International Journal of Trend in Scientific Research and Development (IJTSRD)
Volume 5 Issue 5, July-August 2021 Available Online: www.ijtsrd.com e-ISSN: 2456 – 6470

Isolation and Screening for Citric Acid Production by *Aspergillus Niger* Using Sucrose as a Carbon Source

Mbah, O. M; Okpalla, J; Osuala, O. J; Okoye, P; Alagbu, P. O

Department of Chukwuemeka Odumegwu Ojukwu University Uli, Anambra State, Nigeria

ABSTRACT

The numerous applications of citric acid have increased its demand. It is of great importance to produce citric acid using adequate monosaccharides and dissacharides as they have proven to be easily catabolyzed and decomposed by Fungi when compared to polysaccharides. The superiority of sucrose to other sugars is a result of strong extracellular mycelium-bound invertase of *A.niger* which rapidly hydrolyses sucrose at low pH. This project was undertaken to isolate, screen and produce citric acid in a submerged fermentation by four different strains of *Aspergillus niger* using sucrose. *Aspergillus niger* was isolated and characterized from garden soil. Citric acid production from *Aspergillus niger* in a submerged basal medium was quantitatively determined using titrimetric method. *Aspergillus niger* strain HUS1 (ASHUS1), *Aspergillus niger* strain HUS7 (ASHUS7), *Aspergillus niger* strain HG49 (ASHG49) and *Aspergillus niger* strain AN8 (ASAN8) showed significant accumulation of citric acid from the submerged basal medium, of which ASHUS 1 significantly ($\alpha<0.05$) recorded the most yield from the production after 9 days of incubation.

KEYWORDS: *Citric acid, Fungi isolation, Identification, Molecular characterization, Aspergillus niger, Sucrose, fermentation*

How to cite this paper: Mbah, O. M | Okpalla, J | Osuala, O. J | Okoye, P | Alagbu, P. O "Isolation and Screening for Citric Acid Production by Aspergillus Niger Using Sucrose as a Carbon Source" Published in International Journal of Trend in Scientific Research and Development (ijtsrd), ISSN: 2456-6470, Volume-5 | Issue-5, August 2021, pp.2315-2324, URL: www.ijtsrd.com/papers/ijtsrd46260.pdf

IJTSRD46260

1. INTRODUCTION

Citric acid ($C_6H_8O_7$, 2 - hydroxy - 1,2,3 - propane tricarboxylic acid) is one of the most common and versatile organic acid. It is also a common metabolite of plants and animals. Citric acid is widely used in the field of food (60%) and pharmaceuticals (10%). It is also used as chelating agent. (Papagianni, 2007).

About 99% of world production of citric acid is carried out using microbial processes, which can be done using surface, solid or submerged cultures. Species of *Aspergillus* such as *A. wenti*, *A. foetidus*, *A. aculeatus*, *A. awamori*, *A. fonsecaeus*, *A. phoenicis* and *A. carbonaries*, as well as *Trichodermaviride* and *Mucorpyriformis*, have been proven to produce significant amounts of citric acid (Berovic and Legisa, 2007).

Besides fungi and bacteria, yeast species such as *Candida*, *Candida guilermondi, Candida tropicalis, Candida parapsilosis, Candida citroformans* (Angumeenal*et al*.,2003) (Angumeenal and Venkappayya, 2013), *Saccharomyces lipolytica* and *Zygosaccharomyces* are efficient in the production of citric acid from *n*-alkanes and carbohydrates (Weyda *et al*.,2014).

Aspergillus niger has so far gained its grounds in citric acid production as it has advantages over bacterial microorganisms such as *Arthrobacterparaffinens*, *Bacillus licheniformis*, *Bacillus subtilis*, *Brevibacteriumflavum*, *Corynebacterium* spp. and *Penicillium janthinellum* (Ikram-ul *et al*.,2004). It is easy to handle, can ferment a broad range of low-cost raw materials and provides high yields (Themelis and Tzanavaras, 2001).

A wide range of substrates have been utilized in the fermentation process of the microorganisms. Materials such as hydrocarbons, molasses and starchy materials are commonly used. The review by Soccol *et al*. (2006) highlighted some examples such as beet molasses, black strap molasses, cane molasses, carob pod extract, *n*-paraffin, glycerol, corn starch,

hydrolysate starch, yam bean starch, wood hemicellulose, olive oil, rapeseed oil, palm oil and soya bean oil.

Monosaccharides and disaccharides are the best choice of carbon source as they are more rapidly metabolized by the fungus than polysaccharides, hence producing higher yield (Mattey, 2002).

Polysaccharides are not adequate raw material and the decomposition process takes too long to meet the rate of sugar catabolism necessary for the production of citric acid. The slow rate of polysaccharide hydrolysis is as a result of reduced enzymatic activity, which affects the pH in the fermentation medium (Hossain *et al.*, 2004; Xu*et al.*, 2009; Papagianni *et al.*, 2005)

Sucrose is better than glucose, fructose and lactose, in order of decreasing citric acid yield (Angumeenal andVenkappayya, 2003). The superiority of sucrose to other sugars is as a result of strong extracellular mycelium-bound invertase of *A. niger*, which rapidly hydrolyses sucrose at low pH (Kubicek-Pranz *et al.*, 2000)

Pure sucrose or glucose may not be economically reliable on an industrial scale. Hence, low-grade carbon sources such as wastes from sugar refineries, for example cane and beet molasses are used. Adding to the various sources from which these raw materials are obtained, there may be a need for pretreatment. Cations are mostly the major contaminants, and the commonly used method of pretreatment is by precipitation using potassium ferrocyanide or cation-exchange resins (Angumeenal and Venkappayya, 2003). The concentration of the carbon source is important to the success of citric acid production as the type of carbon source. Since citric acid production relates directly to sugar concentration, therefore, as the concentration increases so does the amount of citric acid produced (Xu *et al*., 2009). However, results have shown that the maximum concentration can be achieved with 14-–22% of sugar. A research conducted by Xu *et al.,*(2009) using sucrose, glucose, fructose, mannose and maltose obtained a great yield at a sugar concentration of 10% (w/v), except for glucose, where a 7.5% (w/v) maximum yield was obtained (Amenaghawon and Aisien, 2012).

1.1. Aim of Research

The aim of this research is to isolate and screen in order to get the best strain of *Aspergillus niger* for citric acid production using sucrose as a carbon source.

