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BIOTECHNOLOGY OF ALCOHOL FERMENTATION WITH YEAST RECIRCULATION

L. Levandovsky, professor
O. Vitriak, associate professor
M. Demichkovska, Ph.D., senior teacher
Department of Technologies and Organization of Restaurant Business
Kyiv National University of Trade and Economics,
St. Kioto, 19, Kyiv, Ukraine, 02158

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Correspondence:

L. Levandovsky
E-mail: levandov7@gmail.com

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Abstract. In recent decades, there has been a tendency in the world to increase ethanol production significantly in order to solve energy problems, that is, to use it as a biofuel. The factors determining the production cost of targeted biotechnological products include the output of these products from the raw materials used. One of the modern and effective ways to intensify alcoholic fermentation and reduce the cost of fuel ethanol is yeast recirculation. The research objects were: raw material (sugarbeet molasses), molasses wort, yeast *Saccharomyces cerevisiae* of the strain M-5, fermented wash and its distillates. In the raw materials, intermediate products, and fermented wash, the techno-chemical parameters recommended by the current technology regulations for obtaining spirit from molasses have been determined. Acoholic fermentation was carried out in an industrial environment, in a battery of series-connected fermentors. Recirculation of yeast was carried out by separating it from the final stage of fermentation, concentrating it on the separator, and introducing it into the first fermentor. The experimental data obtained prove that for wort fermentation, it is effective to use yeast that recirculates in the anaerobic stage. It has been established that the alcohol-forming power of recycled yeast increases as the yeast adapts to the environment in which it has been staying for a long time. The yeast becomes more active biochemically, with more efficient metabolism. Its need for continuously cultured biomass is reduced, the share of aerobically assimiliated sugars decreases, and, consequently, the losses during yeast generation are fewer. At the same time, accelerating the initial period of anaerobic fermentation helps inhibit the biosynthesis of glycerol, the formation of which consumes the largest amount of sugar among all the secondary products. The parameters of molasses wort fermentation, with yeast biomass recirculating, have been determined in an industrial environment. It has been established that the alcohol output from the raw materials increases as the synthesis of secondary metabolic products weakens. The advantages of this fermentation method will be used in further studies, namely when fermenting molasses wort, with an increased concentration of dry matter, in order to reduce the specific heat energy consumption in production and to make it cheaper. The developed biotechnology of alcohol can be usefully employed to produce fuel ethanol, and increasing its production will contribute to Ukraine's energy self-sufficience.

Keywords: yeast, molasses wort, fermentation, yeast recirculation, ethanol.

Introduction. Formulation of the problem

In recent decades, there has been a tendency in the world to increase ethanol production significantly in order to solve energy problems, that is, to use it as a biofuel [1-3]. The problem is important and global, so any steps and improvements to make this product more efficient in manufacturing are topical, as they lay the groundwork to make the products of processing enterprises more competitive [4,5]. The factors determining the production cost of targeted biotechnological products include the output of these products from the raw materials used and the specific energy consumption.

Alcoholic fermentation of sugar-bearing substrates is made the basis for the process when sugars obtained

from plant raw materials or sugar production waste (molasses) are transformed into ethanol.

Using yeast recirculation is one of the modern and effective methods to intensify alcoholic fermentation. In this paper, it is shown that increasing the concentration of yeast by its repeated use in fermentation improves the technological, kinetic, and environmental characteristics of the technology [6,7].

Analysis of recent research and publications

The factors determining the production cost of targeted biotechnological products include the output of these products from the raw materials used and the specific energy consumption. Besides, production of alcohol from plant raw materials is not low-waste: obtaining a litre of alcohol is accompanied by the formation of 12–14 litres of waste water with a high concentration of organic substances, harmful to natural ecosystems [4,8]. Solving the problem of rational utilization or recycling of these wastes requires significant material costs [1,3].

To solve these problems, scientists from different countries have suggested a number of technological improvements aimed at making ethanol production cheaper and more environmentally-friendly. The main way to reduce energy consumption at the fermentation stage in alcohol production is increasing its concentration in the fermented wash [4,9]. This can be achieved in the following ways:

– gradual introduction of the substrate at the main stage of fermentation [4,10,11];

– increasing the concentration of the dry matter (DM) of the fermented substrate, using selected osmotolerant strains of alcohol producing microorganisms [4,8,12], with vacuum created for fermentation [1], with immobilization of yeast on various carriers in the fermentation zone [6,8,13], etc.

