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Utilization of *Schizosaccharomyces pombe* for Production of Ethanol from Cane Molasses

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Abstract The objective of the present study was using the molasses to produce ethyl alcohol by *Schizosaccharomyces pombe* as a fermenting microorganism and evaluation of the final product. Chemical and microbiological analyses were carried out for molasses samples which contained 49.9% soluble sugars. To produce ethanol the fermentation and distillation processes were controlled, and the results showed that optimum urea, sugar concentrations and the temperature were 0.25%, 20% and 32℃, respectively. This formulation gave 9.9% (w/v) ethanol in fermented mash while 11% (w/v) ethanol was produced using *Saccharomyces cerevisiae* as fermenting microorganism using similar production method. The physio-chemical characteristics of the ethanol which included purity, density and viscosity were 95.5%, 0.804 gm/ml and 0.81 cip, respectively, and the final ethanol appeared to be clear, bright and free from turbidity indicating its as high specification quality. The study recommended the utilization of both yeast *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* in large scale production of ethanol.

Keywords Ethanol, sucrose, yeast, mash, fermentation, macro-minerals

1. Introduction

Molasses is defined as the final effluent obtained in the preparation of sugar by repeated crystallization; It is residual syrup from which no crystalline sucrose can be obtained by simples means[1]. World molasses production has increased markedly over the past 40 years from around 15 million Metric Tons (MT) in the early 1960s to around 45 million MT by 2000. The yield of molasses per tone of cane is approximately 2.7% but it is influenced by a number of factors and may vary within a wide range (2.2% - 3.7%)[2].

The molasses is considered as a perfect feedstock for production of alcohol. All the sugars are present in a readily fermentable form[3]. One-half of the fermented sugar is converted to carbon dioxide, which can be used for industrial application or in green houses for increasing plant growth. The other half of the fermented sugar is converted to ethyl alcohol. Since it contains all the fusel oil, esters and aldehydes, it is not good for drinking but good as burn fuel. In modern times ethanol intended for industrial use has also been produced from by-products of petroleum refining[4].

People have used yeast for fermentation and baking throughout history. The useful physiological properties of yeast have led to their use in the field of biotechnology. Fermentation of sugars by yeast is the oldest and largest application of this technology. Many types of yeasts are used for making many foods: baker's yeast in bread production; brewer's yeast in beer fermentation and yeast in wine fermentation[5] *Shizosaccharomyces pombe,* also called "Fission Yeast", is a species of yeast. It is used as a model organism in molecular and cells biology. It is a unicellular eukaryote, whose cells are rod-shaped. Cells typically measure 3 to 4 micrometers in diameter and 7 to 14 micrometers in length.

Ethanol is known as ethyl alcohol or grain alcohol a flammable, colorless, mildly toxic chemical compound with a distinctive perfume like odor, and the alcohol is found in alcoholic beverages. In common usage, it is often referred to simply as alcohol. Its molecular formula is variously represented as ETOH, (C_2H_5OH) or as its empirical formula C_2H_6O[5].

Ethanol is used in many different products ranging from perfumes to explosives. The most popular use of ethanol is in the automotive fuel industry. It is mixable in all proportions with water and most organic solvents. Ethanol in the presence of an acid catalyst makes an ethyl ester. It is also used to make up such products as paints, coatings, adhesives and the most popular household product is nail polish remover. Ethanol is used worldwide and known mostly for getting people intoxicated. Many social gatherings involve ethanol in the form or beer, wine and liquor (6).

The objective of the present study was to use molasses for production of ethyl alcohol using *Schizosaccharomyces pombe* yeast as a fermenting microorganism and evaluation of the product.

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2. Materials and Methods

2.1. Materials

Molasses samples were collected from Elguneid Sugar Factory during the production season 2007-2008. *Shizosaccharomyces pombe* (yeast) which had been isolated from fermented honey beverage from beer (Doma) was supplied by Department of Food Science and Technology, Gezira University.

2.1.1. Analysis of Molasses

The molasses samples were analyzed for pH using a pH-meter (PHS-3C Digital) at ambient temperature according to ICUMSA[7], total soluble solids using a hand refractometer and reducing sugars content according to ICUMSA[8].