2. MATERIALS AND METHOD

2.1. Isolation of Fungi

2.1.1. Sample Collection: This was carried out using the modified method of Suleiman and Omafe (2013). Soil samples were collected using a sterile spatula by scrapping the upper layer of the soil and dug 5 cm of the soil. The collected sample were put inside a sterile polythene bag and put inside cooler containing ice packs, and taken to the lab for immediate analysis.

2.1.2. Isolation of the Fungi Isolates: This was carried out using the modified method of Suleiman and Omafe (2013). A 0.1 ml aliquot of the suspension was plated on Sabouraud Dextrose Agar (SDA) containing chloramphenicol antibiotics (0.05%) and incubated at room temperature (30±2ºC) for five days. After the five days, the fungal isolates were aseptically sub-cultured on a sterile petri plates containing 20mL of SDA with (0.05%) chloramphenicol and incubated at room temperature to obtain the pure isolates.

2.2. Identification of Fungal Isolates:

The fungal isolates obtained were identified to the genus/species level based on macroscopic, microscopic and molecular characteristics of the isolates obtained from pure cultures (Watanabe, 2002).

2.2.1. Macroscopy: The colonies were carefully checked for fungal characteristics. The rate of growth, color, shape, texture, consistency of the growth and other peculiar features of the colonies were examined according to the method of Watanabe, (2002).

2.2.2. Microscopy: This was carried out using Needle mount technique. A drop of lactophenol cotton blue (LCB) solution was placed on the center of a clean grease-free slide. A fragment of the colony was placed in the drop of the LCB using sterile wire loop and covered with a cover-slip to prevent air bubbles. Excess fluid from the outside of the cover slip was cleaned with cotton wool and slide was passed through the flame to warm the staining so as to remove the remaining air bubbles and facilitate staining of the fungal element. The slide was then examined under the microscope, using low-power objective of ×10 magnifications, and followed by high-power objective of ×40 magnifications to reveal the nature of the hyphae, shape, size, texture and arrangement of the conidia. The pictorial nature of the fungal organisms was confirmed using the fungal atlas (Watanabe, 2002).

2.2.3. Slide culture technique: This was carried out using Riddel's method as described by Umedum and Iheukwumere (2013). A filter paper was cut and placed on the bottom of Petri-dish. Two slides were crossed over each other on top of the filter paper and the filter paper was moistened. The set-up was sterilized by autoclaving at 121^0C for 15 minutes.

Approximately one centimeter square agar block was cut from already prepared Potato Dextrose Agar (PDA) and placed on the intersection of the two slides.The four edges of the agar block were inoculated with the test organisms. It was then covered with sterile cover slip and incubated at room temperature for 5 days. After 5 days of growth, the cover slip was removed and inverted over a slide containing a drop of lactophenol cotton blue (LCB). The agar block was removed and discarded. A drop of LCB was also placed on top of the adherent colony on the slide and covered with sterile cover slip.The edges of the cover slip were sealed with nail polish to prevent evaporation of the stain. The slides were examined under the microscope using x10 and x40 objective lenses.

2.3. Screening

The qualitative screening of the selected isolates of *A. niger* (A – M) were carried out on petri plates using Czapek-Dox agar medium. The medium is composed of 30 g of sucrose, 2.0 g of $NaN0_3$, 1.0g of K_2HP0_4, 0.5 g of $MgS0_4.7H_2O$, 0.5 g of KCL, 0.01g of $FeS0_4$, 20 g of Agar and 40mL of bromocresol purple dye. The medium was prepared by dissolving all the ingredients excluding the agar in 900 mL distilled water. The pH was adjusted at 6.0. The agar was dissolved by heating the medium for 15 mins with constant stirring. The volume was raised up to 1000 mL with distilled water and sterilized in an autoclave. 10 - 12 mL of this medium were poured into individual sterile petri plates and allowed to solidify at room temperature. A small fragment from each of the pure cultures labeled were collected using a sterile wire loop and spread into the solidified Agar. The plates were incubated at 30ºC for 3-5 days. Yellow zones due to acid hydrolysis were formed. On the basis of larger zones the best strains of *A. niger* were picked and transferred to the SDA slants. The cultures were incubated at 30ºC for 5-7 days for maximum sporulation. (Narasimha *et al.*, 2012)

2.4. Citric acid production

The submerged fermentation was carried out using Sucrose as carbon source to obtain the best isolate for citric acid production. The basal medium was supplemented with Sucrose, nitrogen supplement (ammonium phosphate, potassium hydrogen phosphate and peptone 0.5%) and moistened with distilled water to a desired moisture level of 70% (21mL) in a 100mL Erlenmeyer flask. The pH of the medium was adjusted to pH 4.0 using dil. HCL and NaOH. The flask was cotton plugged and autoclaved at 15psi for 15 minutes. After cooling at room temperature, a sterile 6.0 mm sized mycelia plug was used to collect a fraction from the selected four strains of *Aspergillus niger* and inoculated into a basal medium. A flask was not inoculated with the organism, this served as a negative control. The inoculated flasks were incubated at 30 ^oC for 7days. (Dhandayuthapani, 2008).

2.5. Citric acid determination:

At the end of the fermentation, 10 mL of fermentation media from each of the inoculated flasks will be removed aseptically using a sterile 10 mL syringe into a sterile 250 mL beaker containing 90 mL of distilled water. Citric acid concentration was determined titrimetrically by collecting 20 mL from the beaker using a sterile pipette into a flask followed by the addition of6 drops of phenolphthalein as the indicator and titrated against 0.1 M NaOH. Titrations were done in triplicates and average values were obtained and recorded.

The citric acid concentration will be calculated according to the following:

$$C_6H_8O_7 + 3NaOH_{aq} \rightarrow C_6H_5O_7Na_3 + 3H_20$$

$$C = nV$$

Where;
n= Concentration of 0.1M NaoH
V=Volume of NaOH titrated
C= Concentration of citric acid,

Concentration of citric acid obtained was multiplied by the molar mass of citric acid from the equation below to obtain equivalent weight of citric acid.

$$C_6H_8O_7 + 3NaOH_{aq} \rightarrow C_6H_5O_7Na_3 + 3H_20$$

Percentage of citric acid was calculated as follows;

$$\frac{W_A}{W_S} X\ 100$$

Where,
W_A = Weight of Acid
W_S = Weight of Sample (Dhandayuthapani,2008).

