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THE COMPARATIVE STUDY ON TWO COMMERCIAL STRAINS OF *SACCHAROMYCES CEREVISIAE* FOR ETHANOL PRODUCTION FROM HARD-TO-FERMENT SUGAR-CONTAINING RAW MATERIALS

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Abstract. The growth of bioethanol production in Ukraine is practical for both environmental and economic reasons. To reduce the cost of bioethanol production from sugar-containing raw materials, in particular, sugar beet molasses, it is effective to use high gravity wort and osmotolerant yeasts strains. Due to long exposure to high temperatures during sugar production and because of changes in the composition during storage, molasses accumulates compounds that adversely affect the function of yeast cells. For this type of raw material, a yeast strain should be found that will not only withstand high concentrations of sugars or the finished product, but will also be more resistant to inhibitors. The use of two industrial yeast strains (Deltaferm® AL-18 and Y 5007 (K-7)) for fermentation of high gravity molasses wort has been considered. It has been determined that sugar beet molasses, which is hard-to-ferment, can be used to produce ethanol from high gravity wort if some corrective measures are taken, in particular, resistant producer strains are used. It has been found that the yeast Deltaferm® AL-18 has a longer lag phase and consumes 50–60% of carbohydrates from the medium by 24 hours later than the yeast strain Y 5007 (K-7) does. It has been established how the parameters of the wash change depending on what yeast strains ferment high gravity wort based on hard-to-ferment molasses. It has been found that when using high gravity wort obtained from low-quality molasses, the yeast strain of foreign selective breeding does not allow achieving the calculated alcohol content in the fermented wash. According to the research results, under the same fermentation conditions, the distiller's yeast strain Y 5007 (K-7) is more effective in fermenting high gravity wort based on hard-to-ferment molasses. Unlike it is with dry yeast, the industrial use of this strain according to the classical two-stream fermentation scheme does not require additional investments.

Keywords: fermentation, hard-to-ferment molasses, *Saccharomyces cerevisiae*, high gravity wort, glycerol, bioethanol.

Introduction. Formulation of the problem

The development of renewable energy sources is one of the priorities for achieving high energy security in Ukraine. Increasing bioethanol production is a topical task not only because the national legislation is being adapted to European standards prescribing that all motor fuels must contain a certain proportion of bioethanol, but also because bioethanol can be exported to Europe, which looks promising now due to the aggravation of the global food crisis and the limited farmland area [1].

Products of sugar beet processing, including sugar beet molasses, are a source of raw materials for bioethanol production in Ukraine. Molasses can be used efficiently, because this industrial waste is inexpensive and sugars for yeast are easily available, so preparing it for fermentation requires no energy-intensive technological methods. One tonne of bioethanol produced from molasses is cheaper by 15–20% than the same amount produced from maize [2].

Analysis of recent research and publications

When obtaining bioethanol from sugar-containing raw materials, an effective measure to reduce the cost of the end product is the use of high gravity wort [3,4]. However, a significant increase in the dry matter content of the original wort has a negative effect on the

yeast cell functioning [5-7]. Besides, high ethanol content in the fermented wash, too, adversely affects the growth and the alcohol-forming power of the biomass [8-10].

A way to ferment high gravity wort effectively is to use osmotolerant strains of *Saccharomyces cerevisiae*.

In Ukrainian enterprises, bioethanol is produced mostly with the use of dry distiller's yeast from abroad. This yeast is easy to introduce and involves no expenses on biomass growth in a pure culture apparatus, so it helps reduce the cost of the end product, if raw materials of good quality are used.

But sugar beet molasses is a multicomponent system, and living cultures of microorganisms cannot be used in ethanol production without taking into account that molasses contain substances inhibiting the effective functioning of yeast cells (in particular, melanoidins and products of caramelisation of sugars). The high content of these compounds in sugar beet molasses results from prolonged action of high temperatures during sugar production [11], which, in turn, is due to the low efficiency of the sugar beet processing technology at most enterprises of the Ukrainian sugar industry [12].

