THE OPTIMIZATION OF NUTRITIVE MEDIUM COMPOSITION AND CULTIVATION CONDITIONS FOR *SACCHAROMYCES CEREVISIAE* CNMN-Y-18 YEAST STRAIN – MANNANS PRODUCER

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Abstract. This paper represents synthesis of results that refers to the optimization of nutritive medium composition and cultivation conditions for *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain for maximum accumulation of mannans. New nutritive medium for cultivation of yeast strain selected as active mannan producer was elaborated by the application of mathematical methods of experimental planning. The optimized medium YP(GA) with the following composition: yeast extract - 10.0 g/l, peptone - 20.0 g/l, glucose - 46.0 g/l, ammonium monohydrogen phosphate - 2.32 g/l assure the obtaining up to 12.07% or 1.050 g/l mannans. Optimum conditions for mannans biosynthesis – the temperature of 25^0C, aeration mode 81.0 mg/l, pH 5.5, duration of cultivation 120 hours were established for *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain. The procedure of directed cultivation of selected yeast strain assures the obtaining 12.65% opposite to 10.15% dry cell weight of mannans obtained on initial medium.

Keywords: *Saccharomyces cerevisiae;* mannan; nutritive medium; mathematical methods of experimental planning; cultivation conditions.

INTRODUCTION

Currently, microbial polysaccharides obtained from yeasts find their application in various fields of human activity: medicine, pharmacy, hydrometallurgy, food and cosmetic industries [7, 19, 23, 28]. Because of its properties, mannans can be used as stabilizers, thickeners agents, replacing raw materials of bacterial and plant origin. Another important aspect is the use of yeast cell wall polysaccharides as gelling agents in the production of syrups and jams, cosmetics [8]. In addition, due to the ability of flocculation, mannans can be used in metallurgy during purification, concentration and separation of metals, including wine production - to stabilize the protein and improve the color of wine [11, 12], and also to prevent the deposition of salts of tartaric acid [6].

The mannans that form the outer cell wall layer (40% of dry substances) are highly glycosylated with a carbohydrate fraction that often amounts to over 80-90% are covalently linked with different proteins through glycosidic bond (di-N-acetyl-chitobiose and asparagine part of the protein) forming mannoproteins. Located mainly in the outer layer of the cell wall mannans are associated with β-1,3 and β-1,6 glucans through phosphodiester bonds [18, 26]. The basic role of mannans is to protect the protoplast from mechanical damage, providing rigidity yeast cell, maintaining its shape according to the phases of the cell cycle, also, mannans are involved in the transport of macromolecules from the environment to the periplasmic space and vice versa, in the yeast agglutination - important technological properties [17].

According to the literature papers, the content of mannans in yeasts depends on the composition of nutrient medium. With this purpose, yeasts are cultivated on the special medium containing sources of carbon, nitrogen and other growth factors. The basic source of carbon for yeasts is presented by hexoses (glucose, mannose, galactose, sucrose) which are necessary for biosynthetic reactions and energy obtaining [15, 27].

The presence of some sources nitrogen and phosphorus in the form of inorganic salts (ammonium sulphate and ammonium monohydrogen phosphate) or natural products (peptone, yeast extract) are necessary for yeasts cultivation [25]. As a consequence of the fact that the composition of yeast biomass might be significantly changed by the modification of nutritive medium, it is important to optimize selected factors for mannan producers.

Essential factors that influence yeast growth rate and the intensity of the processes of polysaccharides biosynthesis are following: the duration of cultivation, the temperature, the aeration, the pH of the growth medium [3, 10, 14]. It is known that the content of mannans depends on yeast strain, composition of nutritive medium and cultivation conditions [1, 22]. Therefore, cultivation parameters settle down in agreement with specific requirements of yeast strain.

In this way, the aim of this study was to optimize the composition of nutritive medium and cultivation conditions of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain selected previously as active mannan producer.

MATERIALS AND METHODS

Strains. *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain previously selected as a producers of mannans [4].

