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DEPENDENCE OF GLYCEROL ACCUMULATION AND STARCH HYDROLYZATES FERMENTATION ON WORT CONCENTRATION

S. T. Oliynichuk[1]
T. I. Lysak[1]
L. V. Marynchenko[2]

[1]Institute of Food Resources of the National Academy of Agricultural Sciences of Ukraine, Kyiv
[2]National Technical University of Ukraine «Kyiv Polytechnic Institute»

E-mail: oliynichuk@ipr.net, lolitamar@ukr.net

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The purpose of this work is to study the dependence of ethanol accumulation by-products and secondary products (glycerol and propionic acid) during the fermentation in the case of increasing the wort concentration from 12 to 21% by weight of sugar as an example of commonly used in the alcohol industry the commercial dry yeast company «Danisco» and experimental osmophilic strain *Saccharomyces cerevisiae* DS-02-E, isolated from a concentrated (80% DM) of rye malt wort which spontaneously fermented. The enzyme preparations "AMYLEX 4T", "ALPHALASE AFP" and "DIAZYME SSF" were used for the liquefaction and saccharification of starch wort). The finished industrial of both yeast strains were added to the fermentation flasks in an amount of 10% by volume of the primary wort. In the mature brew the unfermented carbohydrates content was determined by colorimetric method with anthrone reagent, alcohol — by glass areometer-alcoholometer, acidity — potentiometrically, the concentration of dry matter — by areometer, glycerol content — by photocolorimetry method. In the brew distillate a volatile impurities content, namely propionic acid, was determined using gas chromatography. Statistical processing of the results of three series of experiments were carried out by calculating the arithmetical mean value of 5 measurements, their standard deviations and errors. To determine the probable differences between the mean values were used Student's test. Differences were considered statistically significant at $P < 0.05$.

Reduction for accumulation of glycerol (between 38 till 53%) at higher concentrations of nutrient medium in the case of the *Saccharomyces cerevisiae* yeasts DS-02-E as compared with commercial dry yeast, reduction the formation of unwanted by-product of fermentation — propionic acid (up to 34%), a better ability of the experimental strain to accumulate sugar of wort and to accumulate ethanol (up to 0.1–0.25% vol.) were shown. It was concluded that the involvement of other mechanisms for osmoadaptation not related to HOG (high-osmolarity glycerol) way, or less active glycerol synthesis system in response to osmotic stress. The practical significance of research using a new experimental osmophilic yeast strain consists of increasing the depth of substrate utilization and ethanol yield from the starch of grain raw materials that have a positive impact on the economy and ecology of ethanol (bioethanol) production.

Key words: brew, osmophility, yeasts, glycerol.

One of the main technological stages in ethyl alcohol bio-technology is yeast cultivation, viability of which greatly influences on fermented wort characteristics during the starch-containing feedstock processing. The source kind of alcohol yeasts is *Saccharomyces cerevisiae,* which has been the main object of ethanol production for many years. Yeast strains XII, XII-T and dry yeasts of different manufacturers have become the most popular to ferment wort from starch-containing feedstock in Ukraine. They fully assimilate sugars, create secondary and by-products, which influence on alcohol quality and sustainability to relatively high ethanol concentrations in fermented wort. The ethanol technology is quite costly when fermented wort contains 9–10% vol. of ethanol. According to this fact, the increase of alcohol yield if fermented wort even up to 12–14% vol. is a real energy efficient measure and it allows to improve the production profitability [1–3].

The major obstacle to achieving this goal is the issue of yeasts osmoadaptation to higher nutrient concentration in mash and ethanol accumulated in fermented wort. The solution of this problem lays in selection of new strains which have osmoprotectoral abilities, and are capable of high fermentation activity.

The increased osmophilic pressure, which occurs in case of increasing the carbohydrates concentration in cultivation media, causes the loss of free cell energy to provide osmoregulation, providing transportation systems and synthesis of osmoprotectors [4–5]. According to this fact, under the conditions of high osmolarity, lag-phase extension, growing speed reduction, ethanol and biomass accumulation level decrease take place. All this happens because yeasts are in osmotic stress.

Efficient yeast function under conditions of high sugar and alcohol concentration in media is caused by various metabolism ways activation, adaptive proteins expression and eventually yeast cell biochemical composition and modified profile of metabolism products.

