Citric Acid Production by *Aspergillus niger and Aspergillus awamori* Isolated from Soil in keffi, Nigeria

I. K. Ekeleme*, M. D Makut, J. E Owuna, S. O Obiekezie, and F. H Muhammed

Department of Microbiology, Nasarawa State University, P.M.B 1022, Keffi, Nigeria
*Corresponding Author**

Abstract: **Citric acid is an Organic acids, commercially valuable product extensively used in different industries for various purposes. This study aimed at citric acid production by *A.niger* and *A. awamori* isolated from soil in Keffi using waste starch from corn milling factory. *A.niger* and *A. awamori* was isolated from soil in Keffi and identified using standard microbiology methods. Starch production media was prepared by following standard fermentation conditions. The citric acid produced was estimated using Gas Chromatography/Mass Spectrometry (GC/MS) method. The occurrence of *Aspergillus* species showed that Pyanku had the highest percentage occurrence with 100%. The screening for citric acid producing *A.niger*and *A. awamori* showed that isolates from location A, C and D showed ability to produce citric acid. Effect of different temperature on citric acid production showed that *A.awamori*A1produced highest at 28^{o}C with 3.10 mg/ml and *A.awamori*A2 produced lowest at 26^{O}C (2.08mg/ml). *A. niger* F4 produced the highest at32^{O}C with 5.03mg/ml and *A.niger* F5 lowest at 26^{O}C with 3.10mg/ml. Fermentation duration showed duration by *A. awamori* A2 after 144hours with 5.00mg/ml and *A. awamori* A1 showed lowest yield after 24hours with 0.71mg/ml while *A. niger* F5 yield highest after 168 hours with 5.02mg/ml and *A. niger* F4 yield lowest after 24hours with 0.91mg/ml. Effect of pH on citric acid production showed that *A. awamori*A1 yield highest at pH 4.5 with 5.19mg/ml while *A. niger*F5 produced highest at pH 5.0 with citric acid yield of 5.69mg/l.**

***Keywords: A.niger*and *A. awamori*, pH, citric acid, Temperature**

I. INTRODUCTION

Citric acid is an Organic acid,commercially valuable product extensively used in different industries for various purposes such as: acidulent, antimicrobial preservatives, antioxidant, prevent crystallization, anticoagulant, flavour stimulator, metal cleaner, (Kim *et al.*, 2007; Alam*et al* 2009). The acceptability of this organic acid in food products mainly depends on the flavor components, which are complex as well as type specific. These flavor components are influenced by the presence of organic acids and other substances like sulphur compounds, lactones, methyl ketones, alcohols and phenolic substances (Urbach, 2013)

The largest amount of citric acid is consumed in food industry using almost 70% of the total production, followed by about 12% in the pharmaceutical industry and 18% for other applications. The production of citric acid by *Aspergillus niger* is one of the most commercially utilized examples of fungal overflow metabolism. Many microorganisms such as fungi and bacteria can produce citric acid. The various fungi, which have been found to accumulate citric acid in their culture media, include strains of *A. niger, A. awamori, Penicilliumrestrictum, Trichodermaviride, Mucorpiriformis* and *Yarrowialipolytica*(Arzumanov *et al*., 2010). However, *A. niger* is considered as the organism of choice for the production of citric acid because of the fact that this organism has the capacity to utilize varieties of substrates due to its well-developed enzymatic system (Munshi*et al*., 2013). Agricultural waste or biomass poses serious disposal problems in the environment that can be used as raw material for commercial production of citric acid.

Therefore, the aim of this study was to produce citric acid from wastewater corn starch by *Aspergillus niger* and *Aspergillus niger. Awamori* isolated from soil.

II. MATERIALS AND METHODS

A. Study Area

The study was in Keffi local government area, Nasarawa State, Nigeria. Keffi is approximately 68km from Abuja, the Federal Capital Territory and 128km from Lafia, the Capital of Nasarawa state. Keffi is located between latitude 8^{o}5'N of the equator and longitude 7^{o}8'E and situated on an altitude of 850m above sea level (Akwa*et al*., 2007).

