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Review Article

THE STUDY OF ETHANOL PRODUCTION BY NEW STRAIN OF YEASTS "HANSENIASPORA OPUNTIAE MK 460485", INVESTIGATION OF ITS ETHANOL PRODUCTION IN PRESENCE OF DIFFERENT CARBON AND NITROGEN SOURCES, AND OPTIMAL CONDITIONS

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Abstract
42 native yeast strains isolated from 5 wine grapes of the Tehran Center for Fruits and Vegetables were studied, among which 6 strains were selected according to the results of tests for resistance to high concentrations of ethanol and glucose; according to the results of screening for the amount of ethanol produced, one strain was left in the study, fermenting the maximum amount of alcohol in a given culture medium 16%. Strain number Af was a new strain of yeast, identified in another study and named by the author of this article as Hanseniaspora Opuntiae MK 460485. Quantitative chromatographic evaluation of ethanol production by Hanseniaspora opuntiae MK 460485 strain was carried out for various carbon sources (glucose, sucrose, fructose syrup, malt, molasses), which showed that the highest amount of ethanol is fermented with yeast in the presence of molasses (17%). strain with various sources of nitrogen (ammonium phosphate, ammonium sulfate, urea, ammonium nitrate, peptone and ammonium chloride) showed that the best production of alcohol goes to culture media x to ammonium nitrate (17.3%). The main factors for ethanol fermentation were screened and optimized based on the mathematical method of experiment planning using the Minitab© program and the Plackett-Burman package. It was shown that the main factors in ethanol production by this strain are sources of carbon, nitrogen, and temperatures, and pH does not have a significant effect. A mathematical model is obtained, based on which the optimal conditions for the cultivation of the strain are revealed: molasses concentration 56.5% ammonium nitrate 3 g/l; cultivation temperature 30.4 °C. Ethanol production under optimal conditions increased upto 19.6%, that is considerable result and investigation in the area of ethanol production.

Key words: Yeast, Ethanol, Carbon, Nitrogen, Hanseniaspora opuntiae MK 460485.

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INTRODUCTION

Today's world is faced with many problems that can easily turn into complex crises, critical for future generations, if now we do not try to reduce their impact [1]. People, even at the beginning of the formation of the alcohol industry did not pay attention to the negative consequences of its development, and only at the end of the 20th century, scientists warned of some negative consequences of rapid industrial development [9].

One of the most negative aspects of the industrial development of the world over the past two centuries is environmental pollution, which includes various types, including pollution of air, water and soil. The growth of carbon dioxide emissions into the atmosphere of our planet over the past 260 years, indicating an increase in the concentration of this toxin in the atmosphere by 38,000 million times, shown in Figure 1.1, indicates the catastrophic state of the atmosphere on our planet [3]. On the other hand, the unreasonable use of fossil fuels will not only reduce the access of future generations to these universal resources that have been created over many millions of years, but also lead to their depletion, and also leave a legacy for future generations in the form of irreversible pollution of the planet [4].

In this regard, one of the main solutions that will help reduce environmental pollution and reduce the overuse of fossil fuels is to switch to alternative fuels from renewable resources [5].

The production of biofuels based on the use of renewable biological resources is currently a priority in the fuel industry of many European and American countries [2]. One of the most important types of biofuels is bioethanol, which is produced mainly from plant materials containing starch and cellulose. These are: sugarcane, wheat, barley, rye, corn, sugar sorghum, sugar beets, Jerusalem artichoke and others [6]. Bioethanol can be produced in large quantities from cellulose, various agricultural and forestry waste are best suited for this: straw of grain crops (wheat, rye, and rice), grain processing waste, sugarcane biomass, sawdust, etc. [7, 8].

The basis of the production of edible alcohol and bioethanol as a fuel agent is the alcoholic fermentation of organic products containing carbohydrates (starch or cellulose) under the action of yeast and bacteria enzymes. Fermentation results in a solution containing not more than 15% ethanol, since in more concentrated alcoholic solutions, yeast usually dies [5]. Ethanol obtained in this way needs to be purified and concentrated, which is achieved traditionally by distillation. To obtain ethanol by this method, various strains of Saccharomycetes Class yeast are most often used, and pre-treated wood chips and/or a solution obtained from them are used as a nutrient medium [9]. A disadvantage of the known strains is the insufficiently high yield of alcohol, the use of low concentrations of fermented wort, low competition of fermentation and respiration (the process must be conducted under anaerobic conditions or mutant yeasts that have lost mitochondria and are unable to breathe); high sensitivity to ethanol, which reduces the yield of the target product per unit volume of the bioreactor (mutants resistant to ethanol, characterized by an altered structure of cell membranes, are necessary) [10]. That is why the purpose of this

study been to achieve an effective strain for producing alcohol and to find optimal conditions for its growth and fermentation.

MATERIAL AND METHODS

In this study, five different grape varieties were used to breed yeast producing bioethanol and optimize the conditions of fermentation production. Samples were collected in sterile plastic bags and transferred to a laboratory at 4 ° C for further research. After transferring the sample to the laboratory, in order to separate the yeast flora, 200 g from each selected grape variety was transferred to water bottles designed to carry out the fermentation process and increase the microbial load of the samples. Under these conditions, the samples were kept for 14 days at 25 °C.

