

Production of Bio-ethanol from Molasses by *Schizosaccharomyces* Species

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Authors' contributions

This work was carried out in collaboration between both authors. Author SEAB designed the study, wrote the protocol and interpreted the data. Author MAM anchored the field study, gathered the initial data and performed preliminary data analysis. Author SEAB managed the literature searches and produced the initial draft. Both authors read and approved the final manuscript

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ABSTRACT

Aims: The aims of this study were isolation of *Schizosaccharomyces* species and production of bio-ethanol from local sugarcane molasses.

Study Design: The study was designated as an experimental study.

Place and Duration of Study: This study was conducted at the Department of Microbiology and Molecular Biology, Faculty of Science and Technology, Al-Neelain University, Khartoum – Sudan “1st February to 30th May 2014”.

Methodology: *Schizosaccharomyces* species were isolated from three different sources (Lentils, Banana and Sorghum Fermented dough) using poured plate technique consisting Yeast Extract Agar (YEA) medium. Physical and microbiological analyses were carried out for molasses samples. Raw Molasses (RM) in range of 100-500 ml and Sucrose-determined molasses in range of 10-50% sucrose were fermented using three isolates for each concentration separately. The bio-ethanol was determined and evaluated.

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Results: The moisture content of molasses was found to be 65%. The ash was 6.50%. The pH value was decreased by one unit during the fermentation processes due to the molasses degradation with acid production. Bio-ethanol was produced from two types of molasses preparations (raw molasses and sucrose determined concentration samples). *Schizosaccharomyces* spp. fermented molasses samples at all concentrations except 100% because the solution was hypertonic and the microorganisms did not tolerate that concentration. The highest volume of ethanol obtained at concentration of 3:300 ml of molasses/row. While the lowest one obtained at concentration of 4:10% of sucrose / row. The final bio-ethanol was appeared to be colourless, clear, bright, and free from turbidity indicating its high specification quality.

Conclusion: The best conditions to obtain a highest volume of bio-ethanol are appropriate concentrations of molasses and suitable pH. The highest volume of bio-ethanol was 23.51 ml which obtained at 85.5 g/solids (molasses) and pH 6. While highest volume of bio-ethanol is sucrose-determined concentration sample was 16.03 ml at 71.25 g/solids and pH 6. We recommended the utilization of *Schizosaccharomyces* species in large scale production of ethanol to manage the industrial wastes.

Keywords: *Schizosaccharomyces*; molasses; bio-ethanol; biofuel; distillation; sucrose.

1. INTRODUCTION

Sugarcane molasses is a viscous, dark and sugar-rich by-product of sugar extraction from the sugarcane (*Saccharum officinarum* L.) [1]. It contains about 62% of carbohydrates in the form of 30% un-crystallized sucrose and about 32% of invert sugar which is a mixture of glucose and fructose [2].

The sugar, which is converted into molasses, is adjusted to 14-16%, which permits an alcohol content of 8 - 10 volume percentage in the fermented worts [2].

The term 'molasses' is applied to the final effluent obtained in the preparation of sugar by repeated crystallization. The amount of molasses obtained and its quality (composition) provide information about the nature of the beets (local conditions of growth and effects of the weather) and the processing in the sugar factory, such as the efficiency of the juice clarification, the method of crystallization during boiling, and the separation of the sugar crystals from the low-grade massecuite [3].

In white sugar factories the yield of molasses is in the neighbourhood of 4% on beets, corresponding to up to 25% on sugar. With average sugar content in the beets of 16-18% only 13 to 14% of the sugar will be recovered as a commercial product. As an average, 2.2-2.6% sugar on beets will go into the molasses when raw sugar is produced. The yield of molasses is affected by various factors and differs from batch to batch [3].

Sugarcane molasses has several important roles in livestock feeding, due to the nutritive, appetizing and physical properties of its sugar content. Molasses is rather difficult to handle because of its viscosity: it is rarely fed directly in its liquid form but instead mixed to other ingredients [1].

