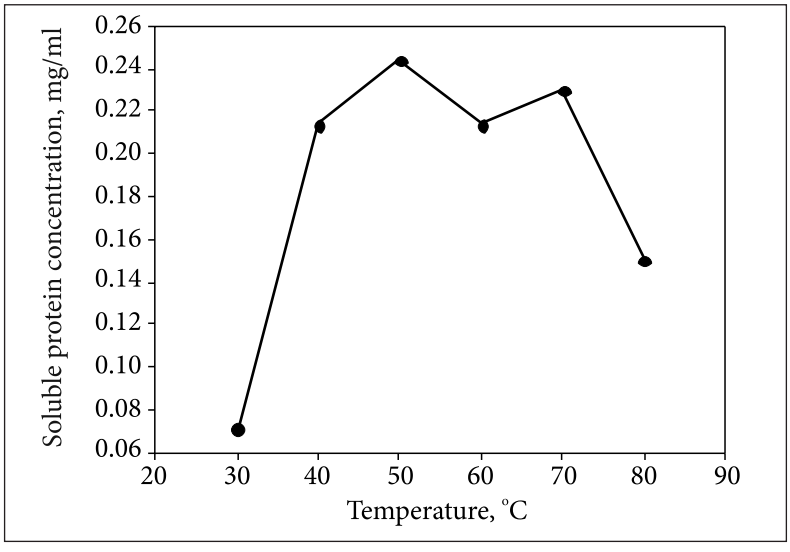

Fig. 1. Yeast lysis activity dependence on reaction t °C

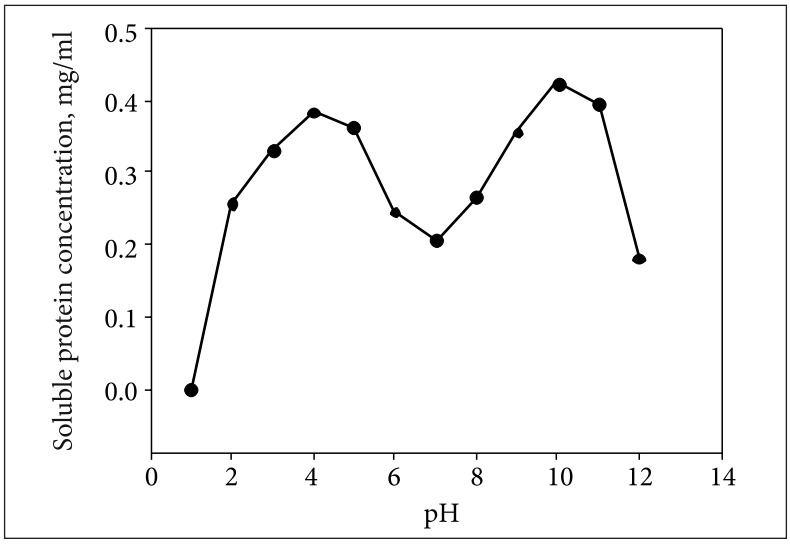

Fig. 2. Yeast lysis activity dependence on reaction pH

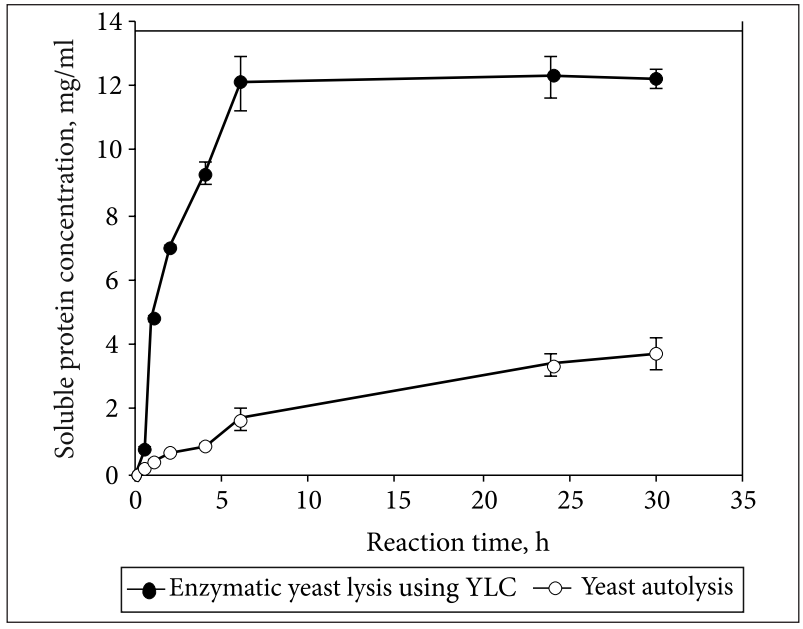

Fig. 3. Soluble proteins concentration in reaction solution. Data represent middle point of 3 replicates with standard error