Medium. The following mediums were used in this study: seed culture medium with beer wort, medium YPD (as control) (g/l): glucose - 20.0, peptone - 20.0, yeast extract - 10.0; pH 5.0-6.0 [2].

Culture conditions. Cultivation was carried out in Erlenmayer 250 -1000 ml flasks containing 200 ml of the culture medium at 200 rpm agitation rate, 25-30ºC, for 48-168 hours.

Dry cell weight was determined gravimetrically [9].

Preparation of yeast cell walls was effectuated according to [29]. Yeast cell walls were prepared by autolysis of yeast suspension (adjusted to 15% w/w solids content and pH 5.0) at 50^0C for 24 h with moderate agitation in a jacketed kettle. The autolysate was then heated at 80^0C for 15 min, cooled to room temperature and centrifuged at 3565 g in a centrifuge for 10 min at room temperature. The supernatant was discarded and the yeast cell walls were collected and stored at 4^0C until used. Yeast cell walls were resuspended in distilled water to obtain a suspension containing 15% solids content. The suspension was homogenized using a high pressure homogenizer (model Heidolph Silent Crusher M) at 600 bar for six passes. **Alkaline extraction of mannan:** 50 g of *Saccharomyces cerevisiae* CNMN-Y-18 (30% dry cell weight) yeast autolysate was treated with 250 ml 2% NaOH added to the cell wall sediment. This was placed in a boiling water bath and agitated at 150 rpm/min for 2 h. The preparation was centrifuged and the supernatant was collected. The residue was washed with little deionized water and combined with supernatant extracts. After that, the pH was adjusted to 6.5 with 10% acetic acid, and the supernatant was concentrated to one fifth of the original volume, triple absolute ethyl alcohol was added to precipitate mannan. The precipitated mannoprotein was dissolved in water and centrifuged; the supernatant was precipitated again by the addition of triple ethanol and recentrifuged. The obtained white sediment was washed twice with absolute ethanol and once with ether, then dried at 70^0C [23]. **The optimization of nutritive medium** was effectuated by mathematical methods of experimental planning. The optimization of culture medium for the obtaining of maximum content of mannans has been carried out in some consecutive stages: the experience according to plan „Fractional factorial experiment ($FFE2^2$)" with the determination of direction of variation of concentrations (increase or decrease) and the experiment according to plan „Movement along the gradient" during which the most effective combination of the essential and nonsential factors has been selected [24]. **Statistical analysis.** Results were expressed as the mean ± standard deviation (SD). Statistical evaluations were performed using t test. A value of $P<0.05$ was considered significant. The results were performed according to Student's t-test.

RESULTS

Previously, the effect of carbon (the glucose, sucrose, mannose, fructose, molasses, ethanol) and nitrogen sources (ammonium sulphate and ammonium monohydrogen phosphate) on the mannans accumulation at *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain that depends on type and concentration of administered compound has been established. It was proposed to add glucose in the concentrations of 30.0 – 50.0 g/l and 1.0 – 4.0 g/l of ammonium monohydrogen phosphate to the nutritive medium in view of the obtaining of increased amounts of mannans.

Resulting from the fact that each component of nutritive medium has been investigated separately, it was important to study the interaction of these factors and their effect on mannans accumulation at *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain. Medium YPD with the following composition, g/l: yeast extract - 10.0, peptone - 20.0, glucose - 20.0 was selected as control. For optimization of nutritive medium with balanced formula were used mathematical methods of experimental planning.

Method of optimization of nutritive medium is based on statistical analysis and the utilization of regression equations [24].

With the aim to obtain increased content of mannans in yeast biomass, it was effectuated nutritive medium optimization which included the following stages: initially, the components of medium with maximum effect on mannans biosynthesis were selected (in the base of the obtained dates from monofactorial experience) and lower and upper levels of concentration were established; then the experience according to the factorial fractional plan $EFF2^2$ was effectuated (Table 1).

In accordance with the obtained results, regression coefficients were calculated by algorithm Iets (Table 2).

