



## ISOLATION OF CITRIC ACID-PRODUCING FUNGI AND OPTIMIZATION OF CITRIC ACID PRODUCTION BY SELECTED ISOLATES

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### ABSTRACT

The aim of the present research work was to screen for fungal isolates with potential for citric acid production. Submerged fermentation was carried out using 1.0%w/v soluble starch in a 250 ml Erlenmeyer flask during the screening. A total of thirty three strains of fungi were isolated. *Aspergillus specie* EGN006 exhibited the highest citric acid yield of 3.456g/L followed by *Aspergillus specie* EGN004 with 2.432g/L and *Aspergillus specie* EGN003 with 2.304g/L after 96 hours incubation period on 1.0%w/v soluble starch. Based on the result of the screening investigations, *Aspergillus species* EGN003, EGN004 and EGN006 were selected for optimization studies. The citric acid productivity was strongly affected by fermentation conditions. The optimal starch concentration, temperature, pH and fermentation period were 60g/L, 30°C, 5.5 and 144 hours respectively. Under optimal culture conditions, the maximum productivity and yield of citric acid produced by *Aspergillus specie* EGN006 were 23.261 ± 1.447g/L and 65% respectively. The productivities and yields of *Aspergillus species* EGN003 and EGN004 were respectively 15.998 ± 2.343g/L, 50% and 19.072 ± 1.327g/L, 54%. Under the same optimum culture conditions, citric acid production by *Aspergillus specie* EGN006 using cassava starch and fed-batch fermentation were 24.712 ± 2.430g/L and 23.444 ± 1.379g/L respectively. The factors such as energy source, incubation period, initial pH, aeration, agitation and temperature strongly affected citric acid production. Moreover, the results suggest that cassava starch could be potentially utilized in citric acid production. This would stimulate cassava production, increase earnings from it and provide employment opportunities for the teeming unemployed youths.

**KEYWORDS:** isolation, citric acid, fermentation, culture conditions, Cassava starch etc.

### INTRODUCTION

Citric acid (2-hydroxypropane-1, 2, 3-tricarboxylic acid) is an intermediate of tricarboxylic acid (TCA) cycle which is obtained when carbohydrates are oxidized to carbon IV oxide. It has three carboxylic acid functional groups with three pKa values at pH 3.1, 4.7 and 6.4. It is a ubiquitous intermediate product of metabolism and its traces are found in virtually all plants and animals (Papagianni, 2007). Citric acid is known as the most important organic acid produced in tonnage by fermentation and is the most exploited biochemical/biotechnological product (Yalcin *et al.*, 2009). It has an annual production of 1.6 million tons (Sauer *et al.*, 2008) with annual growth demand/consumption rate of 3.5-4.0% (Nadeem *et al.*, 2010). Citric acid was first found as a fungal product in cultures of *Penicillium glaucum* on sugar medium by Wehmer in 1893. It is an important multi-functional organic acid with a broad range of versatile uses in household and industrial applications. Citric acid, being a commercially valuable microbial product, is widely used in food, pharmaceutical, biomedicine, biopolymer synthesis, bioremediation and agricultural industries. It also has application in beverage, cosmetic, textile, chemical, electroplating, bioleaching, toiletry and detergent industries (Imandi *et al.*, 2008).

Citric acid is responsible for the tart taste of various fruits in which it occurs (*i.e.* lemons, limes, figs, oranges, pineapples, pears and goose-berries). Hence, citric acid is used to impart a pleasant tart flavours to foods and

beverages. It is used in the industries to achieve acidulation, antioxidation, emulsification, preservation, flavour enhancement, and as plasticizer and synergistic agent (Soccol *et al.*; 2006). The wide applicability of citric acid in industries is attributed to its low/non-toxicity, high solubility, biodegradability and palatability (Ali *et al.*, 2002). It is a product adjudged to be GRAS (Generally Recognized As Safe). The food industry consumes about 70% of total citric acid produced and pharmaceutical industries consume about 12% and the remaining 18% are consumed by other industries (Nadeem *et al.*, 2010, Soccol *et al.*, 2003). The demand for citric acid is increasing faster than its production and hence, more economical processes are required (Kim, 2004). The supply of natural citric acid is very limited and the demand can only be satisfied by biotechnological processes (Lofty *et al.*, 2007). Many microorganisms such as fungi, bacteria and yeast can produce citric acid. A large number of these microorganisms have been employed for citric acid production, but only a few of them can produce citric acid in industrial scale (Soccol, *et al.*, 2006). It is reported that *Aspergillus niger* is almost exclusively used for industrial scale production of citric acid (Lofty *et al.*, 2007). This is due to its high citric acid productivity at low pH, without secretion of toxic metabolites, ease of handling, and ability to ferment a variety of cheap raw materials. Citric acid is commercially produced by large scale fermentation mostly using selected fungal or yeast strains in aerobic bioreactors.

