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Optimization of Citric Acid Production by *Aspergillus niger* isolated from different Habitats.

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ABSTRACT

Citric acid is known as one of the most important organic acids produced by microbial fermentation. *Aspergillus niger*has remained the fungus of choice for citric acid production. Our study is concerned with optimizing the conditions for the highest production of citric acid by *A. niger* isolated from different natural sources. *Aspergillus niger*was isolated from fresh orange fruits and soil. They were screened for their citric acid productivity at different conditions. The obtained results showed that of the highest production citric acid by both fungi was achieved at pH 5.0 and 6.0 respectively, temperature 30 ^{0}C, inoculum size 1%, and incubation period of 168 h. Also, the maximum amount of citric acid produced by the two fungi was demonstrated at molasses containing medium (15 %). Hence, Cane molasses was selected as the best carbon source for the further study. Moreover, it was found that citric acid production by immobilized spores of both fungi was higher than the free ones.

Keywords: Citric acid production, *Aspergillusniger*, optimization, conditions, immobilization.

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INTRODUCTION

Citric acid (2-hydroxy propane 1,2,3 –tricarboxylic acid) is abundant in nature and present as an intermediate in the tricarboxylic acid cycle [1-2]. It is solid at room temperature, melts at 153°C and decomposes at a higher temperature [3]. This organic acid exists as a natural constituent of a variety of citrus fruits, pineapple, pear, peach, oranges and fig. The worldwide request for citric acid is about 6.0×10^5 tons per year [4].

Many microorganisms were reported as a citric acid producer including bacteria e.g., *Bacillus licheniformis, B. subtilis*, *Brevibacterium flavum*, *Arthrobactorparaffinens* and *Corynebacterium spp.* [5]. Fungi such as, *Aspergillus niger*, *A. awamori*, *A. foetidus*, *Pencilliumrestricum, Trichodemaviride* and *Mucor pyriformis* [6] and yeasts e.g., *Candida lipolytic*, *C. intermedia*, *C. citrica* and *Saccharomyces cerevisiae* [7-8]. However, *Aspergillusniger* has remained the organism of choice for citric acid production [9]. *Aspergillus niger* is considered as the most important organism for commercial production of citric acid due to its (i) ease of handling; (ii) ability to ferment a wide variety of cheap raw materials and (iii) production of high yields [10].

Various methods of fermentation have been established for citric acid production using specific substrate and microbial strains. The good yield of citric acid in any technique depends upon several factors including the microbial strain used, substrate, employed fermentation conditions (temperature, pH, etc). Other factors like the used carbon and nitrogen sources in the fermentation process have a pronounced effect on the yield of citric acid in the medium [11]. *A. niger* has been immobilized for citric acid production on supports as calcium alginate and polyacrylamide [12]. Furthermore, luffa sponge, a natural material consisting of a fibrous network, obtained from the mature dried fruits of *Luffa cylindrica* L., is used to immobilize various plant [13] and microbialcells [14]. Immobilization in luffa sponge is cheap and the material is highly porous. Also, it is resistant to autoclaving, pH and temperature changes; so, it is reported to be an ideal carrier for industrial fermentation in developing countries [16].

This study aimed to obtain the highest yield of citric acid by adjusting the cultural conditions affecting the production of citric by different strains of *Aspergillus niger* in submerged fermentation.

MATERIALS AND METHODS

Isolation and maintenance of the organism:

The experimental organisms were isolated from different sources (orange and soil) by serial dilution technique [16].

Preparation of spore suspension:

The spore suspension was prepared from 7-10 days old culture on PDA slants. Spores were harvested by flooding the slants with a sterile saline solution containing (8g NaCl and 1ml tween 80/L) as a wetting agent. Then spores were scraped from the surface of the colonies with a sterile needle. The obtained spore suspension was collected in a sterile Erlenmeyer flask and was further diluted in a sterile saline to achieve the desired concentration (5×10^7 CFU /mL).

Screening of selected strains for citric acid production:

Primary screening

The isolated fungi were screened qualitatively for citric acid production by plate method on Czapex-dox agar medium containing Bromocresol purple as an indicator. Disk (0.7 cm) of each fungus was placed on the surface of the medium plates and was incubated for 5 days at 30°C. Appearance of yellow zones indicated citric acid production.