The regression equation took the form: $\mathbf{Y = 10.99 + 0.39X_1 - 0.075X_2 + 0.28X_1X_2}$.

In accordance to obtained equation the experiment „Movement along the gradient" which allows selecting optimal concentration of nutritive medium components was effectuated (Table 3).

Table 1. Plan for fractional factorial experiment $EFF2^2$

Factors	Codification	Concentration, g/l			Units of variation
		Lower level (-)	Base (0)	Upper level (+)	
Glucose	X_1	30.0	40.0	50.0	10.0
Ammonium monohydrogen phosphate	X_2	1.0	2.5	4.0	1.5

Table 2. Regression coefficients by algorithm Iets

Experimental plan		Experimental results (Y)	Algorithm Iets		Regression coefficient *(bi)*	Codification
			1 step	2 step		
X_1	X_2					
-	-	10.96±0.12	22.14	43.98	10.99	1
+	-	11.18±0.09	21.84	1.56	0.39	X_1
-	+	10.25±0.03	0.22	- 0.3	- 0.075	X_2
+	+	11.59±0.01	1.34	1.12	0.28	X_1X_2

Table 3. Plan of the experiment „Movement along the gradient"

Value	Factors		Experimental results	
	Glucose X_1	Ammonium monohydrogen phosphate X_2		
Regression coefficient, *b(i)*	0.39	-0.075		
Units of variation, $\lambda(i)$	10.0	1.5		
$b(i) \cdot \lambda(i)$	3.9	-0.11		
Coefficient of proportionality, K_i	1	-0.03		
Step, *H(i)*	3	-0.09	**Mannans, % dry cell weight**	**Mannans, g/l**
Concentration	g/l	g/l		
1	40.0	2.50	10.30±0.03	0.840±0.04
2	43.0	2.41	10.62±0.12	0.947±0.05
3	46.0	2.32	12.07±0.02	1.050±0.13
4	49.0	2.23	10.58±0.03	0.904±0.04
5	52.0	2.14	10.45±0.10	0.885±0.09
control	20.0	-	10.15±0.05	0.564±0.02

Thus, new nutritive medium has been elaborated with the following composition (g/l):

yeast extract - 10.0
peptone - 20.0
glucose - 46.0
ammonium monohydrogen phosphate - 2.32
distilled water - 1 litre
pH - 5.0…6.0

The utilization of this nutritive medium for the cultivation of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain contributes to obtaining up to 12.07% dry cell weight or 1.050 g/l of mannans, opposite to 10.15% dry cell weight or 0.564 g/l of mannans at the cultivation on control medium YPD [5].

Therefore, it is possible to conclude that the application of mathematical methods of experimental planning in process of mannans producing by yeasts allow not only to optimize the composition of nutritive medium in the goal to obtain maximum amount of the finite product, but also to establish the interdependence of the different factors at biosynthetic process accomplishment.

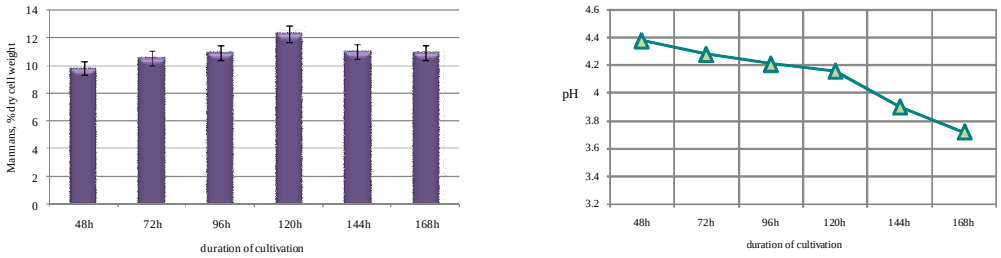
The study of the effect of cultivation conditions on mannans production at *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain

Further the study of the effect of cultivation conditions on mannans production at *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain has been carried out.