For determination of sucrose content, 13 g of molasses were weighed in 100 ml volumetric flask. About 2 g of basic lead acetate were added and mixed for 2 minutes. Then the mixture was filtered using a Whatman (No. 1) filter paper. The first 10 ml of the filtrate were discarded. A 200 mm tube was filled with the filtrate and put inside the polarimeter chamber. Sucrose contents was calculated from the following equation:

$66.5 = \alpha \times 1000 / C \times L.$

α = rotation angle.

C = sucrose. concentration %.

L = tube length.

66.5 = constant.

The total sugar content was calculated according to ICUMSA[8], in which total-reducing sugars in molasses after hydrolysis (inversion) is equal to sucrose plus invert sugars.

Total sugars = (Reducing sugars + sucrose).

The ash content was determined according to Chen and Chou[9], ash is the residue remaining after incinerating the product under specified conditions.

2.1.2. Macro Minerals Determination

The minerals determined were Sodium (Na^+), Potassium(K^+) and Calcium(Ca^{++}). The method used was that described by AOAC[10] in which Flame photometer was used for analysis.

2.2. Microbiological Analysis

The total viable count per ml of molasses was determined following the method of APHA[11]. Incubation was accomplished at 30˚C for 48 hours. .plates containing between 30 to 300 colonies (c.f.u/ml) per ml of sample was used to calculate the total.

C.F.U = Colonies counted × 1/D

Where:

C.F.U = colonies forming unit

D = dilution factor

The yeast and mould strains were enumerated by culturing them on potato dextrose agar (PDA) medium and incubating for 72 hours at 25℃.

2.3. Mash Preparation and Fermentation

Different amounts of urea (0.15, 0.25, and 0.5) % (w/v) were added to three volumetric flask containing molasses diluted with distilled water to about 8% sugar concentration to determine the best nutrient concentration which produces high yield of alcohol. The pH of mash was then adjusted at 4.8 by the addition of sulfuric acid. The mash was then seeded with the yeast culture in the proportion of 5% by volume. Then the mash was transferred into the incubator at 33℃ for 72 hours under anaerobic conditions.

Different temperatures (28, 29, 30, 31, 32, 33 and 34)℃ were used to incubate the mash to determine the optimum temperature which produces high yield of alcohol. The molasses was diluted to about 15% sugar concentration. The best nutrient concentration (urea) was added in the proportion of 0.25% (w/v) for each treatment. The pH of mash was then adjusted at 4.8 by the addition of sulfuric acid. The mash was then seeded with the yeast culture in the proportion of 5% by volume. Then the mash was transferred into the incubator for 72 hours under anaerobic conditions.

The molasses was diluted to different sugar concentrations (10%, 15%, 20% and 25%) in four volumetric flasks to determine the optimum sugar concentration which produces the highest yield of alcohol. The best nutrient concentration (urea) was added in the proportion of 0.25% (w/v) for each treatment. The pH of mash was then adjusted at 4.8 by the addition of sulfuric acid. The mash was then seeded with the yeast culture in the proportion of 5% by volume. Then the mash was transferred into the incubator at 32℃ (optimum temperature) for 72 hours under anaerobic conditions.

2.4. The Yield of Ethanol

The yield of ethanol in fermented mash was measured by Gas Liquid Chromatography (GLC) as follows:

(I) Preparation of standard solution and internal standard Solution:

15.7 ml of ethanol and acetonitrile were dispensed into 1000 ml volumetric bottle and then distilled water was added to the 1000-mL mark. This was the 1% (w/v) ethanol standard solution or internal (acetonitrile) standard solution.

(II) Relative response factor (RRF) of ethanol to acetonitrile Ethanol, 1% (w/v), was mixed with 1% (w/v) acetonitrile in various ratios (ethanol: acetonitrile = 15:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, and 1:15). A linear regression line was generated with the GC peak area-under-curve (AUC) ratio of ethanol to acetonitrile (Y-axis) against the concentration ratio of ethanol to acetonitrile (X-axis). Relative response factor (RRF) is the slope of the regression line, as in the Equation 1: RRF = (AS/WS) ÷ (AIS/WIS); in which, AS = ethanol AUC, AIS = acetonitrile AUC, WS = ethanol weight (mg), WIS = acetonitrile weight (mg).

(III) Direct injected GC method:

Beverage sample solution (0.5 ml) was dispensed into an l-ml caped sample vial, and then 5 ml of 1% internal standard

solution (equivalent to 50 mg) was added. After mixing, 0.1 µL of the sample solution was injected directly into a GC with syringe. Ethanol content was calculated according to the Equation:

Ethanol (mg/ml) = (AS/AIS) X (WIS/RRF) X 1/V

in which, V = sample volume (ml).

