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RESEARCH ON THE ULTRATHIN STRUCTURE OF CELLS OF DIFFERENT DISTILLERS' YEAST RACES AND ITS DEPENDENCE ON THE CONCENTRATION OF DRY MATTER IN WORT

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Abstract. There are a number of directions of introducing the resource-saving and energy-efficient technology of alcohol washes into alcohol production. One of them is the use of highly concentrated wort from grain raw materials. Application of highly productive strains of distillers' yeast is the basis of resource-saving and energy-efficient technologies, a way to reduce the cost of ethanol and increase the profitability of its production. To develop the technology of highly concentrated wash from grain raw materials, it is necessary to select the appropriate yeast races and study their morphological and physiological properties. Diagnostics of the physiological state of microorganisms has been performed. It has been studied how the concentration of dry matter in the wort effects on the specific morphological and cytological features of the structure of yeast cells (distillers' yeast *S. cerevisiae*, races DO-16, DO-11, K-81, XII) when they are cultured on media from starch-containing raw materials. The concentration of dry matter in the wort was 20 and 28%. It has been found that the *S. cerevisiae* race DO-16 bred by selection synthesises the largest number of yeast cells when the dry matter concentration is 28%. The osmophilic *S. cerevisiae* races DO-16 and DO-11 had smaller sizes and areas of their cells in comparison with the thermotolerant and mesophilic races of *S. cerevisiae* K-81 and XII at the 28% concentration of DM in the wort. During fermentation, these parameters characterise the increase in the working surface of the yeast in the medium fermented. This allows accelerating the fermentation process and ensuring microbiological purity of the medium, which is especially important for highly concentrated wort. The morphological and cytological studies of the *S. cerevisiae* race DO-16 have proved its advantages over the races DO-11, K-81, XII in fermenting highly concentrated wort. The studies of the intracellular structure of the yeast *S. cerevisiae* DO-16, DO-11, K-81, XII have allowed establishing the relationship between the formation of glycogen in yeast cells and the DM concentration of the wort. When culturing industrial yeast at the DM concentration 28%, the glycogen content in the cells of *S. cerevisiae* DO-16 was significantly higher compared with the races under study. This indicates that these conditions of the culture medium are favourable for this race.

Keywords: distillers' yeasts, ultrathin structure, highly concentrated wort, dry matter, fermentation, culturing.

Introduction. Formulation of the problem

New directions in the alcohol technology development require the following: increasing the concentrations of dry matter in wort; fermentation at higher temperatures and with higher concentrations of alcohol in the wash; making alcohol more economical to produce by saving raw materials and energy resources. To meet these conditions, one needs highly productive yeast races with increased osmophilicity, thermotolerance, and fermentation activity [1]. So, it is a topical issue for the alcohol industry to conduct research aimed at finding new ethanol-producing strains and to develop a technology of highly concentrated wash from grain raw materials [2-4].

Selective breeding of new ethanol-producing races with high osmophilicity, alcohol-forming ability, and alcohol resistance leads to intensifying the biochemical processes of sugar transformation during alcohol production [5-6]. Scientists of the National University

of Food Technologies are constantly working in this direction. In particular, the thermotolerant and osmophilic races DO-16, DO-11, K-81 of the distillers' yeast *S. cerevisiae* were selected at the Department of Biotechnology of Fermentation and Winemaking Products, which are now successfully used in distilleries [7-9].

Analysis of recent research and publications

The technology of ethanol production is based on microbiological processes of alcohol fermentation. Its effectiveness depends on the yeast, the properties of which significantly affect the entire production cycle. Therefore, selection of alcohol races is very important.

Using highly productive distillers' yeast strains is the basis of resource-saving and energy-efficient technologies, a way to reduce the prime cost of ethanol and increase the profitability of its production. In present-day science, there are the following directions of search for possible ways to increase the efficiency of fermentation:

– improving the technological modes;

– selecting more productive strains of yeast and bacteria.

For distilleries that process starch-containing raw materials, yeast must have the following characteristics:

– to withstand high dry matter and alcohol concentrations;

– to ferment completely the carbohydrates of the wort;

– to accumulate the maximum amount of alcohol and the minimum biomass;

– to resist foreign microflora and increased acidity [10].

