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Fermentative Production of Citric acid by *Aspergillus niger* strains Using Sugarcane Bagasse as the substrate

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Abstract:

An essential organic acid, citric acid is employed in many pharmaceutical and other commercial food items. A rise in the demand for citric acid has prompted researchers worldwide to look for high yielding microorganism strains and less expensive fermentation substrate. The current work examines the isolation, screening, and synthesis of citric acid by A. niger (MTCC 281) and local (isolates), with sugar cane bagasse serving as the substrate. The yield was compared after two distinct inoculums were used in the fermentation process: set 1 had spore suspension as the inoculum, while set 2 contained the entire mycelium. The fermenting process took six days to complete. Right after autoclaving, following the addition samples of the inoculums were taken, and the levels of sugar and citric acid were first determined. Every 48 hours, samples were taken and analysed to estimate the amount of residual sugar and citric acid produced. Citric acid production was calculated spectrophotometrically using the pyridine acetic anhydride method, and residual sugar was calculated using the 3, 5 DNS method. The MTCC culture with intact mycelium had a higher yield than the spore suspension and was determined to be more suited.

***Keywords*:** Citric acid, submerged fermentation, *Aspergillus niger*, sugarcane bagasse.

Introduction:

The six-carbon tricarboxylic acid molecule known as citric acid was originally extracted from lemon juice and crystallised by Scheele in 1784. Citric acid is a 2-hydroxyl acid.

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tricarboxylic acid, 2, 3, and propane are all present in nature. An intermediate byproduct of metabolism is citric acid (Dhillion et al., 2011). It melts at 153oC and is solid at room temperature (Ana Maria, 2011). While the acid found in fruits is referred to as natural, the acid created by microbial fermentation is regarded as synthetic. The most widely used biotechnological product and one of the most significant organic acids produced by various fermentation processes is citric acid (Dhillion et al., 2011). It is a mild organic acid that is also used as a natural preservative and to impart an acidic or sour flavor to meals and soft drinks. It is a versatile chemical that is widely used, with 70% of its usage going to the food industry, 12% to the pharmaceutical industry, and the remaining 18% going to other industries.

There is a 6x105 tons annual demand for citric acid worldwide.

1. Numerous microorganisms, including bacteria like Bacillus licheniformis, B. subtilis, and Corynebacterium spp., have been studied for the generation of citric acid (Kapoor et al. Penicillium restrictum, Aspergillus niger, Aspergillus awamori, Aspergillus foetidus, and others (Mattey and Allan, 1990; Kubicek, 1998) are examples of fungi. Candida lipolytica, C. intermedia, and Saccharomyces cerevisiae are examples of yeast (Crolla and Kennedy, 2001; Archer et al., 2001; Kamzolova et al., 2003). However, due to its simplicity of handling, propensity to ferment a wide range of affordable raw materials, and high yields, the filamentous fungus A. niger continued to be the organism of choice for citric acid synthesis (Schuster et al., 2002). . Citric acid manufacturing can be made more affordable by employing inexpensive agricultural wastes such apple and grape pomace, orange peel, and kiwi Fruit peel, cotton waste, Okara soy residue, cane molasses, and okara soy residue are examples of waste materials (Kiel et al., 1981; Hang and Woodams, 1986; 1987; Khare et al., 1995; Haq et al., 2004). The sugar industries produce a large amount of sugarcane bagasse worldwide. Many scientists have successfully utilized suagarcane bagasse as the substrate for the fermentation of citric acid (Kumar et al.,2003and Vandenberghe et al., 2000). In India sugar cane bagasse is mainly used as a fire fuel, so this agricultural waste can be easily used for citric acid production. The main objective of this study was to employ sugarcane bagasse as an inexpensive substrate for the Citric acid is produced by *A. niger* (Abhishek Sharan,et al; October 2015).

Since it was approved by the Joint FAO/WHO Expert Committee on Food Additives, citric acid is acknowledged as GRAS all over the world (Ashish Kumar et al., 2008). As a result, it is utilised in a wide range of biomedical applications in addition to the food and pharmaceutical industries, biopolymers, and medication delivery (Ashish Kumar et al., 2016).