The presence of furfural, oxymethylfurfural, volatile organic acids, etc. in the molasses is no less harmful [13-15].

This molasses is slowly fermented by yeast, and fermented wash obtained from it has a high content of non-fermented sugar and a low alcohol content, which increases its cost due to its loss with non-fermented sugar and increased energy consumption during distillation [16,17].

During bioethanol production, the use of low-quality sugar-containing raw materials to obtain high gravity wort makes it necessary to establish whether osmotolerant producer strains can effectively function in a growth medium of this composition.

The purpose of the research is to compare how efficiently the two commercial yeasts strains can ferment high gravity wort from low quality molasses.

The research objectives:

– to determine whether the quality characteristics of the sugar beet molasses samples comply with the regulatory requirements;

– to compare the dynamics of fermenting different concentrations of wort based on low-quality sugar beet molasses, using osmophilic yeast strains of foreign and domestic selective breeding;

to compare the quality of the fermented wash obtained by fermentation of wort of different concentrations based on low-quality molasses.

Research materials and methods

Microorganisms. The sample of the yeast *S. cerevisiae* Y 5007 (K-7) was obtained from the Ukrainian research institute *Spyrtbioprod*'s collection of cultures (Kyiv, Ukraine). The dry yeast sample *S.cerevisiae* Deltaferm® AL-18 (manufactured by WeissBioTech GmbH, Germany) was obtained from the manufacturing company Bio PEC Ltd. (Lutsk, Ukraine). The characteristics of the yeast strains are shown in Table 1.

Table 1 – Characteristics of the yeast strains used in this study

Yeast strain	Characteristics
Y 5007 (K-7)	Moist paste with the mass fraction of DM 30-35% The number of viable cells per 1g of the dry product not fewer than 1×10^9 Effective at the temperature 30-33°C
Deltaferm® AL-18	Dry granules with the mass fraction of DM >95% The number of viable cells per 1 g of the product not fewer than 1×10^{10} Effective at the temperature 30-37°C

The sample of Y 5007 (K-7) was kept on an agar medium (containing 120g/dm^3 of malt wort, 20g/dm^3 of agar) for 24 hours at 30°C and then stored at 4°C. The culture was transferred onto a fresh agar medium every 2 months.

Malt wort was prepared as follows: 250–300g of coarsely ground dry barley malt was mixed with 1dm^3 of tap water, and kept at a specified temperature during the three following stages, with continuous stirring: for 30minutes at 45–50°C, for another 30minutes at 55–58°C, and then at 62–63°C, until full saccharification was reached (1–2 drops of the cooled mixture with iodine do not colour the mixture blue). The prepared medium was strained through a cloth filter, then through a folded paper filter. The concentration of solids was determined in the filtrate at 20°C with a refractometer. The filtrate was diluted with tap water to the required concentration. To ensure sterility of the medium, it was autoclaved at 1 atm for 30 minutes.

The sample of Deltaferm® AL-18 was dry and stored at 4°C.

The yeast was cultivated using malt wort with the concentration of solids 17–18% and the temperature 30°C (303.15°K), in three stages lasting 18–20 hours each. At each stage, the volume of the medium increased tenfold: 5ml, 50 ml, 500 ml. The yeast biomass was separated through a paper filter using a vacuum pump. Before being placed into a flask for fermentation, the raw yeast, in a quantity equivalent to the yeast content in the wort with the concentration 18g/dm^3, was suspended in 10cm^3 of sterile water (rehydration time before introducing into the wort 20 minutes).

A certain quantity of dry yeast Deltaferm®AL-18, equivalent to the raw biomass of K-7, was also diluted with 10 cm^3 of sterile water before being placed into the fermentation flask.

*Raw materials***.** The dry barley malt for the malt wort was obtained from Lviv Brewery, Lviv, Ukraine**.** The molasses samples used as the fermentation

medium were obtained from Hnidava Sugar Factory, Lutsk, Ukraine (the quality characteristics of molasses are shown in Table 2).