Sugarcane molasses are also used for alcohol (rum or fuel ethanol) production and the distillery process yields vinasses that can also be used in animal feeding [4].

Ethanol known as ethyl alcohol or grain alcohol is a flammable, colourless, mildly toxic chemical compound with a distinctive perfume-like odour and the ethanol is found in alcoholic beverages. In common usage, it is often referred to simply as alcohol [5].

Traditionally ethanol is produced from cane molasses by fermentation with yeasts. Due to product inhibition ethanol concentration is usually limited to 8-9% by volume [2].

Ethanol fermentation is a continuous process, the molasses flow in and fermented wash flows out of the fermentor. The concentration of yeast cell cycle can be segregated in different fermentors for the yeast cell growth and carbon dioxide evolved. The process is continued and yeast cells remain in suspension. Finally the yeast cells are removed and clear wash is taken for distillation. The yeast strains normally employed in industrial process show a limited tolerance to ethanol, temperature and high osmotic pressure of the medium [6].

Fermentation of sugar-based raw materials is referred to as "first generation" use of lignocelluloses raw materials is commonly called "second generation" bio-ethanol. The "third generation" of algal bio-ethanol is at an early stage of investigation [7].

Microorganisms play an important role in biotransformation of waste products into human, animal and plant consumables. Yeast cells are used in household fermentation, food production, industrial fermentation and biotransformation process. Fermentation of sugars by yeast is the oldest and largest application of this technology, it process involves conversion of sugars to alcohol and carbon dioxide by the yeasts *Schizosaccharomyces* and *Saccharomyces* [8].

Schizosaccharomyces 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 [6].

Schizosaccharomyces pombe is usually found in sugar-containing fermentations of alcohol from the subtropical regions [9].

Even though its origin dates back to quite a long time ago, it was not widely known before the 1890's. It was discovered in 1893 when a group working in a Brewery Association Laboratory in Germany was looking at sediment found in millet beer imported from East Africa that gave it an unsavory acidic taste [9]. P. Lindner was the first to describe *Schizosaccharomyces pombe*. He chose as its epithet the Swahili word for beer, pombe. It was identified as yeast, and it became known as the fission yeast because it reproduces by means of fission unlike its relative *Saccharomyces cerevisiae*. The name *Schizosaccharomyces* was assigned to it because Schizo- means "different," which had been previously used to describe other fission species [9].

The sequencing of its genome was significant since *S. pombe* is a single-celled living archiascomycete fungus that shares many features with cells of more complicated eukaryotes [10]. Researchers have identified fifty genes of *S. pombe* associated with human diseases including cystic fibrosis, hereditary deafness, and diabetes [10]. Researchers state that the largest groups of human disease-related

genes are those implicated in cancer. There are 23 such genes, and they are involved in DNA damage and repair, checkpoint controls, and the cell cycle. All these processes are involved with maintaining genomic stability [10]. These discoveries are important because it will allow researchers to find out more about the evolution of one-celled and multi-celled eukaryotic organisms compared to others such as bacteria, which do not have nucleated cells. Further analyses and comparisons should reveal which genes define eukaryotic cells and the transition from one-celled to multi-celled organisms [10].

Schizosaccharomyces pombe is a chemoorganotroph, so it uses organic compounds as a source of energy and does not require light to grow. These fission yeasts can grow under both aerobic and anaerobic conditions. Fission yeasts are facultatively fermentative and exhibits aerobic fermentation in the presence of excess sugar [11]. Alcohol dehydrogenase (ADH) catalyzes the reduction of acetaldehyde to ethanol in the last step of alcohol fermentation. This reduction is coupled with the oxidation of NADH and provides the NAD⁺ essential for the glyceraldehyde-3-phosphate oxidation in glycolysis. Therefore, ethanol production is important to maintain the redox balance in the cytoplasm [11]. For a while, it has been widely assumed that *S. pombe* does not contain a mitochondrial ADH isoenzyme, and therefore does not have ethanol-dependent respiratory activity in the mitochondria [12]. Ethanol-dependent respiratory activity is generally attributed to the presence of mitochondrial ADH isoenzymes. However, it was shown recently using genetic knockout strains that *S. pombe* does exhibit mitochondrial ADH activity, but the physiological function of yeast mitochondrial ADH enzymes is unclear [12].

