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GRADIENT-CONTINUOUS YEAST CULTIVATION FOR THE ALCOHOL PRODUCTION FROM MOLASSES

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This work's objective is to find the technological conditions for the intensification of yeast growth in the gradient-continuous yeast cultivation process. Experiments on four sequential yeast chemostats (connected into a battery) demonstrated broad possibilities to influence the metabolic activity of yeast and the alcohol production depending on the content of molasses introduced into the second, third and fourth yeast generators.

Adding the molasses according to the 3:2:1 scheme in quantities, which are sufficient to achieve the initial concentration of solids of 26.5 g/100 cm^3 to the end of the process resulted in high accumulation of yeast in the medium (up to 99 g/dm^3).

It is demonstrated that the highest ratio of economic effect of biomass synthesis from molasses sugars (88 g/100 g) is achieved when molasses is added according to the 1:2:2.5 scheme and the initial solids concentration in the medium is near 12 g/100 cm^3.

Key words: yeast, ethanol, molasses.

The biotechnology of the joint production of alcohol and baker's yeast from molasses develops towards the intensification of the synthesis of yeast biomass. At the end of the 20th century, a new approach to the cultivation of yeast for the anaerobic fermentation of molasses, called gradient-continuous method, was launched.

An effective way of processing molasses in the alcohol production in Ukraine and other countries is simultaneous production of two target products, rectified alcohol and pressed baker's yeast, from this raw material [1]. The practice of industrial production has proven that such a technological solution has a number of economic advantages compared to separate productions of alcohol and specialized (strictly baker's) yeast [2]. At the same time, the entire technological process of producing two target products consists of two stages: the cultivation of the alcohol (yeast) producer fermenting carbohydrates into the alcohol is followed by the production of yeast biomass and rectified alcohol as finished commodity products.

Unlike specialized yeast production, the main feature of biomass cultivation in joint production of alcohol and baker's yeast is combining two paths of carbohydrate catabolism: glycolysis (resulting in alcohol) and citric acid cycle (with the formation of elements for the yeast cells). Despite the fact that the main objective of the yeast production stage in this technology is the accumulation of yeast biomass, the producer's metabolism should not be completely directed into aerobic oxidation of carbohydrates. The reason for this, according to [3–5], is that the functional capacity of aerobically grown biomass does not fully meet the requirements of effective anaerobic fermentation of the substrate into alcohol with the maximum yield of the target product. Thus, biotechnology for the molasses-based alcohol production has proved that the yeasts for the alcohol fermentation of sugars should be cultured in partially aerobic conditions in order to accumulate sufficient producer biomass, and to ensure its high alcohol-forming ability to ferment carbohydrates [1].

In the biotechnology of microbial synthesis, the principle of sequential introduction of the substrate is known to reduce the inhibitory

effect of the increased concentration of substrate at the beginning of the process [5, 6–12].

A progressive trend in the implementation of yeast generation biotechnology for the alcohol production from sugar-based raw materials is the use of battery principle (sequential connection) of the yeast generator scheme.

Ukrainian scientists conducted a complex of studies optimizing the process of yeast cultivation in the technology of co-production of alcohol and baker's yeast [7, 8], finding the optimal parameters of periodic cultivation of the producer.

At the same time, the capacity reserves of the yeast production process to increase the yeast accumulation are far from exhausted, according to the analysis of existing achievements in this field. The most progressive technologies of co-production of alcohol and baker's yeast are characterized by the accumulation of biomass in quantities not exceeding 40 g/dm^3 [1, 6, 7].

The purpose of our research was to achieve high biomass accumulation by regulating the stepwise introduction of the substrate in the process of continuous yeast production alcohol and baker's yeast from molasses.

Materials and Methods

Sugar-molasses containing 78.5% dry matter (DM) and 50.3% digestible carbohydrates were used as the main component of the nutrient medium. The wort was enriched with nitrogen (carbamide) and phosphoric (H_3PO_4) nutrition according to [1], and then diluted with water to the required DM concentration (about 4%). Active acidity of the medium was pH 5.2 by acidification with H_2SO_4.

As a producer of alcohol and yeast biomass, *Sacharomyces cerevisiae* strain U-563 [1] was used.