2.6. Effect of Time Course on citric acid production

The basal medium using sucrose as the parent carbon source was prepared and autoclaved. The inoculated flasks were incubated at 30 ^oC for different periods i.e. 5, 6, 7, 8, 9, 10 and 11 days. (Abeer *et al.,* 2017)

3. RESULTS

3.1. Isolation and Macroscopic Characteristics of the Fungal Isolates

A total of 13 isolates were isolated from soil sample. The fungal isolates showed similar cultural characteristics on Sabouraud Dextrose Agar (SDA) as shown in Table 1. They have similar growth rates, texture with slight variations in their appearances on the agar plates and colour of their mycelia. The

macroscopic characteristics of the fungal isolates pointed to that of *Aspergillus* species.

3.2. Microscopic Characteristics of the Fungal Isolates

The fungal isolates exhibited similar microscopic characteristics as shown in Table 2. They have septate hyphae, hyaline with long and smooth conidiophores which were aseptate. They were biseriate with globose versicle. Their conidia head were radiated with finely wrinkled texture. Their metula covering was entire. Isolates C and H showed ellipsoidal conidia whereas isolates L and G showed globular conidia. They varied slightly on the diameter of their vesicles.

3.3. Molecular Characteristics of the Isolates

The molecular characteristics of the fungal isolates are shown in Tables 3 and 4 and Figure 1. The quality of the nucleic acids extracted from the fungal isolates were within the stipulated value (1.80-1.90) for DNA (Table 3). The gel characteristics of the amplicons reviewed amplification of the selected regions for sequencing of the fungal nucleic acids (Figure 1). The Fungal sequencing of the amplified regions of the fungal isolates showed 100% identities of the isolates, and revealed the presence of *Aspergillus niger* strain HUS1 (ASHUS1), *Aspergillus niger* strain HUS7 (ASHUS7), *Aspergillus niger* strain HG9 (ASHG49) and *Aspergillus niger* strain AN8 (ASAN8) as shown in Table 4.

Table 1: Macroscopic characteristics of the fungal isolates

Parameter	Isolate C	Isolate G	Isolate L	Isolate H
Initial Appearance on SDA (2-3 days)	White	White to pale yellow	White	White to pale yellow
Final appearance on SDA (5 days and above)	Dark brown	Black	Black	Dark brown
Reverse color of the colony	Pale yellow	Pale yellow	Yellow	Pale yellow
Growth rate	Rapid	Rapid	Rapid	Rapid
Texture of the colony	Powdery	Velvety	Powdery	Powdery
Colour of the mycelia	Dark brown	Black	Black	Dark brown

Table 2: Microscopic Characteristics of the Fungal Isolates

Parameter	Isolate C	Isolate G	Isolate L	Isolate H
Nature of the hyphae	Septate	Septate	Septate	Septate
Colour of the conidiosphores	Hyaline (Brownish)	Hyaline (Brownish)	Hyaline (Brownish)	Hyaline (Brownish)
Texture of the conidiosphores	Smooth	Smooth	Smooth	Smooth
Length of Conidiophores	Long	Long	Long	Long
Seriation	Biseriate	Biseriate	Biseriate	Biseriate
Diameter of vesicle (mm)	39	41	43	40
Shape of vesicle	Globose	Globose	Globose	Globose
Shape of conidia	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal
Colour of conidia	Dark brown	Black	Black	Black
Conidia head	Radiate	Radiate	Radiate	Radiate
Texture of Conidia	Wrinkled	Wrinkled	Wrinkled	Wrinkled
Metula covering	Entire	Entire	Entire	Entire

Table 3: Purity of the Nucleic Acids Extracted From the Fungal Species

Isolate Code	Conc (ng/µL)	A_{260}	A_{280}	A_{260}/A_{280}
C	197.36	3.144	1.672	1.88
G	182.44	3.102	1.686	1.84
L	201.62	3.115	1.666	1.87
H	116.45	3.007	1.652	1.82