Schizosaccharomyces pombe has evolved as a natural inositol auxotroph. Inositol is essential for the growth of all eukaryotic cells because it is a precursor of a major membrane phospholipid, sphingolipids, and glycosylphosphatidyl-inositol. These phosphorylated metabolites of inositol play an important role in the signal transduction pathways [9]. It was discovered that *S. pombe* might have evolved as a natural inositol auxotroph because the natural environment of *S. pombe* contains a significant amount of phytic acid, which can be utilized as a source of inositol under very specific conditions. However, more research needs to be done in this area [9].

Isolates of *S. pombe*, *T. delbrueckii*, and *Z. bailii* exhibit tolerance up to 60% glucose concentration and are commonly associated with alcoholic fermentation for wine and champagne production. As the fermentation progressed, species with low acid tolerance decreased in population [13]. Species such as *S. pombe*, with moderate tolerance to acidic conditions, die off after day 10. In general, Kombucha fermentation is initiated by osmo-tolerant species of yeast, which are capable of growing in the presence of high concentrations of sugar. The process is then succeeded and ultimately dominated by acid-tolerant species [13].

The fission yeast *Schizosaccharomyces pombe* is a harmless, rapidly growing eukaryote. Therefore, there are no pathologies associated with this particular organism [9].

The main objective of the current study is to produce bio-ethanol from molasses using the fission yeast (*Schizosaccharomyces*) and detect and determine the bio-ethanol.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Forty litres of sugarcane molasses sample were obtained from the Distillery Unit of Kennan Sugars (D.U.K.S) Company– White Province – Sudan. The sugarcane molasses were collected in clean, durable plastic container and stored at room temperature for further uses.

The banana, lentils and sorghum fermented dough which used to isolate yeast were collected from Sudanese local markets for formers and home for later. These sources were very cheap in Sudan, spoiled mainly by yeast and known as the main habitat of *Schizosaccharomyces*.

2.2 Isolation of *Schizosaccharomyces*

The samples were labelled alphabetically (A= Lentils, B= Banana, and C=Sorghum Fermented Dough). The lentils and banana samples were swabbed using sterile cotton swab, while a loop full of fermented dough was taken. All the samples were inoculated onto three sterile plates containing Yeast Extract Agar (yeast extract 6.5 g/l, glucose 20 g/l, peptone 10 g/l, agar 15 g/l supplemented by 0.3 g Chloramphenicol Sodium Succinate (BP)) for each one labelled digitally and alphabetically (A₁, A₂, A₃, B₁, B₂, B₃, C₁, C₂, and C₃) then incubated at 36°C for 48 hours [14].

Subsequently plates were examined for yeast growth. The culture characteristics were observed and Gram stain method. The microorganisms were examined microscopically using (Olympus CX21FS1, binocular compound microscope) to verify the results [15].

2.3 Production of Ethanol from Molasses

2.3.1 Physical characteristics of the molasses sample

The physical characteristics of sugar cane molasses such as moisture content, ash measurement and pH were analyzed following standard methods [16].

2.3.1.1 Moisture content and ash measurement

The moisture content and ash measurement of molasses was performed by taken 10 grams of molasses sample and oven dried in a crucible at 104°C for 30 minutes [3]. Then the results were calculated using the following equations:

$$\text{Moisture content (\%)} = \frac{(A - X) \div A}{100} \times 100 \quad (1)$$

$$\text{Ash (unit)} = \frac{\text{Weight of molasses before burning (A)} - \text{Weight of molasses after burning (X)}}{2} \quad (2)$$

Where A is the weight of molasses before burning. While X is the weight of molasses after.