Yeast is a non-motile unicellular eukaryotic microorganism that belongs to the type *Ascomycota*, class *Hemiascomycetes*. The size and shape of its cells depend on many factors, in particular, on the growth phase, methods and conditions of culturing, and on the race. Analysing the morphological changes of cells, scientists believe that an increase in the cell volume leads to a deterioration in exchange of materials with the environment, an increased concentration of metabolic products in the cytoplasm, which results in the death of microorganisms [11].

A lot of metabolic processes take place in a yeast cell. All biochemical reactions in a living cell are strictly localised. The cytomorphological characteristics of a culture clearly reflect its physiological state. Ultrastructural changes in the cell nucleus make it possible, to some extent, to characterise the biosynthetic, genetic, and metabolic processes that occur in it [12-14].

Many authors have established that for microorganisms capable of fermentation, most changes in their cellular structures are of the same type. So, conclusions have been drawn about the fermentation rearrangement of cells. This rearrangement was related to both the cell size and the structural organisation.

Thus, studying ultrathin structures is important when researching the physiological state of anaerobic yeast grown at high dry matter concentrations in the medium.

Significant changes in the structural organisation of a cell occur with changes in the growth conditions: the transition from anaerobic to aerobic culturing, changes in the composition of the medium and in the concentration of limiting factors, as well as after physiological stress [14].

The morphological structure of yeast correlates with the functional characteristics of its cells. The processes of fermentation and respiration are closely related to the state of cellular structures. The main structural elements of the cytoplasm of microorganisms are mitochondria, ribosomes, nucleus. Important enzymatic processes take place in the cytoplasm with its organelles (chondriosomes, microsomes, vacuoles), microscopic and submicroscopic inclusions. Mitochondria contain a number of enzymes, some of them specific, so they are viewed as a "power station" [13-15].

The novelty of our work lies in studying the ultrafine structure of distillers' yeast, in establishing a relationship between the DM concentration of wort and the formation of glycogen in yeast cells, in selecting alcohol races for the fermentation of high-concentration wort.

The morphology and ultrastructure of the distillers' yeast *S. cerevisiae* cultured on a starch-containing medium have been studied but insufficiently. Based on the literature data and taking into account the technical possibilities for diagnosing the physiological state of microorganisms, it is necessary to study how the dry matter concentration of wort effects on the specific morphological and cytological features of the yeast cell structure.

The purpose of the research: investigation of the ultrathin structure of distillers' yeast cells and selection of highly productive yeast races for fermentation of highly concentrated wort.

The research objectives:

1. To study how the DM concentration in wort effects on cultivation of industrial yeast.
2. To study the specific morphological and cytological features of distillers' yeast cells.
3. On the basis of theoretical and experimental research, to select distillers' yeast races for fermentation of highly concentrated wort.

Research materials and methods

Milled maize grain, with a dispersion of 100% of milled material passed through a sieve with the mesh diameter 1mm, and enzyme preparations by Danisco were used for the research. Amylex 4T and glucoamylases Diazyme TGA were used as α-amylase. The enzyme preparations were added by enzyme activity units. The starch content of the maize grain used for the research was 69.0%. The thermoenzymatic

treatment of the starch-containing raw materials was performed at 90–92°C for 3 h, and saccharification of the liquefied mixture was carried out at 50–55°C for 30min. The concentration of thermostable α-amylase was 0.4; 0.60 units of α-amylase ability/g of starch. That of glucoamylase was 5.0 units of glucoamylase ability/g of starch.

The yeast was cultured at 30°C, with the concentrations of wort dry matter 20.0 and 28.0%, using the alcohol races DO-16, DO-11, K-81, XII of the yeast *S. cerevisiae*. The yeast inoculum was added in the proportion 20mln/cm^3 of the wort. The starch content in the initial grain was estimated by Evers's method [16], the grain humidity by drying to constant weight [16]. The granulometric composition of the milled grain was determined by sizing on metal and nylon 6 sieves [16]. The dry matter concentration was determined with a saccharimeter and a refractometer RPL-3 [16]. The total number of yeast cells in 1 cm³ was determined by direct count in a Goryaev chamber.

The average cell volume and area were determined by the formulae 1-3:

$$V = \frac{1}{6}\pi d^2 l \; ; \qquad (1)$$

$$S = \frac{1}{2}\pi \cdot d \cdot l\sqrt{1 - E^2} + \frac{arc \cdot \sin E}{E} \; ; \qquad (2)$$

where V is the average cell volume, μm³;
S is the average area of cells, μm³;
l is the average cell length, μm;
d is the average cell diameter, μm;

$$E = \frac{l^2 - d^2}{l^2} \qquad (3)$$

Studies related to the fixation of cells of microorganisms, their dehydration, replacement, were performed according to the method [17].