romyces cerevisiae yeast lysis. For that purpose, we decided to analyze lysation process dependence on reaction time. Also, it is known that at 50 °C temperature (earlier determined optimal t° condition for YLC) the yeast autolysation process may start. Although it is a very long process (a few days), we had to analyze it and compare with *Actinomyces rutgersensis* 88 culture solution lysis (Vosti et al., 1954; Kwei et al., 1981).

Concentration of soluble proteins in enzymatic reaction solution using YLC rises rapidly till approximately 6 h of the process (Fig. 3) and does not rise anymore. On the other hand, autolysis experiment shows that concentration of soluble proteins in autolysis reaction does not rise so quickly, as is in the enzymatic reaction using YLC (Fig. 3). Furthermore, even after 30 h of a process it does not reach the similar concentration of soluble proteins in reaction solution, what proves the statement about an extremely long time of autolysis process (Vosti et al., 1954; Lowry et al., 1951). Further we investigated whether there are viable cells in lysis solution after 6 h and 24 h of the enzymatic lysis reaction. Enzymatically lysed yeast cells were seeded on *Petri* plates, cultivated for 24 h and compared with the intact yeast cells.

After 24 h of yeast cultivation lawn of the cells had grown on dishes seeded with intact cells (Fig. 5A), but not a single colony had grown on dishes inoculated with lysed cells either after 24 h or after 6 h of lysis (Fig. 5B, C). In addition, we

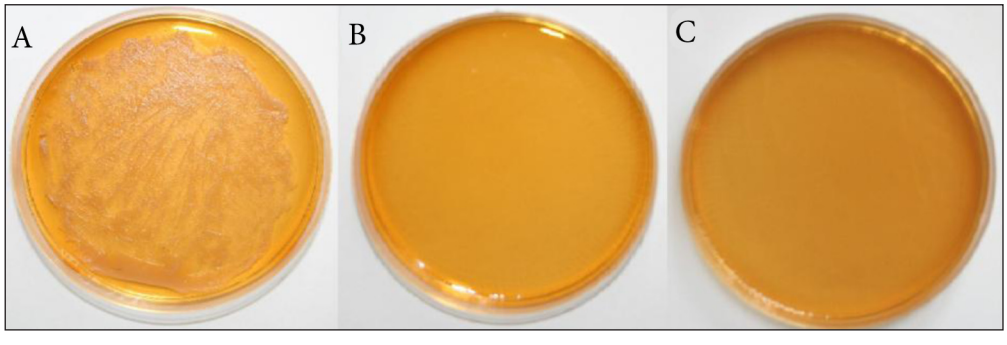

Fig. 4. Lysed cells viability analysis, yeast cultivation on *Petri* dishes, grown colonies after 24 h: A – intact yeast; B – yeast lysed for 6 h; C – yeast lysed for 24 h

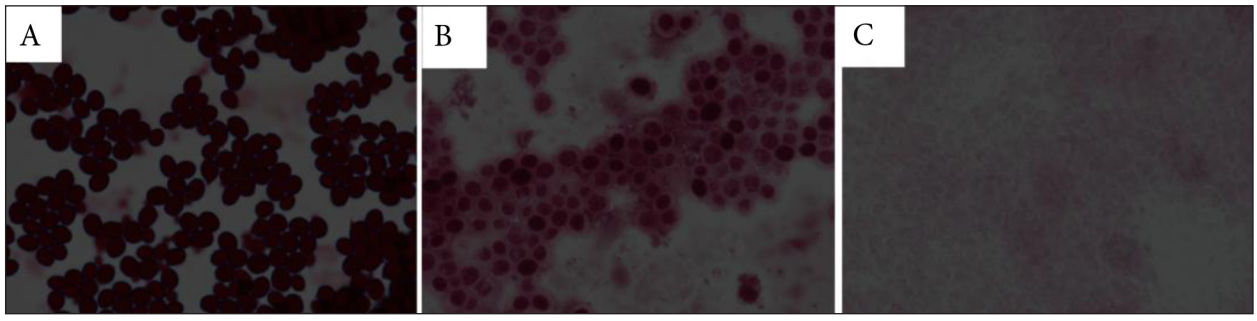

Fig. 5. Intact and lysed yeast cells: A – intact yeast; B – yeast lysed for 6 h; C – yeast lysed for 24 h (magnification × 1 000)

have done photographs of intact and lysed cells after 6 and 24 h of the lysis process, which show that cells after 6h are highly damaged and after 24 h we cannot observe any cells at all.

