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The Study of the influence of various factors on the ethanol production by the *Hanseniaspora opuntiae*

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The amount of ethanol production in fermentation process, depends on environmental factors. In order to study the influential factors in producing ethanol by strain of Hanseniaspora opuntiae, the amount of ethanol production was measured in YPD culture medium (1% yeast extract, 2% peptone, 2% glucose, in distilled water) at different temperature and pH and also in the presence of specific volume of different carbon sources including glucose, sucrose, fructose syrup, glucose syrup, molasses and whey, and different nitrogen sources such as ammonium phosphate, ammonium sulfate, industrial urea, ammonium nitrate, peptone and amine-chloride. In order to optimize the conditions of ethanol production and to gain maximum production rate by yeast strain, test design method with Response Surface Method was used, aiming at achieving better results, significant decrease in the number of tests and optimizing production conditions. Using the Placket-Burman option of Minitab© software, effectual factors were screened with. To determine the amount of ethanol produced used HPLC technique. The results showed that among the measured factors, nitrogen source has the most effects in producing ethanol by studied strain and the minimum effect was related to ph. Also, among the carbon sources, the highest production occurred in the presence of sucrose and molasses, and among nitrogen supplies the best performance in producing ethanol occurred with peptone and ammonium nitrate.

Keywords: alcohol fermentation, ethanol, *Hanseniaspora opuntiae*, nitrogen, carbon.

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Изучение влияния различных факторов на выработку этанола штаммом *Hanseniaspora opuntiae*

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Объем производимого спиртовым брожением этанола во многом определяется факторами окружающей среды. В настоящем исследовании использован штамм Hanseniaspora opuntiae, который был выделен от поврежденного винограда после отбора проб и скрининга. Для изучения факторов, влияющих на выработку этанола этим штаммом, измеряли количество продуцируемого этанола в культуральной среде YPD (состав: 1% дрожжевой экстракт, 2% пептон, 2% глюкоза, дистиллированная вода) при варьировании различных факторов: температура, pH, наличие различных источников углерода, включая глюкозу, сахарозу, фруктозный сироп, глюкозный сироп, патоку и сыворотка в определенных объемах, и также наличие различных источников азота, таких как фосфат аммония, сульфат аммония, промышленная мочевина, нитрат аммония, пептон и хлорид аммония. Для оптимизации условий производства этанола и получения максимальной его производительности по названному штамму дрожжей, применяли метод планирования и оптимизации экспериментов Response Surface Method, направленный на достижение наилучших результатов при значительном уменьшении количества тестов и оптимизацию процесса в производственных условиях. Для этого использовали опцию Placket-Burman

программного обеспечения Minitab©, предварительно были проанализированы наиболее эффективные факторы внешней среды. Для определения количества производимого этанола применяли метод высокоэффективной жидкостной хроматографии (ВЭЖХ). Результаты показали, что среди измеренных факторов источник азота оказывает наибольшее влияние на продуцирование этанола исследуемым штаммом, а минимальный эффект был связан с изменением pH. Наиболее эффективным источником углерода для продуцирования спирта данным штаммом оказались сахароза и патока, а среди источников азота наивысшая производительность в продуцировании этанола была достигнута при использовании пептона и нитрата аммония.

***Ключевые слова*:** спиртовое брожение, этанол, штамм *Hanseniaspora opuntiae*, азот, углерод.

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INTRODUCTION

Unreasonable and extreme use of fossil fuels, will not only deprived the next generation from these sources that had been made during millions of years, but also will cause severe increasing of pollution [1]. In this vein, one of fundamental ways that help us to produce fuel and not to utilize fossil fuel extremely and at the same time reduce environmental pollution, is to use alternative fuels. One of the most important biofuel is bioethanol that is produced from crops like sugar cane, wheat, corn and sugar beet [2].

Ethanol is a kind of alcohols with (C_2H_5OH) chemical formula that is known with other names such as Ethyl-alcohol and Cereal alcohol. Ethanol is the second element in aliphatic alcohols series that solve easily in water and organic solvents. Ethanol is a colorless liquid with a pleasant odor [3]. Freezing point of Ethanol is –115 and its boiling point is +78 centigrade degrees, its specific weight is 0.79 gram on milliliter in 20 °C [4]. In fact, bioethanol is an ethanol that is produced from crops waste product by fermentation processes with bacteria or yeast, sugar cane, sugar beets, wheat and barley are some examples of these crops. Ethanol is utilized in the fuel, food, pharmaceutical and cosmetics industries but its usage as a perfect fuel or in combination with gasoline is increasing [5]. Ethanol fermentation process that is known as alcoholic fermentation, is a biologic process that leads to consume sugars like glucose, fructose or sucrose, and produces the molecule of ethanol as output [6].