Isolation and screening

After 14 days of storage of samples under the described conditions, samples were taken from each already fermented grape variety in a volume of 10 ml, 90 ml of physiological serum containing 1% polysorbate-80 was added to them, the contents were transferred to 250 mm sterilized flasks, which were placed in a centrifuge, where they were for 2 hours at a temperature of 25 °C and a rotation speed of 150 rpm. Thanks to this method, microorganisms are separated from the fermented pieces of grapes. After that, these solutions were stored for 24 hours under standard conditions at a temperature of 25 °C. Then, 50 ml of each of these samples was centrifuged for 5 min. at 200 rpm a series of dilute solutions was prepared from these resulting solutions at a ratio of 1:10 to 10: 10. In the next step, 200 ml of each solution was placed in Petri dishes containing YPD growth medium (composition: 20 g/l glucose, 20 g/l peptone, 10 g/l yeast extract and 17 g/l agar) (This is the main composition of this cultural environment; but at different stages of this study, in accordance with the conditions of that stage, this composition was changed). For each dilution, they were preheated and two plates were held at 30 °C for 24 and 48 hours, respectively. Rotary yeast is isolated based on the morphological characteristics of the colonies. The purification procedure was carried out by re-culturing the colonies appearing in YPD medium.

Screening for yeast strains producing ethanol

The production of ethanol by fermentation is most often carried out using strains of S. cerevisiae yeast and their varieties from new biomass, for example, honey molasses and corn. Among all the characteristics, 2 main factors were used as the main ones for screening, which are the most important for the production of ethanol in an industrial environment. These are: “resistance to high concentrations of ethanol” and “resistance to high osmotic pressure in the presence of glucose” [11], since they are directly related to the production of ethanol by yeast.

Assessment of resistance to various concentrations of ethanol

Ethanol is a toxic substance for yeast, and the ability of the yeast to withstand (tolerate) high concentrations of ethanol is an important parameter for industrial strains producing ethanol.

In this study, YPD medium with slight modifications was used to measure the ability to tolerate different concentrations of ethanol. For this, after the distribution of nutrient media in test tubes and after autoclaving, they were cooled to 24-25 ° C. Then, in the same tubes, different amounts of ethanol were inoculated in each tube to achieve a certain concentration of ethanol. In this experiment, we investigated the effect of ethanol concentrations of 17, 18, 19, 20, and 21% on these yeasts. For this, various concentrations of ethanol with an inoculum from a 24-hour yeast culture medium with a turbidity of 2 according to McFarland were inoculated into test tubes. After 24 h of heating, the growth rate of each isolate was studied by spectrophotometric turbid metric method at a wavelength of 600 nm.

The study of growth in high concentrations of sugar

In this study, to study the growth and tolerance of yeast to high glucose concentrations, which is directly related to the amount of ethanol produced by the yeast, we used YPD culture medium designed for growing strain isolates supplemented with 50% and 60% glucose. After 24 hours of heating, the growth rate of each isolate was investigated using a spectrophotometric method for determining turbidity at a wavelength of 600 nm.

After this step, we evaluated the ability of ethanol production by six selected strains after 48 hours in YPD medium (50% glucose concentration).

Optimization of ethanol production by environmental and internal factors

In this study, in order to optimize the conditions for maximum ethanol production by the selected yeast isolate, we used the experimental design method using the "Response surface method" (response surface analysis method). This method allows you to get results on optimizing production conditions of increased reliability while saving time and materials, significantly reducing the number of tests and analyzes. For this purpose, firstly, use the Plackett–Burman method, in which the effective factors were checked by the Minitab© 17.1.0 version software application. It should be noted that, since in the methods of planning experiments it is necessary to take into account two values for each factor, in order to study the effect on the volume of ethanol produced by such important factors as the carbon source and the nitrogen source, we first conducted a one-factor experiment with varying one factor - time.

Evaluation of ethanol production at various carbon sources

In this study, the following carbon sources were used to evaluate the effectiveness of bioethanol production by yeast: glucose, sucrose, fructose syrup, glucose syrup, molasses and whey. Each of these individual carbon sources was autoclaved at 110 ° C. for 10 minutes and added to the ingredients of the growth medium. Then, a yeast suspension of 3% McFarland turbidity was introduced into these media with various carbon sources and kept in incubator shakers at 30 ° C and a stirring speed of 150 rpm for 48 hours. Further, the efficiency of the process was studied in inoculums by the amount of ethanol produced in each of the fermentation media with different carbon sources.

Evaluation of ethanol production at various nitrogen sources

To determine the best nitrogen source for the production of bioethanol in fermentation media in the presence of an optimal carbon source, 1 such nitrogen sources as ammonium sulfate, urea, ammonium nitrate, and ammonium phosphate were used. After preparing the culture medium and sterilizing it in an autoclave, 5 ml of yeast suspension (3% turbidity according to McFarland) was incubated in it and kept in incubator shakers at 30° C and a stirring speed of 150 rpm for 48 hours. Next, inoculums studied the effectiveness of the process by the amount of ethanol produced in each of the fermentation media with different sources of nitrogen.

Screening for factors affecting ethanol fermentation

At this stage, we used the results of ethanol production optimization with varying the following factors:

the use of various sources of carbon and nitrogen (with varying time factors); the influence of two sources of carbon (sucrose and glucose) and nitrogen (ammonium sulfate and ammonium chloride); the results of five concentrations of the carbon source (range 20-90%) and five concentrations of the nitrogen source (range 1-5%) at temperature ranges of 25-37 ° C and at pH intervals of 4-7;

the effect of inoculum solution concentration values.

All factors were analyzed using the Minitab© software using the Plackett–Burman method.

After developing an experimental design using the Plackett–Burman method, each of the experiments provided by the application was carried out under laboratory conditions. Further, by screening by analyzing the results obtained, the most significant factors affecting the efficiency of ethanol production by yeast were identified.