2.3.1.2 The pH value

The pH value was measured before and after inoculation of molasses samples using pH meter device (pH 213 Microprocessor-based Bench pH/mVIC Meters. HANNAINSTRUMENTS).

2.4 Identification of the Microorganisms Originally Present in Molasses

Serial dilutions were obtained by taking one ml of molasses into sterile test tube and diluted by adding 9 ml of previously sterilized distilled water. This step was repeated 10 times to obtain one 10th of the previous dilution every time. From these serial dilutions the 4th dilution containing 1/1000 parts of molasses was used for culturing fungi on Sabouraud's Dextrose Agar and incubated at 28°C for 5 days. Bacteria was cultured from the 6th dilution containing 1/100000 parts of molasses on Nutrient Agar and incubated at 37°C for 24 hours. All microorganisms were identified microscopically

by Gram stain for bacterial cell and Lactophenol cotton blue stain for fungal cells [3,15].

2.5 Inoculation of Molasses by Isolated Yeast

The molasses samples were prepared as raw material and sucrose determined samples were autoclaved at 121°C for 10 minutes. The raw samples were placed into 15 flasks each three flasks contain equal volume of molasses supplemented with 0.5 g urea as nitrogen source (1:500 ml of molasses/row, 2:400 ml of molasses /row, 3: 300 ml of molasses /row, 4:200 ml of molasses /row and 5:100 ml of molasses / row). Also the sucrose determined samples were placed into 15 flasks (1:50% of sucrose / row, 2:35% of sucrose / row, 3: 25% of sucrose / row, and 4:10% of sucrose / row). All flasks' volume was completed to 500 ml using sterile distilled water. Each flask was inoculated with 10 ml (10^7 - 10^8 CFU/ml) of 24 hours microbial suspension and incubated at 33°C for 5 days [17].

2.6 Distillation and Detection of Ethanol

After incubation period, the flasks were distilled using sample distillation method at 78°C for 3 hours [18]. The distilled volume was detected chemically using $K_2Cr_2O_7H^+$, $KMnO_4H^+$ and iodine with NaOH. Two millilitres of distilled molasses were taken into two test tubes labelled T₁ and T₂ and 1 ml of $K_2Cr_2O_7H^+$ and $KMnO_4H^+$, was added to each tube respectively. While 1ml of distilled molasses was taken in other test tube labelled T₃, 3 ml of iodine were added followed by 3 drops of NaOH. The tube was heated and then cooled using tap water and the white precipitate was observed [19].

3. RESULTS AND DISCUSSION

3.1 Isolation of *Schizosaccharomyces* Species

The culture characteristics of *Schizosaccharomyces* species was appeared as white coloured, semi mucoid, round shaped colonies.

While microscopically, the microorganism was appeared as Gram positive, rod-shaped, thick cell wall, purple colour colonies, some appeared as long rod, thin, purple colour contained true mycelium (Hyphae) Figs. 1 and 2. These findings

were in agreement with the literature data reported by Mandeep and Kocher [6].

