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Влияние параметров процесса культивирования дрожжей *Saccharomyces cerevisiae* в простой периодической культуре на выход биомассы и биосинтез некоторых клеточных компонентов

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Реферат. Рост и размножение промышленно значимых дрожжей Saccharomyces cerevisiae в первую очередь определяется сбалансированностью состава используемой питательной среды. Для предотвращения снижения скорости биотехнологического процесса и достижения оптимального выхода целевого продукта биосинтеза (биомассы) необходимо вносить в питательную среду недостающие питательные вещества (витамины и микроэлементы). В настоящее время имеется много сведений о влиянии различных факторов среды на рост и размножение микроорганизмов. Однако потенциальные возможности микробных культур использованы далеко не полностью, так как при составлении питательных сред до недавнего времени исследователи использовали главным образом методы установления однофакторной зависимости, то есть принцип поочередного изменения в эксперименте каждого фактора среды на фоне постоянного уровня остальных. В данной работе исследуется степень влияния на выход биомассы различных параметров процесса в их взаимодействии, проводится установление многофакторной зависимости, используя методы математического планирования эксперимента. Эти методы дают возможность изучать не только влияние одновременно большого числа факторов, но и позволяют построить математическую модель процесса, а следовательно, выявить количественное значение каждого отдельного фактора и учесть межфакторные взаимодействия в системе. Культивирование дрожжей Saccharomyces cerevisiae проводили в условиях простой периодической культуры. В качестве факторов варьирования использовали: величину инокулята, содержание в среде азота, фосфора и биотина. Используя экспериментальные данные и многофакторный анализ, было обнаружено, что в данных условиях на синтез биомассы более всего влияет содержание биотина в среде. Установлено, что в среде оптимального состава экономический коэффициент не зависит от величины засева. А также показано, что размер фонда свободных аминокислот находится в обратной зависимости от расхода посевного материала: с увеличением начальной плотности популяции количество аминокислот в клетках падает.

Ключевые слова: дрожжи, азот, фосфор, биотин

Influence of the parameters processes of cultivation of yeast *Saccharomyces cerevisiae* in simple periodic culture on the yield and biosynthesis of some cellular components

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Summary.The growth and multiplication of the industrially significant yeast Saccharomyces cerevisiae is primarily determined by the balance of the composition of the nutrient medium used. In order to prevent a decrease in the speed of the biotechnology process and to achieve the optimal yield of the desired biosynthesis product (biomass), it is necessary to introduce such nutrient deficiencies as vitamins and trace elements into the nutrient medium. At present, there is much information about the influence of different environmental factors on the growth and multiplication of microorganisms. However, the potential possibilities of microbial cultures have not been fully used. Researchers during the preparation of nutrient media until recent time mainly use the methods of establishing one-factor dependence that are based on the principle of alternating change of each nutrient media factor with the others being constant. In this work, the impact on the biomass yield of various process parameters in their interactions is investigated. A multifactor dependence is established using the methods of mathematical design of an experiment. These methods allow both to study the effects of a large number of factors and to construct a mathematical model of the process revealing the quantitative value of each individual factor and to take into account the interfactor interactions in the system .The cultivation of the yeast Saccharomyces cerevisiae was conducted in a simple periodic culture. The factors used were: the amount of inoculum, the content of nitrogen, phosphorus and biotin in the medium. Using experimental data and multifactor analysis, it was found that under these conditions, the content of biotin in the medium mostly affects the biomass synthesis. It was established that, in an optimally composed media, the economic coefficient was independent of the size of the seeding. Moreover, it was shown that the size of the fund of free amino acids is inversely related to the consumption of the seeding: with an increase in the initial density of the population, the number of amino acids in cells decreases.

Keywords:yeasts, nitrogen, phosphorus, biotin

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Introduction

In order to achieve the maximum efficiency of the biomass upstream process, i.e. to achieve the maximum intensity of multiplication with a biomass high yield, it is necessary to provide oxidative yeast metabolism and optimal physico-chemical growth environment. The influence of physical and chemical factors is quite thoroughly studied, while the matter of the proper yeast nutrition is still being solved.