Optimization of factors affecting ethanol production

After identifying effective factors affecting ethanol production, the optimization of each of the factors separately and their interactions were investigated using the so-called "surface response" method or RSM method. The surface response method (RSM) is a combination of mathematical and statistical methods used to study the relationship between one or more responses with a set of factors. It makes it possible to find the optimal values of factors in the desired area with a minimum number of tests. One subsection of the RSM method is the Box-Behnken or BBD design method.

At this stage of the study, to optimize the values of the factors selected by the Plackett–Burman method, we developed an experimental design for three key factors. These are: carbon source, temperature and ph.

After the test of each series of experiments presented by the software, the corresponding studies were conducted in the laboratory. Then, by analyzing the results of each test, the best conditions were provided in the next series of experiments. Further, comparing the laboratory results with the obtained software, the optimal fermentation conditions were determined, which were checked in a laboratory environment.

RESULTS AND DISCUSSION

Sample selection

In this study, five different varieties of wine grapes were used to isolate yeast isolates producing ethanol and optimize fermentation conditions. Grapes from Flame Seedless, Sultanina, Fakhri, Muscat Ottonel and Pinot Noir (all from the species Vitis vinifera) were collected at the Tehran Center for Fruits and Vegetables (Tehran; Rd. Azadegan, pr. Behesht Zahra). Photos of used grape varieties are shown in Figure 1.

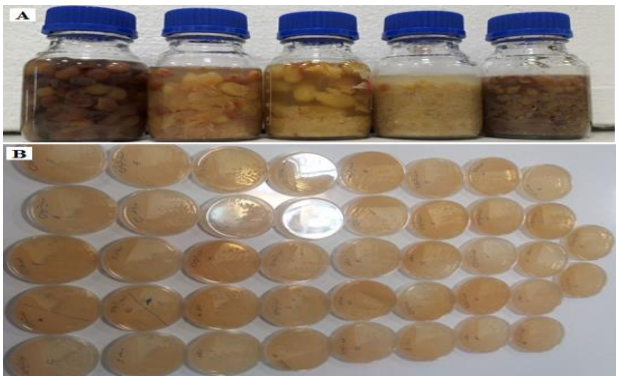

Figure 1. A) Fermented samples of wine grapes of various varieties, designed to isolate yeast that produce ethanol. B) Yeast isolates isolated from five wine grape varieties

Screening for yeast isolates producing ethanol

For screening of yeast isolates, two main characteristics of industrial yeast strains were used, namely: resistance to high concentrations of ethanol and resistance to high osmotic pressure of glucose (which is directly related to the ability to produce ethanol). Isolates that were able to grow at high glucose concentrations (50 and 60%) and also at different concentrations of ethanol were selected as yeast isolates promising for ethanol production. At the first screening stage, 6 were selected from 42 yeast isolates, the yeast of which could grow at high concentrations of sugar and at different concentrations of ethanol. These 6 isolates were then subjected to a second screening step to determine the amount of ethanol produced by them, which was determined by the colorimetric method. The results are presented in table 1.

Table 1. Results of the screening of yeast isolates

Series	Number of isolates	Different ethanol concentrations					Glucose concentration		Series	Number of isolates	Different ethanol concentrations					Glucose concentration	
		17 %	18 %	19 %	20 %	21 %	50 %	60 %			17 %	18 %	19 %	20 %	21 %	50 %	60 %
1	Aa	-	-	-	-	-	-	-	22	Ca	-	-	-	-	-	-	-
2	Ab	+	-	-	-	-	+-	-	23	Cb	-	-	-	-	-	-	-
3	Ac	+	+	-	-	-	+	+	24	Cc	+	+	+	-	-	+	+
4	Ad	-	-	-	-	-	-	-	25	Ce	-	-	-	-	-	-	-
5	Ae	+	+	-	-	-	-	-	26	Cf	-	-	-	-	-	-	-
6	Af	+	+	+	+	+	+	+	27	Cg	+	-	-	-	-	-	-
7	Aj	+	-	-	-	-	+-	-	28	Ch	+	-	-	-	-	-+	-
8	Ah	+	+	-	-	-	+-	-	29	Cj	-	-	-	-	-	-	-
9	Ba	+	+	-	-	-	-	-	30	Ea	+	-	-	-	-	-	-
10	Bb	-	-	-	-	-	-	-	31	Eb	+	-	-	-	-	-	-
11	Bc	+-	-	-	-	-	-	-	32	Ec	+	+	+	-	-	+	+
12	Bd	-	-	-	-	-	-	-	33	Ee	-	-	-	-	-	-	-
13	Be	-	-	-	-	-	-	-	34	Ef	-	-	-	-	-	-	-
14	Bf	-	-	-	-	-	-	-	35	Eg	+	+	+	-	-	+	+
15	Bj	+	+	-	-	-	-+	-+	36	Fa	-	-	-	-	-	-	-
16	Bh	+	+	+	-	-	+	-+	37	Fb	-	-	-	-	-	-	-
17	Bk	+	+	-	-	-	+-	-	38	Fc	-	-	-	-	-	-	-
18	Bl	-	-	-	-	-	-	-	39	Fe	+	-	-	-	-	+-	-
19	Bm	-	-	-	-	-	-	-	40	Ff	+	-	-	-	-	-	-

20	Bn	-	-	-	-	-	-	-	41	Fg	+	+	+	-	-	+	+-
21	Bo	-	-	-	-	-	-	-	42	Fh	+	-	-	-	-	-	-

Six species from these 42 seprated isolates that showed the best resistance were selected for further evaluation. Ethanol concentration in each of the 6 yeast isolates selected during screening from various fermented media was determined (Figure 2).