The process of growing yeast was carried out in a serial chemostat unit (Fig.). The installation is assembled from 4 glass cylindrical series of connected vessels 1 with flat lids and bottoms, connected at the top. Each chemostat (working volume 2.5 dm^3) is equipped with an air-distributing device 2, a heat exchange surface 3, and a thermometer 4. The molasses wort with a DM concentration of 4% was continuously fed by peristaltic pump 5 to the first chemostat, and the undiluted molasses were pumped into the 2nd, 3rd and 4th chemostats. Thus, a continuous medium flow (from the first to the last chemostats over the tube tubes), continuous cultivation of yeast and the removal of the culture liquid (mashing) from the installation were provided.

The wort was fed with peristaltic pumps out of the mineral cylinder 6 into the first chemostat of the battery at 0.4 dm^3/hr, the medium thus staying in a chemostat for approximately 5–6 hr. The medium in all chemostats was aired with 0.1–0.2 dm^3 air/dm^3 per minute. The medium temperature was maintained at 29–30 °C by introducing cooling water from the ultra-thermostat into the heat exchange device (coil).

The molasses was dosed in the 2nd–4th units with the measuring cylinder 8. According to the substrate quantities, several options were examined (Table 1), differing in distribution principle between the chemostats.

The actual concentration of DM in the media was determined by the isometric method after alcohol distillation; pH of the medium was measured potentiometrically; yeast biomass was weighted at 75% humidity. Alcohol concentration was measured arometrically. Unfermented carbohydrates were measured by the colorimetric method with resorcinol reagent [1].

In the existing technologies, the DM concentration in molasses solutions (or molasses wort) is determined directly in the wells by sugar-measuring areometers, which are used traditionally in the biotechnology of alcohol production [13]. However, if the substrate, containing sugar is introduced not in the beginning, but during the process (when a continuous decrease in the DM quantities due to sugar metabolism occurs), this method is not exact. Therefore, an "initial concentration of dry matter" index, *Ci*, was developed and introduced for controlling the technological process of fermentation [13]. It is determined in the fermented environments as follows. Two indicators are measured in a collected sample: alcohol content (a) and the actual concentration of dry substances ($A_{d.m.}$) according to the above methods. Ci is calculated according to the formula:

$$Ci = A_{d.m.} \cdot g + 1.541 \cdot a,$$

where g is solution density at a given DM concentration (g/cm^3), $g = 200/(200 - n)$, and n is the value of sugar-measuring aerometer at a given $A_{d.m.}$ in percents.

The yeast yield in dependence of the content of alcohol, the productivity of yeast biomass and the coefficient of yeast production on molasses sugar were calculated by [4, 13].

