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Production of Citric Acid by *Aspergillus niger* Using Sugarcane Molasses as Substrate

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Abstract

Citric acid ($CH_2COOH.COH.COOH.CH_2COOH$) is a tricarboxylic acid, soluble in water with a pleasant taste; it is an important acid used in food Industries. It exists in nature when carbohydrates are oxidized to carbon dioxide. Because of its high solubility, palatability and low toxicity it can be used in food, biochemical, and pharmaceutical industries. The aims of this study are citric acid production from fungi (*Aspergillus niger*) using by-product of sugar (sugarcane molasses) and to evaluate its concentration. Indigenous strains of *A. niger* were isolated from soil (depth 15cm), air and bread and identified using ordinary medium Sabouraud's dextrose agar medium supplemented with Rose Bengal. A pure culture of tested microorganisms were inoculated into different flasks containing different concentrations of molasses and incubated for 144 hrs at 28°C. The production of citric acid determined by the appearance of air bubble and colour's change; the mixtures were distilled at 175ºC for one and half hr. After the distillation process; the citric acid was detected and titrated to determine its percentage by adding bromocryesol green and NaOH (N 0.1), respectively. Citric acid production from the soil sample was of high amount, when compared with air, and bread. The soil sample produced 9.6 % of citric acid compared with air 6.7% and bread 7.7 %. The maximum citric acid production was produced on the 6th day of fermentation in all samples. By recycling and reusing waste material from cane molasses citric acid production can be easily achieved by using microorganisms that have the ability to produce citric acid efficiency such as *Aspergillus niger*.

Keywords: *Aspergillus niger*, Sugarcane molasses, Citric Acid production, Sucrose, Distillation, Fermentation.

1. Introduction

Citric acid is a weak organic acid with the formula $C_6H_8O_7$. It is a natural preservative conservative and is also used to add an acidic or sour taste to foods and drinks. In biochemistry, the conjugate base of citric acid, citrate, is important as an intermediate in the citric acid cycle, which occurs in the metabolism of all aerobic organisms. It consists of 3 carboxyl (R-COOH) groups (Berovic *et al*., 2007). The basic substrates for citric acid fermentation using submerged technique of fermentation are beet or cane molasses (Pazouki *et al*., 2000). Other different methods are being used for citric acid production, extraction of citric acid from fruits and its chemical synthesis, citric acid from whey and other dairy product wastes, citric acid from beet molasses as substrates. But the most commercially used method for the production of citric acid is by *Aspergillus niger* using cane molasses as an example of fungal over flow metabolism (Kabera *et al*., 2010). Many microorganisms, such as fungi and bacteria, can produce citric acid but *A. niger* remained the organism of choice for the production of citric acid due to its genetic stability, high yields, capacity of using cheaper raw material (like cane molasses) and absence of undesirable reactions (Murad *et al*., 2003). Many useful enzymes are produced using the industrial fermentation of *A. niger*. For example, *A. niger* glucoamylase is used in the production of high fructose corn syrup, and pectinases are used in cider and wine clarification. Alpha-galactosidase, an enzyme that breaks down certain complex sugars, is a component of beano and other products that decrease flatulence. Another use for *A. niger* within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for nuclear magnetic resonance (NMR) analysis (Hess *et al*., 2000).

The objectives of this study are: to produce citric acid from sugarcane molasses as a substrate using *A.niger* with charcterize and to determine citric acid yield and concentration.

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2. Material and Method

2.1. Area of study

This study was conducted at the Department of Microbiology and Molecular Biology, Faculty of Science and Technology, Al-Neelain University, Khartoum – Sudan. All experiments were accomplished aseptically in the laboratory of microbiology.

2.2. Collection of sample

Three samples were collected from air, bread, and soil in depth of 15 cm from Tuti islands' farms (Erika et al., 2013). 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.

