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Citric Acid Production by *Aspergillus niger* and *Trichoderma viride* Using Hydrolysed Potato Peels Substrate

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Authors' contributions

This work was carried out in collaboration between both authors. Author MDM designed the study, performed the statistical analysis. Author IKE wrote the protocol and the first draft of the manuscript, managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aim: Production of citric acid by *Aspergillus niger* and *Trichoderma viride* isolated from soil in Keffi, Nigeria using Hydrolysed potato peels substrate.
Place and Duration of the Study: This study was carried out in the Department of Microbiology Faculty of Natural and Applied Sciences Nassarawa State University Keffi, Nigeria, between February and July 2017.
Methodology: *Aspergillus niger* and *Trichoderma viride* were isolated from soil in Keffi and identified using standard microbiology methods. Hydrolysed potato peels substrate - soy beans cake (ps) and potato peel - groundnut cake (pg) production media were prepared by following standard fermentation conditions. The citric acid produced was estimated using Gas Chromatography/Mass Spectrometry (GC/MS) method respectively.
Results: At pH 5.5 after 72 hours of incubation the highest production of citric acid was produced by *T. viride* 176.33±20.10 mg/l on ps and *A. niger* 91.11±21.22 mg/l on ps. At pH 6.0 *T. viride* produced highest on ps 211.40±7.10 mg/l and *A. niger* 170.22±10.81 mg/l on ps while at pH 6.5 *A.*

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niger produced highest 181.09±20.8 mg/l on ps and *T. viride* 98.76±19.8 mg/l on ps with highest total Sugar consumption and dry cell mass were 16.19±20.01 g/l and 13.17±1.04 g/l while after 144 hours of incubation at pH 6.0 *A. niger* produced highest citric acid 404.53 ± 14.32 mg/l on ps and *T. viride* produced 297.60±8.31mg/l on ps highest total Sugar consumption and dry cell mass were 19.91±2.01g/l and 17.0±15.11 g/l.

Conclusion: The maximum citric acid production obtained during the course of study was 404.53±14.32 mg/l on ps by *A. niger* and at pH 6.0. Also potato peels showed that it can be alternative carbon source for citric acid production as cheaper source of carbon.

Keywords: Aspergillus niger; Trichoderma viride; ps and pg substrate.

1. INTRODUCTION

Citric Acid (CA), an intermediary of the tricarboxylic acid cycle, is one of the most vital commercially valuable products due to its commonly used mainly in food industrial (70%), pharmaceuticals (12%), and others (18%) (Ates et al., 2002) [1]. The global manufacture of citric acid has improved to 1.7 million tons in 20017, as estimated by Business Communications Co. (http://www. bccresearch.com). Due to its numerous applications, the volume of citric acid production by fermentation is continually increasing at a high annual rate of 5% Finogenova et al., 2005; Francielo et al., 2008 and Helen et al., 2014) [2,3,4] and also witnessing steadily increasing demand/ consumption. The high demands in market have increased the price of CA, which is about $1.0 to $1.3 per kilogram. Meanwhile, its consumption rate is rising day by day due to its numerous applications; considering the slight increase in price, the market value for this commodity chemical will exceed $2 billion in 2019 (Makut et al., 2012) [5]. It is accepted worldwide as a GRAS (generally recognized as safe), before the application of citric acid in food industry approved by the Joint FAO/WHO Expert Committee on Food Additives Carlos et al., 2006) [6]. CA and its salts (primarily sodium and potassium) are used in many industrial applications: as a chelating agent, buffer, pH adjustment, and derivatization agent. Applications include laundry detergents, shampoos, cosmetics, enhanced oil recovery, and chemical cleaning (Varsha and Shetty) [7]. Aqueous solutions of CA are excellent buffer when partially neutralized, as citric acid is a weak acid and has three carboxylic groups; hence, three pKa's at 20°C pK1=3.15, pK2=4.77, and pK3=6.39 (Vergano et al., 1996; Ikram-ul-Haq et al., 2002) [8,9], resulting in buffering action in the pH range 2.5–6.5 Blair et al., 1991) [10]. Nowadays, there is a growing trend to increase CA production due to many advanced applications coming to light. Studies have reveal the potential use of CA in biopolymers, for drug delivery, tissue engineering for culturing a variety of cells, and many other promising biomedical applications, besides environmentally sustainable use of CA for the efficient removal of post-soldering flux residues by the military (Guillermo et al., 2010) [11]. Most of the citric acid is manufactured through biological means, mainly through submerged fermentation of starch/sucrose-based media (molasses) exclusively by the filamentous fungus *Aspergillus niger* (Lesniak et al., 2002) [12] due to its high citric acid productivity at low pH without the secretion of toxic by-products. CA is regarded as a metabolite of energy metabolism whose concentration will rise to appreciable amounts only under conditions of substantial metabolic imbalances. In recent years, various agricultural waste residues and by-products have been investigated for their potential to be used as a substrate for CA production.