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Material and Methods

Sample Collection and Isolation of the Organism:

Different soil samples were collected into clean sterile sample bags from five different places in Telengana. 1gm of each soil sample was serially diluted in sterile distilled water and these dilutions were pla ted on to petriplates with potato dextrose agar. These plates were incubated at 28˚C for 4-7 days. Five different fungal cultures obtained in this primary screening were identified by observing under the microscope and sub cultured on the selective media. The selected media for screening of citric acid produced was Czapecks dox agar with Bromothymol blue as the indicator. The fungal growth with should yellow color zones around them were selected as citric acid producers. These colonies were stained and identified under the microscope by referring Prof. Manohar Chariya Manual. For further identification these cultures were given to Zeal biologicals. The cultured were processed for 18srRNA sequencing and identified as *Aspergillus niger*. These cultures were preserved on potato dextrose agar slants for further use. One standard culture of *Aspergillus niger* was procured from MTCC, microbial type culture collection centre, Chandigarh

Substrate:

In this present study sugarcane bagasse used as substrate which is the most common agro industrial waste found in India. Peels were then ground to 1x1cm in size (Kareem et al).

Inoculum Preparation

Two different types of inoculums were used in this type of study.

1. Spore suspension

2. Intact Mycelium

1 Spores suspension: All the *Aspergillus niger* strains were grown on PDA plates at a temperature of 30˚C for 5-7 days. After incubation these plates were washed with sterile 0.1% of tween 80 solution and these suspensions were collected in different tubes. The spores in these suspensions were counted using Haemocytometer or Neubauer's chamber. The suspension in which we get 10^{7}-10^{8} spores were collected and preserved for further use. 1ml of these suspensions were used as the inoculums.

2 Intact Mycelium: The isolated cultures of *Aspergillus niger* strains were grown on potato dextrose agar plate at temperature of 30˚C for 5days.The disc of the colonies from the plates were inoculated in 250ml Ehrlenmeyer flask containing potato dextrose broth and incubated for 48-72 hours at 30˚C till the mycelial mat develops. This mycelial used as the inoculums.

Fermentation:

Simultaneous saccharification and fermentation were followed.

The saccharification of sugarcane bagasse used for the production of citric acid is comprised of 5% substrate in distilled water (w/v).

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Fermentation process was carried out in 250ml Ehrlenmeyer flask. The fermentation media consisted of 5% substrate in 100ml of distilled water. This flask with the media was cotton plugged and kept for autoclaving at 15lbs for 15minutes at 121˚C. Sterilization was done for two purposes

a. To prevent from contamination
b. b. For mild hydrolysis

Immediately after autoclaving, the initial sugar was estimated by 3,5DNS Method and the initial citric acid was estimated by Pyridine acetic acid anhydride method. Then the two different inoculums of spore suspension and intact mycelium were added in the sterilized media.

To check the efficiency of two different inoculums the fermentation processes were carried out in two different sets; set I consists of spores suspension as the inoculum and the second set consisted of intact mycelium as the inoculum.

Set I: 1ml of inoculums (spore suspension) was inoculated in all the flask and incubated at 30˚C for 6days.

Set II: A mat of intact mycelium was added in all the flasks and incubated at 30˚C for 6days.

Result and Discussions: The following figures shows the pictures of Aspergillus niger on czapeckdox agar and the selective media of czapeckdox agar with bromothymol blue as the indicator.

Fig1: *A. niger* on Czapeckdox Agar

Fig2: *A. niger* on selective media

The following figures are the microscopic observation of *Asperillus niger* and the counting of spores in haemocytometer for set I inoculum.

