Citric Acid Production by *Aspergillus niger* using cane Molasses as Substrate.

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Abstract: Citric acid production by *Aspergillus niger* isolated from spoilt beans using cane molasses was investigated. The spoilt bean on SDA media produced *Aspergillus niger* from which Citric acid was produced using submerged fermentation technique. The sensory properties of the Citric acid produced from *A. niger* indicated dark coloration with pH values of 3.55 as the minimum and 5.19 as the maximum. The total titrable acidity (TTA) in solid state fermentation by *A. niger* during Citric acid production was carried out, and the data obtained are mean values, with minimum value of 1.03 and maximum 2.55. Hence, the curve of TTA against the fermentation days showed that, as the fermentation day increases the volume of NaOH required to neutralize the acidity content in the fermentation medium also increases, but decreases at the 7th day. The concentration of Citric acid observed for the number of days of fermentation showed that the yield of the Citric acid increases with the number of days across the flasks, which have the minimum concentration value to be 0.05g/L at day 1, but different maximum values which are 0.24, 0.12, 0.13 and 0.12g/L at days 7, 5, 7 and 7 respectively. The microbial load in flasks 1, 2 and 4 were within the range of 10-19 cfu/g, while flask 3 showed an increase up to 38 cfu/g which is a significant increase compared to the other 3 flasks. The confirmatory test showed that flask 1, 2 and 4 produced Citric acid, while the flask 3 gave a negative result. This study showed that cane molasses is a suitable medium for biosynthesis of Citric acid by *Aspergillis niger* due to its nutritional content.

Key words: *Aspergillus niger*, cane molasses, Citric acid,

Introduction

The organic acids are extensively used in a variety of food products as preservatives, pH adjuster, sweetness enhancer, leavening agents and stabilizers (Majumder *et al.*, 2010). The acceptability of food products mainly depends on the flavor components, which are complex as well as type-specific. These flavor components are influenced by the presence of organic acids and other substances like sulphur compounds, lactones, methyl ketones, alcohols and phenolic substances. The important flavor substances are formed as a result of the hydrolysis of fatty acids or by the bacterial growth, or enhanced by the addition of acidulants during processing (Adda *et al.*, 1982).

Citric acid is a weak organic acid with the chemical 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, (Frank, 2005). Until about 1920, all commercial citric acid was first isolated from lemon and juices (Kings and Cheetaml, 1987). Citric acid was first isolated in 1784 by chemist Carl Wilhem, who crystallized it from juices (Frank, 2005). Also, in 1893, C. Wilhem discovered penicillin mould could produce citric acid from sugar. However, microbial production of citric acid did not become industrially important until World War I disrupted Italian citrus exports. In 1917, an American food chemist James Currie discovered strains of mould *Aspergillus niger* could be efficient citric acid producers using this technique two years later, followed by Critique Belgian 1929. In this production technique, which is still the major industrial route to citric acid used today, cultures of *A.niger* are fed on sucrose or glucose –containing medium to produce Citric acid. So therefore, Citric acid is mostly produced from starch or sucrose based media using liquid fermentation, a variety of raw materials such as molasses, several starch materials and hydrocarbons have been employed (Rohr *et al.*, 1983). Citric acid is the most important organic acid produced in tonnage by fermentation. Citric acid is a versatile and innocuous alimentary additive. It is accepted worldwide as GRAS (Generally recognized as safe), and approved by the joint FAO/WHO, Expert Committee on food Additives (Schuster *et al.*, 2002). Citric acid is a normal component of human cells that is metabolized, degraded, and eliminated from the body. Within the European Union, it is denoted by E number E330. It is one of the main ingredients in the food and beverage industries, because it is easily obtained in large quantities, healthy and cheap. It is often used for proper mineral supplementation of food, as an acidity regulator, and as a flavor compound. Citric acid enhances the activity of many beneficial antioxidants, but is not, itself, an antioxidant. Citric acid undergoes Krebs cycle in the human body, and it is named after Hans Adolf Krebs, who received a Nobel Prize in

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Physiology or Medicine for its discovery in 1953. It actually refers to the physiological oxidation of fats, proteins and carbohydrates into carbon dioxide and water. And it is in turn referred to as the central metabolic pathway. During this cycle, eight enzymes help produce energy through aerobic respiration. Citric acid is an alkalizing agent and is able to decrease the level of acidity in body fluids. It can bind with excess calcium and allow it to leave the body. Despite its many health benefits, citric acid should not be taken without first consulting your physician.