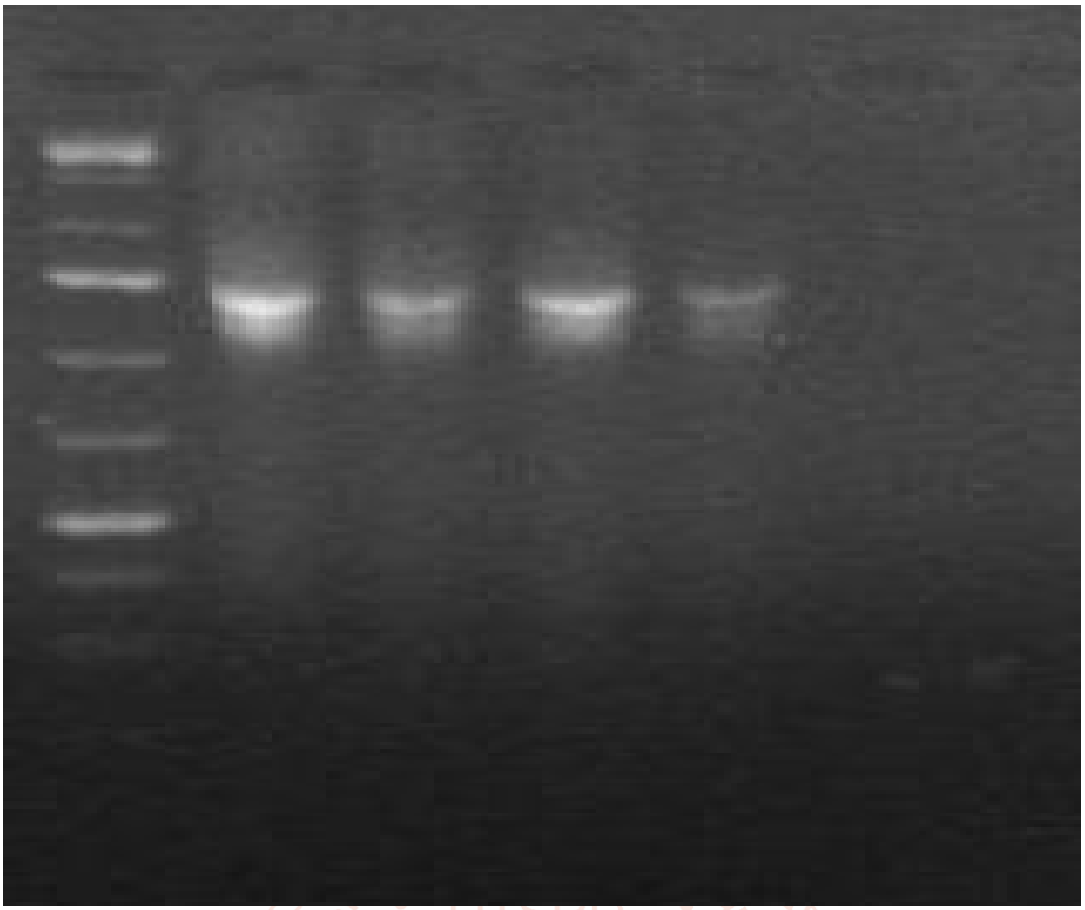

Figure 1: Agarose gel representation of the amplicons

Table 4: Molecular Characteristics of the Fungal Isolates

Isolate code	Max score	Total score	Query cover (%)	E – valve (%)	Identity (%)	Accession Number	Description
C	819	819	100	0	100	MF163445.1	*Aspergillus niger* strain HUS1
G	705	705	100	0	100	MF163444.1	*Aspergillus niger* strain HUS7
L	784	784	100	0	100	KX099669.1	*Aspergillus niger* strain HG49
H	811	811	100	0	100	KU527793.1	*Aspergillus niger* strain AN8

3.4. Screening of Fungal Isolates for Citric Acid Production.

A total of 13 fungal isolates were screened and four isolates were recovered as active citric acid producers. The fungal isolates secreted citric acid in czapedox agar medium. The degree of the growth of the four isolates were shown in Table 5, with isolate C indicating high citric acid production.

3.5. Citric Acid production by *Aspergillus niger* using Sucrose in a Submerged Medium.

The study showed significant production of citric acid from different strains of *Aspergillus niger.* Citric acid production after 5 days incubation period using ASHUS1, ASHUS7, ASHG49 and ASAN8 when sucrose was used as the substrate. It was observed that ASAN8 produced the highest percentage yield of citric acid at 24.81% while ASHUS1 produced the lowest percentage yield of citric acid at 18.42% as shown in figure 2.

Table 5: Screening for Citric acid Production on Czapedox agar medium.

Fungal Isolate code	Degree of growth
C	+++++++
G	++++
L	+++++
H	+++++

Results interpretation: + citric acid producers

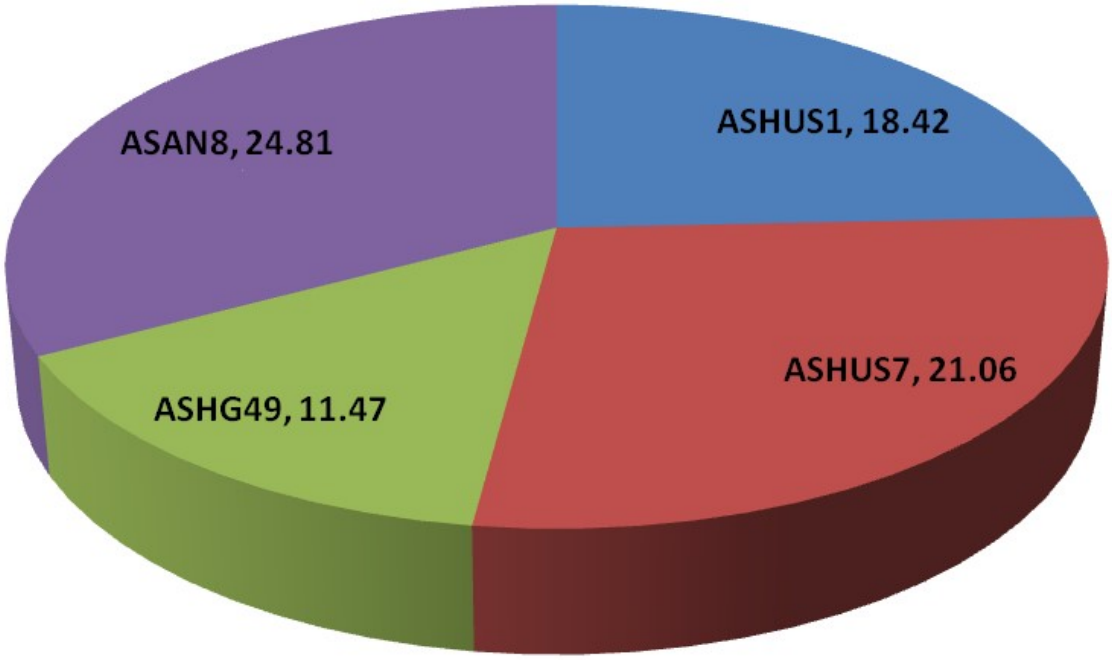

Figure 2: Amount of citric acid produced by four strains of *Aspergillus niger* using sucrose after 5 days of incubation period.

3.6. Effect of Time course on Citric acid produced from *Aspergillus niger* in a Submerged Medium

The study showed significant production of citric acid from different strains of *Aspergillus niger* (Figures 5-8). Citric acid production increased from day 5 to 7, and then variation in production started after day 7. ASHUS1 produced 64.33% of citric acid on day 9, ASHUS7 produced 45.33% citric acid on day 7, ASHG49 produced 43.42% citric acid on day 9 and ASAN8 produced 49.00% citric acid on day 9. Nine days was the best fermentation period for the studied organisms except for ASHUS7 that work best on 7 days. The study further showed that the percentage of citric acid produced by ASHUS1 was significantly ($\alpha<0.05$) greater than those produced by ASHUS7 and ASHG49 but non significantly ($\alpha>0.05$) greater than the citric acid produced by ASAN8. Citric acid produced by the fungal isolates increased non-significantly ($\alpha>0.05$) daily until the 9th day when the maximum production was recorded except ASHUS7 that recorded the maximum production on the 7th day.