2.3. Isolation of test microorganism (Aspergillus niger)

One gram of the soil sample was placed in the test tube containing 10 ml of sterile distilled water to make a soil suspension and tenfold serial dilution was made by transferring one ml of the soil suspension to another test tube containing 9 ml of sterile distilled water. This step was repeated ten times to obtain a dilution of 10-10. An amount of 0.1 ml from each of the first three test tubes (10-1, 10-2, and 10-3) was taken and placed on the plate containing Sabouraud dextrose agar medium supplemented with rose bengal to inhibit the growth of saprophytes fungi other than *A. niger*. Another plate was opened inside the laboratory of microbiology to isolate *A. niger* from the air; the third plate was inoculated with the *A. niger* from infested bread; all plates were incubated aerobically at 25°C for 72 hrs. After the incubation period, the culture characteristics were observed and the growth was examined microscopically to confirm its purity using lactophenol cotton blue stain technique (Cheesbrough, 2008).

2.4. Physical characteristics of the sugarcane molasses sample

The physical characteristics of sugar cane molasses, such as moisture content, ash measurement and pH, were analyzed following standard methods (APHA, 2000).

2.4.1. 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 (Hubert, 2006). Then the results were calculated using the following equations:

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

Ash (unit) = Weight of molasses before burning (A) - Weight of molasses after burning (X) (2)

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

2.4.2. 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/mV/C Meters. Hanna Instruments).

2.5. Production of citric acid from raw sugarcane molasses

Isolates of *A. niger* were transferred to the 15 flasks containing raw sugarcane molasses media with different concentrations, i.e., each three flasks have the equal amount of molasses (20%, 30%, 40%, 50%, and 60%) by taking 100 ml, 150 ml, 200 ml, 250 ml, and 300 ml of sugarcane molasses and the volume was completed to 500 ml using sterile distilled water. The flasks were autoclaved at 115°C for 10 minutes. An amount of 50 ml of distilled water was added to the fungal pure culture to make a fungal suspension and then 10 ml from this suspension was transferred to the sugarcane molasses media. All flasks were incubated at 28°C for 144 hrs till 10 days. After incubation, the suspension was distilled to monitor the growth and observe the results (Elholi and Al-Delaimy, 2003).

2.6. Production of citric acid from sugarcane molasses with determined concentration of sucrose

In another experiment, different concentrations of sucrose (10%, 25%, 35%, and 50%) were measured using a hand refractometer device. After sterilization, an amount of 0.5 grams of urea powder was added to each flask containing sugarcane molasses with known concentration. Then 3 ml from pure *A. niger* were added to the media and the culture was incubated at 28°C for144 hrs till 10 days. After incubation, the suspension was distilled to monitor the growth and observe the results (Dubey, 2003).

2.7. Detection of citric acid

The detection of citric acid was done chemically by the addition of three drops of bromocrysol green indicator to the 10 ml of distillation yield (Soccol1 *et al*., 2006).

2.8. 2.8. Determination of citric acid concentration

Citric acid was determined by titration using 0.1N NaOH and Phenonphthalein as indicator and calculated as percentage according to the following formula (Soccoll *et al.*, 2006):

- Normality of Citric acid = normality of NaOH× NaOH volume ÷ volume of Citric acid
- Concentration of Citric acid = Citric acid normality ×equivalent ×100 ÷ volume of distillation
- (Equivalent = 96, volume of distillation = 10)

3. Results and Discussion

3.1. Isolation of Aspergillus niger

Three isolates were isolated from three different sources air, bread, and soil using sterile culture media, the isolates were purified, examined microscopically to show its purity and characterized by its culture characteristics.

3.2. Physical characteristics of the sugarcane molasses sample

The physical characteristics of sugarcane molasses were determined and calculated. The present study shows that the percentage moisture content was 65%. The ash was calculated as 6.50%.While the pH shown 6.0±0.2.

These findings were in disagreement with the findings of Gasmalla *et al.* (2012) 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 (2011) who reported that the pH value of obtained molasses was 5.1. The ash was 8.24%.