At present, the fundamental exploitation of food waste, which participates in pollution, is the controlled biological degradation of the waste by microorganisms for the production of valuable compounds such as enzymes, citric acid and others as raw materials for medical and industrial uses (Munshi *et al.*, 2013). Citric acid can be used for the following purposes:

Food Production: It strengthens the gelatin in jams and slows down the oxidation process in fruits and fruit products, by combining itself with the naturally present metals and preventing their discoloration. Citric acid is often used for creating the proper environment for the enzyme activity in the process of cheese making. It can also be used for making ice cream, because it helps the fat cells in dairy separate.

Pest Control: Citric acid is known to have the ability to destroy bacteria, mold, viruses and rust, and therefore was used in the production of pesticides, fungicides and disinfectants solutions.

Drugs: Citric acid is used in the manufacture of medicinal products and in bio-technology industries. It is combined with sodium bicarbonate for medicines such as Alka-Seltzer. As it has been established that citric acid has beneficial effects, if an excess of the acid was used or consumed, some of these side effects include stomach cramps, diarrhea, nausea and vomiting. People with sensitive skin should avoid using creams containing citric acid as it will cause irritation or a rash to form. The acid is also believed to erode the tooth enamel when consumed frequently, which leads to an increased susceptibility of tooth decay, infections and other complications.

The production of citric acid by *Aspergillus niger* is one of the most commercially utilized examples of fungal overflow metabolism. Many microorganisms such as fungi and bacteria can produce Citric acid. *Aspergillus niger* is considered as the organism of choice for the production of citric acid because of the fact that this organism has the capacity to utilize varieties of substrates due to its well-developed enzymatic system (Munshi *et al.*, 2013). *Aspergillus niger* is a fungus and one of the most common species of the genus *Aspergillus*. It causes a damage called black mould on certain fruits and vegetables such as grapes, onions and peanuts, and is a common contaminant of foods. It is ubiquitous in oil and is commonly reported from indoor environments, where its black colonies can be confused with those of starch botrys (Pazouki *et al.*, 2008).

Sugar molasses is viscous, dark and sugar-rich byproduct of sugar extraction from the sugarcane (*Saccharum officinarium* L.). It is a major ingredient used as an energy source and as binder in compound feeds. Molasses provide readily fermentable energy that promotes lactic acid bacteria development and subsequently reduces pH and improve quality (Adesogan *et al.*, 2010). The basic substrates for Citric acid fermentation using submerged technique of fermentation are beet or cane molasses (Pazouki *et al.*, 2008). Molasses vary by amount of sugar and method of extraction, and age of plant. Cane sugar is obtained by the way of successive evaporations/ crystallization/ centrifugation. Both the sugar extraction process and the sugar refining process yield molasses, and each step of those processes output specific types of molasses. Sugarcane molasses has several important roles in livestock feeding, due to the nutritive, appetizing and physical properties of its sugar content.

Methodology

Sample Collection

The spoilt bean where the *Aspergillus niger* is expected to be isolated was collected into a sterile polythene bag from Lapai market. Sugarcane molasses (probable substrate for *A. niger*) was obtained from the National Cereal Research Institute (NCRI) Baddegi along Lapai-Bida road, Niger State.

Media preparation

Exactly 7.9g of the Sabouraud dextrose agar (PDA) was dissolved in 120ml of distilled water, very insignifant grams of Streptomycin was added to the media and then sterilized using an autoclave at 121°C for 15 minutes, and transferred into Petri dishes to gel (solidify).

