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Research Article
Production of Citric Acid from Corn Stalk through Submerged Fermentation Using *Aspergillus niger*

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Abstract

Background and Objective: Many agro wastes have been explored for the production of citric acid. However, information on the use of corn stalk is scarcely available, hence, there is need to explore the use of corn stalk in the production of citric acid. This study is aimed at producing citric acid from corn stalk by submerged fermentation using *Aspergillus niger* isolated from the soil. **Materials and Methods:** Dried and pulverized corn stalk and *A. niger* obtained from soil samples were used in this study. The effects of carbon source, nitrogen source, pH, temperature, incubation time and methanol on citric acid production were studied. **Results:** Eight fungal isolates were characterized and isolate Asp 2 had the best citric acid production efficacy. Corn stalk powder mixed with 50% sucrose and 1 g of ammonium nitrate gave the highest yield of citric acid when compared to the other carbon and nitrogen sources. More so, pH 5, temperature of 30°C and 7th day of fermentation offered the highest yield of citric acid. Furthermore, a higher yield was obtained when 3% (v/v) methanol was added to the medium. **Conclusion:** Observation in this study deduced that corn stalk is a suitable and inexpensive substrate for the production of citric acid and could serve as value added agro wastes in the production of citric acid.

Key words: Agro wastes, citric acid, submerged fermentation, corn stalk, *Aspergillus niger*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Agricultural waste is an abundant, inexpensive and renewable material for the production and sustainability of value added organic acid such as citric acid. The increased demand of citric acid by industries coupled with the search for an efficient raw material from agricultural waste residue for its production is of great concern[1]. Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is a tribasic organic acid commonly found in some fruit juices, animal bones, muscles and blood and its chemical structure[2] is shown in Fig. 1. It has many applications in food, pharmaceutical and cosmetic industries as an acidulant, flavour enhancer, preservative, antioxidant, emulsifier and chelating agent[2]. Due to its high demand by pharmaceutical, chemical, food, beverage and cosmetic industries, it is mass produced worldwide at the rate of 1,000,000 Mt per annum[3].

Citric acid (CA) can be produced through chemical processes, but microbiological processes using *A. niger* are still preferred to commercially produced citric acid[4]. Although many micro-organisms have been assessed for citric acid production[4,5], however, the fungus *A. niger* is more preferable due to the ease in handling, ability to utilise a variety of substrate and produce large amount of citric acid[6-9].

Significant attention has been focused on the proficient use of waste and its management. A number of value-addition to agricultural waste has been evaluated for this purpose with the aim of reducing environmental littering, pollution control and upholding the concept of recycling[1]. Common agricultural waste residues such as cassava, potato residue, banana peel, bagasse, pineapple waste, apple pomace, soybean, wheat bran, kiwi fruit peel, corncobs, sugar cane have been exploited as raw material for the synthesis of citric acid. Using inexpensive and cheap raw materials from agricultural waste residues as carbon source for citric acid production has provided sustenance in the industrial sector to encourage waste management, cost effectiveness, reduction in expenses and efficient yield and output[10-13].

Corn is the 3rd most important cereal in the world, next to rice and wheat and with highest production potential among the cereals[14]. It is the most heavily cultivated cereal crop globally and one of the main cereals crops of west Africa and the most important cereal food in Nigeria[15]. Every part of the maize plant has economic value: the grain, leaves, stalk, tassel and cob can all be used to produce a large variety of food and non-food products[16]. Currently, the disposal of corn stalks poses considerable economic and environmental problems, hence, there is need for its efficient utilization. This began the quest for its use in citric acid production using submerged fermentation technique. The study was aimed at the screening of the fungal isolates to ascertain the best citric acid producers and to study the effect of various fermentation parameters on the production condition.

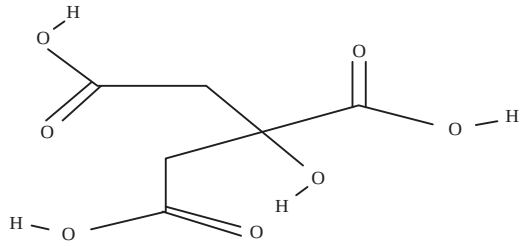

Fig. 1: Chemical structure of citric acid
Source: pubchem.ncbi.nlm.nih.gov

MATERIALS AND METHODS

The study work was carried out at the National Research Institute for Chemical Technology (NARICT), Zaria, Kaduna state, Nigeria between 3/10/2017 to 16/11/2017.