Among them are molasses, fruit pomace waste, wheat bran, coffee husk, and cassava bagasse, among others which otherwise are used in composting or dumped in landfills and cause environmental hazards (Kuforiji et al., 2010) [13]. The production of value-added products like citric acid using agricultural-waste substrates will provide advantages from both the standpoint of waste material management and of lower costs for carbon substrates.

Aspergillus niger is cultivated for most of the industrial product of many valuable substances. Various strains of *A. niger* are used to produce organic acid such as citric acid and gluconic acid and have been assessed as acceptable for daily intake by the World Health Organization (Walid et al., 2007) [14].

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were randomly collected (at the top soil) from Keffi metropolis 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.

2.2 Isolation of *Aspergillus niger* and *Trichoderma viride*

The isolation of *Aspergillus niger* and *Trichoderma viride* was carried out following a method described by (Kuforiji et al., 2010) [13]. Briefly, One (1) gram of the soil sample was suspension 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 to obtain a dilution of 10^{-7}. From each of the first three test tubes, 0.5 ml of the aliquot was spread on Potato dextrose agar plates and was incubated at 35℃ for 4 days.

2.3 Identification of *Aspergillus niger* and *Trichoderma viride*

Identification of *Aspergillus niger* and *Trichoderma viride* was carried out as described by Partos [15]. 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 (Kishore et al., 2008) [16] fungi standard chart.

2.4 Screening for Citric Acid-Producing *Aspergillus niger* and *Trichoderma viride*

Screening for Citric Acid-Producing *Aspergillus niger* and *Trichoderma viride* was carried out as described by (Helen et al., 2014) [4]. The isolates were screened qualitatively for citric acid production. Potato dextrose agar plate method containing Bromocresol green as an indicator 1% at pH 6 was used. *Aspergillus niger* and *Trichoderma viride* were streaked on the plates and incubated for 48 hours. Yellow zones indicate citric acid production by the test organisms.

2.5 Preparation of Potato Peel Starch Substrates

Substrates such as potato peels were collected and sun dried and grinded into powder form using clean grinding machine and sieve. Five hundred gram (500 g) powder form was added into 5 litres of distilled water and sieved to form a homogenous mixture and placed at 4ºC for 24 hours. The settled starch was separated from liquid and dried in an oven at 60ºC, overnight. A starch solution of 20 g/l was prepared and autoclaved at 5.0 lbs (115℃) for 5 min. To liquefy starch, alpha amylase (2.0 µ/ml) was added and heated at 95℃ in a water bath for 15 min. For saccharification, amyloglucosidase (2.0 µ/ml) was added and heated at 55ºC with constant stirring for about 4 hours (Jamai et al., 2006) [17].

2.6 Preparation of Inoculum for Fermentation

Preparation of inoculum for fermentation was carried out as described by Ates et al., 2002) [1]. Five (5 ml) of peptone water buffer solution containing 0.3% sterile tween 80 and glass beads was transferred into four (4) days' slant culture of *Aspergillus niger* and *Trichoderma viride,* the slant were shake thoroughly until spores were homogenized and incubated at 35℃ for 6 hours.

2.7 Starter Culture

The starter culture was incubated as described by (Kishore et al., 2008) [13]. Seed culture, 10 ml of 6 h inoculum were inoculated into 90 ml of freshly prepared potato dextrose broth and incubated at 35℃ for 24 hours before use.

2.8 Fermentation Technique

The batch fermentation was carried out as described by (Soccol et al., 2006) [18] with modification. One hundred millimeter Medium1 (ps)] containing (g/l): 2.5 g soy bean cake, 0.4 g NH_4Cl, 0.1 g $KH_2P\ O_4$, 0.025 g $MgSO_4$, $7H_2O$] was added into Prepared 20 g potato peel hydrolysed starch and water to one liter and Medium 2 (pg)] containing 2.5 g of groundnut cake, 0.4 g NH_4Cl, 0.1 g $KH_2P\ O_4$, 0.025 g $MgSO_4$, $7H_2O$] was added into Prepared 20 g potato peel hydrolysed starch and water to one liter. 200 ml of the formulated media were transferred into separate 250 ml conical flasks. The flasks were plugged with cotton and autoclaved at 15 psi for 15 min. The sterilized flasks were inoculated with 5.0 ml of the inoculum under aseptic conditions. Sterilized ferrocyanide (200 ppm free ions concentration) was added to each flask. The flasks were placed in a shaker incubated at different temperature.