Isolation of A. niger from spoilt beans

Exactly 3.3g of the spoilt beans was disinfected by using 10% NaOCl (sodium hypochlorite) for five (5) minutes and rinsed in sterile distilled water for a desired time to ensure complete removal of NaOCl. The already sterilized beans were then inoculated on the previously prepared media and kept at room temperature for about 5-7 days until growth occurs. After which sub- culturing of the growth was done on another PDA media and kept at room temperature until growth occurs, the spores obtained from it were inoculated on a slant culture for 4-6 days to get a pure culture of *Aspergillus niger*.

Inoculum Preparation

The spore of the isolate was harvested from the slant bottles of 4-6 days of the culture by washing

with 0.8% Tween 80 (polyoxyethylene orbitanmonoleate). Also serial dilution of the isolate were done using normal saline and compared with 0.5 McFarland standards (1% Barium chloride and sulphuric acid). (Lasure *et al.*, 2003).

Fermentation Technique of *A. niger* on Citric acid production

All fermentations were carried out in 1000ml flask, the fermentation medium consists of 40g of the molasses, 1.86g ammonium nitrate (NH_4NO_3), 0.09g Potassium dihydrogen phosphate (KH_4NO_3),1.32g Magnessium sulphate heptahydrate ($MgSO_4 7H_2O$), and 3% methanol in 600ml of distilled water (Lasure *et al.*,2003). The spore inoculum used was 10^6 spores per ml (that was compared with the McFarland standards),1.32g potassium ferrocyanide was added to the fermentation medium to reduce the deleterious effect of trace metals on Citric acid yield, while refined vegetable oil was also added as an antifoam. After the addition/ mixture of all salts, the bioreactor containing the mixture was left on orbital shaker (agitation) for 9 days at pH below 2.0

Estimation of the produced Citric acid

Total titrable acidity (TTA) and Citric acid produced by fermentation were estimated by the method of anthrone-sulphuric acid method (Morse, 1947) and Marrier and Boulet method (Marrier and Boulet, 1958) respectively. The total titrable acid (TTA) was determined daily by filtrating 10ml of the culture medium through Whatman filter paper. 2-3 drops of phenolphthalein as an indicator was added to the filtrate. Titrations of the fermented broth with 0.1M NaOH was done daily for the 9 days to check the volume of the base required to neutralize the acidity of the fermented broth and calculated as % according to the following formula (Al-Delaimy and El-Holi, 2003):

% Citric acid = 192.13 x M_{NaOH}x V_{NaOH} / Weight of substrate

Where,

192.13 = molar mass of citric acid.

M_{NaOH}= molarity of NaOH

V_{NaOH}= volume of NaOH consumed during titration.

Concentration of citric acid in g/L = % citric acid calculated above x 1000 / 100

After the complete fermentation i.e. on the 9th day, little quantity of hydrated lime [Ca $(OH)_2$] was added to the broth and filtered, this was done in order to precipitate calcium citrate, which was isolated and converted to the acid using diluted sulphuric acid (Frank, 2005). The microbial load of the fermented medium was examined using pour plate method, where 0.1ml of the inoculum was dispensed and kept at room temperature for 7 days. After which the observed colonies were counted using colony counter. The pH of the culture medium was maintained to about 3.5 by adding ammonium nitrate in order to avoid the fermentation of oxalic acid and gluconic acid.

Qualitative Analysis of the produced Citric acid

A solution of the liquor (fermented broth) was prepared, to it, little amount of NH_4OH was added and boiled until no smell was perceived. Thereafter, $CaCl_2$ solution was added, and further heated for two (2) minutes. Appearance of white precipitate of citrate confirms Citric acid.

Results

Table 1: Morphological characteristics of fungal isolates from spoilt beans.