Among all the yeasts, *Saccharomyces* family is one of the best options in ethanol production. The yeast of *Saccharomyces* is one of the few yeasts with the capacity to grow rapidly both under aerobic and anaerobic conditions [7]. It is a sort of yeast in *Saccharomycetes* series that has special usage in biological products production [8]. *Hanseniaspora opuntiae* yeast is unicellular fungi that divide asexually by budding or fission and whose individual cell size with a large diameter of 5-10μm and a small diameter of 1-7μm. The cells of *S. cerevisiae* are pigmented, where cream color may be visualized in surface-grown colonies [9]. In order to increase the ethanol production efficiency of yeast, it is necessary to optimize parameters that affect the process of fermentation [10].

MATERIAL AND METHOD

The utilized strain in this study was Iranian native strain of *Hanseniaspora opuntiae*, that was separated from being corrupted grapes after sampling and screening. In order to optimize the conditions of ethanol production and to gain maximum production rate by yeast strain, test design method with Response Surface Method was used, aiming at achieving better results, saving time and material, significant decrease in the number of tests and optimizing production conditions. At first, using the Placket-Burman option of Minitab© software, effectual factors were screened with. It is worth mentioning, as in design method tests, two ranks were considered to study qualitative factors like carbon and nitrogen supply, the effect of the sources was investigated by one factor at the time method.

Evaluating the amount of produced ethanol by the presence of different carbon sources

To choose the best carbon source to produce ethanol, different carbon sources like glucose, sucrose, fructose syrup, glucose syrup, molasses and whey were used. Each of these carbon sources were sterilized in autoclave at 110 °C for 10 min and then they were added to YPD culture medium (1% yeast extract, 2% peptone, 2% glucose, in distilled water). Then, these mediums with different carbon sources, were inseminated with 5 ml yeast suspension with 0.5 McFarland turbidity and 3% compactness, after they heated up in Incubator shaker under 30 °C and 150 rpm. After 28 hours of insemination, each of fermented environments with different carbon sources were evaluated by hplc (High-performance liquid chromatography).

Evaluating the amount of produced ethanol in the presence of different nitrogen sources

In order to determine the best nitrogen source for ethanol production in fermented environments in the presence of optimal carbon source, different nitrogen sources such as ammonium phosphate, ammonium sulfate, industrial urea, ammonium nitrate were used. After preparing culture mediums and sterilizing in autoclave, fermented environments inseminated with 5 ml cell suspension with 0.5 McFarland turbidity and 3% compactness and then heated up in Incubator shaker with 30 °C and 150 rpm. After 28 hours of heating up,

the amount of produced ethanol was evaluated with colorimetric method in hplc. In each case, three samples were prepared for each measurement.

RESULTS

Evaluating the amount of produced ethanol by in presence of different carbon sources

The amount of produced ethanol in each fermented environment with specific carbon sources (glucose, sucrose, fructose syrup, glucose syrup, molasses and whey) were investigated (Fig. 1). The results showed that the amount of ethanol production by this strain in the presence of sucrose (Fig. 2) and fructose syrup was more than other sources. Also, the least amount of ethanol production by this yeast was in culture medium with whey in the fermentation process.

Evaluating the amount of produced ethanol by the presence of different nitrogen sources

In order to define the best nitrogen source for ethanol production in fermentation medium by our strain, in the presence of optimal carbon source, the amount of ethanol production in each fermentation medium measured. For instance, the chromatogram of Ethanol production of ammonium nitrate source has shown in Fig. 3. The results of the tests showed that this strain of *Hanseniaspora opuntiae* has the highest yield for fermentation and ethanol production in presence of peptone sulfate and ammonium nitrate respectively. Moreover, the lowest amount of ethanol production with this strain was when urea used as the source of nitrogen in the culture medium (Fig. 4).