All the experiments were run parallel in duplicates.

2.9 Analytical Methods

Sugar utilization was estimated using gravimetrically by DNS method (Asad-ur et al., 2002) [19]. Photoelectric colorimeter (Model: AE-11M Erma, Japan) was used for measuring colour intensity. Dry cell mass was determined by filtering the culture medium through weighed Whatmann filter paper No. 44.

Mycelium was thoroughly washed with tap water and dried at 105℃ for two hours.

2.10 Quantification of Citric Acid

2.10.1 Estimation of citric acid

The citric acid produced during fermentation was determined using Gas Chromatography/Mass Spectrometry (GC/MS) (Akalin et al., 2002) [20] as detailed below;

2.10.2 Sample preparation

During sample preparation, 7 ml of fermented media was added to 40 ml of buffer-acetonitrile mobile phase of (0.5% (w/v) $(NH_4)_2HPO_4$ (0.038 M) - 0.4% (v/v) acetonitrile (0.049 M), at pH 2.24 with H_3PO_4), 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/MS analysis. All the samples were analysis in Duplicate.

2.10.3 GC and MS analysis

The analysis was carried out by GC & MS detector ("SHIMAZU GC-14, Gas Chromatograph) equipped with flame-ionization detector. The column used for the separation of solvent was PEG (2.1 m x 3.0 mm). 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/MS -grade reagents were 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 (192℃), Nitrogen gas (30 ml/minutes) was used as carrier gas. The temperatures of injector and detector were 150℃ and 200℃ respectively. The peaks were recorded on "SHIMADZU C-R-4_A, Chromatograph", and were 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.

3. RESULTS

In this study, *Aspergillus niger* and *Trichoderma viride* isolated from soil in Keffi, Nigeria were screened for production of citric acid using potato peel - soy beans cake (ps) and potato peel - groundnut cake (pg) hydrolysates media by submerged fermentation (Tables 1 and 2). The amount of citric acid produced by *A. niger* after 72 hours at pH5.5 was 87.14 ± 19.04 mg/l on pg and 91.11±21.22 mg/l on ps and *T. viride* produced was 105.89±18.27 mg/l on pg and 176.33±20.10 mg/l on ps (Table 1).

At pH 6.0 the ranges of citric acid produced is as follow *A. niger* produced was 143.10±18.11 mg/l on pg and 170.22±10.81 mg/l on ps while *T. viride* produced was 163.61±22.40 mg/l on pg and 211.40±7.10 mg/l on ps. At pH 6.5 *A. niger* produced was 131.11±15.2 mg/l on pg and 181.09±20.8 mg/l on ps while *T. viride* produced was 84.94±2.2 mg/l on pg and 98.76±19.8 mg/l on ps and the highest Sugar consumption and dry cell mass were 16.19±20.01 g/l and 13.17±1.04 g/l respectively (Table1).

Table 2. shows the production of citric acid after 144 hours on different pH and substrates were at pH 5.5 *A. niger* produced highest 286.43±6.1 mg/l on ps and 228.36±7.8 mg/l on pg while *T. viride* produced highest 193.76±8.6 mg/l on ps and 166.76±4.9 mg/l on pg.

The production of citric acid at pH 6.0 by *A. niger* produced 340.03±41.9 mg/l on pg and highest 404.53±14.32 mg/l on ps also *T. viride* produced highest 297.60±8.31 mg/l on ps and 273.73±27.68 mg/l on pg. The amount of citric acid obtained at pH 6.5 showed that *A. niger* produced 277.36±6.1 mg/l on pg and highest 304.63±10.1 mg/l on ps while *T. viride* produced 194.10±9.9 mg/l on pg and 206.46±7.2 mg/l on ps and the highest total Sugar consumption and dry cell mass were 19.91±2.01 g/l and 17.0±15.11 g/l respectively as given in (Table2).