S/No	Isolate and macroscopic characteristics	Texture	Microscopic characteristics	Probable fungi identity
1	Initial white; later turned yellowish to light green.	Velvety	Septate hyphae, unbranched conidiophores foot. Vesicle completely covered by double sterigmata. The conidia are in chains.	*Aspergillus* sp.
2	Conidia appeared black with rough surface.	Velvety	Long conidiophores	*Aspergillus niger*

Table 1 shows the morphological characteristics of fungal isolates from spoilt beans, where the growth was initially whitish and later turns yellowish in which the conidia appeared blackish with rough surface and a velvety texture. Microscopically, the unbranched septate hyphae covered by double sterigmata elongate forming long conidiophores.

Table 2: Result of sensory properties of the product of fermentation.

Flasks	Color	pH
1	Dark brown	3.55
2	Dark brown	4.98
3	Dark brown	5.19
4	Dark brown	5.14

Table 2 shows that the sensory properties of Citric acid produced by *Aspergillus niger* indicated a dark brown coloration with minimum pH of 3.55 (flask 1) and maximum pH 5.19 (flasks 3).

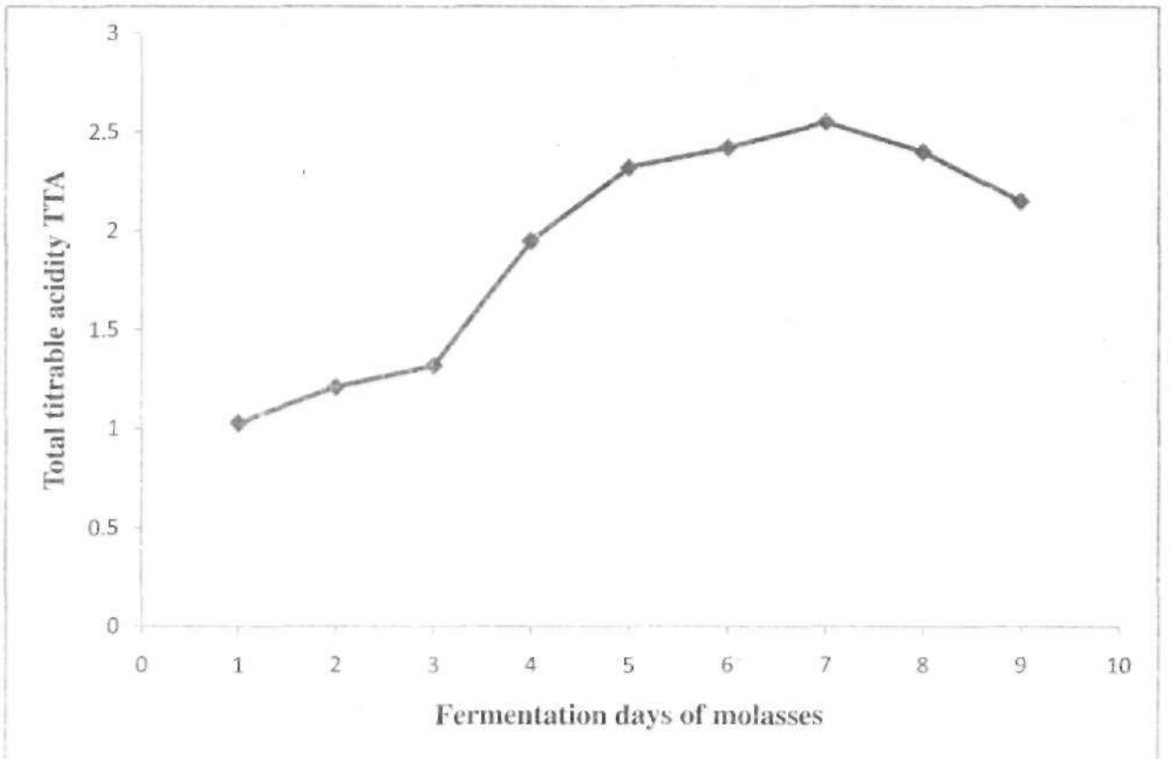

Figure 1: The graph of total titrable acidity against fermentation days.