Screening effectual factors in the process of ethanol fermentative production

In the phase of optimizing the conditions of ethanol production, in order to saving time, significant reduction in the number of tests and therefore reducing the costs of the optimizing stages, screening effectual factors was done by Minitab software with Placket-burman statistical method (Table 1)

Then, in the next stage the designed tests in the previous stage, were empirically studied in the real lab conditions and the results were fed into the Minitab to analyze and determining effectual factors (Table 2).

The results of the designed analysis by the software showed that in considering $\alpha=0.05$, carbon, nitrogen and temperature are three factors that influence ethanol production, while pH doesn't have significant effect on ethanol production by *Hanseniaspora opuntiae* (Fig. 5).

Moreover, the results showed that the amount of ethanol production by this strain in the presence of sucrose was more than glucose, in the presence of ammonium nitrate was more than ammonium sulfate, and increased in 30 °C comparing to 37 °C (Fig. 6).

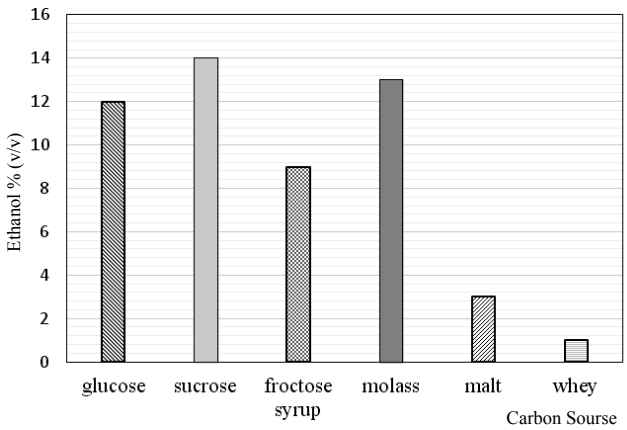

Fig. 1. The amount of ethanol production in the presence of different carbon sources

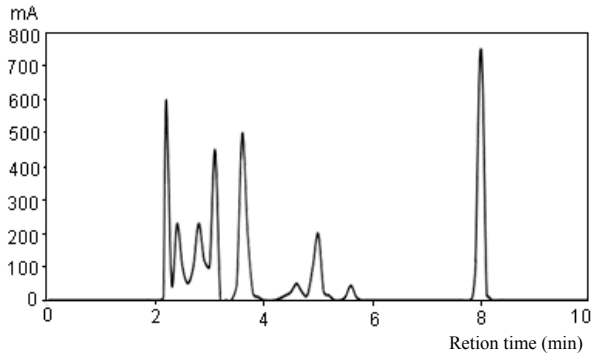

Fig. 2. Chromatogram of HPLC of the sucrose, the peak number 4 caused by ethanol

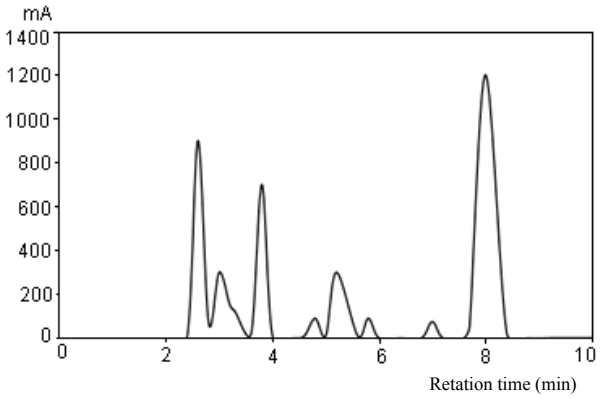

Fig. 3. Chromatogram of ethanol production in the presence of the ammonium nitrate as nitrogen source. The peak number 3 is the peak of evidence of ethanol

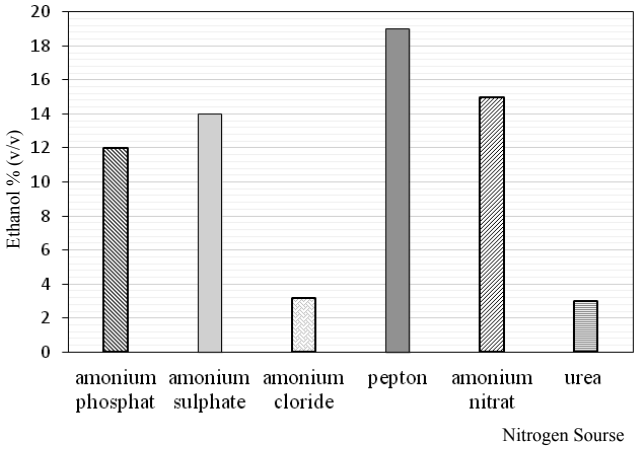

Fig. 4. The amount of produced ethanol in the presence of different nitrogen sources