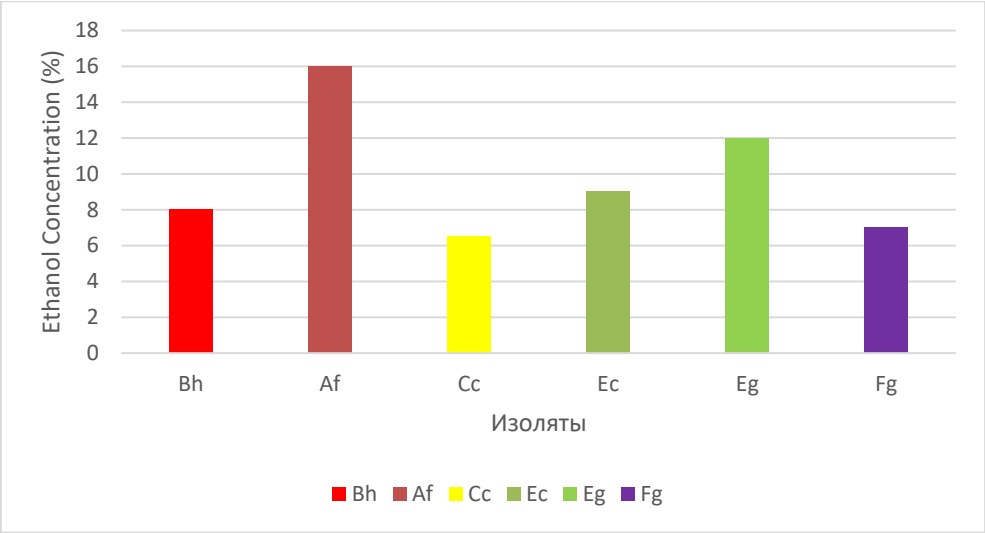

Figure 2 - Diagram of the amount of ethanol produced by selected yeast isolates

Enzymatic production of ethanol is most often carried out using strains of Saccharomyces yeast, as well as using varieties of this type of yeast isolated from other biomasses such as honey syrup, corn, molasses, etc. [12].

Ethanol is a toxic substance for yeast; therefore the ability of the yeast to withstand a high concentration of ethanol is an important property of industrial strains producing it [13]. Ethanol reduces cell growth and, accordingly, the formation of ethanol is reduced, due to inhibition of yeast enzymes and disruption of glycolysis pathways due to changes in the plasma membranes of cells. Recently, the production of ethanol is carried out mainly by the microbiological method using various microorganisms. The most common are S. cerevisiae yeasts; the use of Zymomonas mobilis strains is promising [14]. A search for new effective strains is ongoing. So, Armanul et al., 2017 [15]., Shamim 2013 [16] and Rao et al 2007 [17] isolated and identified yeast isolates with high bioavailability from various organic sources and fruits. The results of their studies showed that isolates of native yeast from organic samples could potentially be used as alternative producers of ethanol [18]. Further, this strain was studied using genetic methods, and it became known that strain number Af is a new strain of yeast. The novelty of identification of the new strain, named by the author of this article, Hanseniaspora Opuntiae MK 460485, is confirmed by the Patent of the International Genetic Center GenBank (Maryland, USA))

Assessment of ethanol production in the presence of various carbon sources.

Continuing to study the ethanol production of this strain, the study of ethanol under the global standard [19] (analysis of productivity results at the end of 48 hours) was on the agenda. As shown by the results in the following graph, the yeast Hanseniaspora opuntiae 460485 in the presence of molasses as a carbon source produces a higher percentage of ethanol (17%) than other carbon sources (Figure 3).

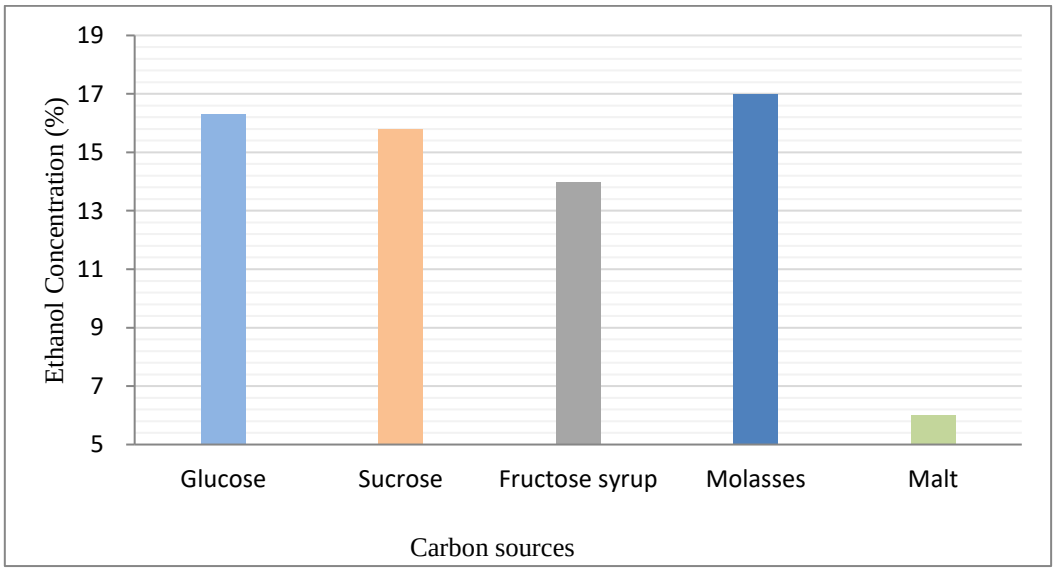

Figure.3 The amount of ethanol produced by yeast in the presence of various carbon sources in 48 hours