There is need to investigate different aspects of fermentation and effects of various environmental parameters on citric acid productivity and yields to meet the ever-increasing demand for this commercially important metabolite. Different techniques for the hyper production of citric acid are continuously being studied for the past few decades. However, there is still a gap between demand and supply. Hence, there is obvious need to achieve industrially sustainable bio-production of citric acid. To meet the rising demand for citric acid in many applications in food and biomedicines, there is need for continual search for more efficient strains from our environment. Although genetic manipulations by classical mutagenesis techniques and rDNA technology are frequently exploited for overproduction of citric acid by microorganisms, there are problems with genetic stability of the strains and safety issues associated with the use of genetically modified organisms. Although a list of screening work for citric acid producing microorganisms have been reported in many developed countries, there are little or no information in many developing countries like Nigeria. Citric acid production can be improved by optimizing the fermentation parameters such as initial substrate concentration, initial pH, nutrient concentration, additives, stirrer speed, incubation period, fermentation temperature, air and O<sub>2</sub>/N<sub>2</sub> supply (Kim, 2004). The optimal conditions vary depending on the species and substrates.

#### AIMS

The aim of this study was to screen and isolate fungal citric acid producers as well as to study the effect of various fermentation parameters on their citric acid production. We believe that the results of this study will give valuable data for generating novel citric acid producers and also add to already existing knowledge.

### MATERIALS AND METHODS

#### Sample Collection

Samples were aseptically collected from refuse dump sites, decaying organic soils, cassava processing sites, rice processing sites, potato waste sites within Abakaliki metropolis, Ebonyi State, Nigeria, using aseptic bags. This was immediately transported to the laboratory and stored at 4°C in the refrigerator.

#### Preparation of Culture Media

Potato Dextrose Agar (Lab M) was prepared according to manufacturer's specification. That is, 39g of powder were dispersed in Erlenmeyer flask containing 1L (1000ml) of distilled water. The flask was covered with cotton plug. The media was sterilized using autoclave at 121°C and 1atm pressure for 15 minutes. After cooling, about 12ml of the sterilized media was distributed to each Petri plates (disposable). The plates were left undisturbed until the agar solidified. The plates were incubated for 24 hours to test its sterility.

#### Preparation of Samples

Ten (10) grams of the samples from different sources were suspended in 100ml of sterilized distilled water. Suitable serial dilutions were carried out.

#### Isolation of Fungi

One millimeter of each diluent was plated on the solidified agar plates by streaking. The plates were incubated at

37°C for 3-5 days to ensure maximum fungal growth. The fungal strains were purified by restreaking on the medium and each pure culture were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C in a refrigerator. Sub-culturing was done monthly.

#### Slant Tubes Preparation

The media was mixed as described in the preparation of Petri-plates. This was heated using Bunsen burner to obtain homogenous mixture. About 6ml of the media was poured into each test tube. The tubes were plugged with cotton ball and autoclaved at 121°C, 1atm pressure for 20 minutes. After cooling, the tubes were placed in a slanted position to allow the agar to gel. Sterility test was conducted by allowing the slants at room temperature for 24 hrs. The tubes that showed no growth after 24 hrs were inoculated with each pure culture of fungal isolates, labeled and incubated at 37°C for 5-6 days. They were then stored at 4°C in the refrigerator and subsequently sub cultured every month.

#### Basal Fermentation Medium

The isolated fungal strains were screened quantitatively for the production of citric acid in liquid culture medium containing (in g/100ml) Soluble Starch, 1.0; NH<sub>4</sub>NO<sub>3</sub>, 0.031; KH<sub>2</sub>PO<sub>4</sub>, 0.01; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.005; MnSO<sub>4</sub>, 0.0014; FeCl<sub>3</sub>.6H<sub>2</sub>O, 0.001 and CaCl<sub>2</sub> 0.005 according Kiriura *et al.* (1987). The basal medium was autoclaved at 121°C for 15 minutes. This basal medium was inoculated with 1.0 ml of the 1.3 x 10<sup>8</sup> cells concentration of inoculums. A total of 33 fungal isolates were screened for citric acid production. Based on the screening results for citric acid yields, three (3) strains EGN003, 004 and 006 were selected for further studies.

#### Preparation of Inoculum

The spore suspensions of the 3 isolates were prepared by adding 10 ml of sterilized distilled water to a 5-6 day old slant cultures having profuse spore growth on its surface. A sterile wire-loop was used to break the spore clumps and shaken vigorously to make a homogenous suspension.

#### Determination of Cell Number

A haemocytometer (Improved Neubauer Counting Chamber) and the cover glass were thoroughly cleaned with moist cloth. The chamber was placed on a flat horizontal surface and the cover glass slide into position using firm pressure. A small amount of the cell suspension was transferred to the chamber by the help of capillary tube held at 45° and allowed to lightly touch the tip against the edge of cover glass. The filled chamber was placed in a Petri-dish containing moist filter paper for the cells to settle. The chamber was placed on the microscope and the cells are counted in the 1mm<sup>2</sup> central square area and four corners 1mm<sup>2</sup> areas using x40 objective lens. The area counted (A) = 5 x 1mm<sup>2</sup>, the depth of the counting chamber (D) = 0.1mm, the number of cells counted (N) and the dilution factor (DF), the number of cells per ml was calculated as:

Dilution was carried out to give a final spore concentration of 1.3 x 10<sup>8</sup> spores per ml. The diluents containing 1.3 x 10<sup>8</sup> spores per ml were stored at 4°C for 2 weeks and used as inoculum in each experiment.

$$\text{Cell} = \frac{N \times DF \times 10^5}{\text{ml} \quad A \times D}$$

### Fermentation Condition

Fermentation was carried out using a 250 ml foam-plugged Erlenmeyer flasks with small hose sealed with masking tape containing 100 ml of fermentation medium. The fungi were inoculated separately to fermentation medium at an inoculum volume of 1.0 ml containing spore concentration of  $1.3 \times 10^8$  cells/ml. Experiments were carried out at constant temperature (room temperature and  $30^\circ\text{C}$ ), with occasional manual shaking. All the runs were performed in triplicate. Mean values of results were plotted on graphs.