Table 1. Citric acid production at 30°C after 72 hours using supplemented potato peels media

Isolates	Substrates 20 g/l	Total sugar consumed (g/l)	Total dry cell mass (g/l)	Citric acid production (mg/l)		
				pH 5.5	pH 6.0	pH 6.5
A. niger	Pg	11.79±0.08	6.1±6.01	87.14±19.04	143.10±18.11	131.11±15.2
A. niger	Ps	14.92±2.01	11.0±5.11	91.11±21.22	170.22±10.81	181.09±20.8
T. viride	Pg	12.31±1.08	9.31±4.01	105.89±18.27	163.61±22.40	84.94±2.2
T. viride	Ps	16.19±20.01	13.17±1.04	176.33±20.10	211.40±7.10	98.76±19.8

KEY: Substrates: pg = potato peels + groundnut cake; ps = peel + soybeans cake

Table 2. Citric acid production at 30°C after 144 hours using supplemented potato peels media

Isolates	Substrates 20 g/l	Total sugar consumed (g/l)	Total dry cell mass (g/l)	Citric acid production (mg/l)		
				pH 5.5	pH 6.0	pH 6.5
A. niger	Pg	18.92±4.08	12.71±22.01	228.36±7.8	340.03±41.9	277.36±6.1
A. niger	Ps	19.91±2.01	17.0±15.11	304.63±10.1	404.53±14.32	286.43±6.1
T. viride	Pg	16.91±1.08	10.31±19.01	166.76±4.9	273.73±27.68	194.10±9.9
T. viride	Ps	17.29±1.21	14.07±22.04	193.76±8.6	297.60±8.31	206.46±7.2

KEY: Substrates: pg = potato peels + groundnut cake; ps = peel + soybeans cake

4. DISCUSSION

The pH of fermentation media is one of the important parameters that have been found to have effect on production of citric acid. Table 1 and 2 shows the effect of different pH (5.5-6.5) on production of citric acid by *Asperillus niger* and *Trichoderma viride* using hydroylsed potato peel medium. Potato peel with soy beans cake was found to be the best substrate. This may be due to that potato peel - soy beans cake contained higher carbohydrate and nitrogen source which favor the production of citric acid which was in agreement with the finding of (Andleeb et al., 2007; Jamai et al., 2006) [21,17] that reported that sweet potato contained upto 80-90% carbohydrates and fermentation media that has good concentration of nitrogen which favors high production of citric acid depending on the choice of organism.

In this study it was observed that at temperature of 30°C after 72 hours the maximum production of citric acid was obtained at initial pH 6.0 with production of (211.40±7.10 mg/l) in ps, total sugar consumption was 16.19±20.01 g/l and dry cell mass was 13.17±1.04 g/l on production of citric acid by *T. viride* Further increase in incubation period at 144 hours enhanced citric acid production with production of (404.53±14.32 mg/l) total sugar consumption was 19.91±2.01 g/l and total dry cell mass was 17.0±15.11 g/l on production of citric acid by *A. niger*. Similar kind of work has also been reported by (Andleeb et al., 2007; Asad-ur et al., 2002; Francielo et al., 2008) [21,19,3] that Initial pH 6.0 was found to be best for citric acid production with the production of 28.40±1.07 g/l with sugar concentration of 150 g/l and temperature of 30°C, and this is in agreement with work reported by (Partos, 2005) [15] that it might be due to that at pH 5.5 – 6.0, the ferrocyanide ions were more suitable for mycelial growth (Partos, 2005; Walid et al., 2007) [15,14]. The fermentation medium with an initial pH 6.0 showed the maximum production citric acid. In batch-wise fermentation of citric acid, the production started after a lag phase of one day and reached maximum at the onset of stationary phase Helen et al., 2014; Soccol et al., 2006 [4,18], so the maximum production of citric acid was achieved, after 144 hours of incubation and this was in agreement with (Andleeb et al., 2007) [21] reported that longer incubation period above 144 hours may have result in the decreased citric acid production.

5. CONCLUSION

Fermentation parameter for citric acid production by *A. niger* and *T. viride* also depend on the type of process. The fermentation conditions are key to high and consistent production of citric acid. In the present study, *Aspergillus niger* and *Trichoderma viride* supported maximum