Secondary screening

Both fungi were tested quantitatively for their productivity of citric acid in a liquid medium containing

(% w/v) sucrose, 3.0; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05; KCl, 0.05; and $NaNO_3$, 0.3 at initial pH 6.5. The experiment was reported in 250 ml Erlenmeyer flasks containing 50 ml of the medium in each flask. The pH of the fermentation medium was recorded daily by using a glass electrode pH meter and the total acidity was determined by the titration of 1 ml of the filtrate against 0.1 N sodium hydroxide using phenolphthalein as indicator.

Fermentation condition

Fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml in each flask. The fermentation medium was inoculated with 0.5 ml spore suspension of the selected isolates separately with concentration (5×10^7 CFU/ml). The flasks were then incubated at 30 ^oC under shaking at (150 rpm) for 7 days.

Clarification of molasses

Molasses was diluted by addition of water in a ratio of 1:1, acidified to pH 3.5-4.0, heated in a water bath at 90 ^oC for 1h and kept overnight to precipitate the undesirable metals. The supernatant was taken and the sediment was discarded [17].

Optimization of fermentation medium

Effect of different initial pH values on citric acid production

Fermentation medium was prepared and adjusted with 1 N HCl and 1N NaOH at different pH values ranged from 2.0 to 6.0 separately. Then the flasks were autoclaved at 120 ^oC for 15 minutes. The sterilized medium was inoculated with 0.5 ml spore suspension of each fungus separately and incubated at 30 ^oC in an incubator shaker (150 rpm) for 7 days.

Effect of different temperature on citric acid production

The tested fungi were inoculated separately into the sterilized medium and incubated at different temperatures i.e. 25, 30, 35 and 40 ^oC in an incubator shaker.

Effect of different inoculum levels on citric acid production

Basal fermentation medium was prepared in 250 ml Erlenmeyer flasks each containing 50 ml. The flasks were sterilized and inoculated with different inoculum levels of 0.5, 1.0, 1.5, and 2.0 ml of the spore suspensions separately. The flasks were incubated for 7 days at 30 ^oC separately.

Effect of different incubation periods on citric acid production

The fermentation medium was prepared and autoclaved. Each flask was inoculated with 0.5 ml spore suspension of the two isolates separately. The inoculated flasks were incubated at 30 ^oC for different periods i.e. 3, 5, 7, 9, and 11 days.

Effect of different nitrogen sources:

Different nitrogen sources were used to detect the best source for citric acid production. Sodium nitrate of the basal medium was replaced by equivalent nitrogen amounts of each the following nitrogen sources (Ammonium nitrate, Ammonium sulphate, Yeast extract and Urea). After sterilization, each flask was inoculated with 0.5 ml spore suspension of the selected fungi and incubated at 30 ^oC for 7 days. Then citric acid and residual sugars were determined after the incubation period.

Effect of different concentrations of sodium nitrate:

Different concentrations of sodium nitrate (1, 2, 3, and 4 g/L) were used in order to investigate the most suitable concentration for citric acid production. Triplicate flasks were made for each treatment. Each flask was inoculated with 0.5 ml spore suspension of the tested fungi after sterilization then incubated at 30^oC

in a shaking incubator at 150 rpm for 7 days. Then residual sugars and citric acid were calculated.

Effect of different carbon sources:

Different carbon sources (glucose, fructose, maltose and lactose and cane molasses) at equivalent carbon contents were prepared in triplicates in 250 ml conical flasks each contains 50 ml of basal medium. After autoclaving at 120 °C for 20 minute, each flask was inoculated with 0.5 ml spore suspension of the selected fungi of concentration (5×10^7). Then the inoculated flasks were incubated at 30 °C for 7days. After the incubation period citric acid, residual sugars, and dry weight were estimated**.**

Production of citric acid by immobilized spores of tested fungi:

As the methods revealed by West and Strohfus[18], the sponge (which obtained from the Egyptian Company of sponge, located in 6th Oct. City) was cut into cubes (1 cm^3) and soaked in water for a minimum of 10 times with continual rinsing, then dried at 80 ºC over night. The dried cubes (0.5 g) were added to Erlenmeyer flasks (250 ml) and autoclaved. Then 50 ml of sterile basal medium and 0.5 ml of spore suspension (5×10^7 CFU/ml) were added. The flasks were then incubated at 30 °C under shaking at 150 rpm. The control treatment was carried out with non-immobilized spore (free culture spore). After 7 days of incubation, the culture medium was filtered and the cubes were washed with distilled water. Citric acid was determined as described before, and the sponge cubes were dried at 80 °C to a constant weight to determine the fungus biomass.