Figure 1 presents the total titrable acidity (TTA), the data are the mean value of the four (4) replicates which show that as the fermentation day increases the volume of Sodium hydroxide (NaOH) required to neutralize the acidity content in the fermentation medium also increases, but decreases at the 7th day because there is no continuous supplement introduced into the medium.

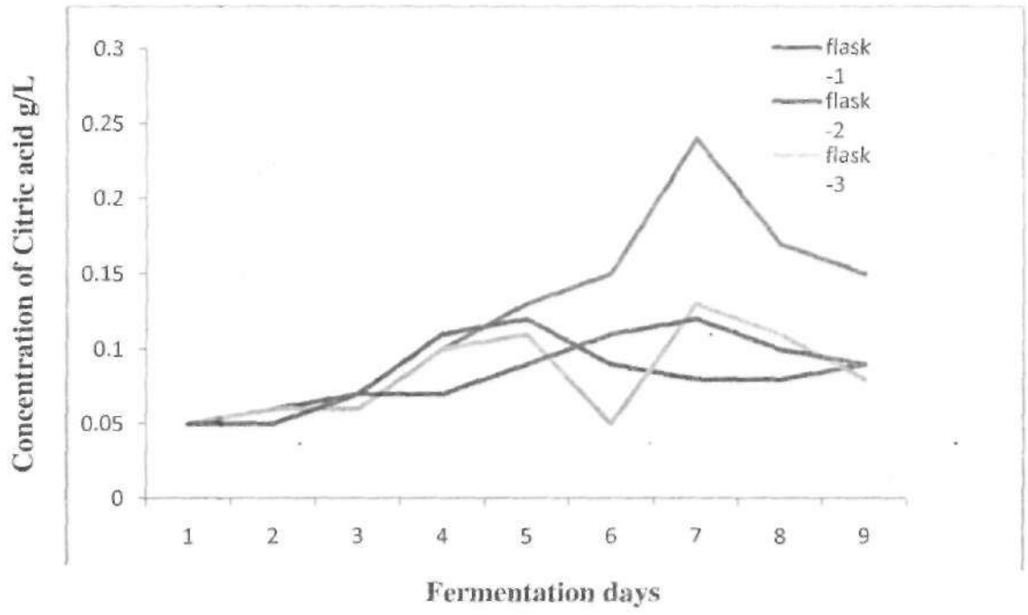

Figure2: The graph of the concentrations of Citric acid production by *A. niger* from 40g molasses against fermentation days.

The graph of concentration of Citric acid production (g/L) by *A. niger* from forty (40g) molasses, shows that there is a steady increase in all the flasks (0.05) from days 1-3 but increases rapidly from the 4th day up to the 6th day with varying concentrations but decreases between the days 7 and 9 as shown in figure 2.

Table 3: Percentages and concentration of Citric acid production by *A. niger* from 40g molasses

Days	Flask1 (%)	Conc. (g/L)	Flask2 (%)	Conc. (g/L)	Flask3 (%)	Conc. (g/L)	Flask4 (%)	Conc. (g/L)
1	0.0048	0.05	0.0054	0.05	0.005	0.05	0.0046	0.05
2	0.0058	0.06	0.0063	0.06	0.0059	0.06	0.0053	0.05
3	0.0061	0.06	0.0067	0.07	0.006	0.06	0.0066	0.07
4	0.0099	0.10	0.0107	0.11	0.0101	0.10	0.0068	0.07
5	0.0125	0.13	0.0115	0.12	0.0112	0.11	0.0093	0.09
6	0.0149	0.15	0.0091	0.09	0.4515	0.05	0.0112	0.11
7	0.024	0.24	0.0082	0.08	0.0125	0.13	0.0123	0.12
8	0.0167	0.17	0.0083	0.08	0.0110	0.11	0.0101	0.10
9	0.0154	0.15	0.0085	0.09	0.0084	0.08	0.0089	0.09