B. Sample Collection

Four soil samples were randomly collected at the top soil from five different locations in Keffi Metropolis such as Angwanzakara, Angwan Jarme, Tudun Wada, Pyanko using a clean hand trowel and stored using disposable black polythene bags and transported immediately to the Microbiology Laboratory, Nasarawa State University, Keffi for analysis.

C. Isolation of Aspergillus species

Isolation of *A.niger* and *A. awamori* was carried out using a method described by Ekeleme *et al*. (2018). Briefly, One (1) gram of the soil sample was suspended in a test tube containing 9 ml of sterile distilled water to make a soil suspension and ten-fold serial dilution was made by

transferring one ml of the soil suspension to another test tube containing 9 ml of sterile distilled water. These steps were repeated seven times to obtain a dilution of 10^{-7}. From the fourth test tubes, 0.2 ml of the aliquot was spread on Potato dextrose agar plates, Malt extract agar and Yeast extract agar and incubated at 28^{o}C for 4 days.

D. Identification of Aspergillus specie

Identification of *A.niger* and *A. awamori* was carried outas described by Makut and Ifeanyi(2017). Identification was based on microbiological standard procedure using cultural and morphological characteristics. The cultural characteristics were determined by their appearance on culture plates while the morphological features were determined microscopically using lactophenol cotton blue staining technique. The isolates were identified with reference to the work of Singh *et al.* (2011) fungi standard chart.

E. Screening for Citric Acid-Producing Aspergillus niger and Aspergillusawamori

Screening for Citric Acid-Producing *A.niger*and *A. awamori*will be carried out as described by Ekelem*et al*. (2018). The isolates were screened qualitatively and quantitatively of citric acid production. Potato dextrose agar plate method containing Bromocresol green as an indicator 1% at pH 6 was used. *A.niger* and *A. awamori* was inoculated on the plates and incubatedfor 48 hours.

F.Preparation of starch substrates

Starch substrate was prepared using a method described by (Ekeleme, *et al*., 2018). Wastewater from corn milling factory were collected and allowed to settle. The starch down was separated from liquid and oven dried at 60ºC, overnight.A starch solution of 20 g/l was dissolved and autoclaved at 5.0 lbs/in2 pressure (115°C) for 5 min. To liquefy starch, alpha amylase (2.0µ/ml) was added and heated at 95ºC in a water bath for 15 min. For saccharification, amyloglucosidase (2.0µ/ml) was added and heated at 55ºC while constant stirring for about 4 hours

Twenty gram (200g) powder form was added into 4 liter of distilled water and sieves to form a homogenous mixture and placed at 4ºC for 24 hours

III. PRODUCTION OF CITRIC ACID

A. Preparation of Inoculum for Fermentation

Preparation of inoculum for fermentationwas carried out as described byAlam et al. Five (5ml) of 0.3% sterile tween 80 contained in peptone water with some glass beads were transferred into four (4) days' slant culture of *A.niger* and *A. awamori* was shaken thoroughly until spores were dissolved and spore suspension was maintained in 1%(w/v) sugar solution and incubated at roomtemperature (28±1^{o}C) for 12 hours.

*B. Media Formulation and Fermentation Technique***:**

The batch fermentation was carried out as described by Bari et al., (2009) with modification. The Starch hydrolysate such as: M [starch 20g/L, 0.09 g/l ZnSO4.7H2O, 0.1 g/l CuSO4.5H2O, 0.4g/l MnSO4 and 5 g/l MgSO4.7H2O, NH_4 2SO4 1.5 g, and KH_2PO_4 4.1 g,] was taken in different conical flasks. The flasks were plugged with cotton and autoclaved at 15 psi for 15 min.