In this regard it is necessary to study the interrelation of energy exchange with physiology and constructive yeast exchange because the intensity of growth and biomass yield will be influenced by reproductive activity of the seed material and by the balance of all components of the nutrient medium, especially the growth factors of nitrogen, macroelements (P, K, Mg) and microelements (Cu, Fe, Mn, Zn).

Nitrogen-containing substances are the main structural components of cells. These are amines, amides, amino acids, proteins and nucleic acids. These compounds account for 37 to 60% of the cell dry solids [1, 2].

Most of them are represented by proteins (40.6–58%) and nucleic acids (15–26%); free amino acids and peptides account for 6.5 to 9.3% [3], i.e. most of them are represented by substances that are associated with both energy metabolism and constructive exchange in the cell.

The nitrogen metabolism is closely related to the phosphorus metabolism, which content expressed in terms of P_2O_5 in yeast varies from 1.9 to 5.5% of dry ingredients in baker's yeast [4] and from 1.4 to 2.0% in brewers' yeast [1, 2].

Phosphorus is a part of nucleic acids, phospholipids, polymers of a cell wall. It can be accumulated in the form of polymethophosphates or volutin. The greatest part of phosphorus is found in ATP.

Other important components of the nutrient medium are growth factors, in particular, it is biotin, the need for which is characteristic of all Saccharomyces yeasts. The lack of biotin in the culture medium entails an imbalance of all types of metabolism: protein, fat, carbohydrate, and nucleic acid synthesis metabolism [5, 6]. Typically, to compensate for the lack of biotin it is added into complex nutrient media for example, to molasses or malt wort on the basis of 0.1 to 0.25 mg per 100 g of growth expressed in terms of absolutely dry biomass of yeast [2, 4].

The amount of associated biotin in enzymes is always constant [7, 8]. Having said so it should be borne in mind that yeast has the ability to accumulate intracellular biotin reserves [9]. The excess of biotin is also undesirable, because in this case there is slowing-down of its transport to the cell, so when calculating the biotin sources flowrate for the cultivation process, the content of biotin in the medium and in the yeast itself should also be taken into account [9].

The relationship of the amount of pitching and the biotin content in the medium, as well as the interrelation of the biotin metabolism with energy and constructive exchange has been poorly studied so far. Meanwhile, this really matters both in science terms and on practical grounds.

The amount of nitrogen and phosphorus salts in the medium determines their physiological state [3].

It is found that the resistance of cells to stress depends on the content of reserve carbohydrates, in particular trehalose and glycogen [10]. The protective effect of trehalose is based on the ability of yeast to maintain the osmotic pressure in the cells, while glycogen is the source of endogenous glucose for initiating the onset of glycolysis. It is shown that the synthesis of trehalose and glycogen in a simple discontinuous culture begins in the phase of cell growth retardation. First, glycogen is synthesized, and then trehalose does [11]. The source of carbon for the endogenous synthesis of trehalose can be the free amino acids of yeast, which content is inversely related to the amount of trehalose in yeast [12, 13].

Directed synthesis of certain cellular components can be achieved by changing the culture conditions. It is known that a shift-down of population growth is accompanied by the synthesis of reserve carbohydrates, by the same token, the growth rate can be regulated by the process limiting with various nutritional components. Synthesis of reserve carbohydrates can be accelerated even when the culture is limited by carbon.

The reproductive activity of yeast during the lag phase of growth probably depends on the inoculum dose, the quality of which is determined by the culture conditions and by the content of reserve carbohydrates in them.

The purpose of these studies is to determine the effect of inoculum dosing on the yield of biomass and the relationship of this index with the composition of the nutrient medium (nitrogen, phosphorus and biotin content) and the chemical composition of the yeast derived.