Chemical analysis:

Determination of pH of the fermentation culture

The pH of the medium was determined by a glass electrode pH meter model AD1030 (pH/mV-ISE).

Estimation of citric acid

The broth culture was filtered to separate mycelia and the filtrate was used for estimation. Citric acid estimated gravimetrically following the recommended pyridine-acetic anhydride method [19]. In brief, 1ml of the sample was added to 1.3 ml of pyridine and mixed well. The solution was added with 5.7 ml of acetic anhydride. The test tubes were placed in a water bath at 32 °C for 30 min. By using a spectrophotometer the optical density was measured at 420 nm and citric acid contents of the sample was estimated with reference (run parallel, replacing 1.0 ml of the culture filtrate with distilled water) to the standard.

RESULTS AND DISCUSSION

Screening of isolated fungi for citric acid production

The isolated fungi were screened qualitatively and quantitatively on solid and liquid Czapex- dox medium. It was appeared from the results that fungi under test were able to produce citric acid on an indicator medium and this indicated by lowering the pH value of the medium hence, the color of the indicator was changed from purple to yellow indicating acid production (Fig.1). On the other hand, the pH of the liquid medium was measured daily and it was observed that the pH was reduced along the incubation period and this may be due to the accumulation of the produced citric acid during fermentation of sugar. The lowest pH value was accompanied with the greatest concentration of citric acid as low pH inhibited contamination with undesirable acids as oxalic acid.

Effect of different initial pH values on citric acid production

The pH is of great importance in citric acid production by *A*. *niger*. During the fermentation process with *A*. *niger,* metabolism of nitrogen causes a release of protons which lower the pH of the medium [20-21]. Results in this study showed that the maximum citric acid was obtained by *A*. *niger*2 at initial pH 5.0 with final pH 2.1. While the highest amount of citric acid produced by *A*. *niger*1 was at pH 6.0 with final pH2.2 (Fig.2). Amenaghawon *et al*. [21] revealed that the decrease in pH as fermentation proceeds is an indication of citric

acid production. The low pH was seen to provide sterile conditions which reduces contamination and inhibits production of unwanted organic acids, like oxalic acid, which makes recovery of citric acid difficult and also improves citric acid production [21-22]. These results were in agreement with [23], they reported that the maximum production of citric acid by *A*. *nigr* (1.5 mg/ml) was obtained at initial pH 5.0. In addition, Ali *et al*. [24] deduced that the highest amount of citric acid (96.12 g/L) was observed at pH 6.0. On the other hand, El-Samragy *et al*. [25] found that the highest citric acid concentration and conversion coefficient were resulted from cheese whey medium at pH 3.5 by two strain of *A*. *niger* (CAIM 111 and CAIM 197).

Effect of different temperature on citric acid production

It appeared from the data illustrated in (Fig. 3) that the maximum amount of citric acid was produced by both *A*. *niger* at 30 ^{o}C. This finding was in agreement with [11]. They found that the highest amount of citric acid by *A*. *niger* AS-6 was obtained at 30^{o} C. Although Ali *et al.* [26] investigated that, the maximum amount of citric acid produced by *A*. *niger* on sucrose salt media was observed at temperature 30 ^{o}C. At low temperature, a lower concentration of citric acid was produced. This may be due to low enzyme activity that causes decrease in citric acid production. Although, when the temperature of the fermentation medium was increased above 30 ^{o}C, the biosynthesis of citric acid decreased. It might be due to the accumulation of different by-products such as oxalic acids. Similar results were also obtained by other workers [27-28].