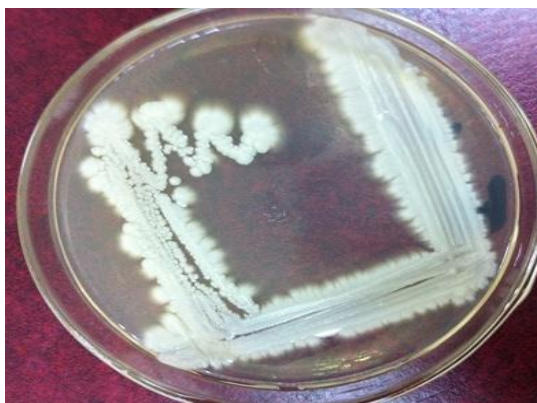


Fig. 1. Culture characteristics of *Schizosaccharomyces* species

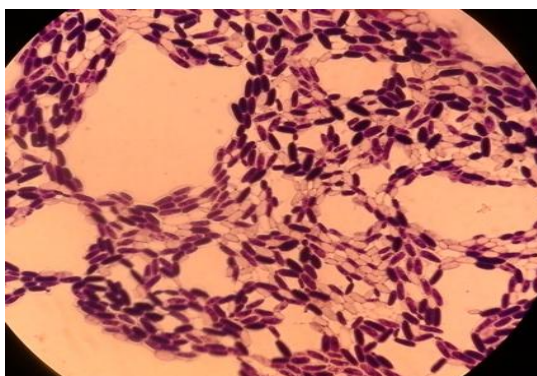


Fig. 2. Microscopic appearance of *Schizosaccharomyces* species using Gram stain technique

3.2 Physical Characteristics of the Molasses Sample

The physical characteristics of sugarcane molasses were determined and calculated. The present study exhibits that the percentage moisture content was 65%. The ash was calculated as 6.50%. While the pH shown $7, 0 \pm 0.2$. These findings were in disagreement with the findings of Gasmalla et al. [20] who reported that the pH value of obtained molasses was 5.8 ± 0.35 . The ash was 12.69% on wet weight basis. Also these findings were in disagreement with the findings of Osunkoya and Okwudinka [21] who reported that the pH value of obtained molasses was 5.1. The ash was 8.24%.

3.3 Microorganisms Originally Present in Molasses

As can be seen in Table 1, the total bacterial and fungal counts were estimated.

Table 1. Microbiological analysis of sugarcane molasses

Dilution	Total bacterial count (CFU/ml)	Total fungal count (CFU/ml)
10^4	2×10^2	1×10^2
10^6	1×10^2	10 yeast cell

These findings were in disagreement with the findings of Gasmalla et al. [20] who reported that the total viable count in dilution of 10^1 was 3×10^2 and the yeasts and moulds count was 2×10^2 .

3.4 Production of Ethanol from Raw Molasses

As can be seen in Table 2, all isolated *Schizosaccharomyces* species (A, B, and C) not produced ethanol at concentrated molasses (100%) in the first row (500 ml molasses). The production of ethanol started at 80% in the second row (400 ml molasses + 100 ml distilled water) resulting in 13.68 ml of ethanol for the samples A and C, while sample B resulted in 12.54 ml of ethanol. At the concentration of 60% in the third row (300 ml molasses + 200 ml distilled water) the three isolates produced almost resemble volume of the ethanol; sample A produced 23.34 ml of ethanol while samples B and C produced 23.51 ml. These findings were highest than that reported by Choi et al. [18]. After distillation, the volume of ethanol produced was 23.51 ml per 85.5 g of molasses. These results were in disagreement with the findings of Gasmalla et al. [20] who reported that after distillation, the volume of ethanol produced was 20 ml per 100 g of molasses. These conditions were considered suitable for yeast activity and high yield of alcohol.

At 40% in the fourth row (200 ml molasses + 300 ml distilled water) all isolates produced similar volumes of ethanol (19.09 ml). The highest production of ethanol was observed at the concentration of 20% in the last row (100 ml molasses + 400 ml distilled water) and the isolates exhibited varies volume as 15.33 ml for isolate A, 11.40 ml for isolate B, and 10.68 ml for isolate C. These findings were higher than that found by Hafiz et al. [22] who reported that in 400

ml molasses mash supplemented with 0.15, 0.25 and 0.50% urea found that ethanol yields were 3.8, 4.3 and 4.2 using *Saccharomyces* and *Schizosaccharomyces*. Also the present study in disagreement with the literature data reported by Choi et al. [18] using of ethanol-producing yeast strain CHFY0201 isolated from soil in South Korea using an enrichment technique in a yeast peptone dextrose medium at 30°C during shaking flask cultivation was 0.59 ± 0.01 g/l/h. *S. pombe* CHFY0201 yielded a final ethanol concentration of 72.1 ± 0.27 g/l and a theoretical yield of $82.7 \pm 1.52\%$ at a maximum ethanol productivity of 1.16 ± 0.07 g/l/h. Also the present study in agreement with findings of Choi et al. [18] who reported that *S. pombe* CHFY0201 is a potential producer for industrial bio-ethanol production.