It is known that in alcoholic fermentation of sugar-bearing substrates by yeast, two processes, each with a different purpose, are applied, one after the other [4]. In the first process (aerobic), there is partial conversion of sugars in the Krebs cycle, which is accompanied by accumulation of yeast biomass and formation of a certain amount of secondary fermentation products. In the following process, anaerobic fermentation of sugars takes place, with the formation of ethanol. Thus, during alcoholic fermentation, part of the substrate is spent on the growth and reproduction of the producer cells and their metabolism, with the formation of acetic acid, acetic aldehyde, glycerol, higher alcohols, esters, etc. [1,5]. These processes are accompanied by using fermented sugars, so creating conditions for reducing the synthesis of these substances provides a reserve of sugars to be converted into alcohol, thus increasing the output of the target product.

An important factor in successful fermentation is the high physiological activity of the producer. Its value corresponds to the amount of alcohol formed by one gram of yeast per unit time [14]. In industrial processes, the specific ethanol formation rate varies widely depending on the physiological characteristics of the producer, the biomass concentration, the type of apparatus, and the mode of fermentation [10].

During the continuous process, it is the the medium dilution rate that mainly determines how completely the substrate is used. In this case, there are contradictions between the two parameters that are based upon when optimizing the fermentation process: the final alcohol output from carbohydrates of the raw materials, and the completeness of their fermentation. Coordination of these parameters determines the duration, productivity, and efficiency of alcoholic fermentation [1].

Disadvantages of alcoholic fermentation (the main ones are its long duration and incomplete use of the substrate) can be to some extent eliminated by multiple use of the yeast biomass [14,15].

This technological method has been studied by scientists from different countries. There are data on the effect of yeast recirculation in alcoholic fermentation of sugar-bearing wort that indicate an increase in the rate of the process and intensification of alcohol formation [16,17]. However, in scientific publications on this problem, there are practically no research results about the metabolism of recirculated yeast compared with the classical process of alcoholic fermentation.

Based on the above, **the purpose** of our research was to determine how recirculation of yeast biomass in the anaerobic stage of ethanol fermentation effects on the direction of saccharide catabolism, biochemical activity, and alcohol-forming power of yeast.

The research **objectives** are:

1. To determine the optimum concentration of the yeast inoculum for molasses wort fermentation.

2. To investigate how the technological parameters of the process depend on yeast recirculation from the final fermentation stage to the initial one.

3. To determine the characteristic features of metabolism and the alcohol-forming power of yeast recycled in the anaerobic stage.

Research materials and methods

The objects researched were raw material (sugar-beet molasses), molasses wort, yeast *Saccharomyces cerevisiae* of the strain M-5, fermented wash and its distillates.

The dry matter content (DM) in the molasses and molasses wort was determined by refractometry, the pH by electrometry, the acidity by electrometric titration. The total of fermented sugars was calculated basing on the values of direct and inverse polarization and invert sugar content [4].

The experimental sugar-beet molasses contained 49.6% of fermented carbohydrates and 78.8% of DM. Nitrogen (carbamide) and phosphorus (H_3PO_4) were

added to the molasses, and it was diluted with water to the required concentration (21–22% of DM). The active acidity of the medium was set at pH 5.2 by acidification with sulphuric acid [4].

Alcoholic fermentation was carried out in an industrial environment, in a battery of series-connected fermentors. Yeast for wort fermentation was grown in three yeast generators that worked in parallel, in low aeration conditions (the medium was air-blown). Recirculation of yeast was carried out by separating it from the final fermentation stage by concentrating it on the separator and introducing into the first fermentor.

The fermented wash was analysed according to the indicators of true DM concentration, acidity, yeast biomass, alcohol concentration, unfermented carbohydrates by the methods used in the theory and practice of alcohol production [18].

In the intermediate products and fermented wash, the perceived and true concentrations of DM were determined areometrically, the yeast concentration by the weight method in terms of 75% humidity, the alcohol concentration by the pycnometer method in the distillate of the wash. The number of unfermented carbohydrates in the wash was determined by the resorcinol-colorimetric method [4]. The glycerol and aldehyde content was determined in wash distillates [18].