It is widely known that yeasts *S. cerevisiae* have quite complex and complete systems of adaptation to the changes of solids concentration in cultivation media. One of them is the presence of osmosensors in cytoplasm membrane. Scientists have claimed that accumulation of some protective compounds in yeast cells is constituent, however under unfavourable conditions the increase of their production may be noticed [4].

Changes in media osmolarity influence on different signal ways of *S. cerevisiae*. A number of researches showed that with increasing of life-dangerous osmolarity, yeasts activate HOG (high-osmolarity glycerol) signal way, the main element of which is Hogl MAP (mytogen activated proteinkinase). Scientists consider that HOG system is the most effective signal way of yeasts reaction on osmotic changes and signal transfer to transcription factors. It activates less than in 1 minute in case of osmolarity increase [6–8]. Activated Hog1 regulates cell cycle, protein translation and gene expression which are essential for glycerol creation and internal osmolarity increase.

On the contrary, alcohol fermentation process, which consists of not less than 13 main stages, is always accompanied by glycerol synthesis. It happens due to the fact that during glucose to ethanol dissimilation one of the stages happens with the help of aldolase transformation of fructose-1,6-diphosphate into two phosphotrioses: phosphodioxyacetone and 3-phosphoglyrerol aldehyde. The last one turns into glycerol after restoration and phosphorus acid cleavage. To sum up, the glycerol formation is constituent in fermentation process.

Glycerol accumulation also plays some a certain role in cytosolic balance of NAD^+/NADH in *S. cerevisiae* (Fig. 1) [9] . Except of glycolitic way (*A*), which leads to creation of glycerol, ethanol and ethyl acetate, scientists consider synthesis of glutamate form 2-oxoglutarate in order to reduce formation of cytosolic NADH and glycerol (*B*). Researchers link overproduction of glycerol by *S. cerevisiae* with over secretion of formate dehydrogenase whish is a stress protein and uses formate to generate additional source of cytosolic NADH (*C*).

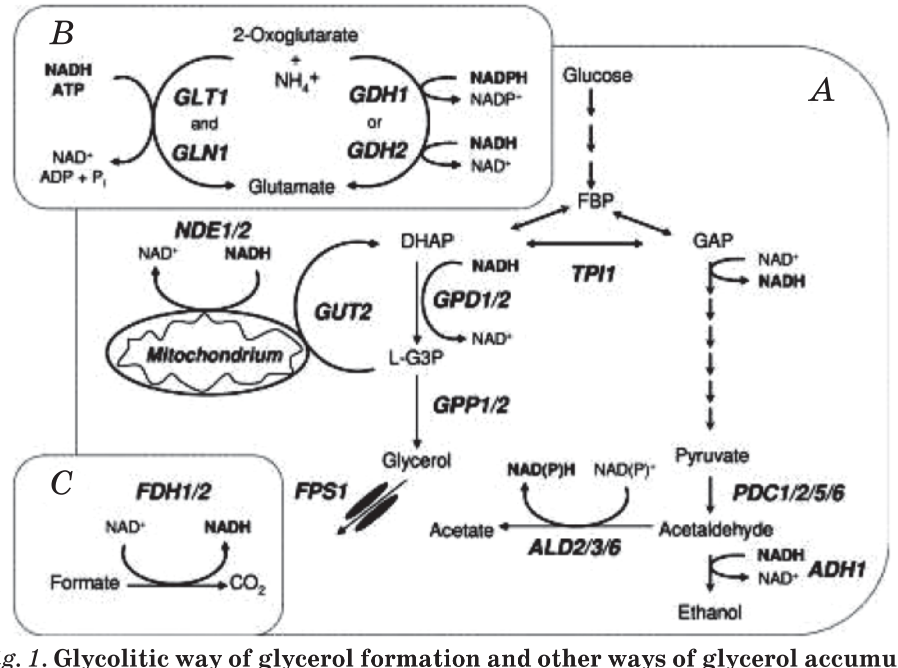

Fig. 1. **Glycolitic way of glycerol formation and other ways of glycerol accumulation, engaged in cytosolic balance of NAD^+/NADH in *S. cerevisiae***

The interest of researchers in *S. cerevisiae* glycerol overproduction is caused by two practical factors. Firstly, presence of glycerol in wine improves its demand and consumer properties. Secondly, it reduces ethanol formation during soft dirnks production which has commercial interest.

On the other hand, in alcohol production the increase of ethanol yield in wort during fermentation is a primal objective. Therefore, the investigation of the way mash solids concentration increase influences the metabolism of *S. cerevisiae* industrial strains is up-to-date task and has practical value.