Cytological studies were performed in the research laboratories of the Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, which is confirmed by the relevant cooperation agreement.

Ultramicrotomy: 50–80nm thick sections were obtained using an ultramicrotome LKB 8800 in automatic mode. Glass knives had been prepared in advance using a device for making glass knives KnifeMaker LKB 7800 according to the procedure described in [18]. The ultrathin sections obtained were transferred to copper grids, which had no coating. Then, according to the method described in [19], the sections were subjected to contrast-enhancing with 1% aqueous alcoholic solution of uranyl acetate for 7 min, and after thorough washing in distilled water, they were painted in lead citrate solution for 2 min and washed again in distilled water. The sections obtained were dried at room temperature, and analysed by transmission electron microscopy using a microscope JEM-1400 (Jeol, Japan) at the voltage 80kV.

Results of the research and their discussion

The effect of wort concentration on the specific morpho-physiological features of yeast cells have been studied. For the study, the races DO-16, DO-11, K-81, XII of the yeast *S. cerevisiae* were used. The results are presented in Table 1 respectively. The yeast was cultured on wort from starch-containing raw materials (maize grain), with the wort concentrations 20.0 and 28.0% of dry matter. It has been found that after 12 h of cultivation at the concentration 20.0% DM, in the yeast of the race

S. cerevisiae DO-16, there were by 16.5% more yeast cells synthesised than in the yeast of the race DO-11. However, when the DM in the wort reached 28.0%, the concentration of yeast cells of the studied races *S. cerevisiae* K-81 and XII decreased, and their cell sizes remained almost unchanged. This means that their cells were not adapted to the high osmotic pressure created by the substrate.

In the osmophilic *S. cerevisiae* races DO-16 and DO-11, with an increase in the concentration of wort DM, a tendency of the cell size to decrease was observed. However, in the races K-81 and XII, the concentration of yeast cells decreased by 31% and 46% respectively (Table 1). The studies have shown that the yeast race *S. cerevisiae* DO-16 can synthesise more yeast cells than the races *S. cerevisiae* DO-11, K-81, and XII can.

Table 1 – Specific morphological features of yeast cells cultured at different concentrations of wort DM

No.	Yeast race	Concentration of wort DM, %	Number of yeast cells, million/cm³	Cell length, l, μm	Cell diameter, d, μm	Ratio of cell length to diameter (l/d)	Cell volume, V, μm³	Cell area, S, μm²	S/V, μm²/μm³
1	*S.cerevisiae* DO-16	20.0±0.2	285±29	7.2±0.5	6.9±0.4	1.04	179.39	79.38	0.442
		28.0±0.2	340±34	3.8±0.5	3.6±0.4	1.05	27.77	27.33	0.984
2	*S.cerevisiae* DO-11	20.0±0.2	238±24	7.7±0.5	6.8±0.5	1.13	186.33	56.34	0.302
		28.0±0.2	292±29	4.5±0.5	4.4±0.3	0.97	46.62	32.87	0.705
3	*S.cerevisiae* K-81	20.0±0.2	183±18	6.7±0.5	6.1±0.4	1.09	130.47	65.27	0.500
		28.0±0.2	118±12	5.9±0.4	4.1±0.5	1.44	58.90	57.52	0.976
4	*S.cerevisiae* XII	20.0±0.2	155±15	8.1±0.5	5.7±0.4	1.42	137.72	81.31	0.590
		28.0±0.2	95±10	6.2±0.4	4.1±0.5	1.51	54.54	60.52	1.109

It has been found that an increase in the concentration of wort DM causes morpho-cytological changes in cells. In the races DO-16 and DO-11, with an increase in the concentration of wort DM, the length and diameter of cells decreased (Table 1). It is characteristic of the race *S. cerevisiae* DO-16 that the l/d ratio did not practically change with a higher wort concentration, and the cell volume and area decreased. A similar tendency is characteristic of the cells of the race *S. cerevisiae* DO-11 (Table 1). The races *S. cerevisiae* K-81 and XII are characterised by an increase in the l/d ratio, which indicates a significant elongation of the cells. This means that the physiological condition of the cells was unsatisfactory. The cell volume in all races decreased with a higher concentration of wort DM. The area of cells of the race *S. cerevisiae* DO-16 decreased by 2.8 times, that of the race *S. cerevisiae* DO-11 by 2.3 times, and by 1.4 times in *S. cerevisiae* K-81, when the concentrations of wort DM increased from 20, 0 to 28.0%. In the race *S. cerevisiae* XII, the cell area remained almost at the same level, but the cell concentration decreased significantly.