The high efficiency of ethanol production in the presence of molasses by this strain can be analyzed as follows: Strain Hanseniaspora Opuntiae 460485, was isolated from grapes growing in Iran. A study by Doulati Baneh and colleagues showed that Iranian tropical grape varieties contain an average of 4.7% glucose, 8.6% fructose, and 13.7% sucrose [20]. Therefore, during the evolutionary process, these strains should be able to decompose the primary carbohydrates used by humans (which are also the main carbohydrates used in this experiment as carbon sources). A higher rate of ethanol production in the presence of molasses compared to ethanol production in the presence of glucose also indicates the importance of elements and compounds [21] that were not covered and studied in this study. Ray, RC 2014 [22], Banach, A 2014 [23] and Vilela A. 2019 [24], in their studies of yeast production or growth factors and yeast quality of life, individually and jointly proved that the amount of zinc and magnesium and thiamine in the environment can have a significant effect on the rate of yeast fermentation. Consequently, an increase in production in the presence of molasses is most likely due to the minerals, elements and compounds present in molasses. The chemical composition of this beet molasses (according to the characteristics measured by the producer - Urmiasugar© sugar factory) is presented in table2.

Table 2. The Chemical composition of molasses (according to the characteristics measured by the producer - Urmiasugar© sugar factory)

The chemical composition of molasses			
Indicators	% of volume	Minerals per 100g	Vitamins per 100g
Solids	86	Calcium, Ca 205 mg	Thiamine 0.041 mg
Sucrose	55	Iron, Fe 4.72 mg	Riboflavin 0.002 mg
Invert sugar	10	Magnesium, Mg 242 mg	Nicotinic acid 0.93 mg
Raffinose	2.5	Phosphorus, P 31 mg	Pantothenic acid 0.804 mg
Fermentable sugar	55	Potassium, K 1.464 mg	Vitamin B-6 0.67 mg
Colloids	4.6	Sodium, Na 37 mg	Choline 13.3 mg
Good quality	75	Zinc, Zn 0.29 mg	
Ash	10	Copper, Cu 0.487 mg	
K2o	5.5	Manganese, Mn 1.53 mg	
Mgo	1.0	Selenium, Se 17.8 mcg	
Cao	2.0		
Total nitrogen	2.3		
Amine nitrogen before hydrolysis	0.5		
Amine nitrogen after hydrolysis	0.8		

In the case of media containing glucose, sucrose, fructose, although they contain pure sugar more, but molasses (as a carbon source), in addition to sugar (although not pure) contains a significant amount of nutrients and other elements, and this point is the point advantages of molasses. The results of this stage of the experiment showed that the production of ethanol in the presence of glucose was higher than the production of ethanol in the presence of sucrose and fructose. This was analyzed as follows: glucose in yeast is the only sugar entering the cycle of intracellular fermentation of yeast [25,26].

Assessment of ethanol production in the presence of various nitrogen sources

The results of the experiment showed that in the presence of ammonium nitrate (17.3) (Figure 4) and peptone (16.2), the greatest amount of ethanol is produced by yeast.

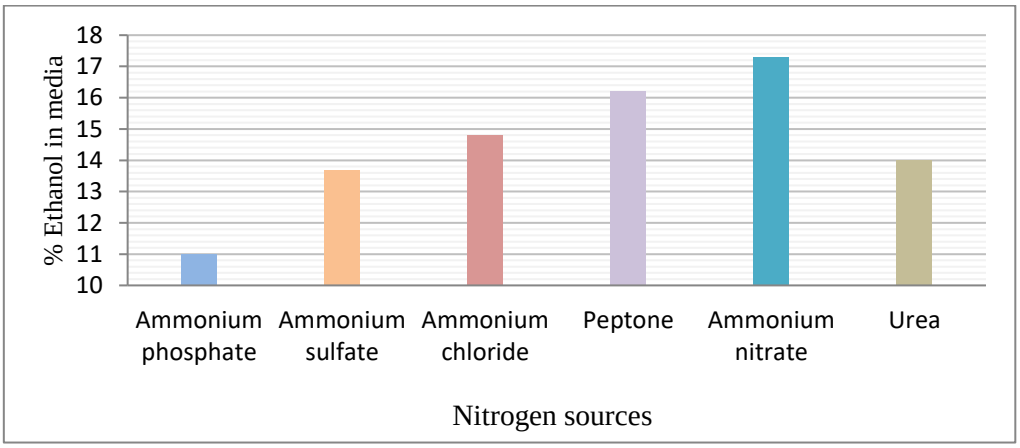

Figure 4. The amount of ethanol formed by isolated yeast in the presence of various sources of nitrogen

Consumables and production volumes, as well as their quantity and even nutritional interests, are different for each type of yeast [27]. That is why every new type of yeast needs new and complete measurements, what are the characteristics of new yeast in terms of growth, nutrition, reproduction, susceptibility, environmental preference, absorbed material and excretion [28].

Measurement of ethanol production in media containing equal volume percentages from different nitrogen sources (in liters) showed that the highest amount of ethanol production by yeast strains occurred in the presence of ammonium nitrate (17.3%). From the author's point of view and according to studies on ethanol production, including Deesuth, O (2012) [29] Laopaiboon, L (2009) [30], the reason for the higher production of ethanol in the presence of ammonium nitrate compared to other materials may be related with nitrogen in the content of each of these compounds. The reason for the higher production of ethanol in the presence of ammonium nitrate compared to other materials may be due to nitrogen in the content of each of these compounds. The percentage of nitrogen from the net mass in each of the compounds is shown in table.3.