The aim of our work was to investigate biological response of *S. cerevisiae* yeasts to osmotic stress, caused by concentration of fermentable compounds and accumulated ethanol. Particularly, we have been studying the influence of mash concentration on secondary and by-products (glycerol and propionic acid) formation in yeasts, using as an example widely used industrial dry yeasts and experimental yeast strain as well.

Materials and Methods

The objects of our investigation are ethanol producers — experimental yeast strain DS-02-E, which is adapted to highly concentrated mash fermentation, and dry yeasts produced by "Danisco" company, which are widely used by alcohol industry enterprises. DS-02-E yeast strain was isolated from concentrated (80% solids) rye malt mash, which self-fermented spontaneously.

For our investigation we used corn mash samples, which contained 12, 15, 18, 21% mas. sugars. To prepare the samples we took corn grind batches of 50, 62.5, 75 and 83 g and transferred them to 250 ml flasks, mixed with water at ratio 1:3, added liquefaction enzyme solution "AMYLEX 4T" in dosage of 1.5 activity units per 1 g of starch and held it for 3 hours at a temperature of 90 °C.

Afterwards, we cooled the liquefied mash to 30 °C, added proteolytic enzyme solution "ALPHALASE AFP" and saccharification enzyme solution "DIAZYME SSF" in dosage of 6.0 activity units per 1 g of starch and added yeast culture, which was cultivated for 24 hours. Yeast ratio was 10% of mash volume (25 ml). The processes of saccharification and fermentation were simultaneous and were held for 72 hours at a temperature of 30 °C.

We propagated pure yeast culture in barley malt mash which contained 17% m/m sugars. We used the received suspension in order to prepare industrial yeasts using saccharified corn mash with sugar concentration equal to their fermentation probe.

To regenerate dry yeasts we took their batch, mixed them with distilled water which contained 0.9% m/m of NaCl and sustained them for 30 min at a temperature of 25–30 °C. Afterwards, we used yeast suspension in the same way we had treated experimental strain DS-02-E.

Mature industrial yeasts of both races were added to fermentation flasks in a ratio of 10% to main mash.

In fermented worts we determined the amount of unfermented sugars using anthrone method; to determine alcohol value we used areometer; we determined acidity using pH-meter; to determine solids concentration we used aerometer [10]; to determine glycerol amount we used photometric method [11].

In wort distillates we determined the amount of contaminants, particularly propionic acid, using gas chromatography [12].

We accomplished the statistical processing of results, obtained from 3 series of researches, calculating the arithmetic means of their standard deviations and mistakes among 5 probes. To determine the probable differences between average amounts we used Student criteria. We took divergences as statistically probable if $P < 0.05$ [13].

Results and Discussion

Increased production of glycerol as a response to osmotic stress, caused by mash concentration advance, has a negative influence on substrate utilization ratio, thus on resource efficiency of alcohol production. According to this fact, the usage of osmophilic yeasts, which have the ability to resist the increased osmolarity due to the synthesis of glycerol, may cause some reduction of economical indexes of the process, particularly, in case of advanced accumulation of other reaction by-products.

In our researches we compared how do dry yeasts of "Danisco" company and experimental yeast race DS-02-E accumulate glycerol in fermented worts under the conditions of increased mash solids concentrations. The results are shown on Fig. 2.

From the diagram we can see, that both yeast races kept the trend to increase glycerol accumulation according to sugar concentration increase. This is natural due to the fact that mechanisms of alcohol and glycerol-pyruvate fermentations are tightly connected to each

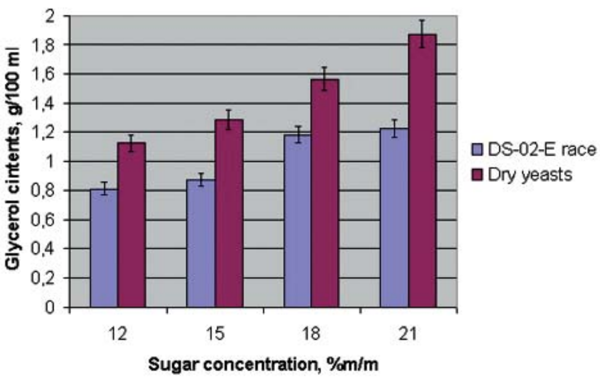

Fig. 2. **The influence of mash sugars concentration on the ability of investigated yeast races to accumulate glycerol in fermented worts**
Here and further: * — $P < 0.05$;
reliably comparatively to columns 2 (dry yeasts)

other. The alcohol fermentation always starts from pyruvate fermentation, which serves as starting period of acetaldehyde synthesis. On the second stage, when the essential level of acetaldehyde in the media is reached, the process of alcohol fermentation and ethanol production starts to prevail.