production of citric acid (297.60±8.31 mg/l) on ps after 72 hours and (404.53±14.32 mg/l) on ps after 144 hours at pH 6.0. It was also observed in this study that ps supported the maximum production of citric acid.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ates S, Dingil N, Bayraktar E, Mehmetoglu U. Enhancement of citric acid production by immobilized and freely suspended *Aspergillus niger* using silicone oil. Process Biochemistry. 2002;38:433–436.
2. Finogenova TV, Morgunov IG, Kamzolova SV, Chernyavskaya OG. Organic acid production by the yeast Yarrowia lipolytica: A review of prospects. Applied Biochemistry and Microbiology. 2005:41: 418–425.
3. Francielo V, Patricia M, Fernanda SA. Apple pomace: A versatile substrate for biotechnological applications. Critical Reviews in Biotechnology. 2008;281–12.
4. Helen Shnada Auta, Khadijat Toyin Abidoye, Hauwa Tahir, Aliyu Dabai Ibrahim, and Sesan Abiodun Aransiola Citric acid production by *Aspergillus niger* cultivated on parkia biglobosa fruit pulp. International Scholarly Research Notices. 2014;21(20):8-19.
5. Makut MD, Ade-Ibijola OB. Citric acid producing fungi found in the soil environment of Keffi Metropolis, Nassarawa State. International Resources Journal of microboiolgy. 2012;3(7):240-245.
6. Carlos RS, Vandenberghe LPS, Rodrigues C, Pandey A. New perspectives for citric acid production and application. Food Technology and Biotechnology. 2006;44(2):141–149.
7. Varsha G Shetty. Production and optimization of citric acid by *Aspergillus niger* Using molasses and corncob. International Journal of Pharmaceutical Science. 2015;7(5):152-157.
8. Vergano MG, N Femndez, MA Soria, MS Kerber. Influence of inoculum oreparation on citric acid production by *Aspergillus niger*. J. Biotechnol. 1996; 12(6):655-656.
9. Ikram-ul-Haq S. Ali MA. Qadeer, J. Iqbal. Citric acid fermentation by mutant strain of *Aspergillus niger* GCMC-7using molasses based medium. Elect. Journal Biotechnology. 2002;5:125-132.
10. Blair G, Staal P. Haarmann & Reimer Corporation. Citric acid. In J. I. Kroschwitz & M. Howe-Grant (Eds.), Encyclopedia of chemical technology. 1991;354–380. New York: Wiley.
11. Guillermo A, Jian Y, Ryan H. New biodegradable biocompatible citric acid nano polymers for cell culture growth & implantation engineered by Northwestern University Scientists. Nano patents and innovations, US Patent Application 20090325859; 2010.
12. Lesniak W, Pietkiewicz J, Podgorski W.. Citric acid fermentation from starch and dextrose syrups by a trace metal resistant mutant of *Aspergillus niger*. Biotechnological Letters. 2002;24;1065–1067.
13. Kuforiji OO, Kuboye AO, Odunfa SA. Orange and pineapple wastes as potential substrates for citric acid production. International Journal of Plant Biology. 2010;1:e4.
14. Walid A., Khaled N, Ghanem Ehab R, Helow EL. Citric acid production by novel from *Aspergillus niger* isolate induced mutagenesis and cost reduction studies: Journal of Chemical Technology and Biotechnology. 2007;98:3464-3469.
15. Partos L. ADM closes citric acid plant as Chinese competition bites. Food Navigator; 2005.
    Available:http://www.foodnavigator-usa.com/news/ng.asp?id=62537-adm-citric-acid-acidulant
16. Kishore Kumar Ganne, Venkata Ratna Ravi Kumar Dasari, Hanumantha Rao Garapati. Production of citric acid by *Aspergillus niger* MTCC 282 in submerged fermentation using *Colocassia antiquorum*. Research Journal of Microbiology. 2008;3:150-156.
17. Jamai L, Ettayebi K, El Yamani J, Ettayebi M. Production of ethanol from starch by free and immobilized Candida tropicalis in the presence of alpha-amylase. Bioresources Technolongy. 2006;98(14): 2765-70.

18. Soccol CR, Vandenberghe LPS, Rodrigues C, Pandey A. New prospectives for citric acid production and application. Food Technology and Biotechnology. 2006;44:141–149.
19. Asad-ur- Rehman, Sikander Ali, Ikram-ul-Haq. Temperature optima for citric acid accumulation by *Aspergillus niger*. Biotechnology. 2002;1:108-110.
20. Akalin AS, Gonc S, Akbas Y. Variation in organic acids content during ripening of pickled white cheese. Journal of Dairy Sciences. 2002;85:1670-76.
21. Andleeb Anwar Sardar, Sikander Ali, Samina Anwar, Ikram-ul-Haq citric acid fermentation of hydrolysed sweet potato starch by *Aspergillus niger* Pak. J. Biotechnol. 2007;4(1-2):119-125.

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