The pH of molasses was decreased through the fermentation period by 1 unit due to the accumulation of acids. These results were in disagreement with literature data [19].

3.5 Production of Ethanol from Molasses with Different Concentrations of Sucrose

The concentration of sucrose in molasses was measured using refractometer device.

As can be seen in Table 3, the isolated *Schizosaccharomyces* species (A, B, and C) started the production of ethanol at 50% of molasses in the first row resulting in 16.03 ml of ethanol for the samples A and C, while sample B resulted 15.68 ml of ethanol. At the concentration of 35% in the second row the three isolates shown variable manner of ethanol production. Sample A produced 11.22 ml of ethanol while sample B produced 9.89 ml and sample C produced 12.47 ml. These results were lower than that produced at the first row (50%). At the concentration of 25% in the third row all isolates produced similar volumes of ethanol (10.69 ml). The lowest production of ethanol was observed at the concentration of 10% in the last row and the isolates exhibited varies volume as 5.34 ml for isolate A, 4.99 ml for isolate B, and 3.92 ml for isolate C. The present results were in disagreement with the findings of Gasmalla et al. [20] who reported that the ethanol yield in 10% and 25% sugar concentration were 5.5 and 10.3 respectively. While the yield was 11.04 in 20% sugar concentration which is almost similar to the present study at the concentration of 35%.

Hemamalini et al. [2] reported that the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used as free cells in continuous ethanol fermentation and the ethanol yield was noted as 5.25% and 7.20% respectively. These findings were in disagreement with the present study.

The pH of molasses was decreased through the fermentation period by 1 unit. These results were in disagreement with the findings of previous study achieved by Mandeep and Kocher [6] and in an agreement with the findings of previous study accomplished by James [17].

3.6 Detection of Ethanol

The presence of ethanol was indicated by change in sample's colour when subjected to different chemical reagents. In the first test when using KMnO_4H^+ purple colour of KMnO_4H^+ reduced to colourless solution figure 3. In the second test and after addition of $\text{K}_2\text{Cr}_2\text{O}_7\text{H}^+$ the yellow colour of $\text{K}_2\text{Cr}_2\text{O}_7\text{H}^+$ changed to green colour Fig. 4. In the third confirmatory test, by using heated iodine followed by cooling and adding of NaOH the yellow colour precipitate called iodo-form was observed Fig. 5. These results were in agreement with Caldwell [1] and Rachel [8].

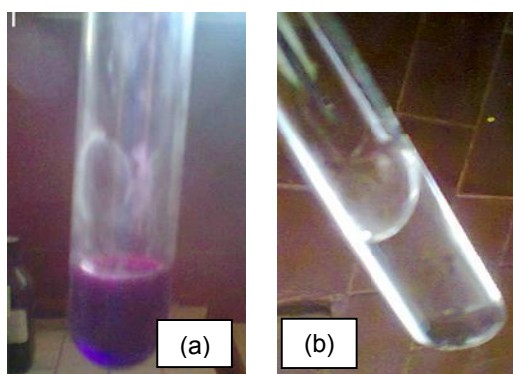


Fig. 3. KMnO_4H^+ before addition of sample (a), Reduction of KMnO_4H^+ to colourless after addition of sample (b)