*Bioethanol production medium***.** The molasses wort was prepared by diluting sugar-beet molasses with water. The initial concentrations of total sugars in the wort were 158, 174, and 190 g/dm^3. These concentrations were calculated to obtain as much ethanol in the fermented wash as 78.9, 86.8, and 94.7 g/dm^3 (corresponding to 10, 11, and 12% vol. respectively). To obtain these concentrations, the hydromodulus of diluting of this molasses sample was, respectively, 2.34, 2.04, and 1.79. Each of the three variants of wort concentration was used for comparative fermentation of the two producer strains.

After that, the mixture was acidified to pH 4.8 by means of sulphuric acid with the molar concentration 1mol/dm^3. Then, it was enriched with nitric and phosphoric nutrition in the form of urea and orthophosphoric acid added in proportion 0.1 and 0.05%, respectively, to the weighed portion of molasses. To prevent contamination of wort with extraneous microflora, the disinfectant *Baktrilon* (*TMA Tristan*, Kyiv, Ukraine) was used in the dosage recommended for ethanol production. The additional nutrition elements and antiseptics were introduced in accordance with the quantities used in ethanol production in Ukraine.

Fermentation process. The process of alcohol fermentation of wort in vitro was studied by "test fermentations" in 500 ml flasks. Fermentation was performed in an incubator at 30°C (303.15°K). The fermentation time was 72 hours. The process was monitored by the amount of carbon dioxide released in the course of fermentation. The amount of CO_2 was measured by weighting the fermentation flasks during the process.

Research methods. Molasses was analysed by the methods described in DSTU 3696-98: the dry matter content (DM, %) was determined with a refractometer ABBE (ULAB, China); the pH of the medium by the potentiometric method using a pH meter MP-512 (ULAB, China); the sucrose content (P) and inversion polarization (I) by the polarimetric method using a saccharimeter SU-4 (*Analitprylad*, Kyiv, Ukraine); the content of reducing substances (I_s) by the iodometric method using Offner's reagent.

The total amount of fermentable sugars (S_{fs}) was calculated by the fraction of sucrose, inversion polarisation, and the content of reducing substances using the formula defined in the standard of DSTU 3696-98:

$$Sfs = 0.68 \cdot P + 0.96 \cdot I + 0.80 * Is$$

The colour value of the molasses was determined colourimetrically using a KFK-03–ZOMZ photocolourimeter, using distilled water as the reference solution [18]. The content of amine nitrogen was measured titrimetrically using a formaldehyde mixture and a pH-meter MP-512[18], the content of calcium and magnesium salts was determined complexometrically using Trilon B [19], the mass fraction of total nitrogen was found by the Kjeldahl method [19], and the ash content by burning with sulphuric acid [19]. The molasses purity was calculated as the ratio of the content of fermented sugars to the total dry matter content.

The content of non-fermented sugars was determined in the fermented wash using the Kulka method, which consists in the ferric ammonium sulphate-mediated oxidation of furanose in concentrated HCl acid to form the furfural product from furanose, followed by reaction with the resorcinol to form a coloured product [20, 21].

The glycerol content was estimated by Lambert and Neish's method [22], which involves quantitative oxidation of glycerol to formaldehyde by periodate under acidic conditions. The formaldehyde thus obtained is then directly determined by the colour reaction with chromotropic acid and is read at 570 nm [23].

The ethanol concentration in distillates was determined by the areometric method [19].

The study was conducted in triplicate, the tables and figures indicate averaged values.

Results of the research and their discussion

Quality of the raw materials for fermentation. One of the indicators influencing the technological properties of molasses is the chemical composition of its non-sugars. It depends on the soil and climatic conditions of vegetation, fertilisers, harvesting methods, conditions of sugar beet storage, processing technology, and other factors. The main quality parameters of sugar beet molasses in Ukraine must meet those specified in DSTU 3696-98 "Sugar beet molasses. Technical specifications." However, its production value for the alcohol industry is determined by an additional set of characteristics in accordance with the regulatory technical documentation on ethanol production, which is active in Ukraine (TTR No.000 32744-3508-2005). If some parameters of molasses do not comply with the standards, this raw material is classified as defective or hard-to-ferment [17,18]. The standards of the molasses quality described in these documents and the results of studying the composition of this raw material are shown in Table 2.