Table 1

Designed tests with Plackett-burman statistical method

Std Order	Run Order	Pt Type	Blocks	Source of C	Source of N	pH	Temperature, °C
6	1	1	1	sucrose	ammonium nitrat	7	30
4	2	1	1	sucrose	ammonium sulphate	7	37
9	3	1	1	glucose	ammonium sulphate	3	37
1	4	1	1	sucrose	ammonium sulphate	7	30
7	5	1	1	glucose	ammonium nitrat	7	37
3	6	1	1	glucose	ammonium nitrat	7	30
10	7	1	1	sucrose	ammonium sulphate	3	30
8	8	1	1	glucose	ammonium sulphate	7	37
12	9	1	1	glucose	ammonium sulphate	3	30
2	10	1	1	sucrose	ammonium nitrat	3	37
11	11	1	1	glucose	ammonium nitrat	3	30
5	12	1	1	sucrose	ammonium nitrat	3	37

Table 2

Output of the Plackett-burman method after inputting the results of the conducting experiments in the real lab conditions

Std Order	Run Order	Pt Type	Blocks	Source of C	Source of N	pH	Temperature, °C	Responce
6	1	1	1	sucrose	ammonium nitrat	7	30	800
4	2	1	1	sucrose	ammonium sulphate	7	37	550
9	3	1	1	glucose	ammonium sulphate	3	37	220
1	4	1	1	sucrose	ammonium sulphate	7	30	560
7	5	1	1	glucose	ammonium nitrat	7	37	600
3	6	1	1	glucose	ammonium nitrat	7	30	670
10	7	1	1	sucrose	ammonium sulphate	3	30	600
8	8	1	1	glucose	ammonium sulphate	7	37	70
12	9	1	1	glucose	ammonium sulphate	3	30	320
2	10	1	1	sucrose	ammonium nitrat	3	37	750
11	11	1	1	glucose	ammonium nitrat	3	30	720
5	12	1	1	sucrose	ammonium nitrat	3	37	750

DISCUSSION

Microorganisms that are used in fermentation process, grow in the which environment that provides its nutrition needs, nutrients in fermentation medium depending yeasts needs and dependences divide into two categories maximum and minimum elements. Carbon is among maximum ones [11]. Main elements specifically carbon sources needed for fermentation process in laboratory scale, are used in pure form and required amount, which is impossible in industry level. In this study, carbon sources like glucose syrup, sucrose, molasses, malt extract and whey were used as carbon sources. The results showed that the amounts of ethanol production in presence of sucrose and then molasses are respectively higher in comparing to other carbon sources. It seems that the better growth in sucrose presence is due to high purity of its sugar comparing to molasses [12, 13]. The reason of high Efficiency of ethanol production due to fermentation process of molasses comparing to other sources, can be considered that molasses in addition to carbohydrate, contains other essential nutritional sources like amino acids and vitamins like biotin which is a required cofactor in reproduction and accelerating fermentation in yeast [12, 14]. As in industrial scale one of considerable factors, is the economic benefits, although the present study showed that ethanol production in culture medium with sucrose is more than ethanol production in culture medium with molasses, considering that molasses is cheaper than pure sucrose [11, 15]. Molasses can be a recommended option in industrial usage for fermentation process. The efficiency of Ethanol production from molasses can be improved in the presence of other organic materials [16, 17].