Effect of different inoculum levels on citric acid production

It was cleared from data illustrated in (Fig. 4) that the maximum amount of citric acid was achieved by both fungi using inoculum of 1% (0.5/50 ml medium). By increasing inoculum levels, citric acid production was decreased this may be due to the over growth of mycelium which increases the viscosity of the medium and hence cause a reduction in citric acid production. Similar results were obtained by Ali *et al*. [24] who revealed that the maximum citric acid production (96.86 g//l) was achieved with 1.0% inoculum size. On the other hand, Maharani *et al*. [29] reported that the highest amount of citric acid was resulted at 5.0% inoculum (83.24 g /L).

Effect of different incubation periods on citric acid production

The data represented in (Fig.5) showed that the best incubation period for citric acid production was achieved after 168 hours (7 days). Any increase in the incubation period did not enhance citric acid production. It might be due to the decrease in amount of available nitrogen in the fermentation medium, the age of fungi, the presence of inhibitors produced by fungi itself, and the depletion of sugar contents. This finding was in agreement with Iqbal *et al*. [30]. While, Rao and Reddy [31] demonstrated that incubation period of 72h was found to be optimum for the production of citricacidusingoatbranassubstrateby*A. niger.*

Effect of different nitrogen sources on citric acid production

Data illustrated in (Fig.7) clarified that the maximum citric acid production by the selected fungi (13.01, 12.24) respectively was achieved in $NaNO_3$ containing medium. These results were in line with the findings of Srivastava and Kamal [3]. Followed by medium containing NH_4NO_3 as a nitrogen source. Whereas, the lowest production of citric acid resulted from urea containing medium. Citric acid production was decreased 3.8 times than in case of $NaNO_3$ containing medium.

Effect of different sodium nitrate concentrations on citric acid production.

It was observed that by increasing the concentration of sodium nitrate, the productivity of citric acid by both fungi increased reached to its maximum (13.56, 12.74 g/L) respectively at 3g $NaNO_3$/L as presented in (Fig.8). Moreover, it was noted that when the concentration of sodium nitrate increased over than 3g/L citric acid production decreased. This may be due to the increase in the mycelial dry weight as a result of the higher concentration sodium nitrate.

Effect of different carbon sources on citric acid production.

Data illustrated in (Fig.9) showed that the maximum amount of citric acid (31.04, 31.80) by both fungi respectively was occurred in Cane molasses containing medium followed by sucrose as a carbon source.

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Sucrose is a relatively low molecular weight organic compound and readily transferred into microbial cells for hydrolysis by intracellular enzymes [33]. Many researchers investigated that sucrose is the best carbon source for the production of citric acid [34-36]. According to Amenaghawon*et al.* [21] sucrose is preferred over glucose due to the fact that*A. niger has* an effective mycelium-bound invertase that is active at low pH. Molasses has been found as a low-cost raw material and it contains 40-55% of sugars in the form of sucrose, glucose and fructose. Therefore, molasses was used as a carbon source for further study.

Production of citric acid by immobilized spores of the selected *Aspergillus niger* strains

The results revealed that citric acid production by immobilized spores of *A.niger* strains was higher than that obtained from the free ones as illustrated in (Fig.10). In addition, the production of citric acid by immobilized spores was increased by 1.5, 1.3 times respectively as compared to free spores. These results were in agreement with findings reported by Roukas and Alichanidis[37] who found that the highest production of citric acid (39 g/L) was obtained by immobilized *A*. *niger* cells after 28days in shaking flasks.