The production value of molasses is characterised by its purity – the ratio of total fermentable sugars to the dry matter concentration. At lower purity, the non-sugar content is higher, which has a negative effect on the enzymatic activity of yeast and on the yield and quality of ethanol. According to this parameter, the molasses samples studied were within normal limits. However, for effective fermentation of this raw material, one should take into account not only the quantitative, but also the qualitative composition of molasses non-sugars.

Table 2 – Physicochemical composition of sugar beet molasses obtained from a production enterprise (n=3, P≤0.95)

Parameter	Results of analysis	Requirements [18]	Requirements (hard-to-ferment molasses) [18]
Dry matter content (DM), %	83.6±0.5	Not less than 75.0	Less than 75.0
pH of the medium	5.65±0.07	6.5-8.5	Less than 6.5
Content of sucrose, %	52.0±0.3	Not less than 43.0	Less than 43.0
Inversion polarisation, %	16.0±0.2	Not regulated	Not regulated
Content of reducing substances, %	2.74±0.06	0.12-1.02	More than 1.02
Total amount of fermentable sugars, %	52.9±0.06	Not less than 44.0	Less than 44.0
Colour value, % of the light transmission of distilled water	1.96±0.03	40-60	Less than 40
Ash content (with sulphuric acid), %	16.1±0.06	10.4-12.0	Less than 10.4
Content of total nitrogen, %	1.65±0.03	1.3-2.06	Less than 1.3
Content of amine nitrogen, %	0.25±0.01	0.35-0.45	0.15-0.34
Calcium and magnesium content, in terms of CaO, %	1.24±0.04	0.17-0.82	More than 0.82
Purity, %	63.3±0.4	58.8-64.0	Less than 58.0

Analysis of the data in the table has shown that the molasses used in the research had the active acidity lower than the prescribed parameters. The low active acidity of a molasses sample results in the increased content of invert sugar (reducing substances) in it and can indicate that the raw material contains harmful products of the vital activity of foreign microflora [17].

Besides, a significant excess in calcium and magnesium content has been established, which, in turn, accounts for too high a content of ash in the molasses. There are two ways in which *S.cerevis*iae metabolises sucrose: hydrolysis by extracellular invertase and hydrolysis within the cell with the use of proton symport [24]. The invertase activity depends on the calcium level in the raw materials and can be reduced to 25% if the content of calcium oxide is higher than 0.72% [25]. An excess of calcium and magnesium salts in the raw materials adversely affects the yeast reproduction rate and impairs their normal functioning [26,27]. Since the raw material processed exceeded the maximum allowable value of this parameter by 51%, it could significantly affect the fermentation process.

It should also be noted that, with the normative level of total nitrogen, a lack of amine nitrogen in molasses has been revealed. The low amine nitrogen content, too, significantly slows down the fermentation dynamics by limiting the yeast biomass formation [28,29].

Deviation from the normative parameters may mean that the sugar beet processed at the factory was of poor quality.

The low colour value indicates long heat treatment of sugar-containing raw materials with the formation of a lot of products of the Maillard reaction (melanoidins). The Maillard reaction products have a negative effect on the activity of the yeast cell enzymes [30].

Analysis of the quality parameters of the molasses samples obtained suggests that this molasses can be classified as hard-to-ferment [17,18]. Accordingly, for its effective fermentation, even with a low sugar content in the initial wort, a complex of corrective measures may be necessary. One of them is the use of yeast resistant to adverse medium conditions.

Using this molasses as a raw material for high gravity wort in ethanol production can lead to significant loss related both to non-fermented sugar and to an increase in the cost of distillation of the fermented wash with a low ethanol content.