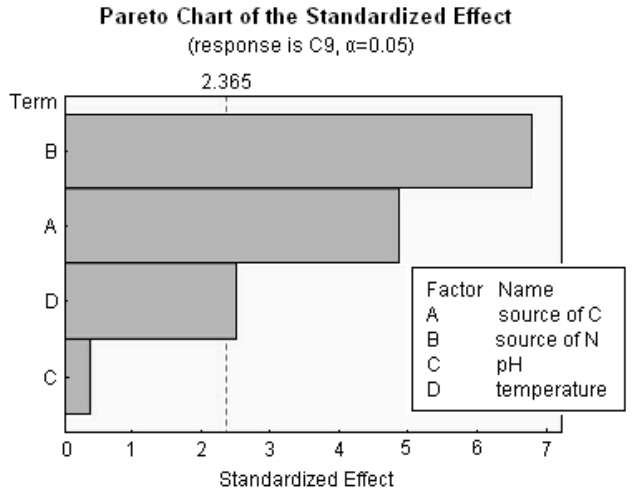

Fig. 5. Effects of carbon, nitrogen, temperature and pH source factors on ethanol production by Hanseniaspora opuntiae

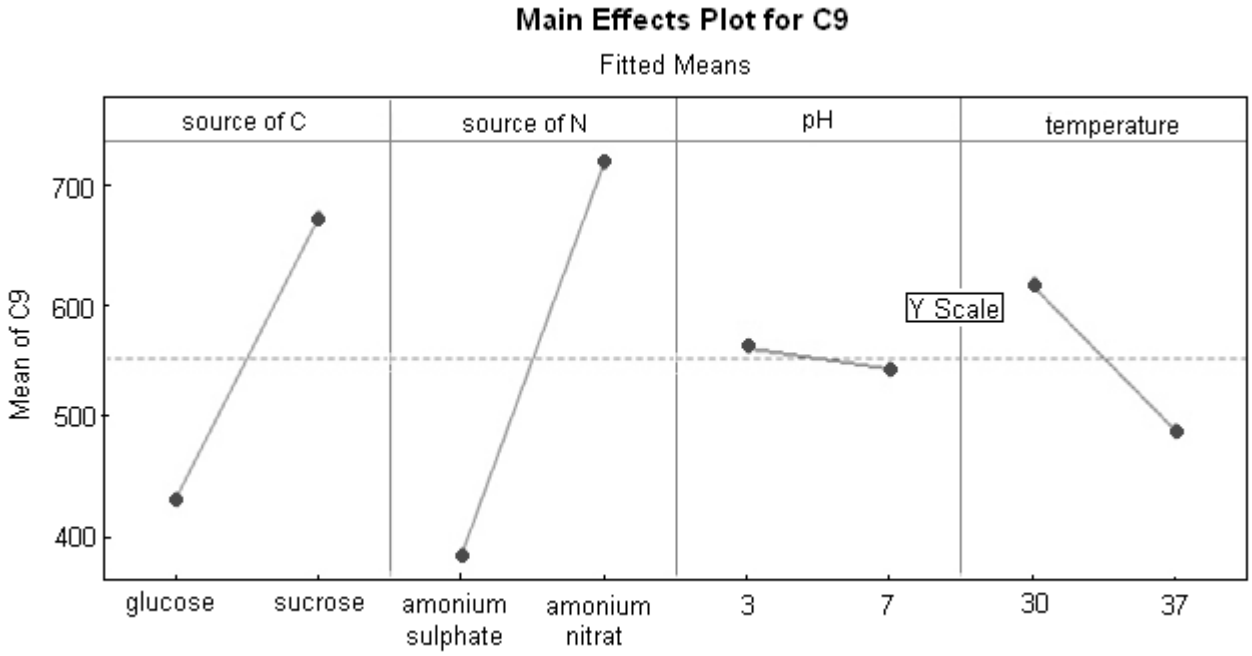

Fig. 6. Presenting the effects of different factors in producing ethanol by Hanseniaspora opuntiae

Yeast extract provides essential necessities like vitamins and cofactors [18], then nitrogen source of yeast extract with second nitrogen source were used for optimization; the results of the study showed that the presence of nitrogen source of yeast extract with peptone leads to the highest ethanol production, in this case the results of the study showed that, although using peptone as nitrogen source cause about 4% outcome increase in ethanol production, in compare with ammonium nitrate, but as its price is several times higher than the price of ammonium nitrate [19]. So here from economical point of view the difference in production efficiency between peptone ammonium nitrate is not enough much to introduce peptone as better source. But it's shown by this study that ammonium nitrate in comparing to urea leads th fifth times more amount of ethanol production by the yeast and it is also cheaper than urea [11, 20]. Then based on gained result ammonium nitrate has preference as industrial source of nitrogen to be used in fermentation process by *Hanseniaspora opuntiae*.

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