Table 3. Chemical formula, the proportion of ethanol produced, and the percentage of nitrogen in the net mass of nitrogen-containing compounds used

Chemical compound	Chemical formula	% The proportion of ethanol produced in the environment	% Nitrogen from the proportion of chemical compounds
Ammonium phosphate	$(Nh_4)_3po_4$	11	6
Ammonium sulfate	$(Nh_4)_2so_4$	13.7	21
Ammonium chloride	NH_4Cl	14.8	25
Peptone	Polypeptides	16.2	50
Ammonium nitrate	NH_4NO_3	17.3	35
Urea	CH_4N_2O	14	46

A research plan was developed to optimize the production of ethanol by an isolated strain while varying the sources of nitrogen, carbon, pH and temperature in the culture medium. The special experiments plan was developed by the Minitab© program with the Plackett-Burman application .At the next screening stage, the experiments planned, were carried out in laboratory conditions. The results were entered into the software as input, on the basis of which the optimization parameter was determined - the effective dimensionless response - response (Figure 5). This dimensionless response (the last column in the table) represents the estimate that the software gave each combination of factors in laboratory experiments to produce ethanol with the Hanseniaspora opuntiae 460485 strain by comparing the effects of various factors.

StdOrder	RunOrder	PtType	Blocks	source of C	source of N	ph	tempreture	responce
6	1	1	1	sucrose	amonium nitrat	7	30	800
4	2	1	1	sucrose	amonium sulphate	7	37	550
9	3	1	1	glucose	amonium sulphate	3	37	220
1	4	1	1	sucrose	amonium sulphate	7	30	560
7	5	1	1	glucose	amonium nitrat	7	37	600
3	6	1	1	glucose	amonium nitrat	7	30	670
10	7	1	1	sucrose	amonium sulphate	3	30	600
8	8	1	1	glucose	amonium sulphate	7	37	70
12	9	1	1	glucose	amonium sulphate	3	30	320
2	10	1	1	sucrose	amonium nitrat	3	37	750
11	11	1	1	glucose	amonium nitrat	3	30	720
5	12	1	1	sucrose	amonium nitrat	3	37	750

Figure 5. The design of experiments to optimize the production of ethanol by an isolated strain of Hanseniaspora opuntiae 460485 while varying 4 factors and the value of the dimensionless response during laboratory tests (results of the first screening stage).

An analysis of the results of laboratory tests performed using the Plackett–Burman application package of the Minitab software allows us to conclude with a 95% probability (α = 0.05) the most effective factors affecting the production of ethanol with this new strain. According to the results, the main factors influencing the ethanol production by the H.opuntiae 460485 strain are sources of nitrogen, carbon, and also the temperature and, in contrast, the pH value is less significant, since its change in the range from 3 to 7 does not significantly affect the amount of ethanol secreted by the yeast strain Hanseniaspora opuntiae 460485 (Figure 6).

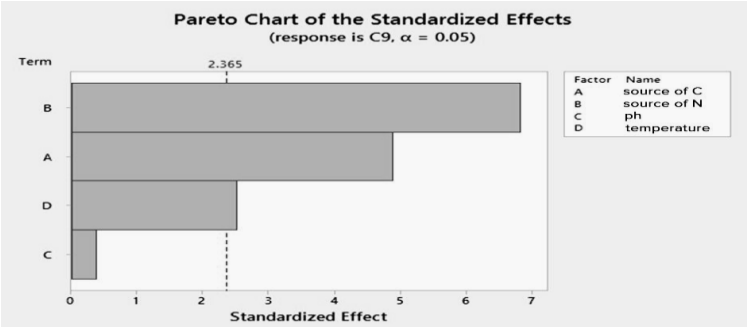

Figure 6. The influence of the main factors: A)The carbon source; B)The nitrogen source, D) The temperature, C) pH on the efficiency of ethanol production by the derived strain- Hanseniaspora opuntiae MK 460485.

After determining the effective factors for ethanol production by the strain by the statistical method of Plackett–Burman, at the next stage, the minimum, maximum and zero levels of each of the factors were determined. The experiment plan for optimizing the ethanol production objective function was developed using the CCD method (Central composite or Central Mix Design), which involves the use of Design Expert 7.0.0.Trial software. Given the maximum level of tolerance for the growth of yeast isolates at a temperature of 42 ° C, quantitative temperature levels of 25 ° C (minimum level or -1) and 37 °C (maximum level or +1) were chosen for optimization experiments. According to the experimental results obtained above, molasses with a concentration of from 20 to 100% was used as a carbon source. When optimizing the source of nitrogen, which was taken as ammonium nitrate (see the rationale above), its concentration varied from 1 g/l to 5 g/l.

Next, an experiment plan was developed, including 20 experiments, in accordance with the variation of all combinations of selected factors by their levels. Of these 20 experiments, 16 were non-centralized tests and 4 were central tests (Response Surface Method). The order of experiments was adopted taking into account the principle of randomness. The amount of ethanol produced by the Hanseniaspora opuntiae 460485 strain, which was established using GC analysis (gas chromatography) , was chosen as the optimization parameter.

After determining the factors affecting the production of ethanol by an isolated strain of Hanseniaspora opuntiae 460485, using the software, we determined the coefficients of the statistical equation, which indicate the influence of each of the factors on the amount of ethanol produced.