The dynamics of the fermentation process. One of the important indicators of fermentation progress is the intensity of releasing carbon dioxide. We determined the intensity of releasing CO_2 every 12 hours throughout the fermentation period with different concentrations of the wort. The findings are shown in Fig. 1 (a, b, c).

When the yeast strain Y 5007 (K-7) was used to fermentation wort with the lowest initial concentration of fermentable sugars, the amount of CO_2 released during the first 12 hours of fermentation was 65.3% of its total amount (Fig. 1a). with an increase in fermentable sugars in the wort, the amount of carbon dioxide released in the first 12 hours reduced to 51.0% (Fig. 1b) and to 42.6% of the final value (Fig. 1c). For comparison, in worts fermented with the yeast strain Deltaferm® AL-18 under the same conditions, after the first 12 hours, this parameter was only 4.0% (Fig. 1a), 2.0% (Fig. 1b), and 1.8% (Fig. 1c). Further intensification of carbon dioxide release for this strain does not ensure achieving the estimated ethanol content within a specified period of time. Increasing the duration of fermentation under production conditions is economically impractical.

According to S. Chniti *et al.* [31], at the initial content of fermentable sugars in the wort, their inhibitory effect should be negligible to obtain the estimated ethanol content 10% vol. However, it has been established that the lag phase of growth of the dry yeast biomass significantly increased and the fermentation activity of its cells decreased at all wort concentrations.