Pre-treatment of corn stalk: The plant material (corn stalks) was obtained from NARICT farm in Zaria, Kaduna state, Nigeria on the 02/10/2017. They were oven-dried at 60°C for 2 h and ground into powder.

Proximate analysis: Proximate analysis was carried out following the method of Association of Official Analytical Chemists (AOAC)[17]. The total carbohydrate was determined by differential method i.e., by subtracting the total protein, lipid, moisture and ash content from 100. Thus:

$$\text{Carbohydrate (\%)} = (100-(\text{moisture (\%)}+\text{ash (\%)}+\text{fat (\%)}+\text{protein (\%)}+\text{fibre (\%)}))$$

Isolation of microorganism: *Aspergillus niger* was isolated from soil samples collected from different locations by serial dilution method. The dilutions were plated on Potato Dextrose Agar (PDA) containing streptomycin and incubated at 37°C for 5 days using the spread plate technique[18]. Fungal growth suspected to be *A. niger* based on macroscopic observation (carbon black or dark brown conidia) were further sub-cultured on fresh PDA plates containing streptomycin. Those that showed characteristic *A. niger* were subjected to microscopic observation according to procedures described by Barnett and Hunter[19]. For the microscopic identification, a drop of lactophenol blue was placed on a clean slide. A loopful of the fungal growth was removed and placed in the drop of lactophenol blue. It was covered with a cover slip and observed under the microscope using x10 and x40 objective lens. The isolates were stored in PDA slants at 4°C.

Inoculum preparation: Spore suspensions of the isolates were prepared by adding 10 mL of sterilized distilled water containing 2 drops of 0.1% tween 80 to the sporulated 5 days old culture. A sterile wire-loop was used to dislodge the spore clusters under sterile conditions and then mixed thoroughly to prepare a uniform spore suspension. The number of spores was counted using a Neubauer's counting chamber as described by Grigoryev[20].

Screening of the fungal cultures for citric acid production: The *A. niger* cultures were screened qualitatively for the production of citric acid using methods described by Ali[21]. Sterile Czapek dox agar medium (20 mL) incorporated with Bromo-cresol green dye and streptomycin to prevent the growth of bacteria was poured into individual sterile Petri plates and allowed to cool at room temperature. The Czapek dox agar contains (g L^{-1}): Sucrose 120 g, $NaNO_3$ 5 g, KH_2PO_4 2 g, $MgSO_4.7H_2O$ 1 g, $CuSO_4.7H_2O$ 0.02 g, $FeSO_4.7H_2O$ 1 g, $ZnSO_4.7H_2O$ 1 g, Agar 15 g and Distilled water 1000 mL. The pH of the medium was adjusted to 6.5. Approximately 1 mL of the spore suspension of *A. niger* was transferred to each of the Petri plates. The plates were incubated at 28±2°C for 7 days. The isolate with the widest yellow zone were used for further studies[22].

Citric acid production by submerged fermentation: Thirty grams of powdered corn stalk was dispensed into an Erlenmeyer flask (500 mL) and 300 mL of Czapek dox medium was dispensed into the flask. The medium was autoclaved at 121°C for 15 min. After cooling to room temperature, the flasks were inoculated with 1 mL of fungal spore suspension, following incubation at 28±2°C for 7 days.

Citric acid determination: Citric acid (CA) was determined titrimetrically using methods described by Khosravi-Darani and Zoghi[23]. In this procedure, 1 mL of the culture filtrate was taken into a flask and then 2 drops of phenolphthalein indicator was added and titrated with 0.1 N NaOH. The end point was noted when the filtrate changed from colourless to pink colour. The CA yield was then calculated using the formula:

$$\text{Citric acid (g L}^{-1}) = \frac{192.13 \times M \times V}{\text{Weight of sample}}$$

Where:

192.13 g mol^{-1} = Molar mass of citric acid
M = Molarity of NaOH
V = Volume of NaOH used during titration

Effect of carbon source: Thirty grams of each carbon source (sucrose, glucose and corn stalk powder) was dispensed into 300 mL of Czapek dox medium, autoclaved, cooled and inoculated with 1 mL spore suspension. The flasks were incubated at room temperature on a rotary shaker at 450 rpm for 7 days. After incubation, the fungal mycelia were filtered using Whatman filter paper, dried in an incubator and the weight of the fungal biomass was recorded[23].