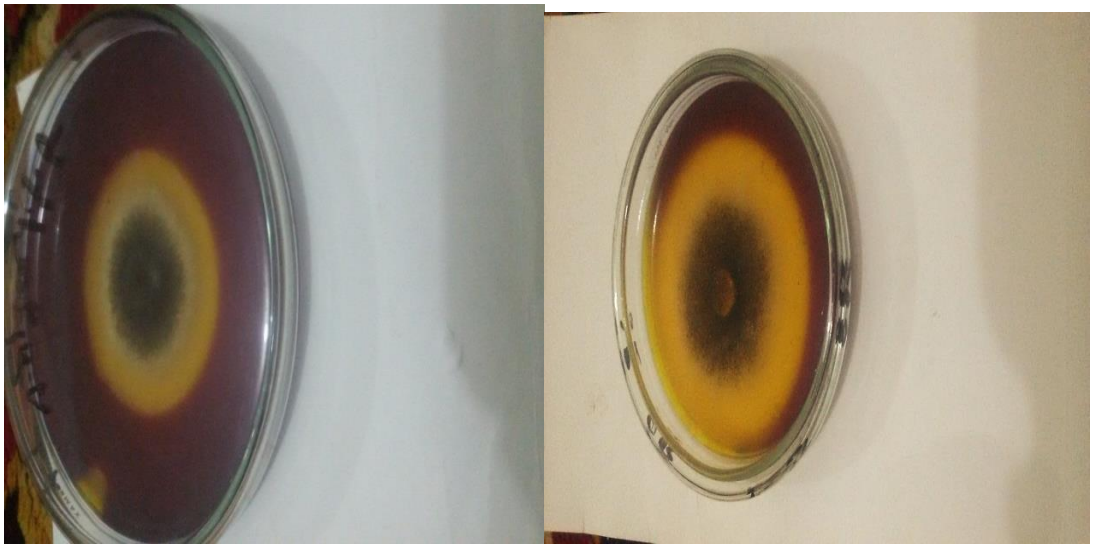

Fig 1: Primary screening of isolated fungi on solid- dox medium in the presence of bromo cresol purple as indicator.

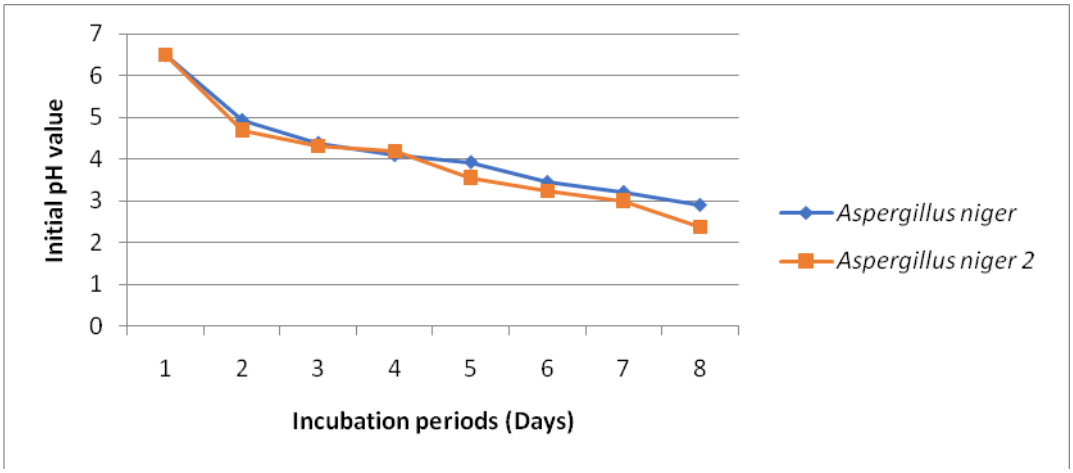

Fig 2: Secondary screening of the isolated fungi byObserving pH during fermentation on liquid -doxmedium.

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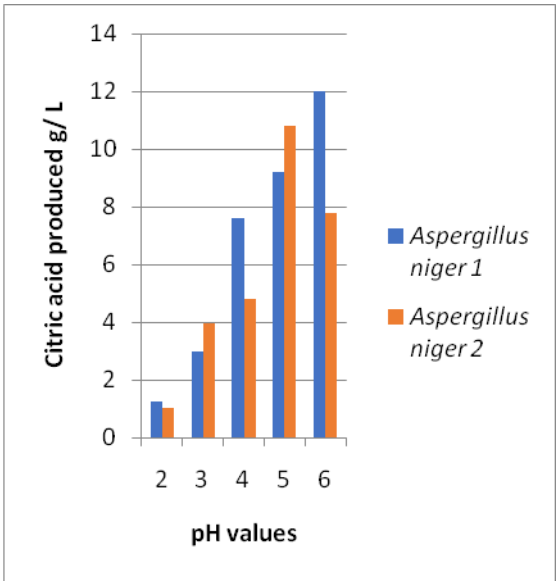

Fig 3: Effect of different pH values on citric acid production by *A. niger* 1and *A. niger* 2