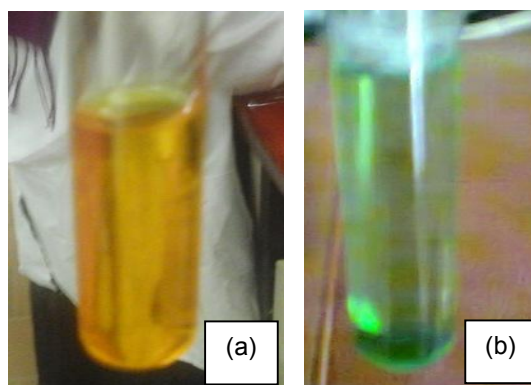


Fig. 4. $\text{K}_2\text{Cr}_2\text{O}_7\text{H}^+$ before addition of sample (a), $\text{K}_2\text{Cr}_2\text{O}_7\text{H}^+$ after addition change into green colour (b)

Table 2. Production of ethanol and pH value from raw molasses

Molasses	Solids/g	Volume of ethanol/ml			pH of molasses	
		A	B	C	Initial	Final
500 ml (100%)	142.5	0	0	0	8	8
400 ml (80%)	114	13.68	12.54	13.68	8	7
300 ml (60%)	85.5	23.34	23.51	23.51	7	6
200 ml (40%)	57	19.09	19.09	19.09	6	5
100 ml (20%)	28.5	15.33	11.40	10.68	6	5

Table 3. Production of ethanol and pH value from molasses with different concentrations of sucrose

Sucrose (%)	Solids/g	Volume of ethanol/ml			pH of molasses	
		A	B	C	Initial	Final
50	71.25	16.03	15.68	16.03	7	6
35	49.88	11.22	9.89	12.47	6	5
25	35.63	10.69	10.69	10.69	6	5
10	14.25	5.34	4.99	3.92	6	5

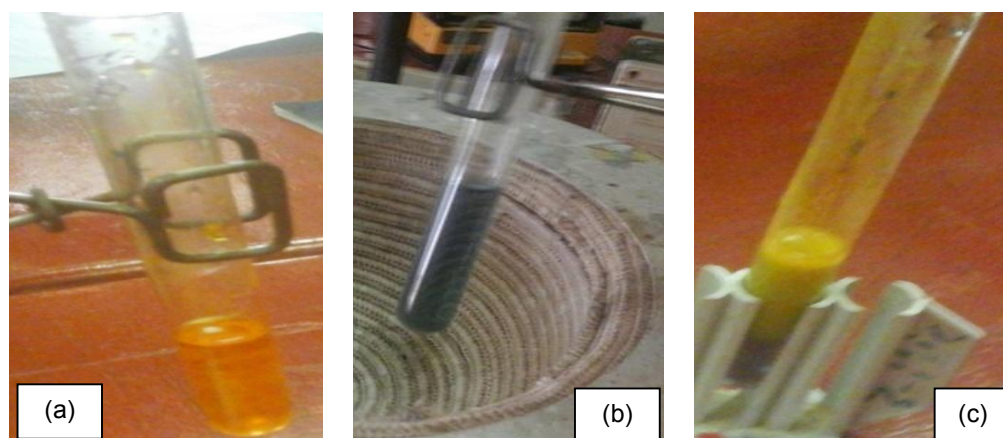


Fig. 5. Iodine reagent before addition sample (a), addition of sample and heating, colour of iodine change into blue colour (b) and when cooling in water and added NaOH, the reaction formed yellow colour precipitate called iodo-form (c)

4. CONCLUSION

Experimental results of producing ethanol from molasses showed that high yield of alcohol (23.51 ml) was obtained especially when the raw molasses of 85.5 g/solids and the pH were 6-5 and 33°C, ethanol in fermented mash; also it could be deduced that *Schizosaccharomyces* species can be used as alternative to *Saccharomyces cerevisiae* to produce high quality ethanol from high sugar concentration. To obtain ethanol in large-scale production, it is highly recommended to control the fermentation and distillation processes using yeast strains of *Schizosaccharomyces* species which have high ability to tolerate high sugar concentration and ethanol concentration too.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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