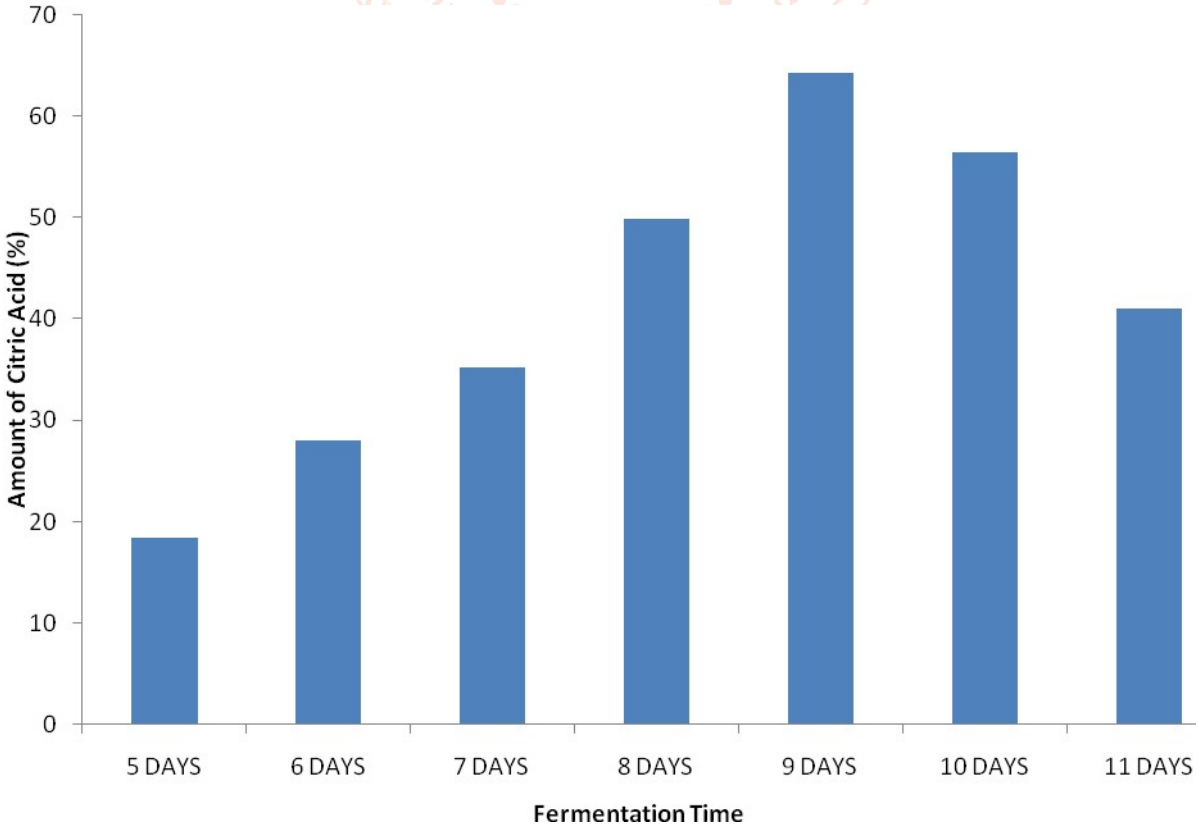

Figure 3: Effect of Time course on Citric acid produced from ASHUS1 in a Submerged Medium.

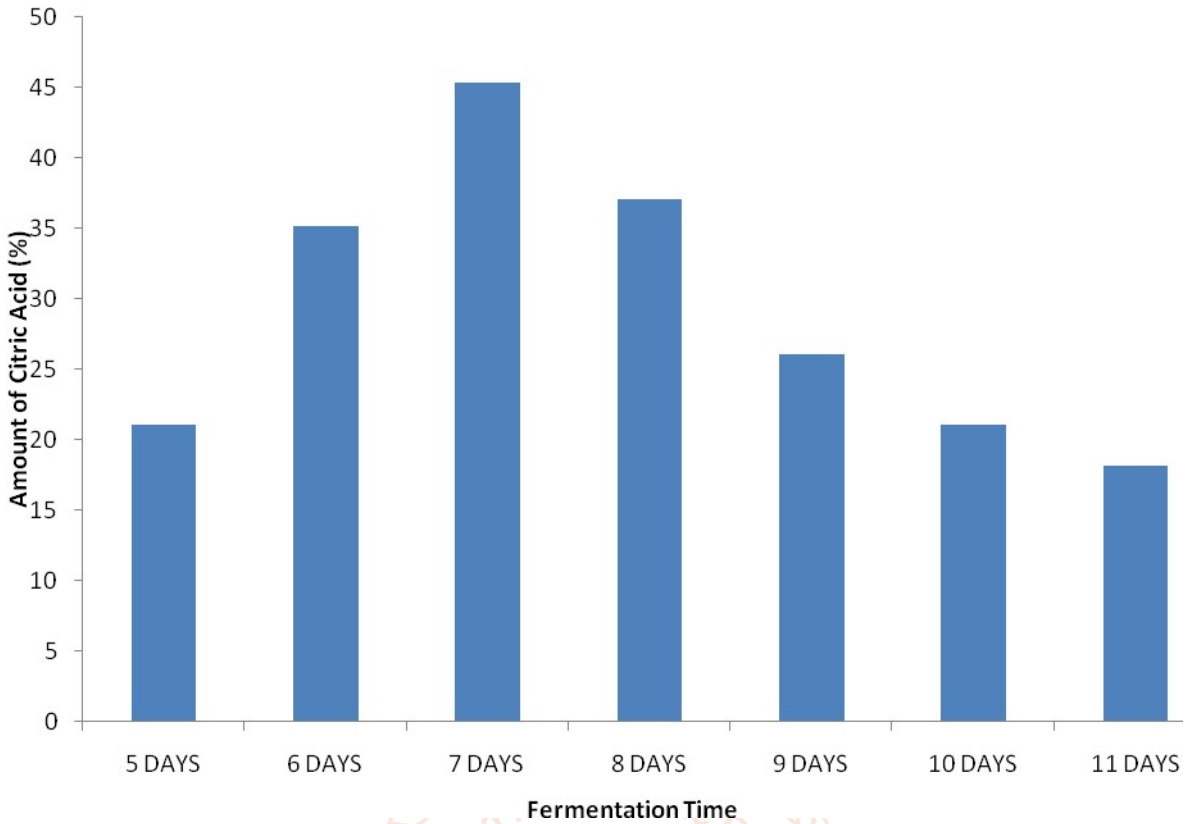

Figure 4: Effect of Time course on Citric acid produced from ASHUS7 in a Submerged Medium.