Materials and methods

Microorganism and cultivation conditions. The *Saccharomyces cerevisiae* yeast strain RCAM 02150 (Russian National Collection of Industrial Microorganisms – VKPM) was cultivated in a simple discontinuous culture with aeration using

a laboratory fermenter (Biostat A, Sartorius) with a working volume of 2L. The temperature of cultivation was 30 °C. The pH value was 4.4 ± 0.2.

For cultivation, a synthetic sterile medium with the following composition per liter was used: 10 g of glucose, 2 g of citric acid, 600 mg of KCl, 500 mg of $MgSO_4 \cdot 7H_2O$, 100 mg of $CaC1_2 \cdot 6H_2O$, 500 μg of H_3BO_3, 400 μg of $ZnSO_4 \cdot 7H_2O$, 200 μg of $FeCl_3 \cdot 6H_2O$, 200 μg of $Na_2MnO_4 \cdot 2H_2O$, 100 μg of KI, 100 μg of $CuSO_4 \cdot 5H_2O$, 2 mg of inositol, 400 μg of pyridoxine-HCl, 400 μg of thiamine-HCl, and 400 μg of calcium pantothenate [14].

The ammonium sulphate was used as the source of nitrogen, the orthophosphoric acid – as the phosphorus source.

The biomass (dry weight) accumulation was determined by gravimetric way after drying of the washed yeast suspension to constant weight at 105 °C.

The economic coefficient. The economic coefficient or biomass yield was calculated by dividing the gather of cells containing 25% of dry matter by the amount of used substrate (glucose) and it was expressed in %.

Determination of protein content in cells. The total amount of protein substances in the yeast cells was determined using the Lowry method [15, 16].

Determination of the yeast amino acid pool. In this paper, a method of extraction of amino acid pool by boiling the cells in distilled water for 15 minutes was applied. Quantification of the pool was carried out using the Swenson & Befts method [17], who modified the Moore & Stein method [18, 19, 20]. This method has the same sensitivity to all amino acids and gives an idea of the total content of free amino acids in cells.

Determination of reserve carbohydrates. The method of step fractionation of carbohydrates [21, 22, 23, 24] was used, followed by the determination of individual carbohydrates in each fraction [22].

Complete factorial experiment [25]. The main advantage of this approach is the possibility of studying the simultaneous impact of a large number of factors on the process efficiency. In addition, this method makes possible establishing the availability of interfactor interactions in the system along with a quantitative consideration of each individual factor and allows to estimate the effect of those.

For the function y = f (x_1, x_2, x_3…, x_n) the regression equation is used, which is the expansion of this function in a power series (Eq.1):

$$y = b_o + \sum_{i=1}^{n} b_i x_i + \sum_{i,j=1}^{n} b_{ij} x_i x_j + \sum_{i,j=1}^{n} b_{iij} x_i^2 x_j + \cdots \quad (1)$$

wherex_{ij} are variable factors, in coded units; bi, b_j – regression coefficients at the corresponding variables.

The number of variants of the experiments that must be put in the initial series depends on the number of initial factors. In total, on two levels for n factors, the number of variants of the experiments will be: 2^n. In the first part of this paper, the number of factors studied was equal 4, respectively, the number of experiments was 16. In the second part of this paper, there were 3 variability factors and 8 experiments.

Regression coefficients calculation formulas (Eq. 2,3) can be presented in a general form:

$$b_o = \frac{\sum \bar{y}_u}{N} \quad (2)$$

$$b_i = \frac{\sum x_{iu} \bar{y}_u}{N} \quad (3)$$

wherex_{iu} is the value of the variable in the corresponding column of the experiment plan; $\bar{y}_u$ – the result of the u-th experiment, the arithmetic mean value; N is the total number of experiments; u is the number of the variant of the experiment; i is the factor's number.

To confirm the validity of the regression equation obtained, the following actions were carried out for the observed process:

– statistical analysis of the significance of regression coefficients b_j;

– validity check of the regression equation.