C.*Optimization of citric acid production by a. Niger and a. Awamori*

Effect of pH on Citric Acid on Production

The effect of pH was carried out following a method described by Makutand Ekeleme (2018). One hundred (100) ml of the fermentation substrate was transfered into different conical flasks. The pH ranges was adjusted to, 4.0 4.5, 5.0 5.5, 6.0, 6.5, 7.0 and 7.5 of fermentation media using 1.0 N HCl to adjusting the pH of the media before autoclaving.

D. Effect of Temperature on Citric Acid Production

Effect of temperatures was carried out following a method described by Asad-ur *et al*. (2002); Ball *et al*. (2001). One hundred (100) ml of the fermentation substrate was transfered into different conical flasks and the fermentation media was incubated at 26^{o}C, 28^{o}C, 30^{o}C and 32^{o}C .

F. Effect of Fermentation Duration on Citric Acid Production

The effect of fermentation duration was carried out as described by Makutand Ekeleme (2018); Ekeleme, *et al*. (2018). Briefly, different time intervals were monitored during the fermentation of the media after 24 hours, 48hours, 72 hours, 96 hours, 120hours, 144hours and 168hours.

IV. QUANTIFICATION OF CITRIC ACID

A. Estimation of Citric Acid

The citric acid produced during fermentation was determined by Gas Chromatography and Mass Spectrometry (GC and MS) (Ekeleme, *et al*. (2018)) as detailed below;

B. Sample Preparation

During sample preparation, 60 mL of fermented media was added to 40 mL of buffer-acetonitrile mobile phase (0.5% (w/v) (NH4)2HPO4 (0.038 M) - 0.4% (v/v) acetonitrile (0.049 M), at pH 2.24 with H3PO4), extracted for 1 hour in orbital shaker and centrifuged at 6000 x *g* for 5 min. The supernatant was collected and filtered once through filter paper Whattman No. 1 and twice through a 0.45 µm membrane filter, and then used directly for GC and MS analysis. Duplicate analyses were performed on all samples.

C. Gc and Ms Analysis

Chromatography equipped with flame-ionization detector. The column used for the separation of solvent PEG (2.1m x 3.0mm). The operating conditions were mobile phase, aqueous 0.5% (w/v) $(NH_4)2HPO_4$ (0.038 M) - 0.2% (v/v)

acetonitrile (0.049 M) adjusted to pH 2.24 with H_3PO_4; flow rate 0.3 mL min-1; ambient column temperature. The mobile phase was prepared by dissolving analytical-grade $(NH_4)2HPO_4$ in distilled deionized water, GC and MS -grade acetonitrile, and H_3PO_4. GC and MS -grade reagents was used as standards (Sigma Chemical Co., St. Louis, MO). Solvents were filtered through a 0.45 μm membrane filter and One hundred and twenty degrees centigrades (120^{o}C), Nitrogen gas (30 mL/minutes) were used as carrier gas. The temperatures of injector and detector were 150^{o}C and 200^{o}C respectively. The Peaks were recorded on "SHIMADZU C-R-4_A, Chromatograph", and was identified by comparison of the retention times with that of standard mixture. The experiment was carried out in duplicate and the means ± standard deviations of the yield of citric acid were recorded.

V. RESULTS AND DISCUSSION

The occurrence of *Aspergillus* species isolated from selected location soil in Keffi is as given in table1, where Pyanku had the highest percentage occurrence of *Aspergillus* species with 100% followed by Nasarawa State University80.0% and the least was from Angwanzakara with 20.0% respectively.

The screening for citric acid producing *Aspergillus* species is as given in table 2 isolates from location A, C and D showed ability to produce citric acid while none of the isolates from location B was able to show ability to produce citric acid.