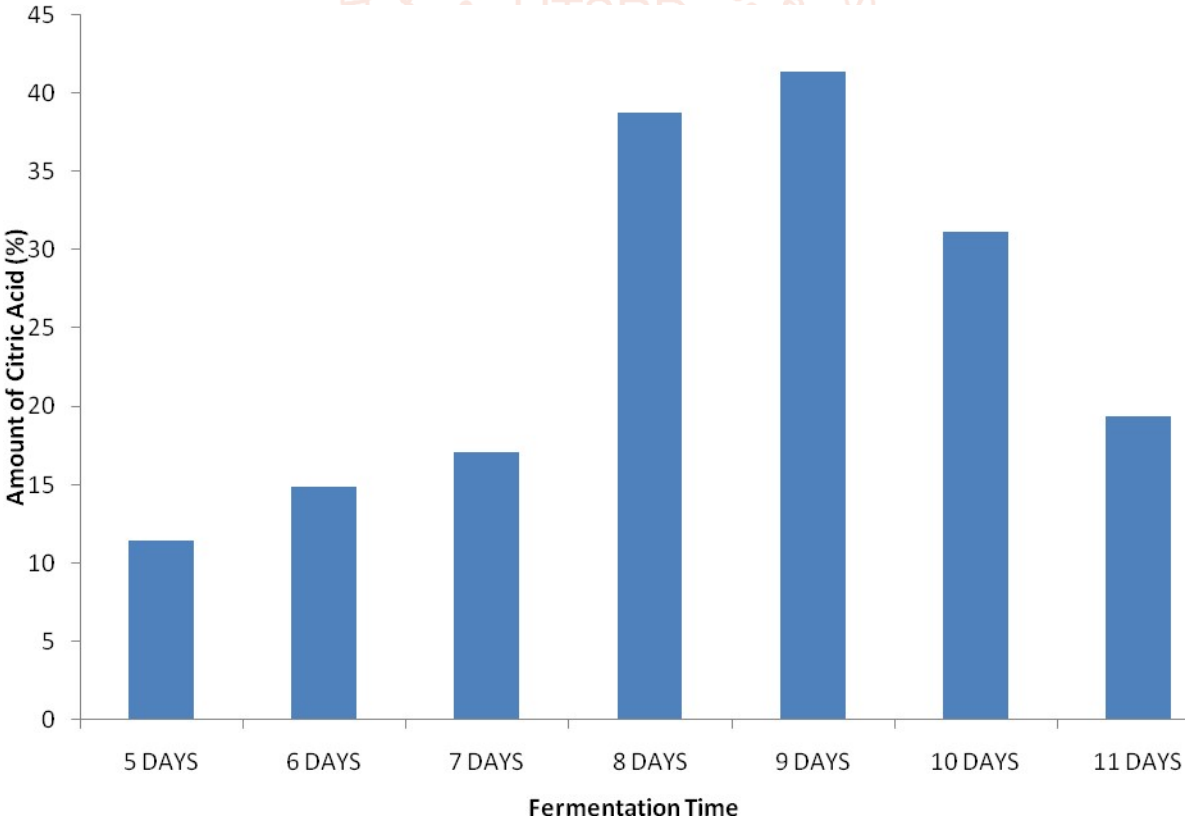

Figure 5: Effect of Time course on Citric acid produced from ASHG49 in a Submerged Medium.

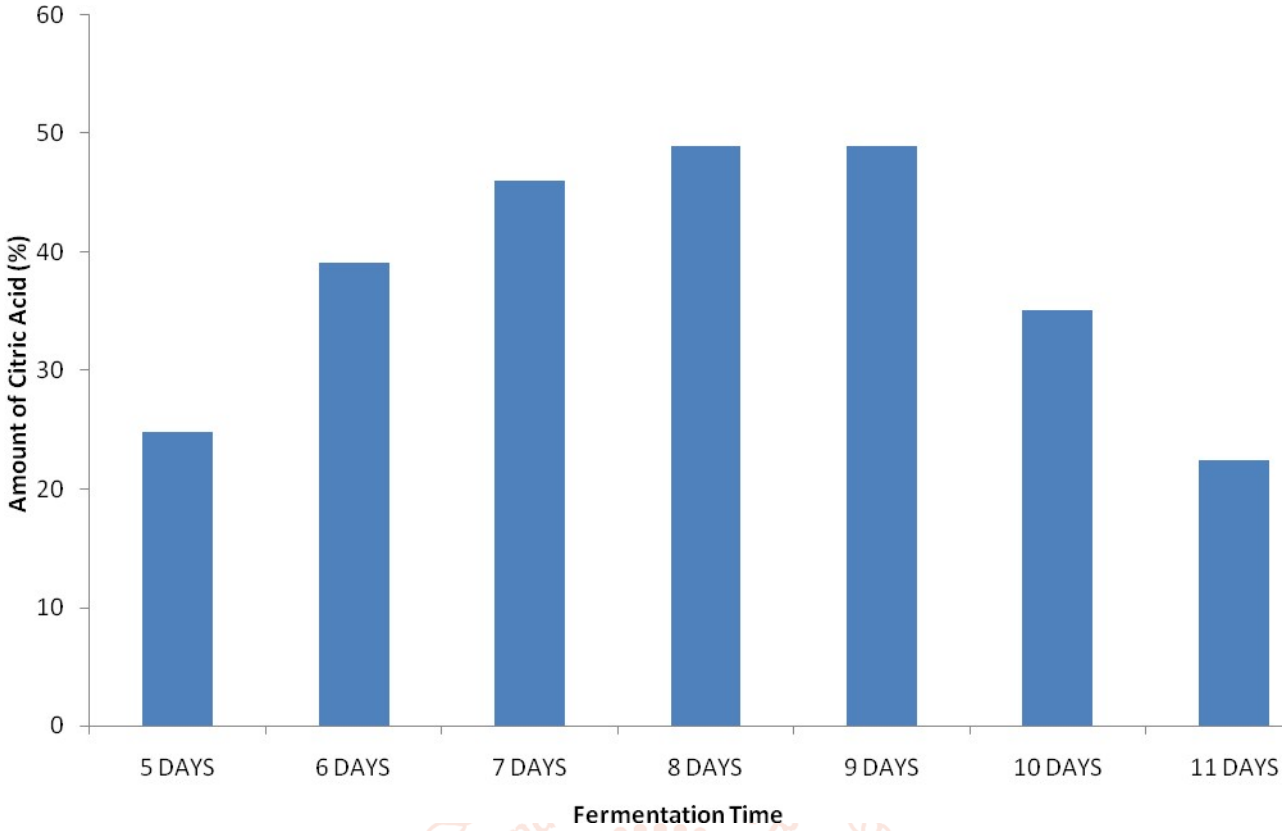

Figure 6: Effect of Time course on Citric acid produced from ASAN8 in a Submerged Medium

4. Discussion

Adding to the numerous diseases and food spoilage, microorganisms have always been known to be harmful while some of them play positive roles in the human body and in the environment. (Tortora *et al*., 2010). Currently these microorganisms are widely utilized in the food industries for the production of fermented food products, most especially in the conversion of agro-industrial wastes into useful products. (Laufenberg *et al*., 2003).