It is known that the synthesis of some compounds is associated with phases of yeast growth. By these considerations, is important to establish the interdependence of the process of yeast multiplication and mannans biosynthesis in dynamic. For this, the optimized culture medium YP(GA) was used at the temperature of 20…25^{0}C for 48-168 hours. As a result of obtained data analysis, referring to the effect of cultivation duration on mannans production, it was determined significant accumulation of these (12.31% dry cell weight) at 120 hours of submerged cultivation (Fig. 1 (a)).

It was demonstrated that *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain possessed its own mechanism of the initial pH regulation of culture medium to the acid reaction. That way, pH modification was established at the submerged cultivation of selected yeast strain, the values of which remain of 4.28 (after 48 hours of cultivation) up to 3.72 (after 168 hours of cultivation (Fig. 1 (b)).

It is known, that microorganisms possess the capacity to modify their metabolism under the influence of cultivation factors, inclusive aeration. Recent studies have demonstrated that oxygen has positive influence on yeasts growth and stimulates polysaccharide biosynthesis [1]. Resulting from this, the effect of aeration on process of mannans accumulation has been carried out.



a) b)

Figure 1. The dynamics of the mannans accumulation in biomass of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain

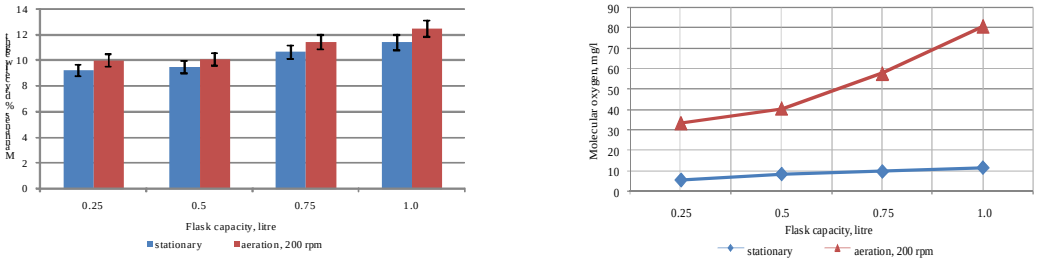


Figure 2. The influence of aeration rate on mannans accumulation at *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain

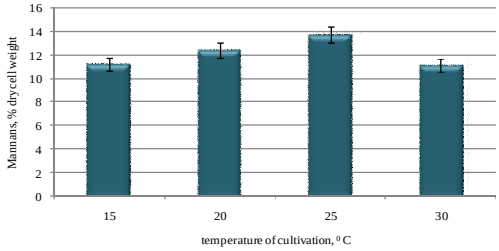


Figure 3. The effect of temperature mode on mannans content in *Saccharomyces cerevisiae* CNMN-Y-18

It was stated, that the molecular oxygen concentration in nutritive medium of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain indicates significant positive effect on mannans content in the biomass. The increase of mannans biosynthesis (12.51% dry cell weight) was revealed at the values of molecular oxygen being included between 57.8-81.0 mg/l (Fig. 2). Therefore, the utilization of flasks with capacity of 1000 ml with 150-200 ml of nutritive medium with the volume of inoculum of 5% it is proposed for yeast cultivation in laboratory conditions.

The temperature, that is one of the basic regulatory factors in regulation of yeasts growth, also, indicates action on biosynthetic processes of cell. According to the literature data, it is well-known, that in the majority of the cases, temperature variation can initiate quantitative modifications of microbial polysaccharides [22].

Some values of temperature -15, 20, 25 and 30^{0}C were used for cultivation of selected yeast strain. According to the obtained results, it was established that the maximum of growth of *Saccharomyces cerevisiae* CNMN-Y-18 strain and the maximum of accumulated mannans (13.70% dry cell weight) took place at the temperature of 25^{0}C (Fig. 3).

The effectuated investigations which refered to the optimization of nutritive medium composition and cultivation conditions for *Saccharomyces cerevisiae* CNMN-Y-18 allowed to elaborate the procedure of increase of the content of mannans in this yeast strain.