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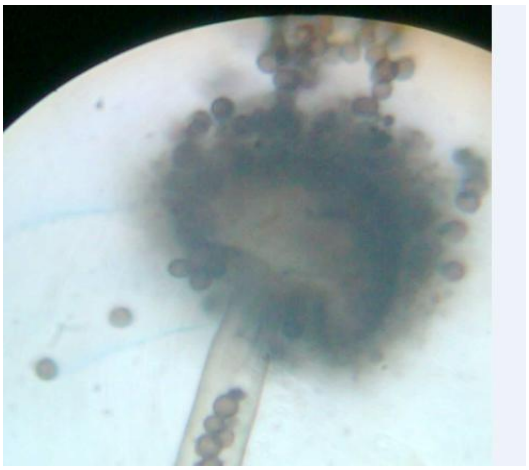


Fig3: Microscopic observation of *A. niger*

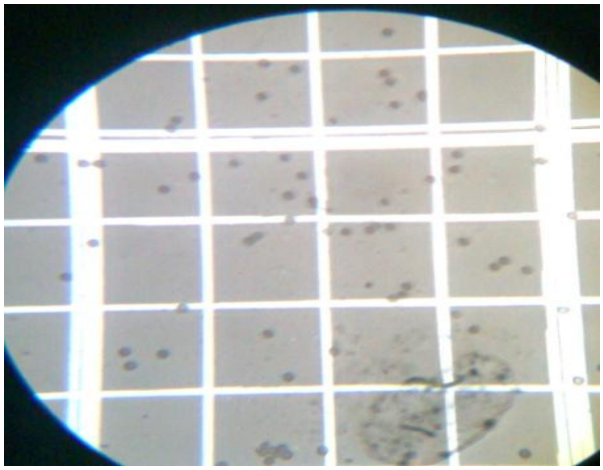
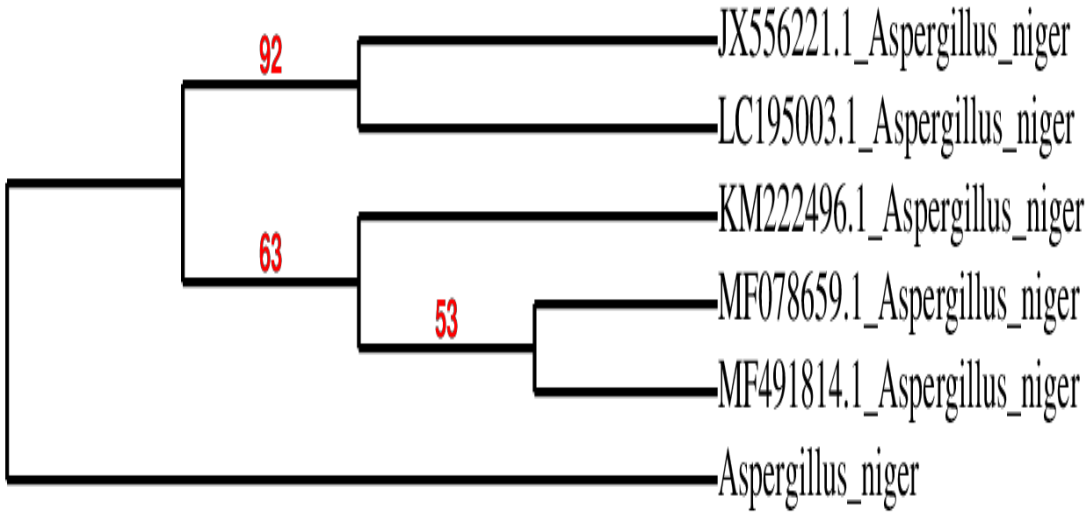


Fig4: Spore counting in Haemocytometers

The 18srRNA sequences of the selected cultures were used to identify them at molecular level. Following is confirmation report froms zeal biologicals along with phylogenetic tree:



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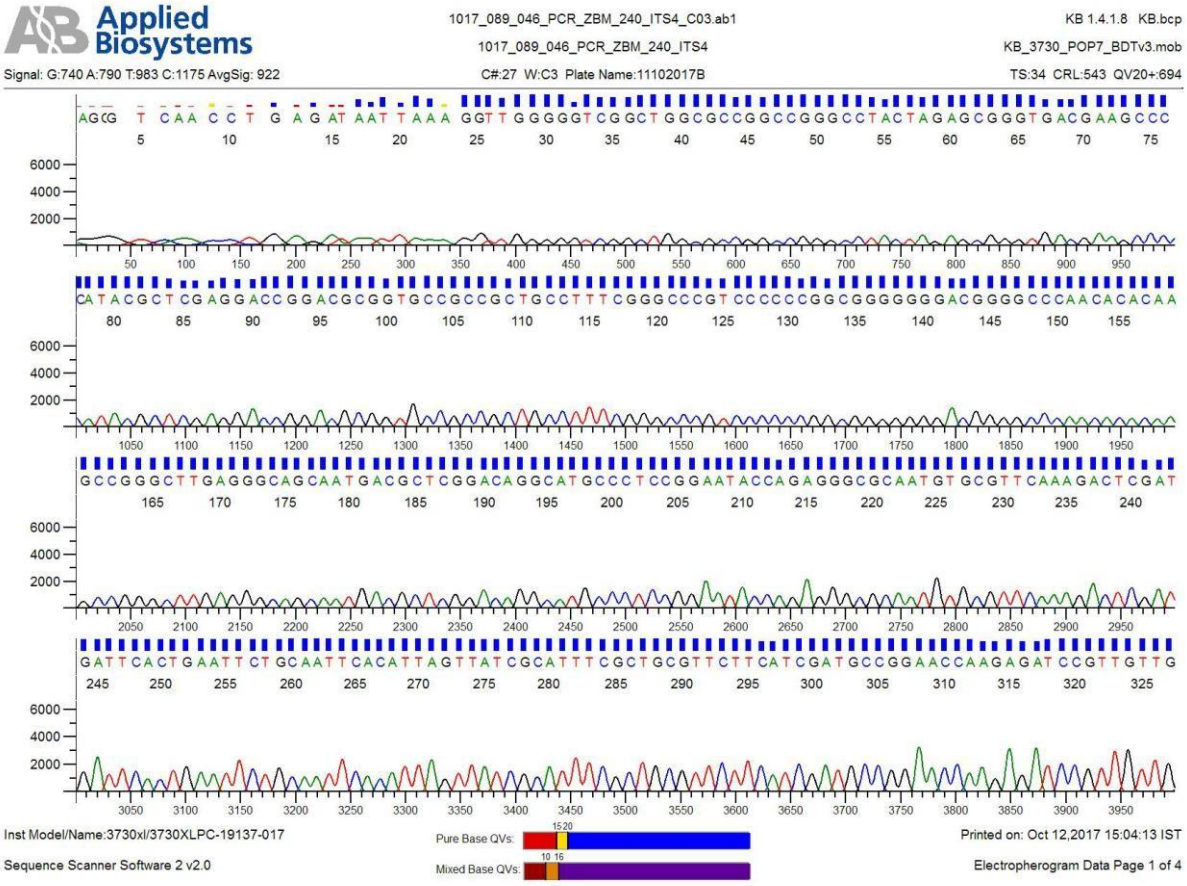

In this present fermentation process, the sugars in the medium were reduced and the citric acid production was increased in proportion to the sugar utilization. The results obtained are given in the following tables:

Table1: Amount of Citric acid produced and left over sugar by *Aspergillus niger* (Set I) using Sugarcane Bagasse as the substrate

			Amount of citric acid produced and left over sugar Spore Suspension					
Strain Employed	Initial sugar	Initial CA	Day 2 CA	Day 2 LOS	Day 4 CA	Day 4 LOS	Day 6 CA	Day 6 LOS
MTCC	23	2.8	5.4	21.2	5.6	17.1	5.7	16.8
Sample 1	23	2.8	5.0	21.7	5.3	17.6	5.3	16.9
Sample 2	23	2.8	4.9	20.7	5.1	17.9	5.1	17.1
Sample 3	23	2.8	5.2	21.3	5.9	18.3	5.0	17.3
Sample 4	23	2.8	5.3	21.5	4.1	17.3	4.8	17.9
Sample 5	23	2.8	4.5	20.1	4.8	17.2	5.0	17.3