### Measurement of Citric Acid Concentration

The concentration of citric acid in culture was estimated titrimetrically (AOAC, 1995) as reported by Imandi *et al.*, (2007) and Khosravi Darani and Zoghi (2008). A clean and dry safety 10ml syringe was used to withdraw 10 ml culture broth and discharged into a 500 ml Erlenmeyer

flask. A 50 ml of distilled water was measured using measuring cylinder and added to the flask. Then, 3 drops of phenolphthalein was added to the culture broth/water solution in each flask from a dropper which was specifically kept for that purpose. Ensuring that the tap on the burette was shut, 0.1M solution of NaOH was poured into the burette until it reached the zero mark using a funnel. The NaOH was slowly titrated into the broth/water solution with continual swirling to keep it thoroughly mixed. The end-point of titration (point of neutrality) was reached when the indicator changes from colourless to light pink. The amount (volume) of NaOH used (titre) on the burette was read off and the figure recorded. The burette was filled for subsequent test.

### Calculation

The acid factor of citric acid is 0.0064g/L citric acid

$$= \frac{\text{Titre} \times 100 \times 10 \times 0.064}{10 \text{ (ml sample)}} = 0.64 \times \text{Titre}$$

### Measurement of Residual Glucose Concentration

The measurement of residual glucose was carried out by the method of Trinder (1969) called the Glucose Oxidase/Peroxidase (GOD/POD) method.

### Measurement of Residual Starch Concentration

The residual starch concentration was determined by Caraway – Somogyi Iodine – Potassium Iodide (IKI) method.

### Measurement of Broth pH

The pH of the culture broth was determined using a digital pH meter. The pH meter was allowed to warm up for about 30 minutes before use. The electrode was removed from distilled water in the storage beaker and dried. The electrode was then placed in a beaker containing a buffer solution of pH 7 and calibrated to the same figure. The electrode was removed and after rinsing in distilled water – was placed in the culture broth to be tested. Care was taken not to allow the electrode have any contact with the glass. The pH was digitally readout from the pH meter and recorded.

## RESULTS

### Screening for citric acid producing fungal isolates

Thirty three cultures of fungi were isolated from samples obtained from different sites in Abakaliki, Ebonyi State, Nigeria and screened for citric acid production. These were grown on 1.0%w/v soluble starch and incubated at room temperature for 72 hours. Thirteen isolates that produced citric acid concentration in the range of 1.024 – 2.342g/L were rescreened by cultivating in a medium containing 1.0%w/v soluble starch and incubating at room temperature for 96 hours. Out of these cultures, *Aspergillus* species EGN003, EGN004 and EGN006 produced higher citric acid (2.304 – 3.456g/L) and were

selected for optimization studies. The cultural and morphological characteristics of these three isolates were examined.

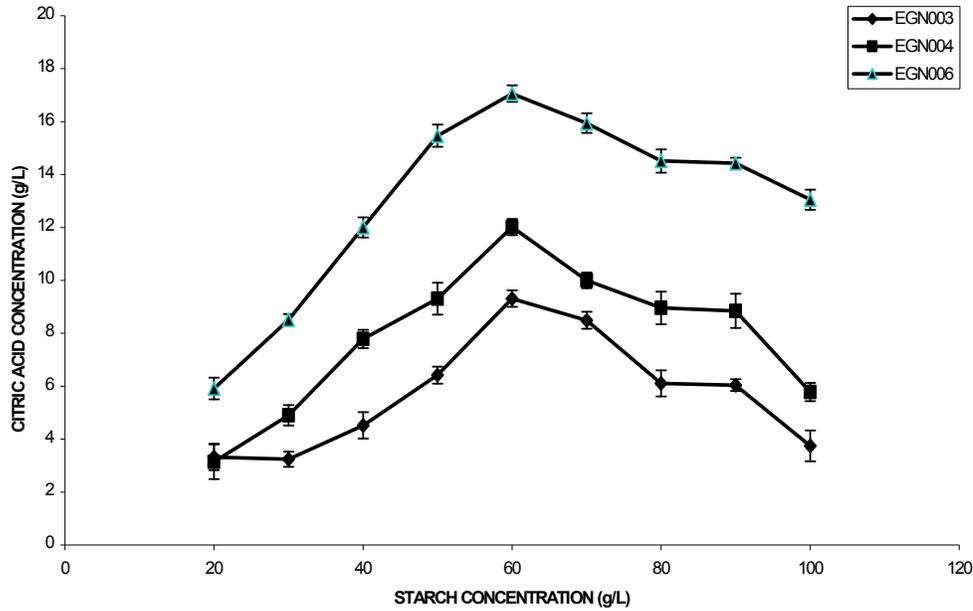
### Effect of starch concentration on citric acid production

The effect of increasing substrate (starch) concentration was estimated by changing the substrate concentration keeping other factors unchanged. The starch concentration optima for citric acid accumulation by the isolates were studied and the result indicated that, the maximum amount of citric acid obtained during the course of study was at 60g/L (figure 1). The present study was carried out with different concentrations of initial soluble starch (20-100g/L) for the three isolates. It was observed that the maximum quantity of citric acid ( $17.059 \pm 0.310\text{g/L}$ ) was obtained with *Aspergillus* species EGN006. For *Aspergillus* species EGN003 and EGN004, the concentrations of citric acid produced were  $9.312 \pm 0.323\text{g/L}$  and  $12.018 \pm 0.205\text{g/L}$  respectively.