Production of Citric acid by *A. niger* on sugar cane molasses as substrate. Minimum concentrations (0.05 g/L) of Citric acid production were obtained across the flasks (1, 2, 3 and 4) with maximum concentrations of (0.24, 0.12, 0.13 and 0.12 g/L) in the flasks respectively. Also, the minimum percentage (0.0048, 0.0054, 0.0050 and 0.0046%) with their respective maximum percentage (0.0167, 0.0115, 0.4515 and 0.0123%) of Citric acid production were obtained across flasks 1, 2, 3 and 4 respectively as shown in Table 3.

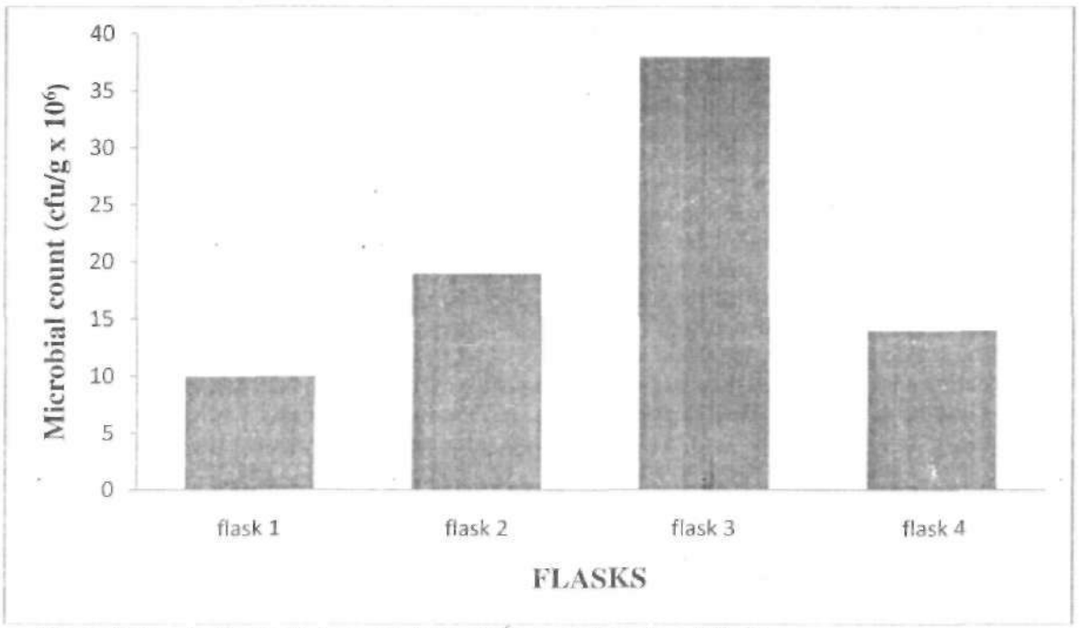

Figure 3: Graph of microbial count (cfu/g x 10^6) against number of flasks.

The microbial load (cfu/g x10^6) against the number of flasks shows that there is a significant increase in the microbial load after citric acid production. The microbial load in flasks 1, 2 and 4 were within 10- 19 cfu/g while flask 3 shows an increase up to 38 cfu/g which is a significant increase compared to the other flasks as shown in figure 3 .

Discussion

Probable Citric acid producing strains of *Aspergillus niger* and other species of *Aspergillus* isolated from spoilt beans were purified and their morphological characteristics were examined. The total titrable acidity (TTA) in solid state fermentation by *A. nigern* during Citric acid production in the four(4) flasks showed increase in the TTA values with corresponding increase in the volume of Sodium hydroxide (NaOH) used for its titration as the day of fermentation increases, this is in agreement with Abdullahi *et al.* (2008) who produced Citric acid using sugar baggase through solid state fermentation. This study is batch culture where there is no further addition of sugar as supplement which might increase the yield of Citric acid. However, media supplemented with sucrose or glucose in higher concentrations yield accumulation of Citric acid (Ali *et al.*, 2004).