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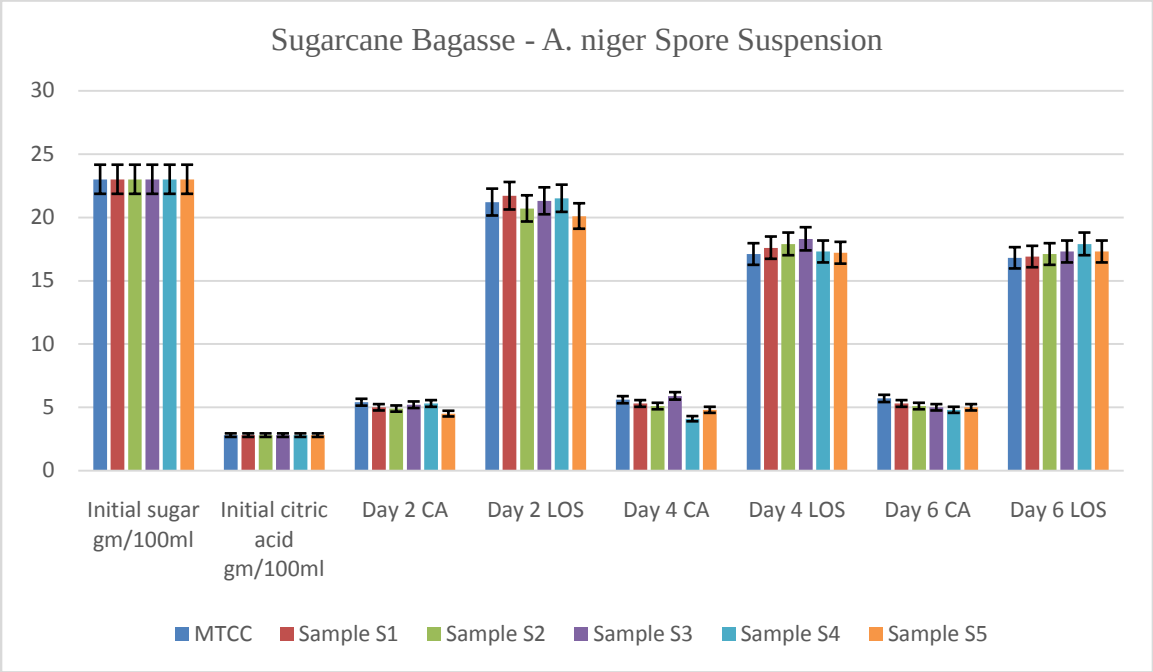

Table:2 Amount of Citric acid produced and left over sugar by *Aspergillus niger* (SetII) Intact Mycelium using Sugarcane Bagasse as the Substrate

			Amount of citric acid produced and left over sugar Intact Mycelium					
Strain Employed	Initial sugar	Initial CA	Day 2 CA	Day 2 LOS	Day 4 CA	Day 4 LOS	Day 6 CA	Day 6 LOS
MTCC	2.3	2.8	5.3	21.5	5.9	19.1	6.0	17.1
Sample 1	2.3	2.8	5.0	21.9	5.5	19.5	5.7	17.2
Sample 2	2.3	2.8	5.2	21.7	5.7	19.2	5.6	17.3
Sample 3	2.3	2.8	5.1	21.6	5.3	19.7	5.3	17.8
Sample 4	2.3	2.8	4.9	22.5	5.0	19.9	5.1	17.9
Sample 5	2.3	2.8	4.8	22.3	5.2	19.8	5.4	17.5