The criteria for evaluating the rate of biochemical reactions of sugar fermentation are kinetic parameters. In our research, the following parameters were determined:

the duration of stay of the nutrient medium in the apparatus, *h*:

$$T = 1/D, \quad (1)$$

where D is the dilution rate of the medium in the apparatus, h^{-1};

sugar fermentation rate, $kg/m^3 \cdot h$:

$$W = S/T, \quad (2)$$

where S is the sugar concentration in the medium, kg/m^3.

Results of the research and their discussion

In order to intensify the fermentation of molasses wort while producing alcohol, different amounts of inoculating yeast (7.5, 15, 30, 60, and 120 g/dm^3) have been used in the research of this process in a laboratory environment (Table 1).

Table 1 – Results of fermenting the molasses wort with different quantities of inoculating yeast
($X \pm m$; $m \leq 0.05$)

Parameters of fermented wash	The quantity of inoculating yeast, g/dm^3				
	7.5	15	30	60	120
Duration of fermentation, hours	62	48	36	27	23
Yeast biomass, g/dm^3	20.3	25.2	37.0	63.8	122.7
Yeast growth, g/dm^3	12.8	10.2	7.0	3.8	2.7
Unfermented sugars, $g/100\ cm^3$	0.65	0.58	0.49	0.47	0.41
Alcohol content, % vol.	11.71	11.82	11.83	11.94	11.79
Glycerol, g/dm^3	10.5	7.9	7.3	6.3	6.1

It has been proved that with an increase in the concentration of the inoculating biomass from 7.5 to 120 g/dm^3, the fermentation time naturally decreased from 62 to 23 hours, the yeast growth from 12.8 to 2.7 g/dm^3, and the amount of unfermented carbohydrates from 0.65 to 0.41 $g/100\ cm^3$.

It has been determined that the accumulation of the target product (alcohol) is extreme in character, and its maximum (11.83–11.94% vol.) was with the initial concentration of yeast biomass ranging between 30 and 60 g/dm^3. The nature of glycerol formation is interesting, too: its content gradually decreased with an increase in the amount of inoculating yeast. This means that the inhibition of the process was becoming weaker as a result of the intensification of alcoholic fermentation. This fact, to some extent, explains the increase in the amount of alcohol in the fermented wash in the cases with higher concentrations of the inoculating yeast.

In an industrial environment, the research was carried out in three variants. As a control, the traditional technology of molasses wort fermentation was used. In the first experimental sample, yeast was recycled without increasing the wort flow into the anaerobic stage, and in the second, this parameter was increased from 0.43 (control) to 0.57 h^{-1}. The results are shown in Table 2.

From Table 2, it is evident that in the control, the yeast biomass was gradually increasing in the medium, from 25.2 g/dm^3 in the aerobic stage of the process to 27.4 and 31.9 g/dm^3 at the beginning and at the end of the anaerobic stage.

Partial recirculation of yeast during fermentation, with other conditions remaining the same (Experiment 1), allowed accumulating 43.8 grams of yeast in 1 dm^3 of the medium in the first apparatus, thus reducing its concentration in the last (eighth) apparatus (up to 10.8 g/dm^3).

As a result, there was a significant increase in the rate of fermentation of sugars from 13.8 (control) to 32.5 $kg/m^3 \cdot h$, and a decrease in the duration of anaerobic fermentation, respectively, from 17.6 to 13.0 hours. So, it was quite logical to increase the ethanol concentration in the first fermentor to 5.9 compared to 5.1% vol. in the control due to the intensification of the process. This also resulted in deeper absorption of the nutritive medium sugars by the yeast: the content of unfermented sugars in the last

fermentor was 1.80 g/dm^3 (Experiment 1) compared to 2.1 g/dm^3 in the control. Experiment 2 was conducted with the amount of aerobically grown yeast significantly reduced: only one yeast generator functioned (three yeast generators functioned in the case of the control and experiment 1). Besides, an additional amount of molasses wort was added to the first fermentor of the anaerobic stage, which resulted in the dilution rate of the medium (D) in the fermentor increased to 0.57 h^{-1} (27%). Thus, in Experiment 2, most of the raw material (over 80 %), in the form of wort, came to the anaerobic stage of the process and was fermented both by grown and by recycled yeast introduced into the first fermentor in the ratio 1:2 (in Experiment 1, 3:2).