It was observed in this study that there is a synchronous increase in concentration (g/L) of Citric acid, with corresponding increase in the number of fermentation days (1-3) which were 0.05, 0.06.0.06g/L for flask 1 and 3, 0.05, 0.06, 0.07 flask 2, and 0.05,0.05, 0.07 for flask 4 respectively. However there was a significant increase in days 7(0.24g/L), 5(0.12g/L), 7(0.13g/L) and 7(0.12g/L) in flask 1, 2, 3, and 4 respectively, these could be as a result contributions by 3% methanol. This observation agreed with Kareem *et al.* (2010) that the addition of methanol to pineapple waste medium led to an increase in Citric acid production by *Aspergillus niger*. Kubicek and Rohr (1986) reported that the final yield of Citric acid in fermentation by *A. niger* is strongly dependent on the type and concentration of carbon source. The inductive effect of methanol for Citric acid production may be due to reduction of the inhibitory effects of metal ions. Also addition of lower alcohols, methanol, ethanol, n-propanol, to crude carbohydrate raw materials could increase the yield of Citric acid. The exact mechanism of the alcohol effect however is unexplained, though it is postulated that addition of methanol increases the tolerance of fungi to Fe^{2+}, Zn^{2+} and Mn^{2+}(kareem and Rahman, 2013).

The pH value maintained at the beginning of fermentation was important for a specific biomass formation. Normally, Citric acid production occurred after 24 h of fermentation. Hence, sensory properties of the product of fermentation indicates a dark- brown coloration. Table3 showed that cells were only maintained and Citric acid was produced as pH decreases. Thus, the drop in pH observed during the process in flask 3 with the maximum pH of 5.19 to the minimum observed in flask 1 which was 3.55 was due to the formation and accumulation of Citric acid in the bioreactors. This is in accordance with Prado *et al.* (2005) that reported Citric acid production by solid-state fermentation on a semi-pilot scale using different percentages of treated cassava bagasse. Also, the microbial counts (x 10^6cfu/g) of the fermented broth after the 9 days were 10, 19,38 and 14 cfu/g for flask 1,

2, 3 and 4 respectively. Thus, in this study, there is highly significant difference in the production of Citric acid at day 7, which is due to their microbial load. This study also showed that cane molasses is a suitable medium for biosynthesis of Citric acid by *Aspergillus niger* due to its nutritional content and this is in agreement with the work of Luciana *et al.* (2005) which reviews recent developments on Citric acid production by presenting a brief summary of the subject, describing microorganisms, production techniques, and substrates, etc. The findings in this study showed that there is a significant increase in the microbial load before (10^6 using 0.5 McFarland standard) and after the Citric acid production. The confirmatory test showed that flask 1, 2 and 4 produced Citric acid, while the flask 3 gave a negative result.

Nitrogen constituent has a profound effect on Citric acid production because nitrogen is not only important for metabolic rates in the cells, but it is also basic part of cell proteins. This report agreed with Kareem *et al.* (2009) that fermentation media for Citric acid biosynthesis should consist of substrates necessary for the growth of microorganism primarily the carbon, nitrogen and phosphorus sources.

Conclusion

Citric acid can be produced by *A. niger* isolated from spoilt beans and supplemented with molasses from Cane sugar. In addition, Citric acid production and biomass increases with steady decrease in sugar along the incubation time, and the pH decreases with respect to increasing fermentations days.

Recommendations

Prior to commercial production of Citric acid, the fermentation medium should be examined in-vitro to observe the microbial loads before and after fermentation, as this explains the validity of the product. Also, the pH of the fermentation medium should be maintained at any value below 2.0, as it could lead to production of another metabolite (oxalate) which would be present as contaminant at pH above 2.0. The fermentation medium should be filtered to remove impurities that could affect total titratable acid.

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