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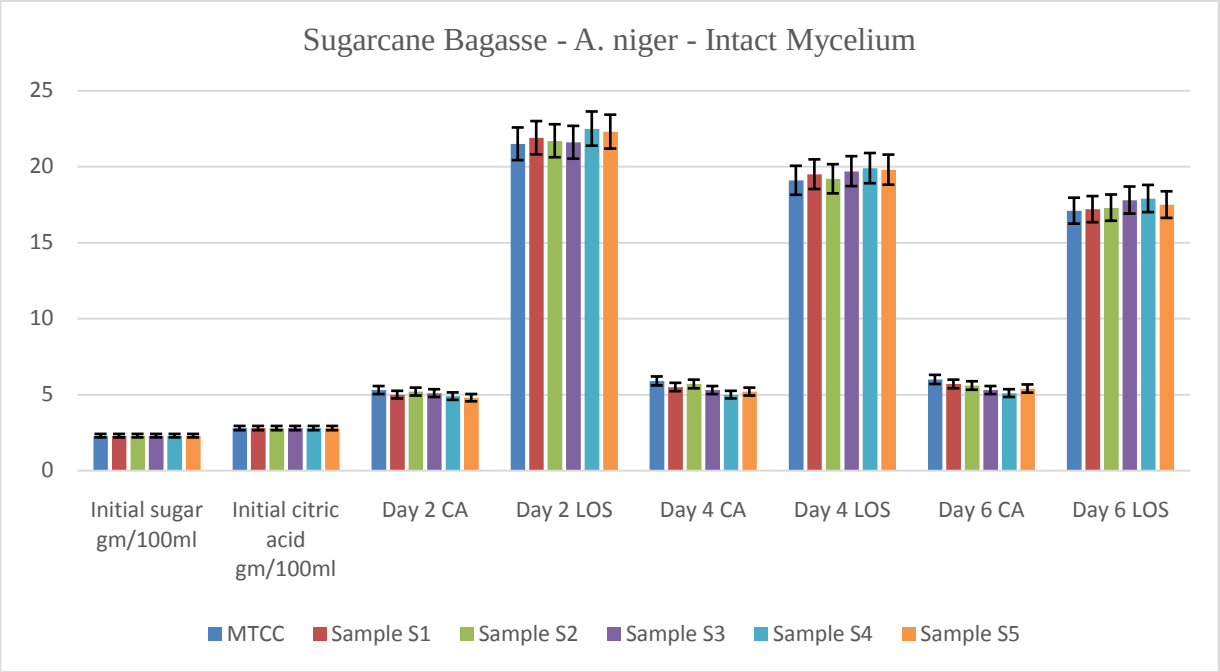

From the above result it is observed there is a parallel relationship between citric acid production and consumption of sugar was observed.

Set I (Spore Suspension as Inoculum): The germination of spores was observed till the second day of incubation and there was gradual development of mycelium in all the flasks, and negligible amount of citric acid was recorded. From the third day onwards there was gradual development in the growth of the mycelium and therefore increase in the amount production of citric acid was observed. The highest yield of citric acid was recorded on sixth day.

Set II (Intact Mycelium as Inoculum): Similar trend was observed in set II except in the development of biomass (mycelium) and the yield. The biomass produced and the yields were comparatively more when compared to set I, as the growth of the organism was complete and did not required more time for the growth of mycelium.

From the above tables, it is noted that there was efficient fermentation in set II where intact mycelium was used as the inoculums with MTCC culture. It is also observed that the intact mycelium present in the media only utilized consumed sugars in the production of citric acid where as in Set I flasks which has spore suspension as the inoculum, sugar is consumed for two purposes one is for the growth of the mycelium and secondly for the process of fermentation of citric acid. Therefore less amount of sugar is left for citric acid fermentation which results in low yield of citric acid in set I.

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It has been observed that the present fermentation process of citric acid occurred in two phases, the first phase it was observed that till the second day of fermentation, the sugars that are readily available in the medium were released by mild hydrolysis (by autoclaving) which are sufficient for the yield mycelial growth. From this point it is observed that more easily available sugars which are present in the media were consumed for the mycelium production without the need of breaking down the cellulosic materials enzymatically. As a result negligible amount of citric acid was produced in both the sets I and II.

The second phase of citric acid production showed a gradual increase in the yield of citric acid in set-I flasks, whereas rapid increase in the yield was observed in set-II flasks. This phase was considered as the production phase. We concluded by observing that the citric acid production increased in proportion with sugar utilization.

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