A. Effect of temperature on citric acid production by A. awamori and A. niger

The effect of different temperature on citric acid production is as shown in figure 1 which showed highest yield of citric acid at temperature of 28^{o}C by *A.awamori*A1 with 3.10 mg/ml followed by 30^{O}C with 3.01mg/ml and lowest at26^{O}C with 1.01mg/ml while *A.awamori*A2 also produced highest citric acid at 28^{O}C with 3.45mg/ml and least was at 26^{O}C 2.08mg/ml. *A.niger* F5 was observed at 32^{O}C with 5.03mg/ml followed by 30^{O}C with 4.09mg/ml and lowest at26^{O}C with 3.10mg/ml while *A.niger*F4 produced the highest at temperature of 32^{O}C with 5.03mg/ml and the lowest was at 26^{O}C with 3.11mg/ml this finding is similar to study reported by Sikander*et al.,* (2002) who reported best yield of citric acid at 30^{O}C by *A. niger.* It has been shown that temperature is an importation factor that determines the yield of citric acid.

B. Effect Fermentation Duration

The time of growth was a very significant parameter in the growth of these fungal isolates towards the fermentation of the media for the production of citric acid. The effect of fermentation duration is as given in Figure2. The highest citric acid produced by *A. awamori* A2 was observed after 144hours with 5.00mg/ml and least was after 24hours with 0.80mg/ml and *A. awamori* A1 produced highest after 144hours with 4.70mg/ml and 24hours with 0.71mg/ml while *A. niger* F5 was observed after 168 hours with 5.02mg/ml followed by 144hours with 4.80mg/ml, 120hours with 4.00mg/ml, 96hours with 2.81mg/ml, 72hours with 1.99mg/ml, 48hours with 1.01mg/ml and the lowest was after 24hours while *A. niger* F4 also produced highest citric acid after 168hours with 4.41mg/ml followed by 144hours with 4.10mg/ml and the lowest was at 24hours with 0.91mg/ml the different in fermentation duration was not surprising but is in agreement with findings reported by Andleeb*et al.,* (2007) 168 hours as the best fermentation duration. The duration showed by *A. awamori* was interesting which showed duration reduction in citric acid production and similar study by Deepika (2015). Further increase in duration resulted in the decreased citric acid production. It might be due to the decreased amount of nitrogen in the fermentation medium, the age of isolates, the presence of inhibitors produced by the isolates itself or depletion of sugar contents.

C. Citric Acid Produced By the Isolates at Different pH

Effect of pH on citric acid production by *A. awamori*as given in Figure 4.5, the highest citric acid produced by *A. awamori*A1 at pH 4.5 with 5.19mg/ml followed by pH5.0 with 5.01mg/ml, pH7.5 with 2.38mg/ml and *A. awamori* A2 produced highest citric acid at pH4.5 with 4.94mg/ml followed by pH5.0 with 4.49mg/ml and lowest at pH 6.5 with 2.90mg/l while *A. niger*F4 at pH5.0 with citric acid yield of 6.51mg/l followed by pH 4.5 with citric acid of yield of 5.46mg/l, pH 5.5 with yield of 5.23mg/l and the least at pH 7.5 with 2.33mg/l. *A. niger*F5 produced highest at pH 5.0 with citric acid yield of 5.69mg/l and the least was on pH 7.5 with 3.00mg/l.

The use of *A. awamori*in the production of citric acid witnessed an increase in the yield of citric acid when pH was reduced (i.e, the lower the pH, the higher the quantity of citric acid produced. In other words, the yield of citric acid was favoured by an increase in acidity). This study is consistent with the report published by Kobomoje*et al*. (2013); Ekelemee*t al*. (2018) that the best growth was observed at pH 4.5 while minimum growth was observed at pH 3. This might be due to the ability of *A. awamori* and *A. niger*to survive more in acidic media.