Table 2 – The effect of yeast recirculation in the anaerobic fermentation stage on the results of the process
($X \pm m$; $m \leq 0.05$)

Technological parameters of the medium	Control (the process without yeast recirculation)	Experiment 1, with recirculation, without increasing D	Experiment 2, with recirculation and with D increased
The dilution rate of the medium (D) in the first fermentor	0.43	0.45	0.57
Yeast biomass, g/dm^3:			
– after the aerobic stage	25.2	25.0	25.5
– in the first fermentor	27.4	43.8	35.1
– in the last fermentor	31.9	10.8	20.2
Ethanol concentration, % vol.:			
– after the aerobic stage	3.8	3.9	3.9
– in the first fermentor	5.1	5.9	5.0
– in the last fermentor	8.21	8.32	8.44
Unfermented sugars, g/dm^3:			
– after the aerobic stage	81.3	81.0	81.9
– in the first fermentor	25.5	42.3	57.8
– in the last fermentor	2.1	1.8	2.1
The rate of fermentation of sugars in the first fermentor, kg/m^3 • h	13.8	32.5	39.6
Duration of anaerobic fermentation, h	17.6	13.0	13.4

As a result, the distribution of biomass in the apparatus changed in character due to the decrease in the total number of aerobically grown yeast and the increase in value D.

Although in Experiment 1, the biomass content in the last fermentor decreased (up to 10.8 g/dm^3), in the first fermentor, this parameter remained at a rather high level of 35.1 g/dm^3 due to continuous yeast recirculation. As a result, the fermentation rate of sugars in the first fermentor increased from 13.8 in the control to 32.5 (Experiment 1) and 39.6 kg/m^3•h (Experiment 2), and the fermentation time, respectively, from 17.6 to 13.0 and 13.4 hours.

The alcohol-forming activity of the yeast population in the first fermentor was determined by the specific rate of ethanol formation by the yeast. This parameter allows comparing processes that differ in the dilution rate, the amount of biomass, and the ethanol content. As can be seen from Table 3, only the use of anaerobically recycled yeast in fermentation (Experiment 1), with all other parameters remaining the same, led to an increase in the specific rate of ethanol formation from 4.12 (control) to 4.46 cm^3/g•day, i. e. 8.3%. Increasing the wort flow in Experiment 2 intensified this effect, which is proved by the further increase in the specific rate of ethanol biosynthesis by yeast up to 4.98 cm^3/g • day (i. e. by 17%) compared with the control. Table 3 contains the research data on the glycerol and aldehydes content.

Table 3 – Effect of yeast recirculation in the anaerobic fermentation stage on yeast metabolism
($X \pm m$; $m \leq 0.05$)

Research variants	Acetaldehyde content, % vol.		Glycerol content, g/dm^3		Specific rate of alcohol biosynthesis by yeast, g/cm^3•day
	First apparatus	Fermented wash	First apparatus	Fermented wash	First apparatus
Control (no yeast recirculation)	0.0195	0.092	3.84	5.46	4.12
Experiment 1, with recirculation, without increasing D	0.0175	0.083	3.38	4.80	4.52
Experiment 2, with recirculation and with D increased	0.0134	0.051	3.05	4.38	4.95

Analysis of the above results allows concluding that processes involving recirculation of yeast biomass differ in glycerol formation, which is lower at the start of fermentation (in the first apparatus): 3.38 (Experiment I) and 3.05 (Experiment 2), compared with 3.84 g/dm^3 in the control. The same tendency was observed in the fermented wash: the glycerol content in the experimental variants involving yeast recirculation was significantly lower than that in the control (i. e. without recirculation). It was, respectively, 4.80 and 4.38, compared to 5.46 g/dm^3.It should be noted that these quantities correlate with the specific rate of ethanol biosynthesis by yeast: the higher the rate, the less glycerol is formed. In Experiment 2, there was less glycerol than in Experiment 1. This confirms the hypothesis of a shorter starting period of ethanol fermentation where yeast is used that is recycled in the anaerobic stage of the process. Such results show an improvement in the physiological state and an increase in the enzymatic activity of recycled yeast when increasing the supply of the nutritive medium.