Yeast cell viability assay showed that there had been already no viable cells after 6 h of enzymatic lysis process. Microscopy (Fig. 6) visually proves that cells are damaged, but, on the other hand, yeast cell walls after 6 h (Fig. 6B) were not completely degraded as they had been after 24 h (Fig. 6C).

Thus, our data show that we have to proceed with the lysis process for 6 h to get *Saccharomyces cerevisiae* yeast cells lysed (under the conditions that we used). Such reaction time and conditions were used in the following experiments. After the enzymatic cell lysis we got yeast cell walls which we used afterwards for β-glucan extraction.

β-glucan extraction from *Saccharomyces cerevisiae* yeast cell walls; extraction optimisation

β-glucan extraction from *Saccharomyces cerevisiae* yeast cell walls was done by one-step procedure using NaOH solution (hot alkali solubilize all other yeast cell wall components and polymers, such as proteins or mannan; insoluble β-glucan stays in solids) (Bacic et al., 2009; Suphantharika et al., 2003). The aim of the main optimization of β-glucan extraction procedure is to reach those results: to get the highest concentration of β-glucan and the lowest concentration proteins in a solid material; to use as low as possible concentration of alkali. Thus extraction conditions were optimized on alkali concentration and reaction time.

Glucose concentration dependence on NaOH concentration used for β-glucan extraction graph

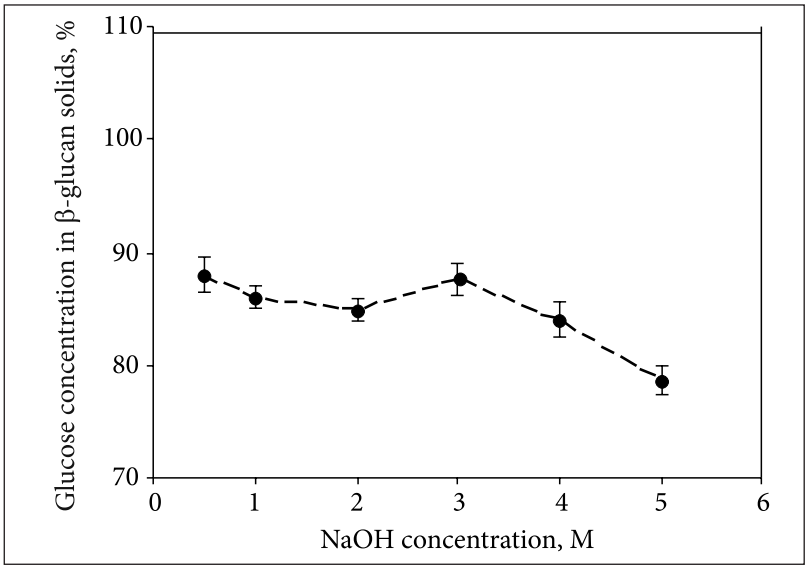

Fig. 6. Glucose concentration dependence on NaOH concentration. Data represent middle point of 3 replicates with standard error

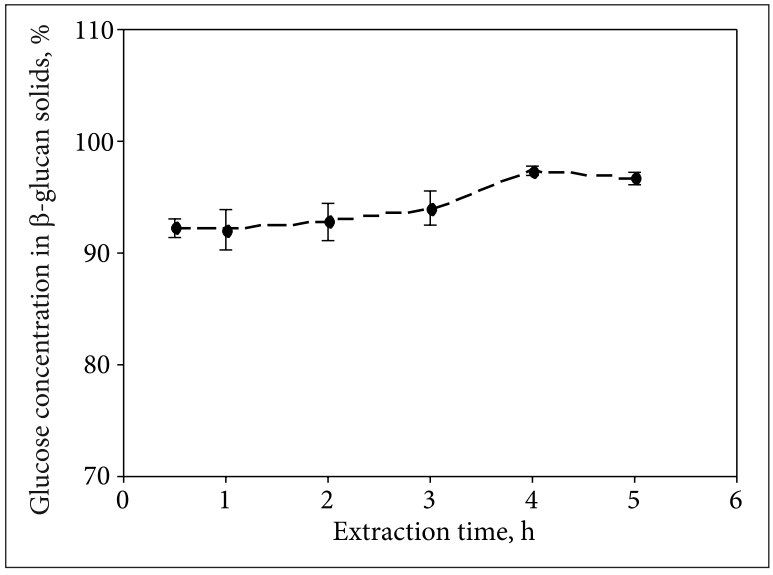

Fig. 7. Glucose concentration dependence on extraction time. Data represent middle point of 3 replicates with standard error