D. Effect of Substrate Concentrations

The effect of substrate concentrations on citric acid production by *A.awamori*as shown in Figure 4.7, the highest citric acid was produced by *A. awawori*A1 at substrate concentrations of 175mg/ml with 6.07mg/ml follow by substrate concentrations of 125mg/ml with 5.65mg/l, substrate concentrations of 150mg/l with citric acid production of 5.63mg/l, substrate concentrations of 100mg/ml with 5.61mg/l, substrate concentrations of 200mg/l with 5.30mg/l and substrate concentrations of 75mg/ml with 5.01mg/l while *A.awamori*A2 yield the highest citric acid at substrate concentrations of 175mg/ml with 5.80mg/l followed by substrate concentrations of 125mg/ml with 5.69mg/l, substrate concentrations of 150mg/ml with 5.66mg/l, substrate concentrations of 100mg/l with 5.24mg/l, substrate concentrations of 200mg/l with 5.00mg/l and substrate concentrations of 75mg/l with citric acid yield of 4.42mg/l

while The highest citric acid was produced at substrate concretion of 175mg/ml with 7.17mg/l followed by concentrations of 150mg/ml with 6.83mg/l, substrate concentrations of 125mg/l with 6.11mg/l, substrate concretion of 100mg/l had 6.09mg/l, substrate concentrations 75mg/l with 5.88mg/l and substrate concentrations of 200mg/l with 5.30mg/l while *A. niger* F5 produced highest at substrate concentrations of 175mg/ml with 6.82mg/l followed by substrate concentrations of 150mg/l with citric acid yield of 6.16mg/l, substrate concentrations of 125mg/l with citric acid yield of 5.99mg/l, substrate concentrations of 100mg/l with citric acid yield of 5.87mg/l, substrate concentrations of 200mg/l with citric acid yield of 5.10mg/l and the lowest was at substrate concentrations of 75mg/ml with citric acid yield of 5.02mg/l

Table 1 frequency of isolation of *Aspergillus* species from soil in Keffi

Locations	No sample	No (%) isolated
A	5	2 (40.0%)
B	5	3(60.0%)
C	5	4(80.0%)
F	5	5(100%)

KEY:A = Angwanzakara; B = AngwanJarme; C = Nasarawa State University; D = Pyanku

Table 2: Screening for Citric Acid Producing *Aspergillus* species Isolated from Soil

Isolates	Citric Acid production
A. niger A1	++
A.awamoriA3	+
A.niger B1	-
A.awamori B2 -	-
B3 -	-
A.awamoriC1	++
A.awamori C2	-
A. niger C3	-
A. niger C4	+
A. awawori F1	-
A. nigerF2	-
A. awawori F3	-
Aspergillus species F4	++
Aspergillus species F5	+

KEY: + = Positive; - = Negative; A = Angwanzakara; B = AngwanJarme; C = Tudun Wada; D = Pyanku

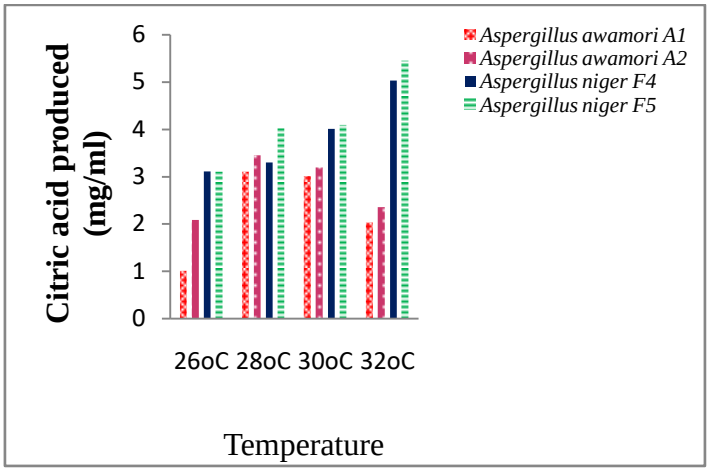

Figure 1: Effect of different temperature on citric acid production by *A. awamori* and *A.niger*