During alcohol fermentation the glycerol accumulation takes place majorly at induction period of glucose utilization, when the first molecule of 3-phosphoglycerol aldehyde recovers and transforms into glycerol, and the second acetifies to 3-phosphoglycerol acid and afterwards to pyruvate and acetaldehyde. Acetaldehyde accumulates and becomes competitor of phosphoglycerol aldehyde in Hydrogen acception. And then the alcohol fermentation and ethanol formation prevails.

In our research, with the initial mash concentration growth, the amount of glycerol in fermented worts was different even for relatively low sugar concentrations: the yeasts of experimental osmophilic race DS-02-E, which were adapted to high concentrations, produced 38% less glycerol, comparing to “Danisco” dry yeasts, in case of 12% m/m sugars wort fermentation. And for sugar concentration of 21% m/m experimental race produced 53% less glycerol.

Therefore, except for common in alcohol fermentation formation of glycerol, the other mechanism of its synthesis is involved. This can be explained by the fact that during the process of selection the yeasts, suitable for drying, which is a strong negative factor, “Danisco” company dry yeasts undertook the impact of substantive stress conditions. Thus, the increased glycerol production is one of achieved abilities of these yeasts. Apparently, the osmophilic experimental yeast race DS-02-E has less active HOG-way mechanisms of protection against stress. However, this has positive impact on alcohol fermentation in the ability to produce such by-product as glycerol.

The tendency of formation the majority of by-products in initial stages of their fermentation is observed not only for glycerol, but for other metabolism products, particularly organic acids [1]. Their amount sometimes even reduces in the end of this process.

Thus, we considered it expedient to investigate the accumulation of organic acids in case of mash concentration increase for selected races, because these metabolism by-products also have a negative effect on the substrate utilization ratio, rectified alcohol storage duration and also worsen their alcohol quality.

For example, propionic acid, the amount of which, along with butyric acid, is the largest among all other acids, gives bitter taste to alcohol. Moreover, during the production of alcoholic beverages it is hardly absorbed by either activated charcoal or other known sorbents [14].

The formation of propionic acid (Fig. 3) grew together with mash concentration increase. But, its amount was smaller in case of experimental yeasts fermenting the mash, comparing with dry yeasts. The amount of propionic acid in distillates was 15.5–20.8 mg/l of pure alcohol for experimental yeast strain respectively to investigated concentrations. In case of using dry yeasts these amounts were 20.6–31.6 mg/l of pure alcohol.

Thus, the experimental yeast race is more appropriate to be used in high concentration mash fermentation than commercial dry yeasts according to glycerol and propionic acid accumulation.

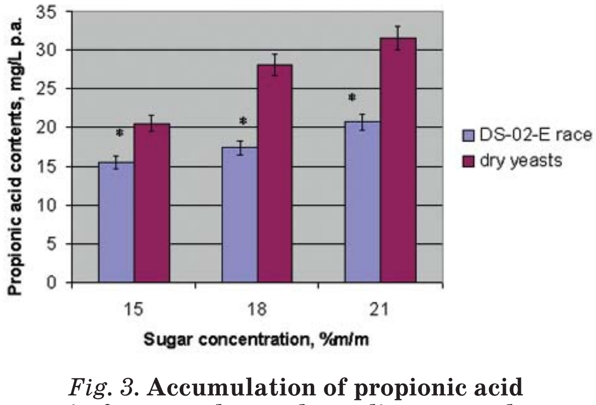

Fig. 3. **Accumulation of propionic acid in fermented wort depending on mash concentration by investigated yeast races**

Considering that glycerol production, as a yeasts mechanism of osmoadaptation to mash concentration increase, and propionic acid, as a secondary product of yeast metabolism, the formation was lower for experimental yeast race. It was natural to foresee the alcohol yield increase from source starch-containing feedstock during the fermentation of high-concentrated mash by experimental yeast race. This happens due to the fact that the majority of sugars transforms into ethanol, and is not spent to produce other metabolites. The results of research are displayed on Fig. 4.