3.3. Production of citric acid from raw sugarcane molasses

As can be seen in Table 1, the best yield by all strains was 37.5 ml, 37.0 ml, 35.0 ml which were obtained at concentration of 20%. At concentration 30% they were 37.0 ml, 37.5 ml, and 33.5 ml, while at concentration 40% the two strains that were isolated from soil and bread gave a similar yield 35.0 ml and the air isolate strains gave 20.0 ml citric acid. The present study was almost in agreement with Sikander *et al*. (2002) who reported four *A. niger* isolates produced citric acid with the concentration of 18.86±1.8 – 42.56±2.0 g/l on 150g/l molasses sugar.

The lowest yields were obtained at the concentration of 50% as 10.0 ml, 5.5 ml, and 8.0 ml citric acid. At 60% sugarcane molasses concentration the microorganism did not exhibited any growth due to the effect of hypertonic solution. These findings were in disagreement with Sikander *et al*. (2002) who stated that three *A. niger* cultures gave concentrations of citric acid ranged between 58.14±2.7 – 78.18±18 g/l on 150g/l molasses sugar. Also, the present study was in agreement with Peksel and Kubicek (2003) who reported that the concentration and type of sugar influence the yield of citric acid production by *A. niger*. The present findings also were in agreement with Laboni *et al*. (2010) who reported that the citric acid production increased with the increase of the fermentation period and the maximum citric acid production was found on day 13. Also, the present results were in disagreement with Helen *et al.* (2014) who stated that the production of citric acid by *A.niger*, cultured on *Parkia biglobosa* fruit pulp, showed that the highest yield (1.15 g/L) of citric acid was obtained at pH 2 and it declined as the pH increased from being acidic to alkaline (pH8) with the yield of (0.86 g/L).

During the fermentation process there was a gradual reduction (Figure 1) of pH noticed in all the experiments and it indicated the production of citric acid. These findings were in agreement with Thangavelu and Murugaiyan (2011) who stated that, in control production medium, the initial pH 6.5 is gradually reduced to 1.5 during fermentation.

Table 1. Production of citric acid from raw molasses

Sugarcane Molasses concentration%	Solids/ g	Soil yield (ml)	Bread yield (ml)	Air yield (ml)
20	28.50	37.5	37.0	35.0
30	42.75	37.0	37.5	33.5
40	57.00	35.0	35.0	20.0
50	71.25	10.0	5.5	8.0
60	85.50	No production	No production	No production

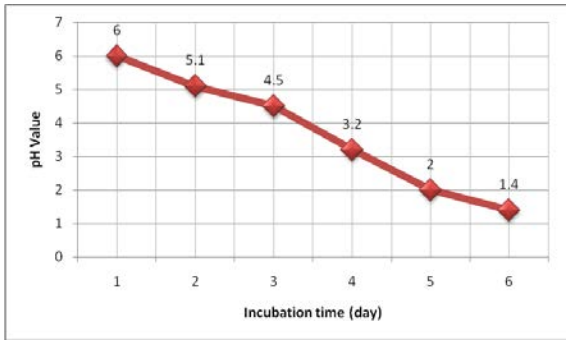

Figure 1. Citric acid indicated by pH reduction

3.4. Production of citric acid from molasses with determined concentration of sucrose

As can be seen in Table 2, the yield of citric acid was high when using the *A. niger* which was isolated from soil at all concentrations compared with other isolates (air, bread), followed by bread isolates, then air isolates which was the lowest yield. These findings were in disagreement with Kareem *et al.* (2010) who stated that the inoculation of *A. niger* on medium supplemented with sucrose (15% w/v) gave the highest citric acid value (36.6 g/kg). Also these findings were in agreement with the same author who stated that *A. niger*, when inoculated on medium containing pineapple peels, gave 17.23 g/kg at 5 days fermentation period. The increase in citric acid production and biomass values was accompanied with a steady decrease in sugar along the incubation time.