### Effect of fermentation period on citric acid production

The quantity of citric acid produced varies with both the type of microorganism and fermentation conditions. To determine the effect of fermentation period, fermentation was carried out for various time periods at optimum starch concentration of 60g/L. In the current experiment, the rate of citric acid biosynthesis was shown in figure 2. Citric acid production increased gradually during the fermentation period and attained its maximum values ( $21.800 \pm 0.216\text{g/L}$ ) 144 hours after inoculation. At the optimum fermentation period, the corresponding pH, residual glucose and starch concentration by *Aspergillus* species EGN006 were  $2.240 \pm 0.198\text{g/L}$ ,  $3.881 \pm 0.190\text{g/L}$  and  $2.270 \pm 0.076\text{g/L}$  respectively (figures 3, 4 and 5).

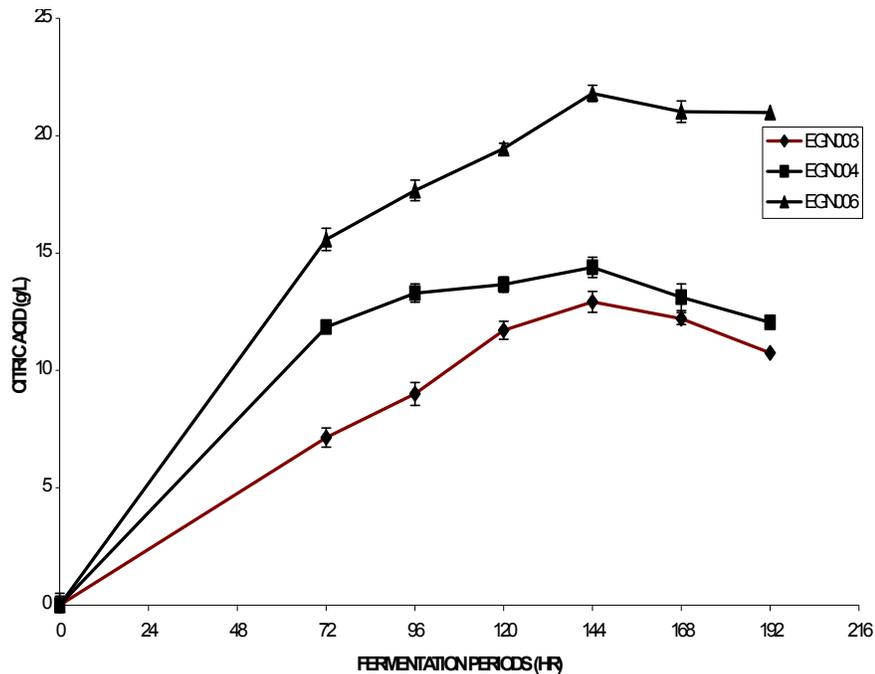
## Isolation and optimization of citric acid production



**FIGURE 1:** Effect of Starch Concentration on Citric Acid Production

The culture conditions were: room temperature and 96 hours cultivation period. The effect of various starch concentrations on citric acid production revealed that the productivity of citric acid peaked at 60g/L starch concentration. Further increase in starch concentration had

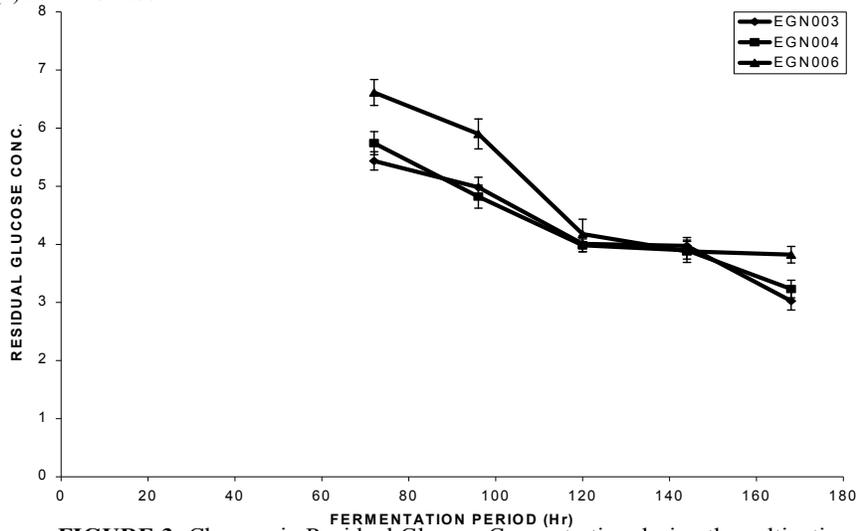
a resultant decrease in citric acid production. Hence, 60g/L starch concentration was found optimum for citric acid production. All experiments were run parallel in triplicates. The values differ significantly at  $P < 0.05$ .