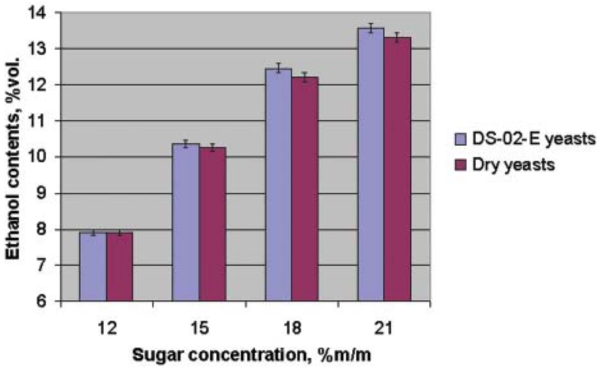

Fig. 4. **The dependence of mash concentration on alcohol contents in fermented wort by investigated yeast races**

As displayed on Fig. 4, when the initial sugar concentration was 12% m/m, alcohol accumulation was equal for both investigated races and reached 7.9 % vol. Yet, when media concentration grew up to 15–21% m/m sugars, DS-02-E yeasts synthesized 0.1–0.25 % vol. more alcohol against dry yeasts. This fact allows increasing the alcohol yield by 4.5–7.5 l/t of corn grain, considering normal ethanol yield per t of corn starch and starch contents in corn grain.

Considering received data, we can affirm, that DS-02-E race yeasts have less active osmoadaptation mechanisms, related to HOG-way, then "Danisco" company dry yeasts. Moreover, they have some other mechanisms, unrelated with glycerol production. This allows them to accumulate much less glycerol in fermented wort. This fact, along with reduced production of fermentation by-products such as propionc acid, reflects positively on fermentation dynamics, allows accumulating more ethanol in fermented wort. It also lets alcohol yield and potable alcohol quality increase.

So, the quality of highly concentrated mash fermentation greatly depends on yeast osmoprotection. The comparison of highly concentrated mash from starch containing feedstock we made showed 53% reduction in glycerol production by experimental yeast race DS-02-E against "Danisco" dry yeast yeasts, which are widely used in alcohol production enterprises. This confirms the engagement of osmoprotection mechanisms, which are different from HOG-way. It also shows less active systems of osmoadaptation, which are linked with glycerol production.

In the same time, using the experimental yeast race showed 0.1–0.25% vol. ethanol content increase. This will positively influence the economics and ecology of ethanol or bio-ethanol production by increasing the substrate (grain feedstock) utilization ratio. We also discovered the 34% reduction in formation of unnecessary fermentation by-product — the propionic acid. This fact also represents changes in metabolism of experimental yeast race, comparing it to widely used dry osmophilic yeasts.

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ЗАЛЕЖНІСТЬ НАКОПИЧЕННЯ ГЛІЦЕРОЛУ ТА ЗБРОДЖУВАННЯ ГІДРОЛІЗАТІВ КРОХМАЛЕВМІСНОЇ СИРОВИНИ ВІД КОНЦЕНТРАЦІЇ СУСЛА

С. Т. Олійнічук[1], *Т. І. Лисак*[1], *Л. В. Маринченко*[2]

[1]Інститут продовольчих ресурсів НААН України, Київ
[2]Національний технічний університет України «Київський політехнічний інститут»

E-mail: lolitamar@ukr.net

Метою роботи було дослідити залежність накопичення етанолу та утворення побічних і вторинних продуктів (гліцеролу і пропіонової кислоти) під час зброджування зі збільшенням концентрації сусла від 12 до 21% мас. цукрів на прикладі широко використовуваних у спиртовій галузі сухих комерційних дріжджів фірми «Danisco» та експериментального осмофільного штаму *Saccharomyces cerevisiae* ДС-02-Е, ізольованого з концентрованого (80% СР) солодового житнього сусла, що спонтанно забродило. Для розрідження та оцукрювання крохмалю сусла застосовували ензимні препарати «AMYLEX 4T», «ALPHALASE AFP» і «DIAZYM SSF». Готові виробничі дріжджі обох рас дріжджів вносили в бродильні колби у кількості 10% від об'єму основного сусла. У зрілих бражках визначали вміст незброджених вуглеводів фотоелектроколориметричним методом з антроновим реактивом, спирту — скляним ареометром-спиртоміром, кислотність — потенціометрично, концентрацію сухих речовин — ареометрично, вміст гліцеролу — фотоколориметричним методом. У дистилятах бражки визначали вміст летких домішок, зокрема пропіонової кислоти, методом газової хроматографії. Статистичну обробку результатів трьох