From the results of the studies, it can be concluded that the osmophilic races of the yeast *S. cerevisiae* DO-16 and DO-11 had smaller cell sizes and areas of their cells in comparison with the thermotolerant and mesophilic races of *S. cerevisiae* K-81 and XII at the wort concentration 28.0%. However, the number of yeast cells in *S. cerevisiae* DO-16 and DO-11 races increased.

During fermentation, these parameters characterise the increase in the work surface of the yeast in the medium fermented. This allows accelerating the fermentation process and ensuring microbiological purity of the medium, which is especially important for highly concentrated wort. Thus, a connection between the concentration of DM in the wort and the morphological and physiological properties of yeast has been established.

The effect of wort concentration on the ultrathin structure of yeast cells of the races *S. cerevisiae* XII, K-81, DO-11, DO-16 has been studied. Electron microscopic examination of the internal structure of yeast cells of the races *S. cerevisiae* XII, K-81, DO-11, DO-16 has shown that at the concentration of wort DM 20%, the cytoplasm of the cells contained an increased number of large gas inclusions (>500nm), with the nuclei noticeably deformed under their influence (Fig. 1a, b – 4a, b). The presence of glycogen grains was also observed in the samples studied. Morphologically, they are granules up to 9nm in size, which are dispersed in the cytoplasm [7,8]. Glycogen is a high-molecular-weight polysaccharide in which D-glycosidic residues are linked by α-1.4 and α-1.6 bonds. A particularly large amount of it was in the samples where the yeast *S. cerevisiae* of the races DO-11, DO-16 was used (Figs. 1a, b – 2a, b). The cell mitochondria were often unidentified or severely reduced. No significant differences were observed in the samples grown in the medium from grain raw materials with the DM concentration 20%.

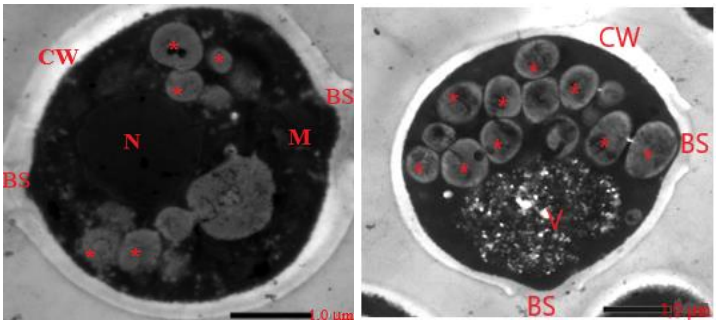
With the increase in the wort DM concentration to 28%, in the samples where the cells of the races *S. cerevisiae* XII, K-81 were studied, glycogen decreased in the amount or was absent, compared with the races *S. cerevisiae* DO-11 and DO-16 (Fig. 5a, b – 8a, b). The sample where *S. cerevisiae* of the race DO-11 was used was more vacuolated in comparison with *S. cerevisiae* DO-16 (Fig. 5a,b-6a, b). The sample of *S. cerevisiae* cells of the race XII is significantly vacuolated, but contains many rounded bodies resembling peroxisomes. Significant vacuolation is a morphological sign of subsequent apoptosis.

The nucleus in the samples of the races S. cerevisiae XII and K-81 is less deformed in comparison with the previous samples. This may be due to the number and size of gas inclusions (Fig. 7a, b – 8a, b). Besides, S. cerevisiae cells of the race XII were markedly deformed, which indicated adverse conditions of the culture medium (Fig. 8a, b).

Based on studying the ultrathin structure of yeast cells of the races S. cerevisiae XII, K-81, DO-11, DO-16 cultured on a medium of grain raw materials with the dry matter concentrations 20 and 28%, we can conclude that significant changes in the ultrathin structure was not observed. Significant changes in the cellular structures obviously occur with changes at the molecular level.