**FIGURE 2:** Effect of Fermentation Period on Citric Acid Production

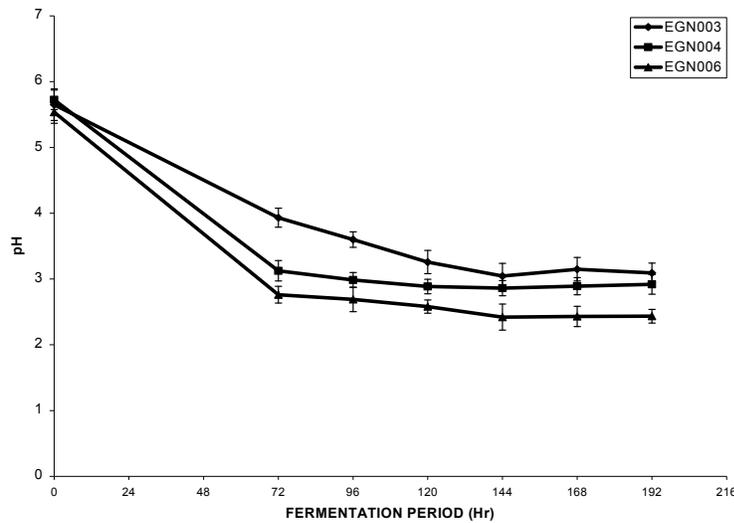
The culture conditions were room temperature; 60g/L starch concentration; 192 hour cultivation and initial pH 5.6. All experiments were run parallel in triplicates. The effect of fermentation period on citric acid production showed that the citric acid concentration tends to increase with increase in fermentation period and reached its

maximum value after 144 hrs of fermentation period. A gradual decrease in citric acid concentration was observed with further increase in fermentation period. Thus 144 hours fermentation period was found optimum for citric acid production.



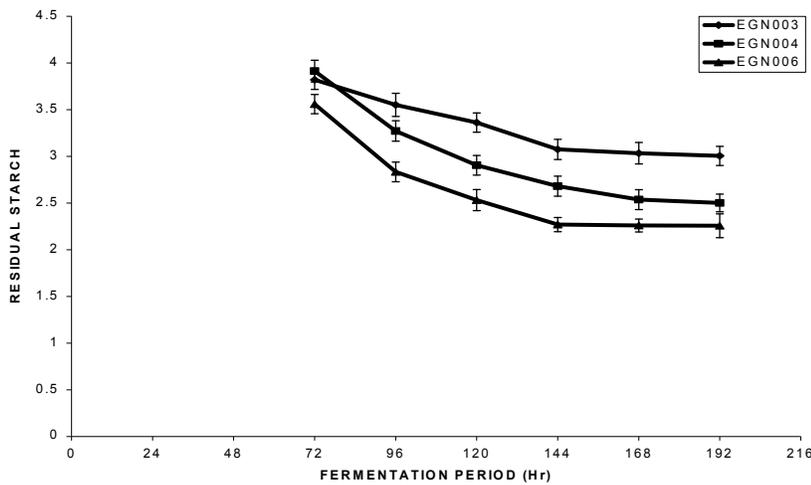
**FIGURE 3:** Changes in Residual Glucose Concentration during the cultivation

The figure depicts a progressive decrease in glucose concentration as the fermentation period increases. All values differs significantly at  $P < 0.05$



**FIGURE 4:** Variation in pH during Citric Acid Production

The figure shows a progressive decrease in pH as the fermentation period increases. The pH was almost constant after 72 hours incubation for EGN004 and EGN006, but was almost steady after 144 hours for EGN003.



**FIGURE 5:** Residual Starch during Citric Acid Production from Soluble Starch

The figure shows a gradual decrease in residual starch concentration as the fermentation period increases for the three isolates. EGN006 showed a high substrate consumption following low residual starch in the medium.

### Effect of initial pH on citric acid production

The pH of the substrate is an important factor that affects the performance of submerged fermentation. In the present study, the optimum initial pH of the medium for citric acid production is shown in figure 6. This implies that the successful production of citric acid depends on maintenance of initial pH of the production medium. A range of initial pH 3.5 to 6.0 was examined to investigate the effect of pH on citric acid production. It was observed that as initial pH increased, there was a significant increase ( $P < 0.05$ ) in citric acid production up till pH 5.5. Further increase to pH 6.0 was associated with a decrease in citric acid yield. The highest citric acid concentration ( $20.679 \pm 0.458$ g/L) was obtained when the initial pH of the production medium was kept at 5.5. At pH less than and above 5.5, fermentation was marked with high residual

starch and glucose (low substrate consumption) in the medium.

### Effect of incubation temperature on citric acid production

Temperature is a critical factor for citric acid production. The effect of temperature on citric acid production was shown in figure 7. In the present study, maximal citric acid production by the three isolates was obtained at a temperature of  $30^{\circ}\text{C}$ . At a temperature of  $25^{\circ}\text{C}$ , lower concentration of citric acid ( $21.690 \pm 0.563$ ) was produced. This may be due to low enzyme activity which makes no impact on citric acid production. When the temperature of the fermentation medium was increased above  $30^{\circ}\text{C}$ , the biosynthesis of citric acid decreased in the three isolates (*Aspergillus specie* EGN0003,  $13.591 \pm 0.357$ g/L, *Aspergillus specie* EGN004,  $15.392 \pm 0.374$ g/L and *Aspergillus specie* EGN006,  $17.263 \pm 0.411$ g/L) at  $35^{\circ}\text{C}$ .