The obtained equations adequately correspond to the experimental results:
R1 = 22.87 + 3.00 * Molasses + 1.03 * Ammonium nitrate -0.38 * Temperature - 0.63 * Molasses * Ammonium nitrate +0.073 molasses * Temperature + 0.085 * Ammonium nitrate * Temperature -0.3.65 * Molasses2 - 1.63 * Ammonium nitrate2 - 1.06 * Temperature2

To verify the established optimal values of the factors, special studies were conducted on the production of ethanol using these cultivation conditions. Finally, the amounts of ethanol obtained experimentally in triplicate amounted to 19.56%, 19.95%, 19.3%, respectively. This indicates that the experimental data coincide with the result of the program forecast, which was 19.6% and shows the adequacy of the model and the reliability of the optimal cultivation conditions for the cultivation of the Hanseniaspora opuntiae 460485 strain calculated on it.

Therefore, at the subsequent stages of the study (including under industrial conditions), the obtained optimized cultivation values of the Hanseniaspora opuntiae 460485 strain were used, which ensured a maximum ethanol yield (56.5% molasses concentration, 3 g/L ammonium nitrate concentration, 30.41 ° C. Cultivation temperature).

The method of analyzing the response surface is one of the scientifically substantiated methods of process optimization, which is useful for identifying the reliable effect of several independent factors on the optimization parameter and the system under study [31]. This method has been successfully used to optimize the alcohol fermentation process [32, 33].

At this stage of the study, the surface response method was used to optimize the cultivation conditions for ethanol production by the Hanseniaspora opuntiae 460485 strain. The method is based on the use of a central composition plan (CCD) when varying the three concentrations of carbon source (molasses), nitrogen source (ammonium nitrate) and temperature at 5 levels of the matrix, which led to 20 experimental tests to obtain optimal ethanol. Based on the study of the response surface, the coordinates of the extreme point were established, which were the optimal values for the cultivation of the yeast Hanseniaspora opuntiae 460485. The maximum ethanol production was obtained under the following cultivation conditions: molasses concentration - 56.5%; ammonium nitrate concentration 3 g/l; temperature 30.41 ° C.

Similar results were obtained by Hoda et al. using the statistical response surface analysis method (RSM by the CCD method) to optimize cultivation of Saccharomyces cerevisiae yeast (PTCC 24860) in molasses of sugar beets in the production of ethanol. In this case, the optimal cultivation conditions were: pH 5.3, incubation time 24 hours, glucose content 35%, and the content of chloride nitrate (NH4Cl) 1.5 g/l [34].

Karuppaiya 2009 et al. using the same method to optimize the cultivation conditions of Zymomonas mobilis yeast using apple juice as the carbon source obtained the following optimal conditions for maximum ethanol yield: pH 4.5, temperature 32 ° C, fermentation time 37 hours. Under these conditions, ethanol production in the culture medium to a concentration was of 12.64% [35].

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REFERENCES

1. Dlugokencky E. and Tans P. Trends in atmospheric carbon dioxide, National Oceanic & Atmospheric Administration, Earth System Research Laboratory (NOAA/ESRL), available at: http:// www.esrl.noaa.gov/gmd/ccgg/trends/global.html, last access: 4 September 2018.
2. Kiran B., Kumar R., Deshmukh D. Perspectives of microalgal biofuels as a renewable source of energy//Energy Convers. Manag.-2014.-№88.-P.1228–1244.
3. Global Carbon Project: Supplemental data of Global Carbon Budget 2018 (Version 1.1) [Data set], Global Carbon Project, https://doi.org/10.18160/GCP-2018, 2018.
4. Kobayashi S., Ota Y., Harada Y., Ebita A., Moriya M., Onoda H., Onogi K., Kamahori H., Kobayashi C., Endo H., Miyaoka K., and Takahashi K. The JRA-55 Reanalysis: General Specifications and Basic Characteristics//J. Meteorol. Soc. Jpn.-2015.-№93.-P.5–48.
5. Demirbas A., Karslioglu S. Biodiesel production facilities from vegetable oils and animal fats//Energy Source A.-2007.-2007.-№29.-P.133–141.
6. Lednev V.P. World trends in the development of bioethanol // Distillery production and winemaking.-2007.-No 10.-S.22-23.
7. K.A. Shestibratov. Bioethanol from wood raw materials is the fuel of the future // Integral.-2007.-No 3.-S.19-21.
8. Hoekman S.K. Biofuels in the US-challenges and opportunities//Renew. Energy.-2009.-№34.-P.14–22.
9. Demirbas A. Use of algae as biofuel sources//Energy Convers. Manag.-2010.-№51.-P.2738–2749.
10. Tofighi A., Assadi M.M., Asadirad M.H.A., Karizi S.Z. Bio-ethanol production by a novel autochthonous thermo-tolerant yeast isolated from wastewater//J. Environ. Health Sci. Eng.-2014.-№12.-P.107.
11. Manwar J., Mahadik K., Paradkar A. Plackett-Burman Design : A Statistical Method for the Optimization of Fermentation Process for the Yeast Saccharomyces cerevisiae Isolated from the Flowers of Woodfordia fruticosa//название журнала.-2013.-№2(1).-P.1–6.
12. Siti Hajar Mohd Azhar, Rahmath Abdulla, Siti Azmah Jambo *et al.* Yeasts in sustainable bioethanol production//A review. Biochemistry and Biophysics Reports.-2017.-№10.-P.52–61.
13. Nofemele Z., Shukla P., Trussler A., *et al.* Improvement of ethanol production from sugarcane molasses through enhanced nutrient supplementation