Aspergillus niger, a filamentous fungi was utilized in the production of citric acid in this study.

A total of thirteen isolates were isolated from soil samples. The macroscopic and microscopic characteristics of the fungal isolates revealed that of *Aspergillus niger* (Table 1 and 2). This supports the findings of many researchers (khan *et al*., 2003; Glare *et al*., 2014; Iqbal *et al*., 2015). The preliminary screening of citric acid production by *Aspergillus niger* using czapedox medium showed that *Aspergillus niger* strains are good producers of citric acid. This agreed to the findings of Iqbal *et al*. (2015); Almousa *et al*. (2018), who screened for citric acid production using the same method. Out of the 13 isolates screened for citric acid production, four isolates were selected as the best citric acid producers (Table 5). The molecular characteristics of the four selected fungal isolates revealed the presence of *Aspergillus niger* strain HUS1 (ASHUS1), *Aspergillus niger* strain HUS7 (ASHUS7), *Aspergillus niger* strain HG49 (ASHG49) and *Aspergillus niger* strain AN8 (ASAN8) (Table 4). This is in line with the findings of Iqbal *et al*. (2015); Khan *et al*. (2003); Glare *et al*. (2014).

The submerged fermentation of sucrose by the four selected strains of *Aspergillus niger* (*Aspergillus niger* HUS1, *Aspergillus niger* HUS7, *Aspergillus niger* HG49 and *Aspergillus niger* AN8) was carried out. The objective of this test was to evaluate and to confirm the suitability of these organisms in citric acid production. It was also carried out to determine the best strain for the production and the best incubation time. Hence, in this study,the result of the production of citric acid from the basal medium by*Aspergillus niger*(Figure 2) agrees with the findings of many researchers (Ganne *et al*., 2008; Dashen *et al*., 2013; Shetty, 2015; Show *et al*., 2015; Sarkar and Das, 2017; Almousa *et al*., 2018; Audu *et al*, 2019; Dutta *et al*, 2019; Aboyeji *et al*., 2020).The production of citric acid by *Aspergillus niger* strains (ASHUS1, ASHUS7, ASHG49 and ASAN8) in a submerged fermentation using sucrose as a parent carbon source gave a significant yield of citric acid with ASAN8 as the best producer after 7 days. This is in affirmation with the works of Almousa *et al*. (2018), Thiruvengadam and Thangavel, (2016), who also used sucrose as a source of carbon for citric acid production.

All the four strains gave their highest yield of citric acid after 9 days except for ASHUS 1 which has

maximum productivity after 9 days. This confirms the report of Iqbal *et al*., 2015 who recorded maximum production after 9 days. Other strains recorded increase in production of citric acid after 9 days with ASHUS1 recording the highest yield. Almousa *et al*. 2018 reported the highest amount of citric acid production after 7 days, and this is at variance with the present study which recorded the highest citric acid production after 9 days. However, according to Ahmed *et al*. (2014) the highest amount of citric acid was produced after 8 days incubation period. The high level of citric acid produced from ASHUS1 among ASHUS7, ASHG49 and ASAN8 could be attributed to the activity of citrate synthase and isocitrate dehydrogenase isoenzymes that exist in this strain. Citrate synthase catalyzes the reversible conversion reaction between acetyl coenzyme A (acetyl coA) and oxoacetate which leads to the production of citric acid. Furthermore, the nature and type of isocitrate dehydrogenase also determines the level of citric acid produced from the fungal isolates as stated by Show *et al*. (2015).

Conclusion

This study has shown that all the four fungal isolates isolated from garden soil and identified as *Aspergillus niger* strain HUS 1, *Aspergillus niger* strain HUS7, *Aspergillus niger* strain HG49 and *Aspergillus niger* strain AN8 are active citric acid producers. This study also revealed that these strains of *Aspergillus niger* were able to utilize sucrose to produce citric acid with ASAN8 having the highest yield after 7 days of incubation period. However, upon the optimization for the effect of time course on citric acid production, the maximum productivity was attained by ASHUS 1 after 9 days of incubation period. The study has shown that ASHUS1, ASHUS7, ASHUS49 and ASAN8 isolated from garden soil produced citric acid from submerged fermentation. ASHUS1 yielded the highest citric acid. It was also observed that 7.0mm mycelial plug of ASHUS1 yielded the highest citric acid at pH and temperature of 1.0 and 55^0C respectively using mango peel extract and soya bean powder as alternative carbon and nitrogen source respectively in a submerged basal medium.

References

[1] Abeer, A., Khattab, Salem, A. A. and Amira, E. S. (2017). Optimization of Citric Acid Production by *Aspergillus niger* isolated from different Habitats. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*. pg 614 – 616

[2] Aboyeji, O. O., Oloke, J. K., Arinkoola, A. O., Oke, M. A. and Ishola, M. M. (2020). Optimization of media components and fermentation conditions for citric acid production from sweet Potato Peel starch hydrosylate by *Aspergillus niger*. *Scientific African* 10:1-9

[3] Almousa, A. A., AbduEl-Ghany, M. N. and Ashour, E. H. (2018). Citric acid fermentation by *Aspergillus niger*. *Journal of Innovations in Pharmaceutical and Biological Sciences*5(4): 20-34

[4] Ahmed, A., Ahmed, Z., Amani, M., Asuka, K., Kuniyoshi, S., Mohamed, A., Momiji, O., Ryuichiro, K. and Saleh El-Sharkawy. (2014). Production of Citric Acid from Corncobs with Its Biological Evaluation. *Journal of Cosmetics, Dermatological Sciences and Applications,* 4:141-149

[5] Amenaghawon, N. A., Aisien, F. A. (2012). Modelling and simulation of citric acid production from corn starch hydrolysate using *Aspergillusniger*. *Environment and Natural Resources Research*. 2:73–85.

[6] Angumeenal, A., Kamalakannan, P., Prabhu, H. J. and Venkappayya, D. (2003). Effect of transition metal cations on the production of citric acid using mixed cultures of *Aspergillusniger* and *Candida gulliermondii*. J Indian Chem Soc. 80:903–906.