2.5. The purity and density of ethanol

The fermented mash was distillated. The purity of ethanol obtained was measured using hydrometer[12].

The density of ethanol was measured as follows, the flacon (100 ml) was filled by distilled ethanol, and then ethanol was weighed and calculated the weight per volume of flacon, Density = weight / volume[13].

3. Results and Discussion

3.1. Chemical Composition of Blackstrap Molasses

The chemical composition of black strap molasses is presented in Table (1). The molasses contained 83.2% total soluble solids, 17.8% reducing sugars, 32.1% sucrose, 49.9% total sugars, 10.25% ash, o.54% calcium, 0.28% sodium, 2.89% potassium and it had a pH value of 5.6. Most of the chemical parameters determined in this study were in close agreement to those reported by Peterson[14], who found that molasses contained (45-55)% total sugars, (20-25)% reducing sugars, (25-35)% sucrose, (10-16)% ash, (0.4-0.8)% calcium, (0.1-0.4)% sodium, (1.5-5)% potassium and pH (5-5.5). On the other hand, Chen and Chou[9] found that molasses contained 52% total sugars, 16% reducing sugars, 34% sucrose, 12% ash and pH 5.0.

Table1. Chemical composition of molasses

Parameter	Value
Total soluble solids	83.2%
Reducing Sugars	17.8%
Sucrose	32.1%
Total sugars	49.9%
Ash	10.25 %
pH	5.6
Purity	38.58%
Calcium	0.54%
Sodium	0.28%
Potassium	2.89%

The most obvious characteristic of cane molasses is its relatively high proportion of reducing sugars. During the crystallisation cycles the reducing sugars increase to such an extent that no more sucrose can be crystallised, because reducing sugars decrease the solubility of sucrose. Mineral matter tends to hold sucrose in solution, so it is the balance of reducing sugar and mineral matter that determines the theoretical yield of sucrose from sugar cane[12]. Molasses is a rich source of minerals. In comparison to other commonly used sources of dietary energy, e.g. cereal grains, the calcium content of cane molasses is high (up to 1%). Cane molasses is also high in sodium, and potassium (which are present as chlorides). Beet molasses tends to be higher in both potassium and sodium but lower in calcium content. Molasses also contains significant quantities of trace minerals, copper for example (7ppm), zinc (10ppm), iron (200ppm), manganese (200ppm)[13].

Soil and climatic conditions, the variety and maturity of the cane and the processing conditions in the factory all influence molasses composition. Consequently, considerable variation may be found in nutrient content, flavour, colour and viscosity of molasses. Sucrose is the major sugar present.

3.2. Microbiological Characteristics

The microbiological analysis of molasses samples (Table 2) revealed, presence of 7×10 and 3×10^{2} (c.f.u/ml) of total microbial counts in 10^{-1} and 10^{-2} molasses residual dilution, respectively. While the other dilutions were devoid of micro-organisms, it seems that the high sugar concentration reduced the total number of micro-organisms to the minimum as a result of reduction in water activity and osmotic pressure. On the other hand, the yeast and mould counts at dilutions of molasses 10^{-1} and 10^{-2} were found to be 8×10 and 2×10^{2} (c.f.u/ml), respectively, other dilutions of molasses were free from yeast and moulds.

Table 2. Microbiological characteristics of molasses

Dilution	Total viable count(c.f.u/ml) bacteria	Yeast and mould count(c.f.u/ml)
10^{-1}	7×10	8×10
10^{-2}	3×10^{2}	2×10^{2}

Hapse and Arabatti[15] reported that contamination resulted in poor fermentation efficiency and low alcohol yield per ton of molasses. Therefore, aseptic conditions in our experiments agree with high efficiency and precision of treatments.

3.3. Measurements of Alcohol

Table (3) shows the yield of alcohol in fermented mash using different amounts of nutrients (urea) (0.15%, 0.25%, and 0.50%). The best nutrient concentration which gave the highest alcohol yield in fermented mash was 0.25%[19]. Barber *et al.,*[3] reported that the use of $(NH_4)_2\ SO_4$ as a nitrogen source in molasses medium is greatly recommended for ethanol production.