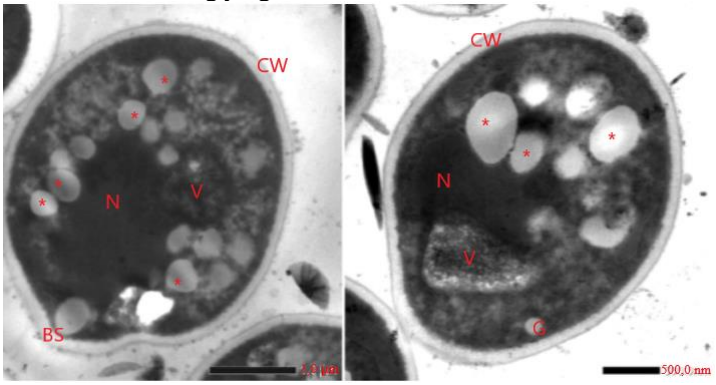
When yeast was cultured at different concentrations of DM in the wort, yeast cells differed in the glycogen content and gas inclusions. The highest amount of glycogen and gas inclusions was observed in the samples using the race S. cerevisiae DO-16, in comparison with other races, regardless of the wort concentration, which indicates a satisfactory physiological state of the cells.

According to the results of studying the effect of the wort concentration on the morphological features of the alcohol races S. cerevisiae XII, K-81, DO-11, DO-16, it can be concluded that a satisfactory state of the ultrathin cell structure at the concentration 28% was observed in the cells of S. cerevisiae DO-11 and DO-16. At the concentration of wort DM 20%, the ultrathin structure of all races studied was in a satisfactory condition. For the S. cerevisiae races XII, K-81, at the wort DM concentration 28%, there were signs of apoptosis, which indicates the maladaptation of the cells to the conditions of the fermented medium.



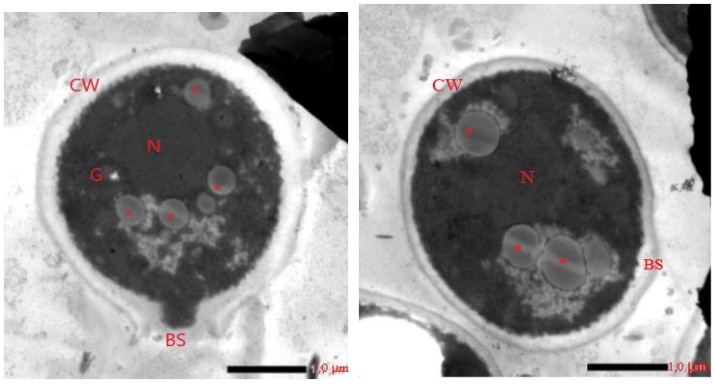
a ***b***

Fig. 1. Ultrastructure of yeast cells of the race S. cerevisiae DO-16 cultured on wort with the concentration of DM 20%: CW – cell wall, M – mitochondria, N – nucleus, V – vacuole, (*) – gas inclusion, G – glycogen inclusions, BS – bud scar



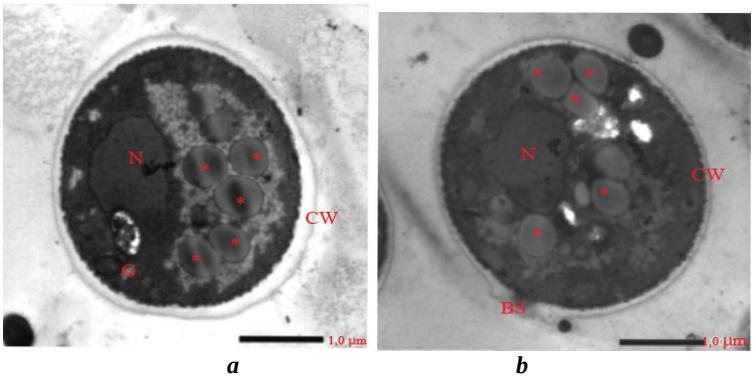
a ***b***

Fig. 2. Ultrastructure of yeast cells of the race *S. cerevisiae* DO-11 cultured on wort with the concentration of DM 20%: CW – cell wall, N – nucleus, V – vacuole, (*) – gas inclusion, G – glycogen inclusions, BS – bud scar