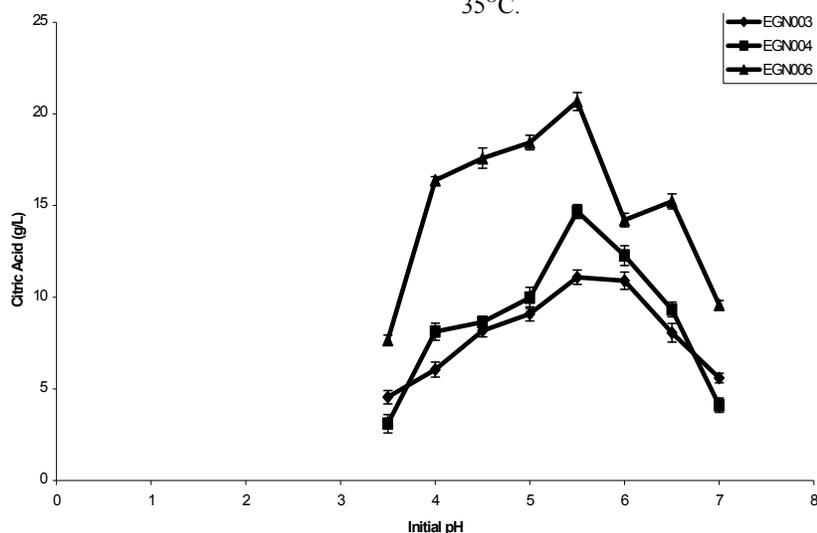


FIGURE 6: Effect of Initial pH Values on Citric Acid Production

Culture conditions: room temperature, 60g/L soluble starch, 144hrs cultivation. All experiments were run parallel in triplicates and values differ significantly at  $P < 0.05$ . The effect of initial pH on citric acid production was investigated. The result showed that increase in pH

brought about increase in citric acid concentration and attained its maximum value at pH 5.5. Further increase in pH was found to decrease citric acid production. Hence, pH 5.5 was found optimum for citric acid production.

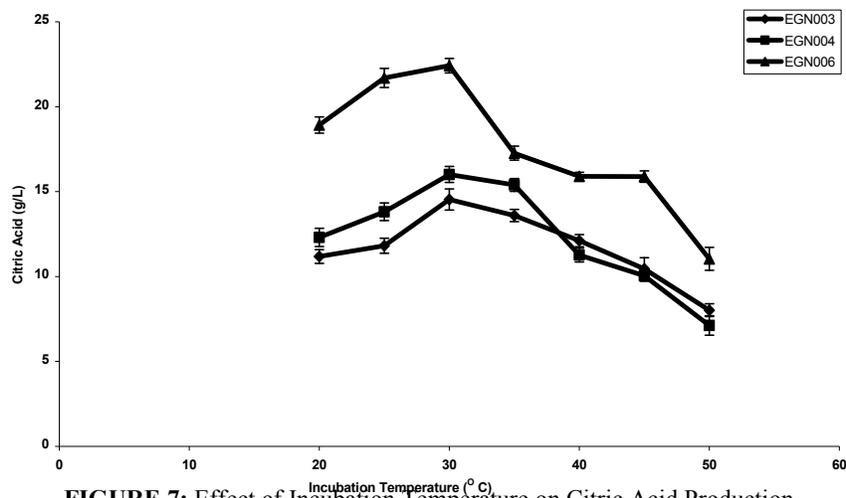


FIGURE 7: Effect of Incubation Temperature on Citric Acid Production

Culture conditions were 60g/L starch concentration, initial pH 5.5, 144 hours incubation period. All experiments were run parallel in triplicates. The effect of different temperatures on citric acid production by the isolates was studied. A temperature of 30°C was found optimal for citric acid production as maximum citric acid was produced at this temperature by the three isolates. Further increase in temperature gradually decreased citric acid productivity by the isolates. All values differ significantly at  $P < 0.05$ .

#### Effect of shaking conditions on citric acid production

The condition under which citric acid is produced by the fungus is of prime importance. In the present study, figure 8 show the effect of shake condition on citric acid production by the three isolates. Maximum citric acid production ( $23.261 \pm 0.447\text{g/L}$ ) was obtained under

shaking condition while that of non-shaking was  $17.31 \pm 0.425\text{g/L}$ .

#### Effect of fed batch culture on citric acid production

The effect of fed batch culture on citric acid production was investigated using *Aspergillus specie* EGN006 (figure 9). The culture was conducted using an initial soluble starch concentration of 50g/L. A subsequent addition of 50g/L after 72 hours incubation was made under optimized condition. Maximum citric acid production under this condition was  $23.444 \pm 0.436\text{g/L}$  after 168 hours incubation period.

#### Effect of cassava starch on citric acid production

The effect of indigenous cassava starch on citric acid production was investigated (figure 10) and the maximum yield of citric acid ( $24.712 \pm 0.430\text{g/L}$ ) was achieved 192 hours, after inoculation.