Along the statistical analysis of the significance of the regression coefficients, the distribution error b_i was calculated. The regression coefficient was assumed to be different from zero, i.e. significant, if the following inequality was satisfied (Eq.4):

$$|b_i| > t\sqrt{S_{b_i}^2} \quad (4)$$

where $\sqrt{S_{b_i}^2}$ – is the error in b_i determining in the experiment; t – Student's criterion for 5% significance level, which allowed us to predicate the significance of b_i values at the 95% credible level.

In order to convince ourselves that the obtained regression equations sufficiently (adequately) describe the process under investigation, in each case the Fisher's criterion F (Eq.5) was determined:

$$F_{ad} = \frac{S_{ad}^2}{S_{\bar{y}}^2} \quad (5)$$

where S_{ad}^2 – is the adequacy variance (the magnitude of error of the reproducible results); $S_{\bar{y}}^2$ – is the variance of the $\bar{y}$ mean value.

The calculated F_{ad} criterion was compared to the tabulated value of F (for 5% significance level). If $F_{ad} > F$, then there is the equation. If $F_{ad} < F$, then this indicates that the process proximity to the

optimal region, i.e. in this case it is not possible to be limited to the linear approximation, it is necessary to take into account the quadratic terms (Eq.1).

Results and discussion

In this study, the following values were taken as factors influencing the main parameters:

X_1 – yeast pitching value with 25% dry matter content, the% of fermentable carbohydrates;

X_2 – content of nitrogen in the culture medium, g dm^{-3};

X_3 – phosphorus content expressed in terms of $P_2 0_5$ in the culture medium, g dm^{-3};

X_4 – the biotin content in the culture medium, mg dm^{-3}.

The yield was calculated by the ratio of biomass increment with a content of 25% solids to the amount of glucose to be disposed and it was expressed in %.

Table 1.

The natural values of factors and the results of experiments at the experimental researches according to the plan of the full factorial experiment

№	X_1	X_2	X_3	X_4	Yield ($\overline{y}$),% of fermentable sugars
1	1,0	0,25	0,1	2,7	44,6
2	12,0	0,25	0,1	10,0	56,7
3	1,0	0,65	0,1	10,0	64,1
4	12,0	0,65	0,1	2,7	52,9
5	1,0	0,25	0,4	10,0	48,2
6	12,0	0,25	0,4	2,7	58,1
7	1,0	0,65	0,4	2,7	49,5
8	12,0	0,65	0,4	10,0	62,6
9	1,0	0,25	0,1	10,0	46,9
10	12,0	0,25	0,1	2,7	49,5
11	1,0	0,65	0,1	2,7	45,3
12	12,0	0,65	0,1	10,0	63,6
13	1,0	0,25	0,4	2,7	43,4
14	12,0	0,25	0,4	10,0	50,7
15	1,0	0,65	0,4	10,0	67,6
16	12,0	0,65	0,4	2,7	62,9

As a result of calculations, the following equation is obtained:

$$y = 54.16 + 5.93X_1 + 4.4X_2 + 1.21X_3 + \\ +3.39X_4 - 2.11X_1X_4 + 2.52X_2X_4 \quad (6)$$

where y – economic coefficient (biomass yield).

During calculation of influence of factors on the biomass yield, it is found that the lower level of factor score X_3 (phosphorus content) is in the optimal region and does not limit the reproduction of yeast.

The relationship between the inoculum value and the economic coefficient at different concentrations of nitrogen and biotin in the medium was found. In that case when both these components, as well as the inoculum amount value, are at the lower level (No. 1, 5, 7, 11, 13 of Table 1), the yield is 44… 48%; when these factors move to the upper level (No. 3 and 15 of Table 1), the yield is increased up to 64… 69%, i.e. up to the value that is achieved in experiments with a large seed material flow (No. 8, 12, 16 in Table 1).

In view of the great importance of the X_1, X_2 и X_4 factors, the effect of seeding, nitrogen and biotin content in the environment on the biochemical composition of cells was studied, upon that the variation interval of nitrogen concentration in the medium made 0.45–0.75 g dm^{-3}. The phosphorus concentration, expressed in terms of $P_2 0_5$made 0.4 g dm^{-3}. The experiments were also carried out according to the full factorial experiment plan.