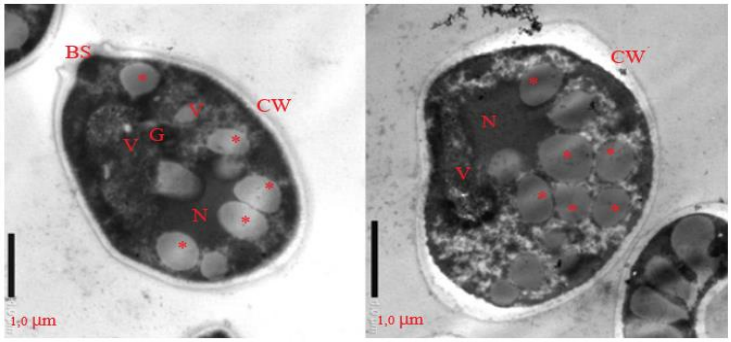
a ***b***

Fig. 3. Ultrastructure of yeast cells of the race *S. cerevisiae* K-81 cultured on wort with the concentration of DM 20%: CW – cell wall, N – nucleus, (*) – gas inclusion, G – glycogen inclusions, BS – bud scar



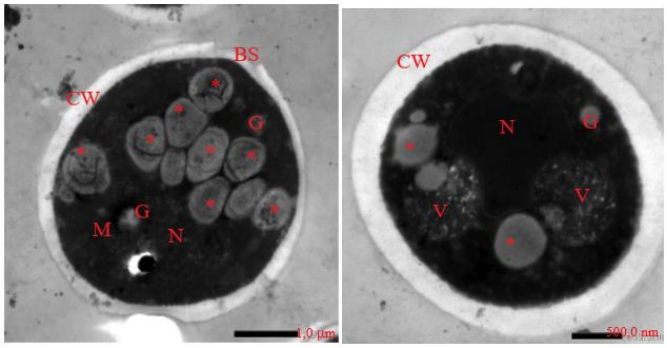
a ***b***

Fig. 4. Ultrastructure of yeast cells of the race *S. cerevisiae* XII cultured on wort with the concentration of DM 20%: CW – cell wall, N – nucleus, V – vacuole, (*) – gas inclusion, G – glycogen inclusions, BS – bud scar.



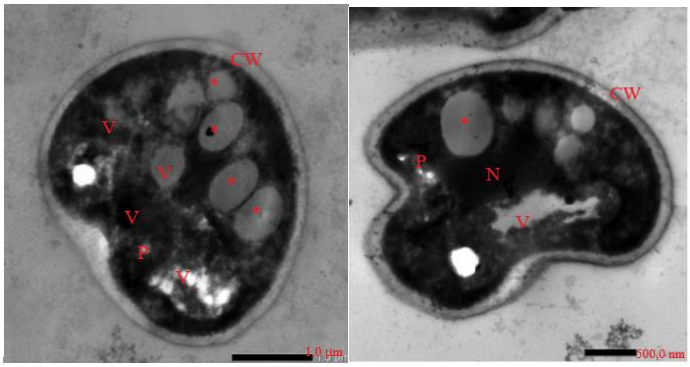
a ***b***

Fig. 5. Ultrastructure of yeast cells of the race *S. cerevisiae* DO-16 cultured on wort with the concentration of DM 28%: CW – cell wall, N – nucleus, V – vacuole, (*) – gas inclusion, G – glycogen inclusions, BS – bud scar



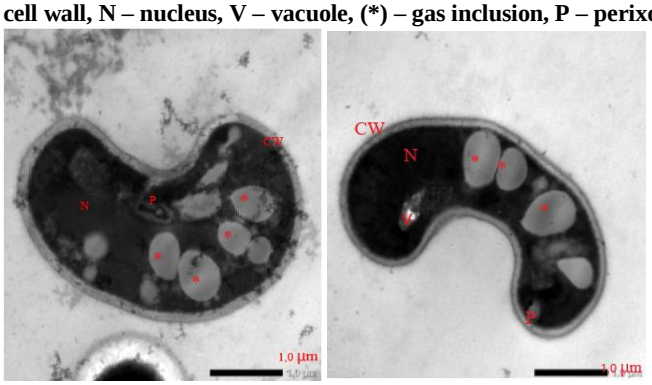
a ***b***

Fig. 6. Ultrastructure of yeast cells of the race *S. cerevisiae* DO-11 cultured on wort with the concentration of DM 28%: CW – cell wall, M – mitochondria, N – nucleus, V – vacuole, (*) – gas inclusion, G – glycogen inclusions, BS – bud scar



a ***b***

Fig. 7. Ultrastructure of yeast cells of the race *S. cerevisiae* K-81 cultured on wort with the concentration of DM 28%: CW – cell wall, N – nucleus, V – vacuole, (*) – gas inclusion, P – perixosomy



a ***b***

Fig. 8. Ultrastructure of yeast cells of the race *S. cerevisiae* XII cultured on wort with the concentration of DM 28%: CW – cell wall, N – nucleus, V – vacuole, (*) – gas inclusion, P – perixosomy