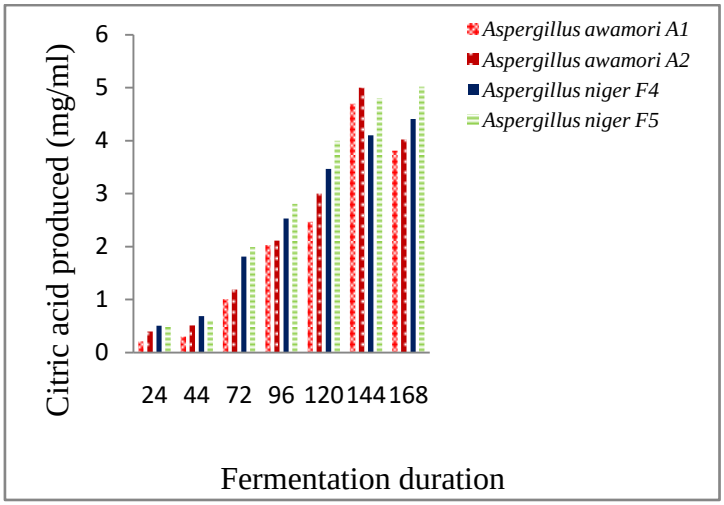

Figure 2: Effect of different fermentation duration on citric acid production

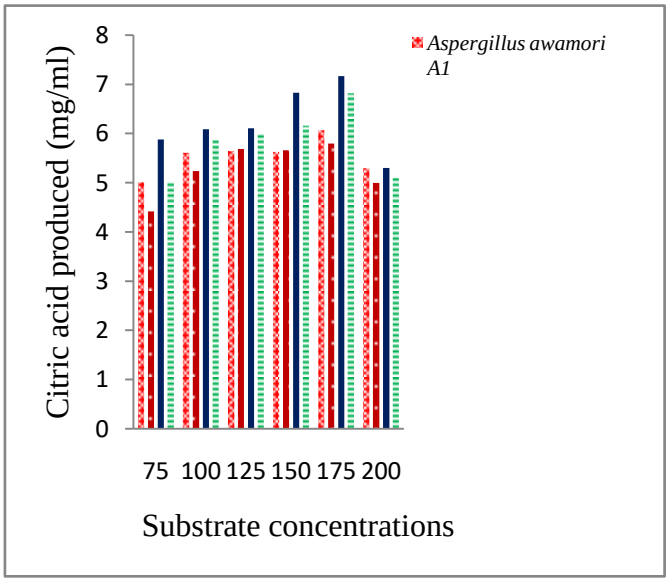

Figure 4: Effect of Substrate concentrations on citric acid production

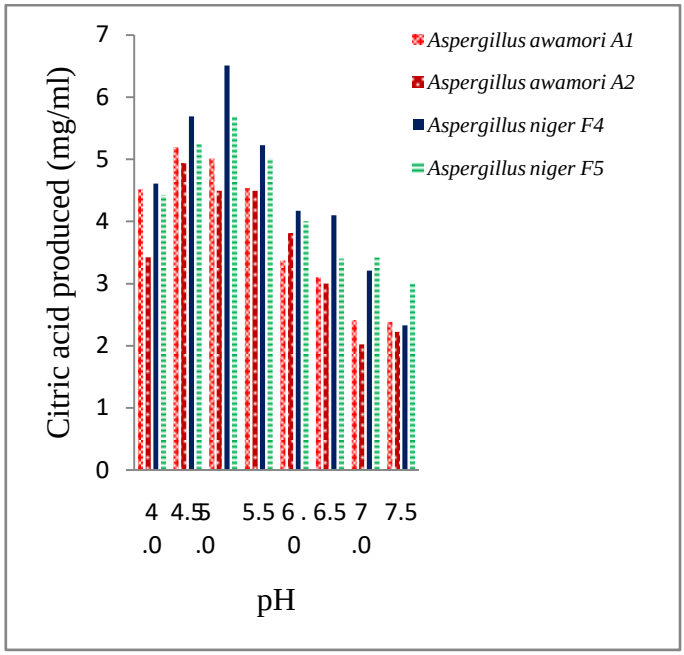

Figure 3: Effect of pH on citric acid production

VI. CONCLUSIONS

The *A. awamori* and *A. niger* isolated from soil in Keffi showed a great ability to produce citric acid in different amount at different fermentation or production parameter. This study also showed that waste can be converted to wealth and help in solving the problem of environmental pollution that come out from factories that process agricultural product such as corn milling factory.