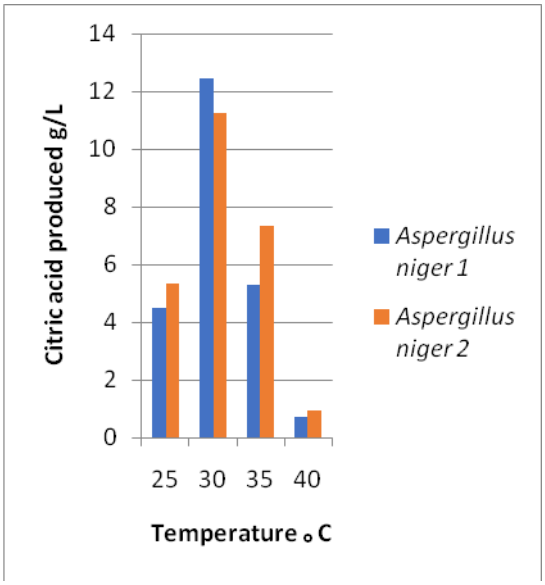

Fig 4: Effect of different temperatures on citric acid production by *A.niger* 1 and *A. niger* 2

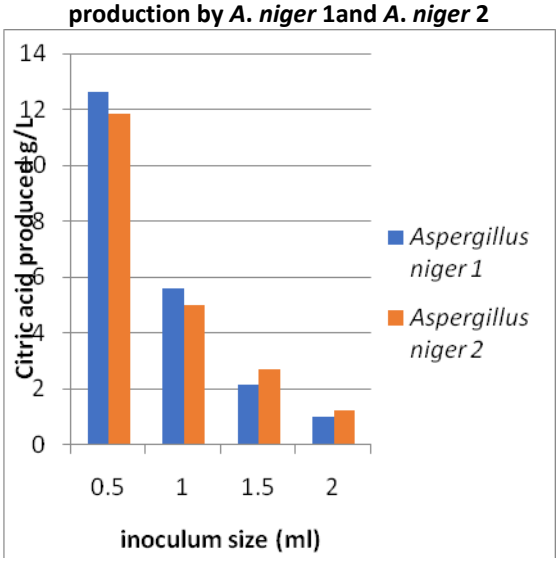

Fig 5: Effect of different inoculum sizes on citric acid production by *A.niger* 1 and *A.niger* 2

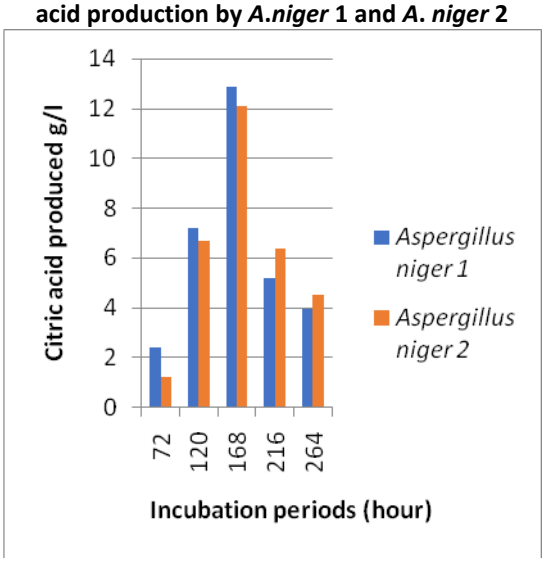

Fig 6: Effect of different incubation periods (hour) on citric acid production by *A.niger* 1 and *A.niger* 2

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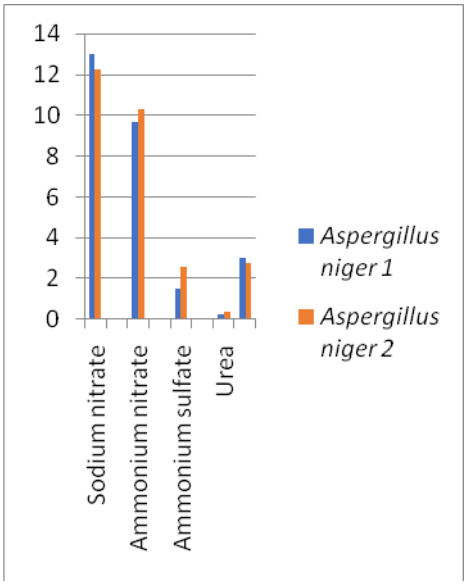