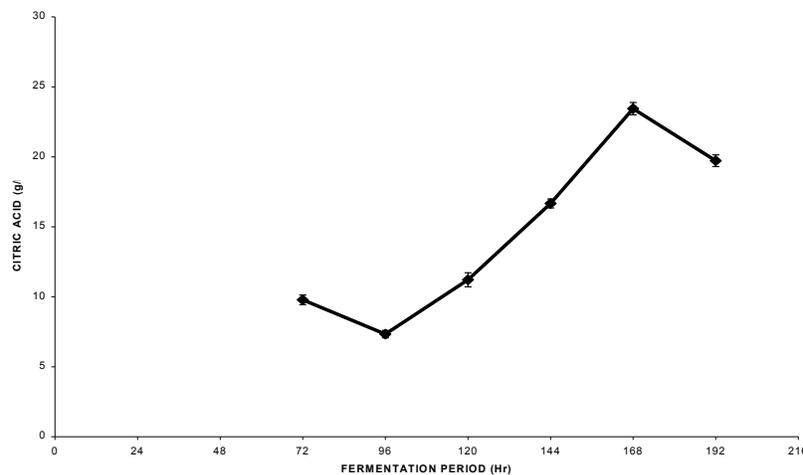


FIGURE 9: Effect of Fed Batch Culture on Citric Acid Production by EGN006

Initial conditions: 50g/L soluble starch, pH 5.5, incubation temperature 30°C, shaking manual, methanol 1.0%v/v and incubation period 192 hours. 50g/L soluble starch was added after 72 hours. The effect of fed batch culture on

citric acid production was investigated. It was observed that citric acid concentration decreased in the medium 96 hours after adding another 50g/L substrate. The citric acid increased again and peaked at 168 hours.

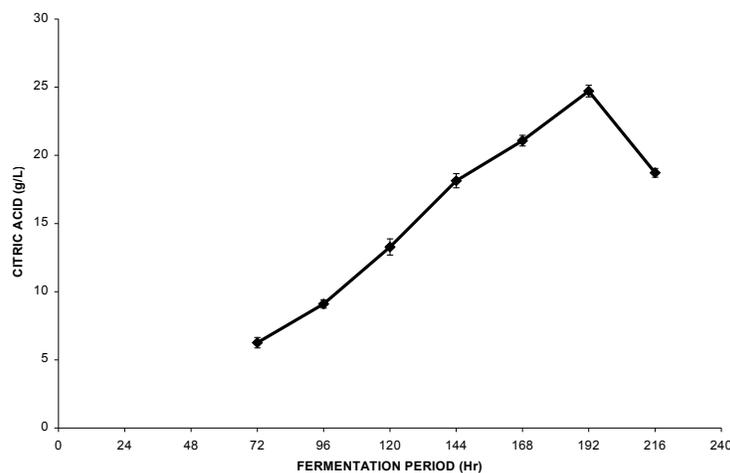


FIGURE 10: Citric Acid Production from Cassava Starch by EGN006

Initial Conditions: cassava starch 60g/L, pH 5.5, incubation temperature 30°C, shaking manual, methanol 1.0%v/v and 216 hours incubation period. The figure

shows that optimum citric acid concentration was achieved at 192 hours after incubation. All experiments were run parallel in triplicates.



Culture flask for citric acid production

## DISCUSSION

### Effect of initial starch concentration on citric acid production

The effect of increasing substrate (starch) concentration was estimated by changing the substrate concentration keeping other factors unchanged. The starch concentration optima for citric acid accumulation by the isolates were studied and the result indicated that, the maximum amount of citric acid obtained during the course of study was at 60g/L. When the starch concentration was higher than 60g/L, citric acid production by the 3 cultures decreased. The decrease in citric acid production after 60g/L soluble starch concentration may be due to polyalcohol formation. The higher initial sugar concentration (18 %w/v) resulted to increase in citric acid yield and productivity as reported by Khosravi- Darani and Zoghi (2008). Kubicek (1998) reported that the final yield of citric acid in fermentation by *Aspergillus niger* is strongly dependent on the type and concentration of carbon source. Current understandings of mechanism by which the carbon source and its concentration influences citric acid accumulation were related to major regulatory points at level of hexose transport and phosphorylation. This means that the higher the amount of carbon source that cross the cell membrane and phosphorylated, the more the citric acid produced.

### Effect of fermentation period on citric acid production

The quantity of citric acid produced varies with both the type of microorganism and fermentation conditions. To determine the effect of fermentation period, fermentation was carried out for various time periods at optimum starch concentration of 60g/L. Citric acid production increased gradually during the fermentation period and attained its maximum values 144 hours after inoculation. Further, increasing fermentation period did not enhance production of additional quantity of citric acid. This decrease in productivity might be due to inhibitory effect of high concentration of citric acid, decreased available nitrogen in the fermentation medium, the age of fungi, depletion of sugar contents and decay in enzyme system responsible for citric acid biosynthesis. The experiment was started with initial pH of  $\approx 5.5$  but decreased gradually to 2.8 (figure 4), showing the generation of citric acid. The increase in citric acid production was accompanied with steady decrease in glucose concentration (figure 3) and residual starch (figure 5) along the fermentation period.

These findings were in agreement with Ali *et al.* (2002) and Wieczorek and Brauer (1998). They reported that maximum productivity of citric acid was achieved after 144 hours of fermentation period. In contrast, investigators like Nadeem *et al.* (2010), Alvarez *et al.* (2007), Arzumanov *et al.* (2000) and Loftly *et al.* (2007) reported that maximum citric acid concentration was obtained after 192 hours of fermentation period at 150g/L sugar concentration and pH  $4.0 \pm 0.2$ .