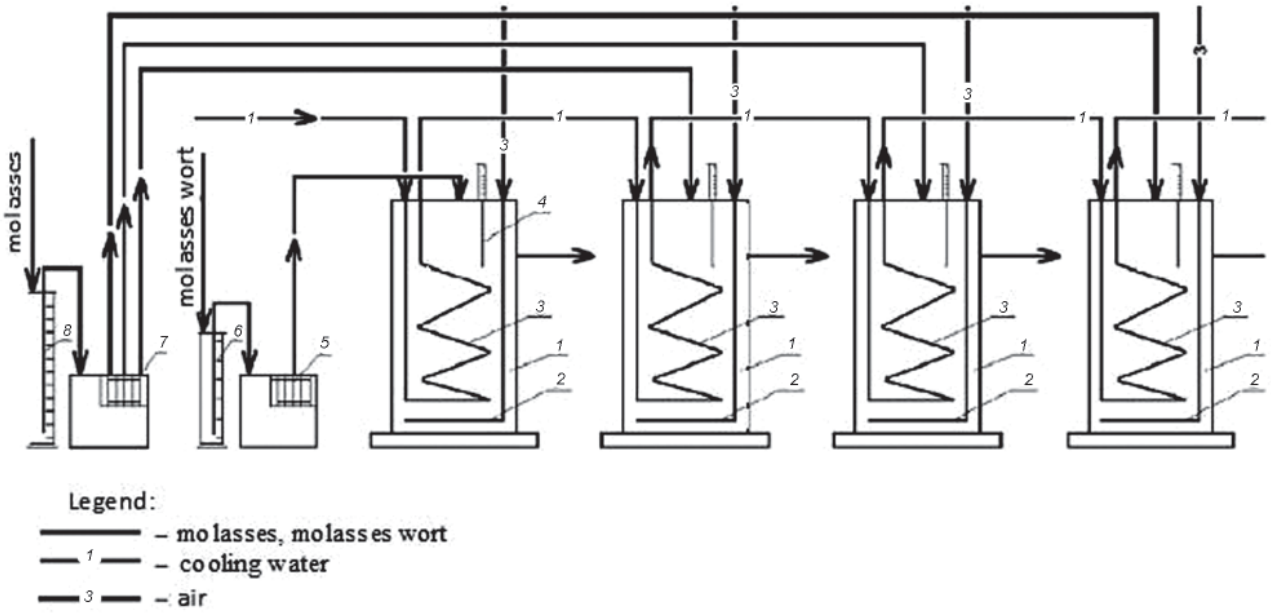

Fig. **Scheme of the chemostat battery for gradient-continuous yeast cultivation:**
1 — glass yeast generator; *2* — air-distributing device; *3* — heat exchange surface; *4* — thermometer; *5* and *7* — peristaltic pump dispensing molasses and wort; *6* — wort measurer; *8* — molasses measurer

Results and Discussion

Five experimental designs of gradient-continuous yeast cultivation in a battery of four chemostats were carried out (Table 1). The designs differed in the introduced amount of molasses, which was characterized by the Ci index in the medium at the output of the last (fourth) unit. This value was 11.1 g/100 cm^3 in the first design and 13.1, 16.2, 19.1, and 26.5 g/100 cm^3 in the second, third, fourth and fifth versions, respectively.

We analyzed the results based on a number of indicators, determininig the overall effectiveness of simultaneous production of alcohol and yeast from molasses, namely:

- accumulation of yeast biomass and alcohol;
- ways of introduction of molasses in the battery of chemostats;
- degree of alcohol removal from the medium during the aeration;
- degree of fermentation of substrate sugars;
- productivity of the yeast biomass generation;
- the ratio between the amounts of yeasts and of produced alcohol;
- economic factor for the yeast production on molasses sugars.

Data in Table 1 indicate that an increase in the quantity of molasses introduced in all designs is accompanied by a gradual increase in the alcohol concentration as well as by a significant increase in yeast biomass at the end of the process. Note, that the biomass concentration in the fourth and fifth designs is 87.8 and 99.0 g/dm^3 respectively. These values are the highest, compared to the known values from the literature on simultaneous production of alcohol and baker's yeast on molasses. Based on the obtained results, these two designs were chosen for further research.

The dynamics of the main parameters of the process in the fourth and fifth research designs are presented in Tables 2 and 3.

The experiments showed the possibility of significant yeast accumulation in the medium, up to 87.8 g/dm^3 (design 4, Table 2) and 99.0 g/dm^3 (design 5, Table 3) due to airing and stepwise increased pH of the medium from 4% (in the initial wort) to 19.1 g/100 cm^3 (in design 4) and 26.5 g/100 cm^3 (in design 5) due to the smooth introduction of undiluted molasses into the second, third and fourth units. The increased yeast concentration provides deep fermentation of wort with a high DM concentration in design 5: only 0.32 g/100 cm^3 unfermented sugars remained. In design 5, molasses was

Table 1. **Dependence of main indexes of gradient continuous yeast cultivation on the quantity of molasses introduced during cultivation**

Design	DM of wort, %	Ci in medium at the end of cultivation, g/100 cm^3	Actual concentration of DM, %	Yeast biomass, g/dm^3	pH of medium	Alcohol, v.%		Yeast output, g/100 cm^3 of produced alcohol	Specific productivity of the chemostat battery by yeast biomass, g/dm^3×hour
						Actual in medium at the end of cultivation	In control anaerobic fermentation of wort with same *Ci* value		
1	4	11.1	4.8	46.9±2.2	4.9	1.8	4.0	117	1.9
2	4	13.1	5.7	59.8±3.0	4.9	2.9	4.7	127	2.5
3	4	16.2	7.0	75.1±3.6	5.0	3.3	5.8	130	3.1
4	4	19.1	8.0	87.8±4.1	5.0	3.7	7.0	129	4.0
5	4	26.5	11.0	99.0±4.9	5.0	6.6	9.7	104	4.75