Fig 7: Effect of different nitrogen concentrations o citric acid production by *A. niger* 1 and *A. niger*2

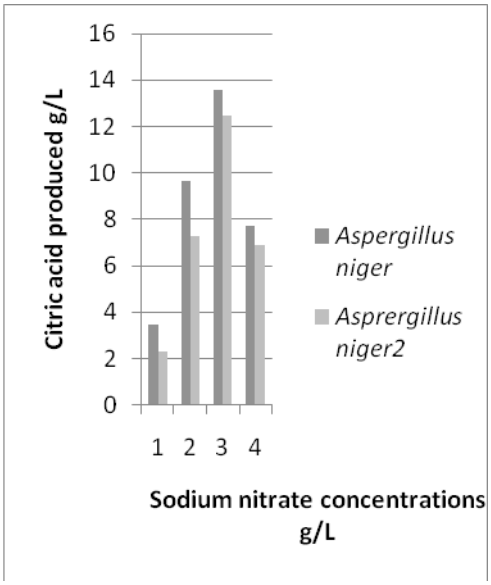

Fig 8: Effect of different sodium nitrate concentrations on citric acid production by *A.niger1* and *A.niger2*

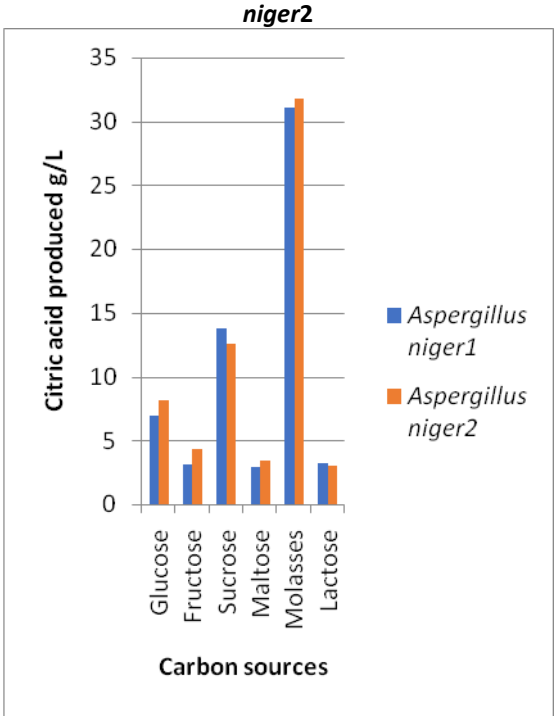

Fig 9: Effect of different carbon sources on citric acid production by *A. niger* 1 and *A.niger* 2

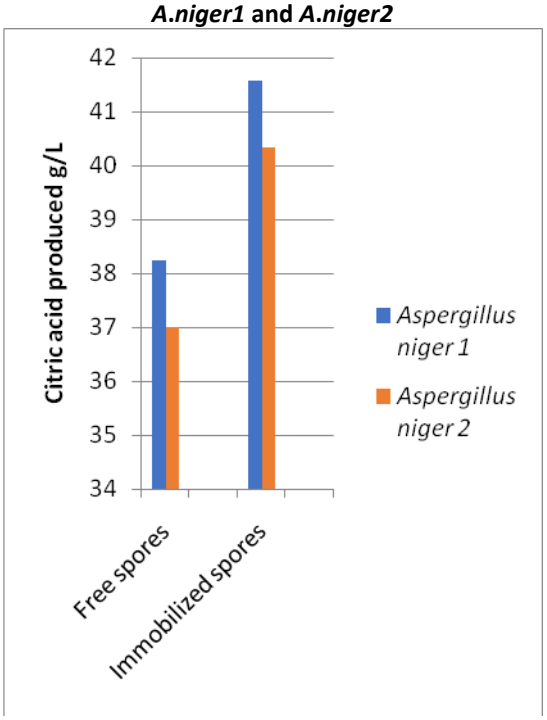

Fig 10: Citric acid production by Immobolized and free spores of A.*niger* 1 and *A. niger* 2

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