The procedure of the increase of mannans content in *Saccharomyces cerevisiae* CNMN-Y-18. It is prepared nutritive medium with the following composition (g/l): yeast extract - 10.0; peptone - 20.0; glucose - 46.0; ammonium monohydrogen phosphate - 2.32; water - 1 litre; pH – 5.5. The inoculum (yeast cells cultivated on beer wort) it was introduced in medium of cultivation in volume of 5% and it was cultivated in agitation mode (200 rpm), the temperature of 25^{0}C, aeration mode 81.0 mg/l, duration of cultivation 120 hours. Biomass was collected by centrifugation, treated by the process of autolysis, followed by mannans extraction. The procedure of directed cultivation of selected yeast strain assures the obtaining 12.65% opposite to 10.15% dry cell weight of mannans obtained on initial medium.

DISCUSSIONS

This paper presents the results of a research on the parameters of directed cultivation of *Saccharomyces cerevisiae* CNMN-Y-18 – mannans producer.

An optimum medium has an important role in regulation of biosynthetic potential of microorganisms which assures the cell with the energy source and corresponding nourishing elements. Parameters of cultivation vary in dependences of physiologo-biochemical peculiarities of microbial strains and are specific for each producer.

The yeasts are cultivated on nutritive mediums that contain necessary substances for the growth. In a lot of cases synthetic and semisynthetic nutrient mediums based on natural extracts, mineral salts and microelements are used for yeasts cultivation [16, 20].

Therefore, nutritive medium composition and cultivation conditions which can activate metabolic pathways, present important factors of yeast cultivation on industrial scale. An important role has the qualitative composition and the concentration of carbon source, since polysaccharides maximum accumulation has been revealed at the yeasts cultivation on the nutritive mediums enriched in carbon. At the same time, the type and the amount of the administered nitrogen source has influenced on the rate of yeast multiplication and polysaccharides quantitative content.

The obtained results are similar with other scientific data. Aguilar Uscanga has demonstrated the accumulation of mannans (4.16...9.14%) in the cell wall of *Saccharomyces cerevisiae* yeasts cultivated on YPD medium [1]. Hong-Zhi Liu has studied the effect of spatial flight on polysaccharides synthesis in some yeast strains *Saccharomyces cerevisiae* and has mentioned that mannans content varied from 46.6 to 90.5 mg/100 ml [21]. Researchers have established the highest value of 94.91…96.41 mg/100ml mannan in *Saccharomyces cerevisiae* yeasts in optimizing culture condition and culture medium respectively [13]. Other scientists have demonstrated that brewer's yeasts contained 5.2% mannan oligosaccharides which have been recently proposed as possible alternatives to antibiotics [30].

So, the optimized by the mathematical methods of experimental planning medium YP(GA) for the cultivation of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain assures the obtaining 12.07% opposite to 10.15% dry cell weight of mannans obtained on initial medium YPD. The effectiveness of new elaborated medium is owed to the fact that the combination of the factors is more favorable, having greater influence on the provision of the growth medium with essential compounds for the accomplishment of the process of biosynthesis of cell components.

The influence of parameters of cultivation such as temperature, pH, aeration and duration of cultivation on mannans synthesis is specific and depends on mode of application.

Factors of cultivation can serve as regulators of the process of mannans accumulation at *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain. The optimum conditions for mannans biosynthesis are the temperature of 25^0C, aeration mode 81.0 mg/l, pH 5.5, duration of cultivation 120 hours.

The procedure of directed cultivation of *Saccharomyces cerevisiae* CNMN-Y-18 yeast strain, based on the use of optimized nutritive medium and optimum cultivation conditions assures the obtaining 12.65% dry cell weight of mannans in comparison with initial procedure (10.15%).

Thereby, let's be able to affirm that the elaborated procedure of the increase of mannans content in yeast biomass allows obtaining of considerable quantities of this compound with high biological value.

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