Effect of nitrogen source: Thirty grams of corn stalk powder was dispensed into 300 mL of Czapek dox broth incorporated with different nitrogen sources (ammonium nitrate, peptone, yeast extract) at different concentrations ranging from 0.5-2 g. The pH of the medium was adjusted to 6.5. The flasks were autoclaved and cooled before inoculating with 1 mL of spore suspension. The flasks were incubated at room temperature on a rotary shaker at 450 rpm for 7 days.

Effect of pH: Thirty grams of corn stalk powder was dispensed into 300 mL of Czapek dox broth. The pH of the medium was varied at different concentrations (4, 5, 6, 7, 8 and 9). An aliquot of *A. niger* spore suspension (1 mL) was inoculated into the flasks containing each pH concentration. The flasks were incubated at room temperature on a rotary shaker at 450 rpm for 7 days.

Effect of temperature: Thirty grams of corn stalk powder was dispensed into 300 mL of Czapek dox broth, autoclaved and cooled. Then 1 mL of spore suspension was inoculated into each flask and incubated at different temperatures ranging from 28, 30, 32, 35 and 37°C on a rotary shaker at 450 rpm for 7 days.

Effect of incubation time on citric acid production: Thirty grams of corn stalk powder was dispensed into 300 mL of Czapek dox broth, autoclaved and cooled. The pH of the media was adjusted to 6.5. Then 1 mL of spore suspension was inoculated into the flask. The flask was incubated on a rotary shaker at 450 rpm for 4-9 days.

Effect of methanol on citric acid production: The effect of different concentration of methanol was determined using concentrations ranging from 1-4% v/v. Exactly 1 mL of the prepared inoculum suspension was added into 300 mL of Czapek dox medium containing 30 g of corn stalk powder and incubated for 7 days at 28±2°C.

Determination of residual sugar and mycelial weight: Exactly 300 mL of Czapek dox broth containing 30 g of corn stalk powder were prepared in an Erlenmeyer flask (500 mL) and sterilized. The pH of the media was adjusted to 5. Then 1 mL of spore suspension was inoculated into the flask. The flask was incubated on a rotary shaker at 30°C for 7 days. The quantity of reducing sugar was determined by 3, 5-Dinitrosalicylic acid (DNS) method using sucrose as standard[24]. The absorbance was measured using HACH DR2400 portable spectrophotometer at 546 nm. The dry mycelial weight was determined by methods described by Haq *et al.*[13]. The mycelial mat was kept in an oven overnight at 70°C to determine the dry weight.

Statistical analysis: Data obtained in this study were expressed as the mean±standard deviation of triplicates (n = 3). Statistical analysis was performed using one way analysis of variance (ANOVA) (SPSS version 20.0). The means were compared using Duncan's multiple test range (MRT) and $p<0.05$ were considered statistically significant.

RESULTS

The proximate composition of the corn stalk powder is shown in Table 1. The results showed that corn stalk has a low quantity of fat (1.37±0.05%) and a high percentage of fibre (50.86±0.01%). The corn stalk has a high percentage of carbohydrate (38.42±0.00%). The percentage crude proteins and moisture contents were 4.46±0.02 and 3.07±0.02%, respectively. However, the ash content recorded was 1.82±0.02%.

Isolation and screening of fungi for citric acid production: Observation of the morphological characteristics of the fungal isolates is shown in Table 2. The growth of *A. niger* on PDA plates revealed the presence of white, brown and black colonies that were either irregular, circular or flat in shape. The elevation of the isolates were either raised or flat while the margins were entire or rhizoid.