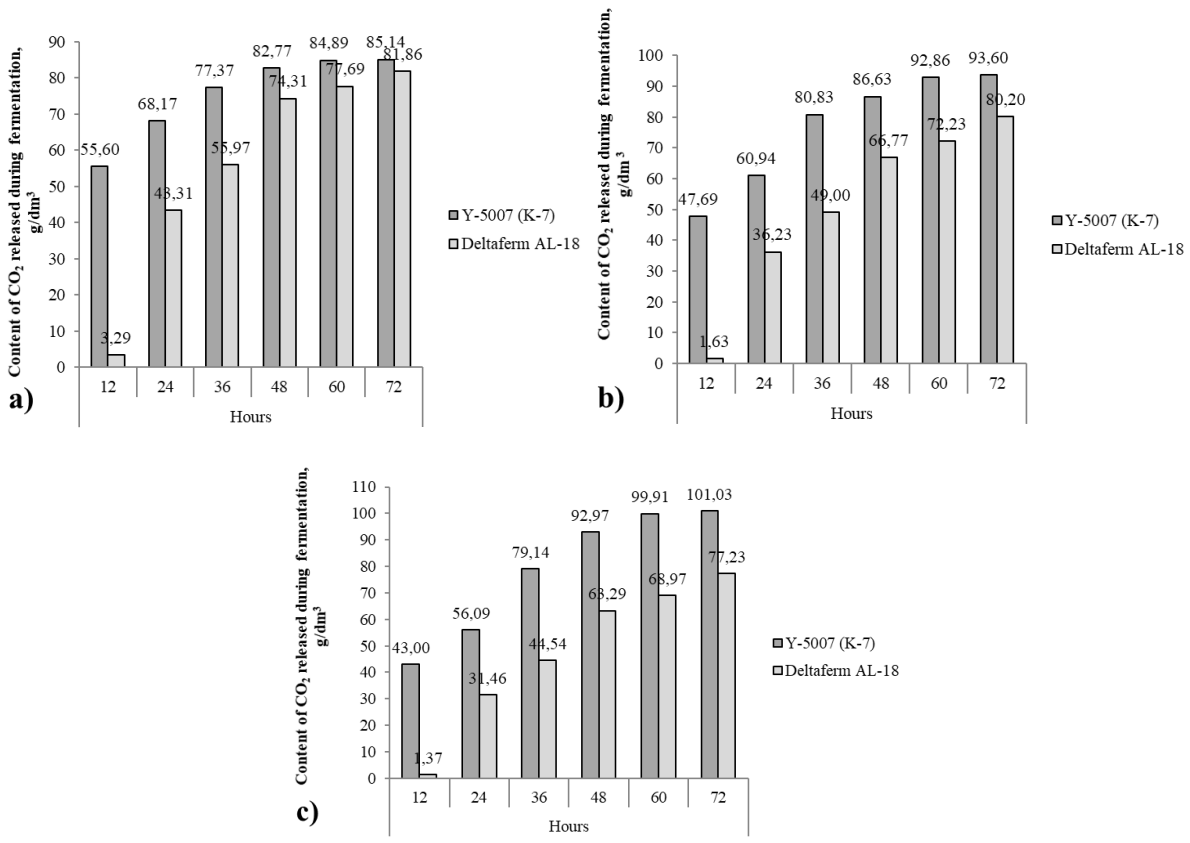

Fig. 1. Dynamics of releasing carbon dioxide during molasses wort fermentation with the strains Y 5007 (K-7) and Deltaferm® AL-18. The estimated ethanol content in the fermented wash: a) 78.9g/dm³ (10% vol.); b) 86.8g/dm³ (11% vol.); c) 94.7g/dm³ (12% vol.)

This is consistent with the data of the previous studies by S. Oliynichuk and O. Koval on the effect of molasses non-sugars on the enzymatic activity of yeast cells [32]. Thus, in all cases, dry yeast reached 50–60% of the level of consuming the carbohydrates of the medium only after 36 hours of fermentation, which is 24 hours later than the yeast of the strain Y 5007 (K-7) did. This indicates lower resistance to osmotic stress, which results in slowing down the processes of adaptation to adverse conditions for the yeast strain Deltaferm®AL-18.

Comparison of the parameters of fermented wash. Ukrainian factories that produce molasses-based ethanol, typically obtain fermented wash with the ethanol content 10% vol. according to this production standard, the strain Deltaferm®AL-18 does not reach the estimated ethanol content. It yields 69.1g/dm³ of ethanol, which may indicate a greater sensitivity of the strain to the presence of non-sugars in a molasses sample (Fig. 2a). On the contrary, the variant using the strain K-7 reached the estimated ethanol content, which, respectively, provided a low content of non-fermented sugars in the fermented wash.

In the variant with the estimated ethanol content 86.8 g/dm³, the strain Deltaferm®AL-18 showed 68.7g/dm³ ethanol (Fig. 2b). With further increase in the concentration of the medium using this strain (Fig. 2c), the content of ethanol in the fermented wash is reduced to 65.5g/dm³. The data obtained during the experiment confirm the sensitivity of the strain Deltaferm®AL-18 to the qualitative composition of the sugars during molasses fermentation.

The use of the strain Y 5007 (K-7) has shown that the calculated ethanol content was achieved in two of the three variants. There was a slight decrease in the biomass content and an increase in the glycerol content in the fermented wash. Therefore, this strain is less dependent on the negative influences of non-sugar compounds in molasses.

The glycerol content in the fermented wash is higher than it is indicated for this content of fermentable sugars [31]. Under these conditions, the concentration of solids in the wort cannot have a significant inhibitory effect on the cells. So, the limiting factor for the growth and functioning of yeast biomass is not the quantitative but qualitative composition of molasses non-sugars.

The same conclusion can be drawn about the fermentation dynamics when the strain Deltaferm® AL-18 is used. Differences in the fermentation process between the two strains can be explained by the fact that the strain of domestic selective breeding was created taking into account the specific features of the raw material base.