ЗАВИСИМОСТЬ НАКОПЛЕНИЯ ГЛИЦЕРОЛА И СБРАЖИВАНИЯ ГИДРОЛИЗАТОВ КРАХМАЛОСОДЕРЖАЩЕГО СЫРЬЯ ОТ КОНЦЕНТРАЦИИ СУСЛА

С. Т. Олийничук[1], *Т. И. Лысак*[1], *Л. В. Маринченко*[2]

[1]Институт продовольственных ресурсов НААН Украины, Киев
[2]Национальный технический университет Украины «Киевский политехнический институт»

E-mail: oliynichuk@ipr.net

Целью работы было исследовать зависимость накопления этанола и образования побочных и вторичных продуктов (глицерола и пропионовой кислоты) в процессе сбраживания при увеличении концентрации сусла от 12 до 21% мас. сахаров на примере широко используемых в спиртовой отрасли сухих коммерческих дрожжей фирмы «Danisco» и экспериментального осмофильного штамма *Saccharomyces cerevisiae* ДС-02-Е, изолированного из концентрированного (80% СВ) солодового ржаного сусла, которое спонтанно забродило. Для разжижения и осахаривания крахмала сусла использовали энзимные препараты «AMYLEX 4T», «ALPHALASE AFP» и «DIAZYM SSF». Готовые производственные дрожжи обеих рас вносили в бродильные колбы в количестве 10% от объема основного сусла. В зрелых бражках определяли содержание несброженных углеводов фотоэлектроколориметрическим методом с антроновым реактивом, спирта — стеклянным ареометром-спиртомером, кислотность — потенциометрически, концентрацию сухих веществ — ареометрически, содержание глицерола — фотоколориметрическим методом. В дистиллятах бражки определяли содержание летучих примесей, в частности пропионовой серій дослідів проводили шляхом розрахунку середніх арифметичних величин із п'яти вимірювань, їх середньоквадратичних відхилень і похибок. Для виявлення вірогідних відмінностей між середніми величинами використовували критерій Стьюдента. Розбіжності вважали статистично достовірними за $P < 0{,}05$.

Показано зменшення накопичення гліцеролу (від 38 до 53%) за підвищених концентрацій живильного середовища у разі застосування дріжджів *Saccharomyces cerevisiae* ДС-02-Е порівняно із сухими комерційними дріжджами, зменшення утворення небажаного побічного продукту бродіння — пропіонової кислоти (до 34%), кращу здатність експериментальної раси використовувати цукри сусла і накопичувати етанол (до 0,1–0,25% об.). Зроблено висновок про існування інших механізмів осмопротекції, не пов'язаних з high-osmolarity glycerol-шляхом, або використання менш активної системи утворення гліцеролу у відповідь на осмотичний стрес. Практичне значення роботи у разі використання нового експериментального осмофільного штаму дріжджів полягає у збільшенні глибини утилізації субстрату та виходу етанолу з крохмалевмісної зернової сировини, що позитивно позначиться на економіці та екології виробництва етанолу (біоетанолу).

Ключові слова: сусло, осмофільність, дріжджі, гліцерол.

кислоты — методом газовой хроматографии. Статистическую обработку результатов трех серий опытов проводили путем расчета средних арифметических величин из пяти измерений, их среднеквадратических отклонений и погрешностей. Для определения вероятных отличий между средними величинами использовали критерий Стьюдента. Расхождения считали статистически значимыми при $P < 0{,}05$.

Показано уменьшение накопления глицерола (от 38 до 53%) при повышенных концентрациях питательной среды в случае использования дрожжей *Saccharomyces cerevisiae* ДС-02-Е в сравнении с сухими коммерческими дрожжами, уменьшение образования нежелательного побочного продукта брожения — пропионовой кислоты (до 34%), лучшую способность экспериментальной расы использовать сахара сусла и накапливать этанол (до 0,1–0,25% об.). Сделан вывод о существовании других механизмов осмопротекции, не связанных с high-osmolarity glycerol-путем, или использовании менее активной системы образования глицерола в ответ на осмотический стресс. Практическое значение работы с использованием нового экспериментального осмофильного штамма дрожжей состоит в увеличении глубины утилизации субстрата и выхода этанола из крахмалсодержащего зернового сырья, что позитивно скажется на экономике и экологии производства этанола (биоэтанола).

Ключевые слова: сусло, осмофильность, дрожжи, глицерол.