(Fig. 7) shows that concentrations of β-glucan (carbohydrates) in solids after alkaline extraction are more or less equal. The largest middle β-glucan concentration was obtained using 0.5 M NaOH solution. However, we cannot state this definitely because of statistical errors. But even so we used 0.5 M NaOH concentration in other experiments as optimal, because middle β-glucan concentration using this NaOH concentration is largest, and at the same time we can claim that β-glucan concentration in solids using 0.5 M NaOH for extraction is not lower than if we used other concentrations. In addition, lower quantity of alkali was used. Also, we have done proteins concentration assay, but in all samples soluble protein concentration was ~0 (therefore we do not show individual sample data).

During β-glucan extraction optimization on reaction time we determined that optimal reaction time for β-glucan extraction procedure is 4 h: performing extraction for 4 h, the middle β-glucan concentration in solids of 3 experiments was largest, statistical error interval was narrowest and highest in values. But once again we have to note that statistical error interval of 4 h and 5 h extraction process results slightly overlap. Protein concentration assay was the same as in the previous experiment – soluble protein concentration in all samples was ~0 (therefore we do not show individual sample data).

Thereby, our experimental data show that optimal condition for alkaline β-glucan extraction after enzymatic lysis is 0.5 M NaOH concentration and 4 h process length.

CONCLUSIONS

As a result of our expirements we have formulated the method of β-glucan extraction using *Actinomyces rutgersensis* 88 yeast digestion complex. The milestones of our method are as follows:

1. Yeast cell lysis for 6 h using *Actinomyces rutgersensis* 88 yeast lysing complex (1.5 a. u.), solid yeast cell walls separation from soluble yeast cytoplasm.

2. Alkaline β-glucan extraction with NaOH 0.5 M solution. Process length – 4 h.

The method can be used to get fast and non-complicated β-glucan extraction for using in different research areas.

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B-GLIUKANO IŠSKYRIMAS IŠ *SACCHAROMYCES CEREVISIAE* MIELIŲ NAUDOJANT *ACTINOMYCES RUTGERSENSIS* 88 MIELES LIZUOJANTĮ KOMPLEKSĄ

Santrauka

β-gliukanai yra gamtiniai gliukozės polimerai, kuriuos sintetina daugelis skirtingų organizmų – bakterijos, grybai, augalai. Šiame gamtiniame polimere gliukozės monomerai sujungti tarpusavyje β glikozidiniais ryšiais (Petravic-Tominac et al., 2010; Bacic et al., 2009).

Nustatyta, kad natūralūs, neceliuliozinės kilmės polisacharidai potencialiai gali būti žmogaus imuninės sistemos moduliatoriai, todėl šie polimerai intensyviai tiriami visame pasaulyje (Novak et al., 2008). Atlikti eksperimentai rodo, kad β-gliukanai gali stimuliuoti žinduolių apsaugines sistemas prieš skirtingas infekcijas, taip pat padidinti imuniteto ląstelių citotoksiškumą vėžinėms ląstelėms (Chen et al., 2007; Vetvicka, 2011).

Vienas tokių imunomoduliuojančių junginių yra iš kepimo mielių *Saccharomyces cerevisiae* ląstelių išskirtas β-gliukanas. Jis sudaro didžiąją mielių ląstelių masės dalį, yra gyvybiškai svarbus ląstelių komponentas – palaiko ląstelių formą (Bacic et al., 2009; Chen et al., 2007).

Straipsnyje pristatome nesudėtingą β-gliukano išskyrimo iš *Saccharomyces cerevisiae* kepimo mielių būdą ląstelių lizei panaudojant *Actinomyces rutgersensis* 88 mikroorganizmo sekretuojamą mieles lizuojantį fermentinį kompleksą ir tolesnę šarminę β-gliukano ekstrakciją.

Raktažodžiai: mielės, β-gliukanas, fermentinė lizė, išskyrimas