[7] Audu, J. A., Arekemse, S. O., Abdulwaliya, I., Bateri, M. L., Napling, H. H, Aronimo, B. D. and Sani, Y. (2019). Production of citric acid from corn stalk through submerged fermentation using *Aspergillus niger*. *Journal of Applied Sciences* 19; 557-564.

[8] Berovic, M., Legisa, M. (2007). Citric acid production. *Biotechnology Annual Review.* 13:303–343

[9] Dashen, M. M., Ado, S. A., Ameh, J., Amapu, T. and Zakari, H. (2013). Screening and Improvement of local isolates of *Aspergillus niger* for citric acid production. *Bayero Journal of Pure and Applied Sciences*6(1): 105-111

[10] Dhandayuthapani, K. (2008). Production of citric acid from banana waste by *Aspergillus niger*, *International Journal on Applied Bioengineering*, 2(1): 58-61.

[11] Dutta, A., Sahoo, S., Mishra, R. R., Pradhan, B., Das, A. and Behera, B. C. (2019). Environmental and Experimental Biology 17: 115-122.

[12] Ganne, K. K., Dasari, V. R. and Garapati, H. R. (2008). Production of citric acid by *Aspergillus niger* MTCC282 in submerged fermentation

using *Colocassia antiquorum*. *Research Journal of Microbiology* 3(3): 100-156

[13] Hossain, M., Brooks,. J and Maddox,. I. (1984). The effect of the sugar source on citric acid production by *Aspergillusniger*. *Appl Microbiol Biotechnol*. 19:393–397

[14] Glare, T. R., Gartiell, B. D., Brookes, J. J. and Parott, J. K. (2014). Isolation and identification of *Aspergillus* spp. Frpm Brown kiwi nocturnal houses in Newzealand. *Avian Diseases* 58: 16-14

[15] Ikram-ul, H., Ali, S., Qadeer, M. and Iqbal, J. (2004). Citric acid production by selected mutants of *Aspergillus niger* from cane molasses. *Bioresour Technol.* 93:125–130

[16] Iqbal, J., Haqq, I. U., Javed, M. M., Hameed, U., Khan, A. M., Parveen, N. and Khan, T. S. (2015). Isolation of *Aspergillus niger* strains from soil and their screening and optimization for enhanced citric acid production using cane molasses as carbon source. *Journal of Applied Enviromental and Biological Sciences*5(4): 128-137

[17] Khan., Ul-Haq, I., Butt, W. A. and Ali, S. (2003). Isolation and screening of *Aspergillus niger* isolates from xylanase biosynthesis. Biotechnology 2(3): 185-190

[18] Kubicek, P. C., Rohr, V. and Schreferl, G. (1986). Inhibition of citric acid accumulation by manganese ions in *A. n i g e r* mutants with reduced citrate control of phosphofructokinase. *Journal of Biotechnology*, 165(3): 1019 – 1022.

[19] Kubicek-Pranz, E. M., Mozelt. M., Rōhr M, and Kubicek, C. P. (1990). Changes in the concentration of fructose 2, 6-bisphosphate in *Aspergillus niger* during stimulation of acidogenesis by elevated sucrose concentration. *Biochim Biophys Acta, Gen Subj*. 10 (33):250–255.

[20] Mattey, M. (2002). The production of organic acids. *Crit Rev Biotechnol.* 12:87–132.

[21] Narasimha, G., Babu, G. V. A. K. and Rajasekhar, R. B. (2012). *Journal of Environmental Biology*, 20: 235-239.

[22] Papagianni, M., Mattey, M. and Kristiansen, B. (2009). The influence of glucose concentration on citric acid production and morphology of *Aspergillus niger* in batch and fed-batch culture. *Enzyme Microb. Technol*25: 710–717.

[23] Papagianni, M., Wayman, F. and Mattey, M. (2005). Fate and role of ammonium ions during fermentation of citric acid by *Aspergillus niger*. Applied and Environmental Microbiology. 71: 7178–7186.

[24] Sarkar, D. and Das, K. (2017). Optimization of citric acid production from *Aspergillus niger* using Pineapple waste as feedstock in submerged fermentation. *World journal of Pharmaceutical Research* 6(17): 810-818

[25] Shetty, V. G. (2015). Production and optimization of citric acid by *Aspergillus niger* using molasses and corncob. *International Journal of Pharmacy and Pharmaceutical Sciences 7(5):152-157*

[26] Show, P. L., Oladeke, K. O., Siew, Q. Y., Zakry, F. A., Lan, J. C. and Ling, T. C. (2015). Overview of citric acid production from *Aspergillus niger*, *Frontiers in Life Sciences* 8(3): 271-283.

[27] Suleiman, M. N. and Omafe, O. M. (2013). Activity of three Medical Plants on Fungi Isolated from stored Maize seeds. *Global Journal of Medicinal Plant Research*, 1: 77-81.

[28] Themelis, D. G., Tzanavaras, P. D. (2001). Reagent-injection spectrophotometric determination of citric acid in beverages and pharmaceutical formulations based on its inhibitory effect on the iron (III) catalytic oxidation of 2, 4-diaminophenol by hydrogen peroxide. *Anal Chim Acta.* 428:23–30

[29] Thiruvengadam, S. and Thangavel, S. (2016). Optimization of Citric Acid Production Using *Aspergillus niger* Isolated from the Leaf Litter Soil of Sathuragiri Hills. *Universal Journal of Microbiology Research* 4(4): 79-87

[30] Umedum, C. U. and Iheukwumere, I. H. (2013). Susceptibility of dermatiaceous fungi isolated from natural sources to conventional antifungal agents. *African Journal of Sciences*, 14(1): 3101-3107

[31] Watanabe, T. (2002). Pictorial atlas of soil and seed fungi. Morphologies of cultured fungi and key to species. 2nd ed. CRC Press, Washington DC, USA. 500

[32] Weyda, I., Lübeck, M., Ahring, B. K. and Lübeck, P. S., (2014). Point mutation of the xylose reductase (XR) gene reduces xylitol accumulation and increases citric acid production in *Aspergillus carbonarius*. Journal of Industrial MicrobiolBiotechnol. 41:1–7.

[33] Xu, D. B., Madrid, C. P., Röhr, M and Kubicek, C. P. (1989). The influence of type and concentration of the carbon source on production of citric acid by *Aspergillus niger*. *Appl Microbiol Biotechnol.* 30:553–558.