Table 2. **The dynamics of indexes of gradient-continuous yeast cultivation at chemostat battery under dosed introduction of molasses (design 4)**

Indexes	The number of the chemostat in the battery			
	1	2	3	4
Actual DM concentration,%	2.1	3.2	5.2	8.0
Yeast biomass, g/dm^3	17.1±0.9	36.5±1.9	77.6±3.8	87.8±4.4
Unfermented carbohydrates, g/100 cm^3	0.10	0.12	0.20	0.29
Actual alcohol in medium, v.% All alcohol, including the aired out, v.%	1.1 1.5	2.0 2.5	2.7 4.5	3.7 7.0
Ci, g/100 cm^3, determined: – by actual alcohol in medium – by total produced alcohol	 3.8 4.3	 6.2 6.9	 9.1 12.0	 13.5 19.1
Doses of molasses per chemostat per hour: – g/hour – % of all introduced quantity	Not added	13.6 17	27.2 34	39.7 49
Chemostat battery productivity by yeast biomass, g/ hour	–			40.2
Specific productivity by yeast biomass in four-unit system, g/dm^3 ×hour:	–	–	–	4.0
Total duration of cultivation, hour	–			21.8
Coefficient of yeast production on molasses sugars, g/100 g	56	68	88	60

Note. «–» — not determined.

Table 3. **The dynamics of indexes of gradient-continuous yeast cultivation at chemostat battery under dosed introduction of molasses (design 5)**

Indexes	The number of the chemostat in the battery			
	1	2	3	4
Actual DM concentration,%	2.4	7.3	9.9	11.0
Yeast biomass, g/dm^3	20.2±1.1	51.9±2.6	85.5±4.4	99.0±5.0
Unfermented carbohydrates, g/100 cm^3	0.42	0.30	0.29	0.32
Actual alcohol in medium, v.% All alcohol, including the aired out, v.%	1.2 1.5	4.2 6.7	5.7 9.0	6.6 9.7
Ci, g/ 100 cm^3, determined: – by actual alcohol in medium – by total produced alcohol	4.2 4.6	13.4 17.2	18.6 23.5	21.1 26.5
Doses of molasses per chemostat per hour: – g/hour – % of all introduced quantity	Not added	64.8 58	32.0 28	15.6 14
Chemostat battery productivity by yeast biomass, g/hour	–			47.5
Specific productivity by yeast biomass in four-unit system, g/dm^3 × hour:	–	–	–	4.75
Total duration of cultivation, hour				20.8
Coefficient of yeast production on molasses sugars, g/100 g	60	44	48	52

distributed in units as follows: 58% in the 2nd, 28% in the third and 14% in the fourth unit, according to 3:2:1 scheme. In design 4 the distribution of molasses was, respectively, 17%, 34% and 49% (1:2:2.5 scheme).

Lesser total amount of molasses introduced in doses during the process in design 4 somewhat reduces the yeast accumulation. However it also contributes to the significant increase in the cost-effectiveness of biomass production from carbohydrates of the substrate. In this design, the yeast yield factor on molasses sugars calculated in units 2 respectively 3 was 68 respectively 88 g/100g. In design 5 these values are 44 respectively 48 g/100 g.

This demostrates the advantage of the cultivating with the stepwise introduction of molasses and Ci of 11–12 g/100 cm^3. In the third unit of the installation, at Ci 12.0 g/ 100 cm^3 (design 4) the highest yeast yield rate is 88 g per 100 g of sugar. If Ci is increased to 17.2–26.5 g/100 cm^3 (design 5), the indicated coefficient does not exceed 52 g per 100 g of sugar. This can be explained by the phenomenon that is called "the Crabtree effect" in biotechnology. Its essence is the activation of the glycolytic pathway of sugar metabolism producing ethanol after introducing a substrate containing carbohydrate to the medium (fermented in anaerobic conditions). That is, the biomass generation is inhibited, and the added sugar is mostly converted into alcohol. This is confirmed by the yeast output index: in the first unit it is approximately the same in both designs (56 and 60 g/100 g), while in the second and third units the designs vary significantly by this index. The yeast yield on molasses sugars in units 2 and 3 in the fourth design (Table 2) was 68 and 88 g/100 g, respectively (molasses were introduced gradually in units 2, 3 and 4, with a slow increase according to the 1:2:2.5 scheme). In the fifth design (the 3:2:1 scheme of introduction), molasses was mostly added to the second and third units. Thus the alcohol formation increased (the Crabtree effect) in contrast to yield growth: in the design 5 their production in units 2 and 3 was 44 and 48 g/100 g respectively (Table 3), which is less than in the design 4.