Table 3. Ethanol yield in fermented mash using different nutrient concentrations

Nutrient urea%	Mash volume/ml	Mash sugar conc.%	PH	Temp ℃	Ethanol Yield %
0.15	400	8	4.8	33	3.8
0.25	400	8	4.8	33	4.3
0.50	400	8	4.8	33	4.2

Table (4) shows the yield of alcohol in fermented mash using different temperature (28, 29, 30, 31, 32, 33 and 34) ℃. The optimum temperature for fermented mash was 32 ℃. Morrison *et. al.*,[18] reported that the temperature of the fermenting mash should be between 29.4℃ to 32℃.

Table (5) shows the yield of alcohol in fermented mash using different Sugar concentrations. Sugar concentration plays an important role in ethanol fermentation by yeast. For economic reasons the residual sugar for maximum ethanol formation should be negligible at the end of fermentation. Therefore, the optimum level of sugar was determined by using (20 %) sugar in molasses medium.

Table 4. Optimum nutrients with different temperatures

Temp ℃	Mash volume\ml	Mash Sugar conc.%	Nutrient urea%	PH	Ethanol Yield %
28	400	15	0.25	4.8	4.3
29	400	15	o.25	4.8	4.6
30	400	15	0.25	4.8	5.5
31	400	15	0.25	4.8	6.0
32	400	15	0.25	4.8	7.3
33	400	15	0.25	4.8	6.7
34	400	15	0.25	4.8	6.1

Table 5. Optimum nutrient and optimum temperature with different sugar concentrations

Mash sugar conc.%	Mash volume\ml	Nutrient urea%	PH	Temp ℃	Ethanol Yield %
10	400	0.25	4.8	32	4.9
15	400	0.25	4.8	32	7.3
20	400	0.25	4.8	32	9.9
25	400	0.25	4.8	32	6.3

Table (5) shows that the maximum amount of ethanol (9.9%) was produced when the sugar concentration was 20 %. Further increase in the sugar concentration, however, resulted in the decrease of its conversion to ethanol. The decrease in fermentation efficiency by increasing the sugar level above 20 % this may be due to the toxic effect of sugar.

According to Gill[19] the low production of ethanol at high concentration of sugar may be due to the toxic effects of ethanol and not by the sugar. Therefore, the sugar level of 20 % was maintained. Monot *et al*,[20] studied the effect of sugar in synthetic medium.

The high yield of alcohol was 9.9% ethanol that is considered lower than that reported by Mohamed[21] who found that the yield of alcohol was 11% which was produced by using *Saccharomyces cerevisiae* as a fermenting microorganism when used similar production method.

Table 6. Physicochemical Characteristics of Ethanol:

Character	Value
Purity	95.5%
Density	0.804 at 30℃
Viscosity	0.81 cip

3.4. Physicochemical Characteristics of Ethanol

Table (6) shows some of the physicochemical characteristics of the examined ethanol after production. The purity, density and viscosity of ethanol were 95.5 %, 0.804 gm/ml and 0.81 cip, respectively. The purity was slightly lower than the most popular method of purification in which the purity (95.6%) as reported by Mills[22]. The density value was greater than the standard density value which was 0.789 gm/ml at 15℃[23]. The viscosity value was greater than the value 0.37 cip reported by Perry[24]. The final ethanol appears to be clear, bright and free from turbidity and considered of high quality.

4. Conclusions

Experimental results of producing ethanol from molasses showed that high yield of alcohol was obtained especially when the urea, sugar concentrations and the temperature were 0.25 %, 20 % and 32℃, respectively; that formulation gave 9,9% (w/v) ethanol in fermented mash; also it could be deducted that the yeast *Schizosaccharomyces pombe* can be used as alternative to *Saccharomyces cereveisiae* to produce high quality ethanol from high sugar concentration.

During the fermentation process, it is important to prevent oxygen from getting into the ethanol, since otherwise the ethanol would be oxidized to acetic acid (vinegar). Also, in the presence of oxygen, the yeast will undergo aerobic respiration to produce just carbon dioxide and water, without producing ethanol.

To obtain ethanol in large-scale production, it is highly recommended to control the fermentation and distillation processes using yeast strains of *Schizosaccharomyces pombe* which have high ability to tolerate high sugar concentration and ethanol concentration too. In addition, it is highly recommended to use 0.25% concentration urea as nutrient to increase the yield of final ethanol produced in large scale production.

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