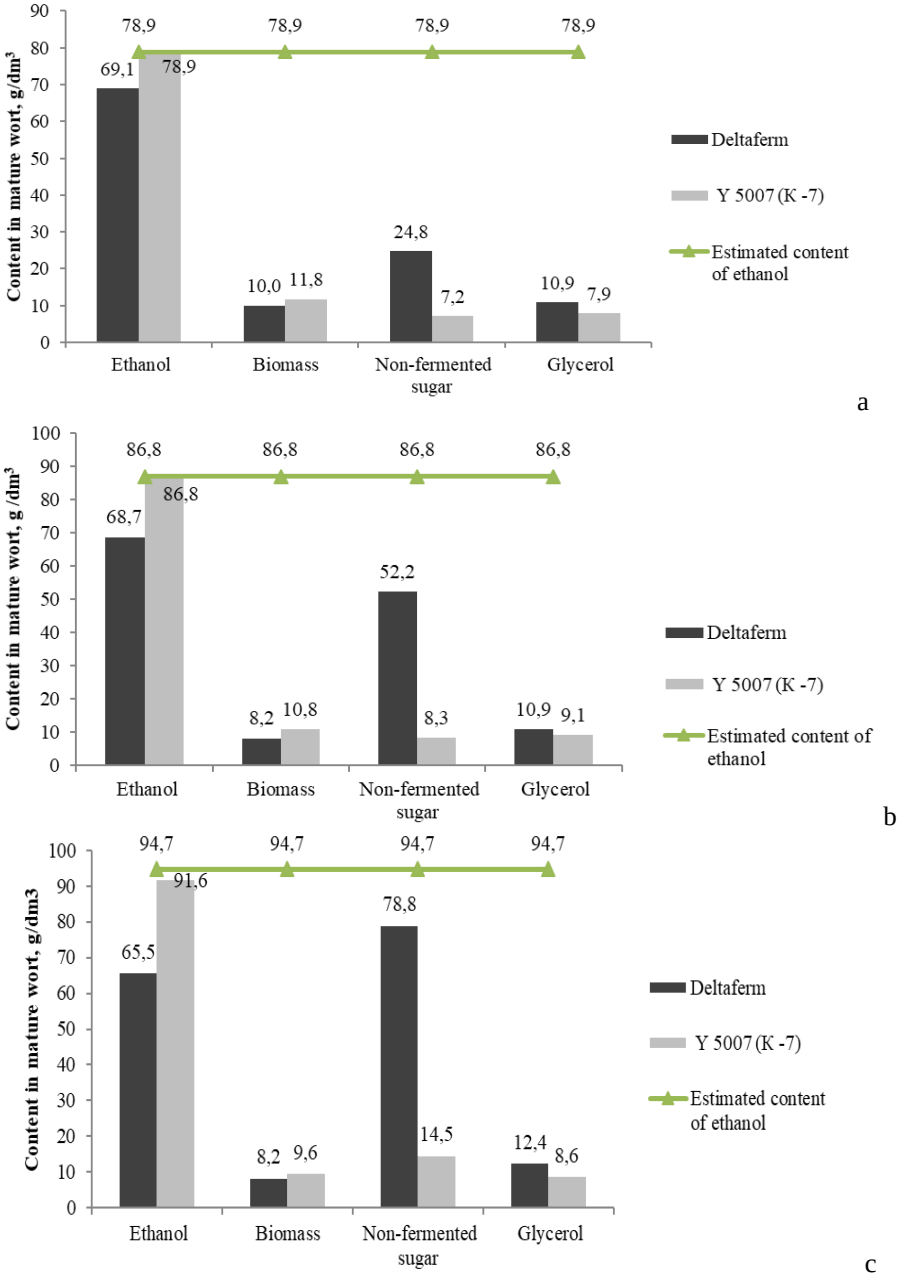

Fig. 2. Parameters of the fermented wash according to the estimated ethanol content in it: a) 78.9g/dm^3 (10%vol.); b) 86.8g/dm^3 (11%vol.); c) 94.7g/dm^3 (12%vol.)

S. Oliynichuk, T. Lysak, L. Marinchenko [33], E. Nevoigt and U. Stahl [34] determined in their studies that a glycerol increase in the fermented wash resulting from an increase in its solids is a biological response of yeast cells to the medium's increased osmolarity. However, under identical conditions, the strain K-7 forms by 16.5–30.6% less glycerol than Deltaferm®AL-18 does. This indicates that the yeast of the strain K-7 have better metabolic algorithms and, to synthesise glycerol, use to a lesser extent the HOG metabolism pathway as an intracellular medium for metabolic processes.