ACKNOWLEDGEMENTS

The contributions of Dodo K, Ezekiel, Atadoga O. Favour, in the areas of sample collection and laboratory work. Special thanks to Nggada, N. Joshua for her help and support in laboratory analysis.

REFERENCES

[1] Alam MZ, M. N. Bari AM. Suleyman, P and Jamal, AA (2009).Mamun, Solid state bioconversion of oil palm empty fruit bunches for production of citric acid by wild *Aspergillus niger*. *Food Biotechnol*, 24(1): 19-36

[2] Sikander A, Ikram-ul, H, Qadeer, MA and Javed I (2002). Production of citric acid by *Aspergillus niger*using cane molasses in a stirred fermentor, Journal of Biotechnology: .5 (3) 1-14,

[3] Andleeb A. S, Sikander A, Samina A and Ikram-ul H (2007). Citric acid fermentation of hydrolysed sweet potato starch by *Aspergillus niger*, Pak. J. Biotechnol. 4 (1-2) 119-125

[4] Deepika H (2015). Citric Acid production by Aspergillusoryzae,Applied Microbiology and Biotechnology, 53(5): 525-529

[5] Kobomoje, OS, Mohammed, AO and Omojasola, PF (2013). The production of citric acid from shea nut shell (vitellariaparadoxa) using *Aspergillus niger*. *Advances in Aplied science Research*, *4*:2:25-28

[6] Ekeleme, IK, Makut, MD., Adoga, MP. Tsaku, PA..Nkene, IH and Oti, VB (2018). Production of Citric Acid by *Trichodermaviride*Isolated from Soil in Keffi, Nigeria Using Glucose Enhanced Substrates, *South Asian Journal of Research in Microbiology;* 1:1: 1-6

[7] Kim JW, Barrington S, Sheppard J and Lee B(2006). Nutrient optimization for the production of citric acid by *Aspergillus niger*NRRL 567 grown on peat moss enriched with glucose. *Process Biochem* 41: 1253-1260

[8] Urbach, G (2013). Relations between cheese flavor and chemical composition.*InternationalDairy* Journal, 3: 389-422

[9] Arzumanov, TE, Shishkanova, NV, and Finogenova, TV (2010). Biosynthesis of citric acid by *Yarrowialipolytica*repeat-batch culture on ethanol. *Appiled Microbiology Biotechnology*, 53: 525-529.

[10] Munshi, M.K., M.F. Hossain, R. Huque, M.M. Rahman, A. Khatun, M. Islam and M.I. Khalil,(2013). Effect of biomass and sugar in citric acid production by *Aspergillus niger* using molasses and jackfruit as substrates. *America Journal of Food Nutrient*, 1: 1-6

[11] Akwa, V.L., Binbol, N.L., Samaila, K.L., and Marcus, N.D. (2007). *Geographical Perspective of Nasarawa State*.Onaiv Printing and Publishing Company, Keffi. pp 3.

[12] Makut M.D. and Ifeanyi U. E. (2017). Screening for citric acid producing fungi from the soil Environment of Jos North, Plateau State, Nigeria**.***FULafia Journal of Science & Technology.* 3 (1):1-12

[13] Bari MN, Alam MZ, Suleyman AM, Jamal P and Mamun AA (2009). Improvement of production of citric acid from oil palm empty fruit bunches: Optimization of media by statistical experimental designs. *Bioresour.Technol*. 100: 3113-3120

[14] Makut, MD. andEkeleme, IK. (2018). Citric Acid Production by *Aspergillus niger* and *Trichodermaviride*Using Hydrolysed Potato Peels Substrate *Asian Journal of Advances in Agricultural Research* 5:1: 1-7

[15] Asad-ur R, Sikander A and Ikram-ul H (2002). Temperature Optima for Citric Acid Accumulation by *Aspergillus niger*.*Biotechnology, 1: 108-110*