using *Saccharomyces cerevisiae*//*J Brewing Distilling*.-2012.-№3(2).-P.29–35.
14. Wang R., Dom R.M., Leonard K., Koutinas A., Webb C. (2002). The Application of a Generic Feedstock from Wheat for Microbial Fermentations//Biodiversity.-2002.- №8(3).-P.1033–1038.
15. Armanul N., Rahman S.S. Choudhury Isolation of Saccharomyces Cerevisiae from Pineapple and Orange and Study of Metal's Effectiveness on Ethanol Production//J. European journal of microbiology & immunology.-2017.-vol. 7.-№1.-P.76-91.
16. Shamim R.S. (2013). Isolation and characterization of Saccharomyces cerevisiae for the production of ethanol from organic sources//Ph.d thesis. University of Dhaka.-2013.-P.207.
17. Rao R.S., Bhadra B., Shivaji S. (2008). Isolation and characterization of ethanol-producing yeasts from fruits and tree barks//J.Lett Appl Microbiol.-2008.-№47(1)-P.19-24.
18. Tikka C., Osuru H.P., Atluri N., Veera P.C., Kumar N., Mannur I.S., Prasad U.V. (2013). Isolation and characterization of ethanol tolerant yeast strains//Indian. Biotech.-2013.-№9(8).-P.225-238.
19. Breisha, Gaber. Production of 16% ethanol from 35% sucrose//J.Biomass and Bioenergy.-2010.- №34.-P.1243–1249.
20. Doulati Baneh,. Mohammadi S., Massimo L., Shafaie F. Genetic diversity of wild grape (Vitis vinifera ssp. Sylvestris) populations in Zagros forests as revealed by SSR markers//J. Acta Horticulturae.-2015.-1082.-P.189-194.
21. Gasmalla M.A.A., Yang R., Nikoo M., Man S. Production of ethanol from sudanese sugar cane molasses and evaluation of its quality//Journal of Food Processing & Technology.-2012.- №36.-P.26-31.
22. Ray, R.C., Joshi, V.K. Fermented Foods: Past, present, and future scenario. In Microorganisms and Fermentation of Traditional Foods// J.CRC Press: Boca Raton.-2014.-№.57.- P.1–36.
23. Banach, A., Ooi, B. Enhancing the Yields of Phenolic Compounds during Fermentation Using Saccharomyces cerevisiae Strain 96581// J.Food Nut. Sci.- 2014.- №.5.-P.2063–2070.
24. 164. Vilela, A. Use of Nonconventional Yeasts for ModulatingWine Acidity//J.Fermentation.- 2019.- №.5.-P.27–35
25. Teoh, A.L., Heard, G., Cox, J. Yeast ecology of Kombucha fermentation//J. Food Microbiol.- 2004.- №.95.- P.119–126.
26. 166. Freire, A.L., Ramos, C.L., da Costa Souza, P.N., Cardoso, M.G.B., Schwan, R.F. Nondairy beverage produced by controlled fermentation with potential probiotic starter cultures of lactic acid bacteria and yeast //J. Food Microbiol.-2017.- №.248.- P.39–46.
27. B. Zoecklein., K. Fugelsang., B. Gump, F. Nury Wine Analysis and Production//Kluwer Academic Publishers.-New York.-1999.- P.152-163.
28. Yiu G., McCord A., Wise A., Jindal R., Hardee J., Kuo A., Shimogawa M.Y., Cahoon L., Wu M., Kloke J., Hardin J., Mays Hoopes L.L. Pathways change in expression during replicative aging in saccharomyces cerevisiae. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences.-2008.-№63.-P.21–34.
29. Deesuth, O., Laopaiboon, P., Jaisil, P., Laopaiboon, L. Optimization of nitrogen and metal ions supplementation for very high gravity bioethanol fermentation from sweet sorghum juice using anorthogonal array design. //J. Energies.-2012.- №.5.- 3178–3197.
30. Laopaiboon, L., Nuanpang, S., Srinophakun, P., Klanrit, P., Laopaiboon, P. Ethanol production from sweet sorghum juice using very high gravity technology: Effects of carbon and nitrogen supplementations//J.Bioresour. Technol.-2009.- №.100.- P.4176–4182
31. Stewart G.G. The Horace Brown Medal Lecture—Forty years of brewing research//J. Inst. Brew.-2009.-№115.-P.3–29.
32. Ratnum B.V.V., Rao M.N., Ayyanna C. (2003). Optimization of fermentation conditions for production of ethanol from sago starch using RSM. Wrld//J. Microbiol. Biotechnol.-2003.-№19(5).-P.523-526.
33. Çam M., Içyer N.C. Phenolics of pomegranate peels: extraction optimization by central composite design and alpha glucosidase inhibition potentials//J Food Sci Technol.-2015.-№52(3).-P.1489–1497.
34. 34. Shafaghat H., Najafpour G., Pouya S.-R., Sharifzadeh M. Optimal growth of Saccharomyces cere-visiae (PTCC 24860) on pretreated molasses for the ethanol production: The application of the response surface methodology//J.Chemical Industry and Chemical Engineering Quarterly.-2010.-№16.-P.199-206.
35. 35. Karuppaiya M., Sasikumar E., Viruthagiri T., Vijayagopal V. "Optimization of process parameters using response surface methodology (RSM) for ethanol production from waste cashew apple juice using Zymomonas mobilis"//Chem. Eng. Comm.-2009.-Vol.196.-P.1-11.