Conclusion

The sugar beet molasses used in the study is defined as being hard-to-ferment according to the regulatory requirements. However, this raw material can be used to produce ethanol from high gravity wort, if corrective measures are taken, in particular, resistant producer strains are used.

It has been found that under identical fermentation conditions, the yeast Deltaferm®AL-18 have a longer lag phase and reach 50–60% of the level of consuming the carbohydrates of the medium 24 hours later than the yeast of the strain Y 5007 (K-7) does. Further intensification of the fermentation process for this strain does not ensure achieving the estimated ethanol content within a specified period of time. Increasing the duration of fermentation under production conditions is economically impractical.

The fermented wash obtained using the strain Deltaferm®AL-18, in all variants, had a lower ethanol concentration than the calculated one, and was high in non-fermented sugar and glycerol. Still, under the same conditions of fermentation in the fermented wash using the strain K-7, the calculated alcohol content was achieved in two of the three variants. The strain K-7 forms by 16.5–30.6% less glycerol than Deltaferm®AL-18 does. This may indicate better resistance to the influence of the qualitative composition of non-sugars in high gravity wort.

The results of the research on the intensity of fermentation and the quality characteristics of fermented wash show that the alcohol yeast strain Y 5007 (K-7) more effectively ferments high gravity wort based on hard-to-ferment molasses. The industrial use of this yeast according to the classical two-stream fermentation scheme, as compared with dry yeast, does not require additional investments, because the pure culture apparatus required for biomass reproduction is part of the equipment of most domestic distilleries working on sugar-containing raw materials.

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ПОРІВНЯЛЬНЕ ДОСЛІДЖЕННЯ ДВОХ КОМЕРЦІЙНИХ ШТАМІВ *SACCHAROMYCES CEREVISIAE* ДЛЯ ВИРОБНИЦТВА ЕТАНОЛУ З ВАЖКОЗБРОДЖУВАНОЇ ЦУКРОВМІСНОЇ СИРОВИНИ

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Анотація. Збільшення виробництва біоетанолу в Україні є доцільним як з екологічної, так і з економічної точки зору. Ефективними заходами для зниження затрат на виробництво біоетанолу з цукровмісної сировини, зокрема меляси цукробурякової, є використання сусла високої концентрації та осмотолерантних штамів дріжджів. Тривалий вплив високих температур у процесі виробництва цукру та зміна складу при зберіганні сприяють накопиченню в мелясі сполук, які негативно впливають на функціонування дріжджових клітин. Для такого типу сировини доцільним є визначення штаму дріжджів, який витримуватиме не лише високу концентрацію цукрів або кінцевого продукту, але також буде більш стійким до інгібіторів. Досліджено використання двох промислових штамів дріжджів (Deltaferm® AL-18 та Y 5007 (К-7)) для зброджування мелясного сусла високої концентрації. Визначено, що меляса цукробурякова, яка має властивості важкозброджуваної, може застосовуватись для виробництва етанолу з сусла високої концентрації за умови використання коригувальних заходів, зокрема застосування стійких штамів продуценту. Встановлено, що дріжджі Deltaferm® AL-18 характеризуються більш тривалою лаг-фазою та досягають 50–60% рівня споживання вуглеводів середовища на 24 години пізніше дріжджів штаму Y 5007 (К-7). Визначено зміни показників бражки в процесі зброджування сусла високої концентрації на основі важкозброджуваної меляси різними штамами дріжджів. Встановлено, що за використання сусла високої концентрації з меляси низької якості штам закордонної селекції не досягає розрахункової концентрації етанолу в зрілій бражці. За результатами досліджень спиртові дріжджі штаму Y 5007 (К-7) більш ефективно зброджують сусло високої концентрації на основі важкозброджуваної меляси та їх застосування у виробничих умовах за класичною двопотоковою схемою бродіння у порівнянні з сухими дріжджами не потребує додаткових вкладень.

Ключові слова: бродіння, важкозброджувані меляси, *Saccharomyces cerevisiae*, сусло високої концентрації, гліцерин, біоетанол.

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