Effect of carbon source on the production of citric acid: The effect of carbon source on the production of citric acid is shown in Table 3. The medium containing corn stalk powder and 50% sucrose produced the highest yield of citric acid (11.68±0.02 g L^{-1}) when compared with the medium containing only sucrose (6.20±0.35), glucose (5.52±0.03) or corn stalk powder (3.76±0.26) (Table 3). Also, a higher mycelial weight (10.24±0.04 g L^{-1}) was recorded in the medium containing corn stalk powder and 50% sucrose. The mycelial weight in the medium containing cornstalk powder, sucrose and glucose were 2.83±0.03, 8.98±0.02 and 8.99±0.08 g L^{-1}, respectively.

Table 1: Proximate composition of corn stalk powder

Parameters	Composition (%)
Moisture content	3.07±0.02
Carbohydrate	38.42±0.00
Crude protein	4.46±0.02
Fat content	1.37±0.05
Ash content	1.82±0.02
Crude fibre	50.86±0.01

Values are expressed as Mean±SD

Table 2: Morphological characteristics of fungal isolates

Isolates	Size	Shape	Pigment	Elevation	Margin
Asp 1	Large	Flat	Black and powdery	Raised	Entire
Asp 2	Large	Irregular	Brown and powdery	Raised	Entire
Asp 3	Moderate	Irregular	White	Flat	Rhizoid
Asp 4	Large	Circular	Brown and powdery	Raised	Rhizoid
Asp 5	Large	Circular	Brown and powdery	Flat	Entire
Asp 6	Moderate	Irregular	White	Flat	Rhizoid
Asp 7	Moderate	Irregular	White	Flat	Rhizoid
Asp 8	Moderate	Flat	White	Raised	Rhizoid

Asp: Isolate code

Table 3: Effect of carbon source on the production of citric acid

Carbon sources	Mycelial weight (g L^{-1})	Yield (g L^{-1})
Corn stalk powder (control)	2.83±0.03	3.76±0.26
Sucrose	8.98±0.02	6.20±0.35
Glucose	8.99±0.08	5.52±0.03
Corn stalk powder+50% sucrose	10.24±0.04	11.68±0.02

Values are expressed as Mean±SD

Effect of nitrogen sources on citric acid production: Effect of nitrogen sources on citric acid production by *A. niger* is shown in Fig. 2. There was a statistically significant ($p<0.05$) increase in citric acid production (11.20±0.02^{b} g L^{-1}) when the medium was supplemented with 1 g of ammonium nitrate. This increase differed significantly ($p<0.05$) when compared with the citric acid yield in the medium containing 0.5, 1.5 and 2.0 g of ammonium nitrate. Supplementing the medium with 0.5, 1.0, 1.5 and 2.0 g of peptone gave a citric acid yield of 4.02±0.03^{a}, 4.50±0.20^{b}, 6.10±0.21^{c} and 5.28±0.21^{d} g L^{-1}, respectively. The lowest yield of citric acid was recorded when the medium was supplemented with 0.5 g of yeast extract and this was significantly different when compared with 1.0, 1.5 and 2.0 g.

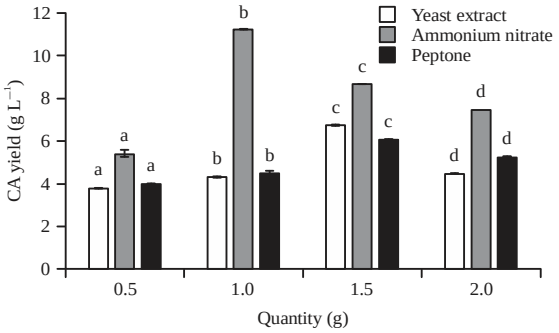

Fig. 2: Effect of nitrogen source on citric acid production
Bars of each nitrogen source with different letter superscript differ significantly (p<0.05)

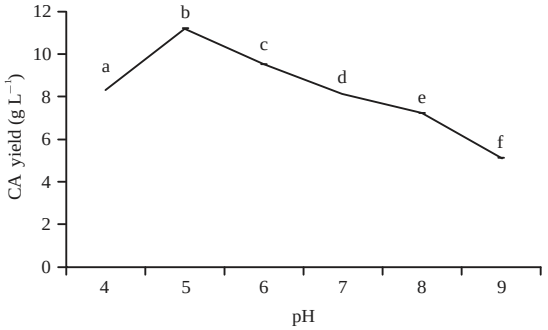

Fig. 3: Effect of pH on citric acid production
Means with different letter superscript differ significantly (p<0.05)