### Effect of incubation temperature on citric acid production

Temperature is a critical factor for citric acid production. In the present study, maximal citric acid production by the three isolates was obtained at a temperature of 30°C. At a temperature of 25°C, lower concentration of citric acid was produced. This may be due to low enzyme activity which makes no impact on citric acid production. When the temperature of the fermentation medium was increased above 30°C, the biosynthesis of citric acid decreased in the three isolates. This could be attributed to the accumulation of by- products such as oxalic acid. This report agrees with previous work by Ali *et al.* (2002), Kareem *et al.* (2010), Kim *et al.* (2002), all reported that 30°C was best for citric acid production. Similarly, Roukas (1999) reported that citric acid concentration increased significantly with an increase in the fermentation temperature from 25-30°C and then decreased above 30°C. In addition, Hang and Woodams (1986) studied the effect of temperature on citric acid production from grape pomace by solid- state fermentation and reported that the temperature of fermentation medium is one of the critical factors that have a profound effect on the production of citric acid in solid- state fermentation of agricultural wastes. In contrast, Szewczyk and Myszk (1994) studied the effect of temperature on *Aspergillus niger* growth on the solid- state fermentation and found that temperature did not have a strong effect on the growth rate within a range of 28-34°C

### Effect of initial pH on citric acid production

The pH of the substrate is an important factor that affects the performance of submerged fermentation. This implies that the successful production of citric acid depends on maintenance of initial pH of the production medium. A range of initial pH 3.5 to 6.0 was examined to investigate the effect of pH on citric acid production. It was observed

that as initial pH increased, there was a significant increase ( $P < 0.05$ ) in citric acid production up till pH 5.5. Further increase to pH 6.0 was associated with a decrease in citric acid yield. The highest citric acid concentration was obtained when the initial pH of the production medium was kept at 5.5. At pH less than and above 5.5, fermentation was marked with high residual starch and glucose (low substrate consumption) in the medium. This would seem to suggest that the initial pH did not directly influence the citric acid production mechanism, but rather affected the enzymes which were active in degrading starch or the permeability of the cell membrane to starch. There was an observable decrease in pH as the fermentation proceeded. In general, low pH is essential for attaining maximum citric acid production (Grewal and Kalra, 1995). A higher initial pH encourages the accumulation of oxalic acid (Haq *et al.*, 2004). Shadafza *et al.* (1976) reported that a low initial pH has the advantage of checking contamination and inhibiting oxalic acid formation. This report agrees with work by Al-Shehri and Mostafa (2006) that studied citric acid production from date syrup using immobilized cells of *Aspergillus niger* and pointed out that the highest value of citric acid was obtained at initial pH 5.5. This present study is also supported by the work of Khosravi-Darani and Zoghi (2008).

#### **Effect of shaking conditions on citric acid production**

The condition under which citric acid is produced by the fungus is of prime importance. Maximum citric acid production was obtained under shaking condition. This revealed that shaking condition is necessary for hyper production of citric acid. Citric acid production is known to be an aerobic process. Hence, shaking helps to supply oxygen to the culture thus, facilitating cell growth and citric acid production. Furthermore, shaking helps to prevent cell sedimentation and generally improve mass transfer, all of which favour citric acid production. More so, shaking is associated with development of thick, short and highly branched filaments that overproduce citric acid. This means that it causes breakage of filaments that result to mycelial fragmentation and regrowth which are beneficial to citric acid production. Kishore *et al* (2008) reported that an agitation speed of 230rpm was found maximum for citric acid production

#### **Effect of fed batch culture on citric acid production**

The effect of fed batch culture on citric acid production was investigated using *Apergillus specie* EGN006. The culture was conducted using an initial soluble starch concentration of 50g/L. A subsequent addition of 50g/l after 72 hours incubation was made under optimized condition. Maximum citric acid production under this condition was obtained 168 hours incubation period. Under the same optimized condition, batch fermentation was found more productive, superior and less costly in terms of energy to fed batch fermentation. This is contrary to expectations since fed-batch culture is known to be suitable for producing high concentration of products. It is therefore necessary to optimize fed-batch culture in terms feed substrate concentrations, time of feedings and control of pH during the fermentation.

#### **Effect of cassava starch on citric acid production**

The effect of cassava starch on citric acid production was investigated and the maximum yield of citric acid was achieved 192 hours, after inoculation. This shows that starch is a good substrate for citric acid production. Cassava is widely produced in Nigeria and its utilization for citric acid production will stimulate cassava production, increase earnings from cassava, and create employment opportunities for the teeming unemployed youths.

#### **CONCLUSION**

The present study established that the environment like that found in Nigeria hold a repertoire of citric acid - producing fungi. The factors such as energy source, incubation time, pH, aeration, agitation and temperature need to be considered in the cultivation of these fungi since it affects the citric acid production. Demonstrating citric acid production at different fermentation parameters with the strains, the results are informative in determining culture conditions for scale-up in a commercial production process. The study also demonstrated the promising utilization of cassava starch for citric acid production. This would offer opportunities for increase cassava production which would guarantee food security, increase earnings from cassava and employment opportunities for our teeming unemployed youths.

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