Table 2.

Influence of cultivation conditions on the economic coefficient and biochemical biomass composition

№	X_1	X_2	X_4	Yield (y),% of fermentable sugars	Proteine (y_p),%of ADB	AA pool (y_{aa}),% of ADB	Trehalose (y_{carb}), % of ADB
1	1,0	0,45	2,7	49,9	29,8	14,4	35,1
2	12,0	0,45	2,7	73,2	27,7	8,3	40,0
3	1,0	0,75	2,7	51,1	33,5	21,8	30,3
4	12,0	0,75	2,7	72,0	34,6	14,3	32,5
5	1,0	0,45	10,0	73,2	25,1	7,8	40,5
6	12,0	0,45	10,0	73,1	26,7	6,9	40,8
7	1,0	0,75	10,0	79,4	34,6	11,5	27,4
8	12,0	0,75	10,0	81,2	34,4	8,5	30,4

After data processing, multiple regression equations were obtained in which the role of each of the factors was demonstrated and, what is especially important for biological objects, the interaction of these factors in the synthesis of important cellular components was demonstrated too. The equations take into account only those factors and their interaction, whose influence on the process exceeds 10%.

$$y = 34.56 + 5.73X_1 + 1.78X_2 + 7.66X_4 - \\ -5.30X_1X_3 + 1.78X_2X_4 \quad (7)$$

$$y_p = 30.8 + 3.48X_2 - 4.01X_1X_2 \quad (8)$$

$$y_{aa} = 11.65 - 2.19X_1 + 2.34X_2 - 2.72X_4 + \\ +1.21X_1X_4 - 1.01X_2X_4 \quad (9)$$

$$y_{carb} = 34.63 + 1.31X_1 - 1.40X_2X_4 \quad (10)$$

The economic coefficient reflects the biosynthetic activity of yeast and in the studied area of factor change the following is established:

– the biomass synthesis is influenced most of all by the biotin content in the medium. Reduction of the dose of inoculum increases the cells biotin requirement;

– the protein synthesis in cells is greatly influenced by the nitrogen concentration in the culture medium;

– the reserve carbohydrates content depends little on the amount of seed material;

– the free amino acids content increases at decreasing in seed material flowrate, at increasing in the concentration of nitrogen content and at decreasing in the biotin level;

– in the biotin-deficient medium, as well as in the medium rich with this growth factor, the value of the free amino acids pool is inversely related to the consumption of the seed material: with an increase in the initial population intensity, the amount of amino acids in the cells decreases.

The increase in the pool value with the increase in the nitrogen level is explained quite simply, because in this case, the amount of exogenous nitrogen significantly exceeds the need for yeast, so the resulting amino acids do not have time to be disposed of in biosynthetic processes and accumulate in cells (Table 2).

In the case when the biotin content is insufficient in the medium, the average content of amino acids in yeast is 14.7%. Increase in the amount of free amino acids with a low seeding value (even in the absence of a limit on nitrogen nutrition) indicates a low activity of biosynthetic processes, as a result of which there is a low yield of biomass (No. 1 and 3 in Table 2). In the biotin-rich medium, the average amino acid content in cells is 8.7%, and the maximum amount of amino acids is contained in yeast obtained in a medium with a high nitrogen content and a low seed material flowrate. In this case, one cannot speak of low yeast activity, because the yields in No. 6 and 7 (Table 2) are almost the same; on the contrary, it follows from these data that when the inoculum content is low in a medium that does not limit cell growth, the yeast reproductive activity will be higher.

Conclusions

The composition of the nutrient medium should be adjusted in accordance with the dose of the seed material. In case of the decrease in the amount of inoculum, the cells biotin and sources of nitrogen nutrition needs are increased. The content of reserve carbohydrates in yeast at the end of the cultivation process doesn't practically depend on the pitching value. The increase in pitching decreases the protein content in cells and reduces the free amino acids pool in them.

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