Conclusion

The efficiency and intensity of the fermentation process, as well as the amount of alcohol largely depend on the race of distillers' yeast. Cultivation of industrial yeast has been studied at the DM concentrations 20% and 28%. The alcohol races *S. cerevisiae* DO-16, DO-11, K-81, XII with thermotolerant and osmophilic properties were chosen for comparative characterisation.

The theoretical and experimental research has allowed selecting the race of distillers' yeast for fermentation of highly concentrated wort from grain raw materials. It has been found that the selected yeast race *S. cerevisiae* DO-16 synthesises the largest number of yeast cells at the concentration 28%. Morphological and cytological studies of the yeast race *S. cerevisiae* DO-16 have proved its advantages over the races DO-11, K-81, XII in fermentation of highly concentrated wort. Studies of the intracellular structure of the yeast *S. cerevisiae* DO-16, DO-11, K-81, XII have allowed establishing the relationship between the formation of glycogen in yeast cells and the concentration of DM in the wort. When culturing industrial yeast at the DM concentration 28%, the glycogen content in the cells of *S. cerevisiae* DO-16 was significantly higher compared with the races studied. Thus, the conditions of the culture medium for this race are favourable.

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ДОСЛІДЖЕННЯ УЛЬТРАТОНКОЇ СТРУКТУРИ КЛІТИН РІЗНИХ РАС СПИРТОВИХ ДРІЖДЖІВ, ЗАЛЕЖНО ВІД КОНЦЕНТРАЦІЇ СУХИХ РЕЧОВИН СУСЛА

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Анотація. Одним із напрямів впровадження у виробництво спирту ресурсо- та енергозберігаючої технології спиртових бражок є використання висококонцентрованого сусла із зернової сировини. Застосування високопродуктивних штамів спиртових дріжджів – це основа ресурсо- та енергозберігаючих технологій, спосіб зниження собівартості етилового спирту та підвищення рентабельності його виробництва. Для розробки технології висококонцентрованих бражок із зернової сировини, необхідно виділити та підібрати відповідні раси дріжджів і вивчити їхні морфологічні і фізіологічні властивості. Проведено діагностику фізіологічного стану мікроорганізмів. Досліджено вплив концентрації сухих речовин сусла на морфологічні та цитологічні особливості структури дріжджових клітин спиртових рас дріжджів *S. cerevisiae* ДО-16, ДО-11, К–81, XII в умовах культивування на середовищах із крохмалевмісної сировини. Концентрація сухих речовин сусла складала 20 та 28%. Встановлено, що селекціонована раса дріжджів *S. cerevisiae* ДО-16 синтезує найбільшу кількість дріжджових клітин за концентрації СР 28%. Осмофільні раси дріжджів *S. cerevisiae* ДО-16 та ДО-11 мали менші розміри клітин у порівнянні з термотолерантною та мезофільною расами *S. cerevisiae* К-81 та XII при концентрації сухихи речовин сусла 28%. При зброджуванні ці показники характеризують збільшення робочої поверхні дріжджів у зброджуваному середовищі, це дозволяє прискорити процес зброджування та забезпечити мікробіологічну чистоту середовища, що особливо важливо для висококонцентрованого сусла. На основі морфолого-цитологічних досліджень раси дріжджів *S. cerevisiae* ДО–16 доведено її переваги над расами ДО-11, К-81, XII для зброджування сусла високих концентрацій. На основі досліджень внутрішньоклітинної структури дріжджів *S. cerevisiae* ДО-16, ДО-11, К-81, XII встановлено залежність між утворенням глікогену в дріжджових клітинах та концентрацією сухих речовин сусла. При культивуванні виробничих дріжджів за концентрації СР 28% вміст глікогену у клітинах раси *S. cerevisiae* ДО-16 був значно більшим у порівнянні з досліджуваними расами, що свідчить про сприятливі умови культивованого середовища для даної раси.

Ключові слова: спиртові дріжджі, ультратонка структура, висококонцентроване сусло, сухі речовини, культивування.

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