The addition of urea as a nitrogen source did not affect the production of citric acid; this is in agreement with Sadia *et al.* (2011) who reported that all concentrations (0.1 to 0.6%) of ammonium sulphate, peptone and yeast extract, used as a nitrogen source, were found to be inhibitory to fungal growth, sugar utilization and citric acid production. Also, the findings of this study were in agreement with Laboni *et al.* (2010) who stated that in the presence of prescott salt, citric acid production was found lower than it is with the absence of prescott salt and mixed substrate prepared with molasses and pumpkin media proved to be the best and potential for citric acid production. The present study was in disagreement with Nehad (2002) who reported that the natural oils with high unsaturated fatty acids content when added at concentrations of 2% and 4% (v/v) to Beet Molasses (BM) medium caused a considerable increase in citric acid yield from *A. niger*. The maximum citric acid yield was achieved in surface culture in the presence of 4% olive oil after 12 days incubation.

Table 2. Production of citric acid from molasses (sucrose + urea)

Sucrose%	Solids/g	Urea /g	Soil yield (ml)	Bread yield (ml)	Air yield(ml)
10	14.25		10.6	10.0	9.0
25	35.63	0.5	18.0	15.0	13.0
35	49.88		19.5	17.0	10.5
50	71.25		9.0	7.5	8.5

3.5. Determination of citric acid concentration

As can be seen in Table 3 and figure 3, the percentage of citric acid was determined. The concentration of citric acid varied due to the sucrose percentage; it showed high at the concentration of 35% (9.6%) for soil isolate, and (7.7%) for bread isolate, similar to the concentration 25% (7.7%) for soil isolate. At the concentration 25% of sucrose, bread and air isolates showed a similar concentration of citric acid (6.7%). At 10% of sucrose the soil and bread isolate exhibited a similar result as (4.8% citric acid percentage) followed by 3.8% of air isolate. The lowest concentrations of citric acid were shown at concentration 50% (2.9%) for both soil and bread isolates and 1.9% for air isolate.

Table 3. Titration of citric acid of three different isolates

Sucrose %	Citric acid %		
	Soil isolate	Bread isolate	Air isolate
10	4.8	4.8	3.8
25	7.7	6.7	6.7
35	9.6	7.7	4.8
50	2.9	2.9	1.9

Comparing the three isolates, the percentage of citric acid which was estimated as the lowest percentage was obtained by the *A. niger* isolated from air, shown in Figure 2.

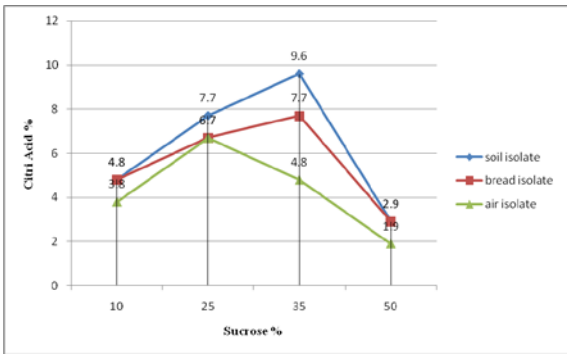

Figure 2. The citric Acid concentration by different *A. niger* isolates

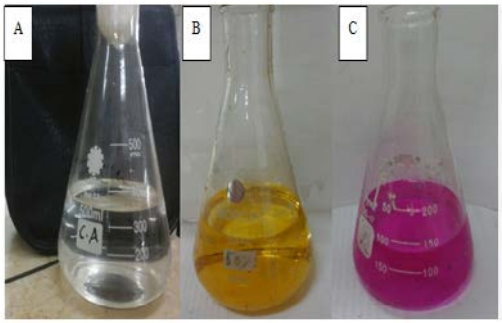

Figure 3. A. Citric acid yield, B. Citric acid after addition of three drops from bromogresol green, C. Citric acid after titrated by NaOH (0.1)

4. Conclusion

By recycling and reusing waste material from sugarcane molasses, citric acid production can be easily done by using microorganism that has the ability to produce citric acid efficiency such as *Aspergillus niger*. The result of this study indicates that the use of Sugarcane molasses for fungal production of citric acid might represent an efficient method of cost reduction in the production and concomitantly producing organic acid of valuable importance.

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