The data on the acetaldehyde content (Table 3) can be interpreted in the following way: intensifying the initial fermentation period contributes to rapid acetaldehyde conversion, too. If in the first fermentor of the control variant, there was 0.0190 % vol. of it, then in Experiments 1 and 2, its content decreased, respectively, to 0.0166 and 0.0129% vol.

Reduction in the formation of these products and a smaller amount of sugars spent on their synthesis became a reserve source to increase ethanol synthesis in the fermented wash to 8.32% vol. (Experiment I) and to 8.44% vol. (Experiment 2), compared to 8.21% vol. in the control (Table 2).

Conclusion

The experimental data presented prove that yeast recycled in the anaerobic stage are practical in alcoholic fermentation, since it has a higher biochemical activity and alcohol-forming power. This reduces the need for large amounts of continuously grown biomass, decreases the proportion of aerobically assimilated sugars, and, consequently, their losses in yeast generation. At the same time, the acceleration of the initial period of anaerobic fermentation inhibits glycerol biosynthesis that consumes most sugar among all secondary products.

So, the use of inoculating yeast recycling in anaerobiosis can be a means of regulation to increase the yield of ethanol and is an effective way to increase the productivity of fermentation equipment.

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БІОТЕХНОЛОГІЯ СПИРТОВОЇ ФЕРМЕНТАЦІЇ З РЕЦИРКУЛЯЦІЄЮ ДРІЖДЖІВ

Л.В. Левандовський, доктор технічних наук, професор, *E-mail*: levandov7@gmail.com
О.П. Вітряк, кандидат технічних наук, доцент, *E-mail*: vitrox@ukr.net
М.П. Демічковська, кандидат технічних наук, старший викладач, *E-mail*: mira_d2011@ukr.net
Кафедра технології і організації ресторанного господарства
Київський національний торговельно-економічний університет, вул. Кіото, 19, м. Київ, Україна, 02158

Анотація. В останні десятиліття в світі спостерігається тенденція до суттєвого збільшення об'ємів виробництва етанолу для вирішення енергетичних проблем, тобто використання його як біопалива. Одним із сучасних і ефективних способів інтенсифікації спиртового зброджування і здешевлення паливного етанолу є використання рециркуляції дріжджів. Об'єктами досліджень були: сировина (цукробурякова меляса), мелясне сусло, дріжджі *Saccharomyces cerevisiae* штаму М-5, дозріла бражка та її дистиляти. У сировині, напівпродуктах і дозрілій бражці визначали технохімічні показники, що рекомендовані діючим технологічним регламентом виробництва спирту із меляси. Процес спиртової ферментації здійснювали у промислових умовах в батареї ферментерів, що з'єднані послідовно. Рециркуляцію дріжджів здійснювали шляхом виділення їх із кінцевої стадії бродіння, концентрування на сепараторі та введення у головний ферментер. Отримані експериментальні дані свідчать про ефективність застосування для ферментації сусла дріжджів, що рециркулюють в анаеробній стадії. Встановлено факт посилення спирторутворювальої здатності рециркульованих дріжджів за рахунок їхньої адаптації до умов середовища внаслідок довготривалого перебування у ньому. Вони мають більш високу біохімічну активність і економічність метаболізму. При цьому скорочується потреба у безперервно вирощуваній біомасі, зменшується доля аеробно асимільованих цукрів і скорочуються за рахунок цього їхні втрати при дріжджегенеруванні. У той же час, прискорення початкового періоду анаеробної ферментації сприяє інгібуванню біосинтезу гліцерину, на утворення якого витрачається найбільша кількість цукру серед усіх вторинних продуктів. Визначено параметри процесу зброджування мелясного сусла з рециркуляцією дріжджової біомаси в промислових умовах і встановлено, що має місце збільшення виходу спирту із сировини за рахунок послаблення синтезу вторинних продуктів метаболізму. Переваги цього способу ферментації будуть використані в подальших дослідженнях, а саме, при зброджуванні мелясного сусла підвищеної концентрації сухих речовин з метою скорочення питомих витрат теплової енергії у виробництві і його здешевлення.

Ключові слова: дріжджі, мелясне сусло, зброджування, рециркуляція дріжджів, етанол.

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