Notably, the amount of alcohol, volatilizing from the medium, is significant.

Moreover, it grows with the increasing levels of its biosynthesis. The corresponding values in Tables 2 and 3 exceed those established by the current regulations of the simultaneous production of alcohol and baker's yeast from molasses [1]. However, the mentioned technologies provide the accumulation of yeast biomass in a mature mash less than 40 g/dm^3. Our results demonstrate significantly bigger value. Therefore, for the process to be implemented with high accumulation of yeast biomass, appropriate technical measures for catching the alcohol must be taken to prevent excessive losses. Modern technical devices for this are not complicated, easily available [2, 3] and are able to prevent excessive losses of ethanol.

Therefore the results of research on the technology of simultaneous production of alcohol and baker's yeast from molasses on a battery-type chemostat device show the possibility of widely influencing the metabolic activity of the producer of alcohol and yeast. High accumulation of yeast in the simultaneous production (up to 99 g/dm^3 of medium) is achieved by the stepwise introduction of molasses during the yeast production process. This value is significantly bigger, than the values, known in the literature. It is established that for the intensification of the synthesis of yeast biomass in the battery of chemostat units it is necessary during the process to introduce the substrate (containing carbohydrates) slowly, with the gradual increase. The highest coefficient of synthesis of biomass from molasses sugars is achieved at an initial concentration of 12 g/100 cm^3 of dry matter in the medium.

Implementing the proposed approach in order to optimize the cost-effectiveness of simultaneous production requires appropriate measures to catch alcohol to be taken.

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ГРАДІЄНТНО-НЕПЕРЕРВНЕ КУЛЬТИВУВАННЯ ДРІЖДЖІВ ДЛЯ ВИРОБНИЦТВА СПИРТУ ІЗ МЕЛЯСИ

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Метою роботи було створення технологічних умов для інтенсифікації синтезу дріжджів у процесі градієнтно-неперервного культивування.

Експериментами на стендовій установці батарейного типу з чотирьох апаратів показано можливість регулювання метаболічної активності продуцента спирту та дріжджів у широких межах залежно від співвідношення кількості меляси, що вводять у другий, третій і четвертий апарати. Досягнуто високе накопичення дріжджів у середовищі (до 99 г/дм^3) шляхом введення меляси за схемою 3:2:1 у кількості, що забезпечує досягнення початкової концентрації сухих речовин наприкінці процесу 26,5 г/100 см^3.

Доведено, що найбільший економічний коефіцієнт синтезу біомаси з цукрів меляси — 88 г/100 г – спостерігається у разі внесення меляси за схемою 1:2:2,5 за початкової концентрації сухих речовин середовища 12 г/100 см^3.

Ключові слова: дріжджі, спирт, меляса

ГРАДИЕНТНО-НЕПРЕРЫВНОЕ КУЛЬТИВИРОВАНИЕ ДРОЖЖЕЙ ДЛЯ ПРОИЗВОДСТВА СПИРТА ИЗ МЕЛАССЫ

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Целью работы было создание технологических условий для интенсификации синтеза дрожжей в процессе их градиентно-непрерывного культивирования. Экспериментами на стендовой установке батарейного типа из четырех аппаратов показана возможность регулирования метаболической активности продуцента спирта и дрожжей в широких пределах в зависимости от соотношения количества мелассы, которая вводится во второй, третий и четвертый аппараты. Достигнуто высокое накопление дрожжей в среде (до 99 г/дм^3) путем введения мелассы по схеме 3:2:1 в количестве, которое обеспечивает достижение начальной концентрации сухих веществ в конце процесса 26,5 г/100 см^3.

Доказано, что наибольший экономический коэффициент синтеза биомассы из сахаров мелассы — 88 г/100г — наблюдается при внесении мелассы по схеме 1:2:2,5 при начальной концентрации сухих веществ среды 12 г/100 см^3.

Ключевые слова: дрожжи, спирт, меласса