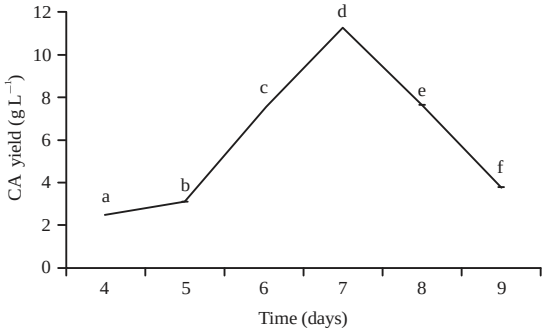

Fig. 4: Effect of Incubation time on citric acid production
Means with different letter superscript differ significantly (p<0.05)

Effect of pH on citric acid production: The maintenance of a favorable pH is a very essential factor for the successful production of citric acid. Figure 3 shows the impact of pH on citric acid yield. The pH 5.0 gave a statistically significant ($p<0.05$) increase in citric acid yield (11.16 ± 0.04^{b} g L^{-1}). This was followed by pH 6 (9.5 ± 0.01^{c} g L^{-1}), pH 4 (8.31 ± 0.00^{a} g L^{-1}), pH 7 (8.08 ± 0.01^{d} g L^{-1}), pH 8 (7.23 ± 0.01^{e} g L^{-1}) and 9 (5.11 ± 0.02^{f} g L^{-1}). The increase in pH led to a decrease in citric acid yield (Fig. 3).

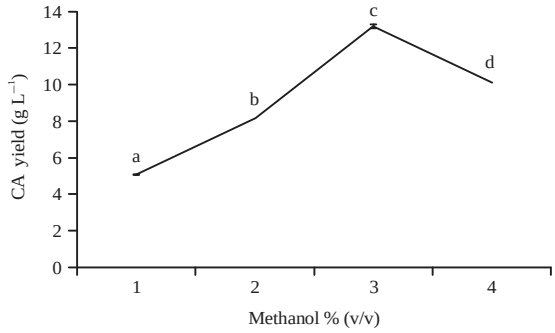

Fig. 5: Effect of methanol on citric acid production
Means with different letter superscript differ significantly (p<0.05)

Table 4: Effect of temperature on citric acid production using corn stalk powder

Temperature (°C)	Yield (g L^{-1})
28	8.11 ± 0.10^{a}
30	11.32 ± 0.28^{b}
32	9.26 ± 0.02^{c}
35	6.40 ± 0.10^{d}
37	5.41 ± 0.09^{e}

Values are expressed as Mean±SD, Means with different letter superscript differ significantly (p<0.05)

Effect of temperature on citric acid production: The temperature of a fermentation medium is a critical factor that has a profound effect on the production of citric acid. In this study, there was a significant increase ($p<0.05$) in citric acid yield at a temperature of 30°C (11.32 ± 0.28^{b} g L^{-1}) (Table 4). Increase in temperature led to a significant decrease in citric acid yield with 37°C giving the lowest yield (5.41 ± 0.09^{e} g L^{-1}) (Table 4).

Effect of incubation time on citric acid production: Figure 4 illustrates the impact of fermentation period on citric acid yield after 9 days of incubation at 28±2°C. At day 0, citric acid was not produced, but an increase in the fermentation period to 4 days significantly ($p<0.05$) enhanced citric acid synthesis to 2.49 ± 0.00^{a} g L^{-1}. The 7th day of fermentation gave a significantly ($p<0.05$) high yield of citric acid (11.24 ± 0.00^{d} g L^{-1}) after which production declined to 3.81 ± 0.0^{f} g L^{-1} on day 9 (Fig. 4).

Effect of methanol on citric acid production: The effect of different concentrations of methanol on the production of citric acid (Fig. 5) showed that the addition of 3% (v/v) methanol gave a statistically significant ($p<0.05$) high yield of citric acid (13.20 ± 0.1^{c} g L^{-1}). There was a significant reduction in citric acid yield (10.13 ± 0.00^{d} g L^{-1}) when 4% (v/v) methanol was added to the medium (Fig. 5).

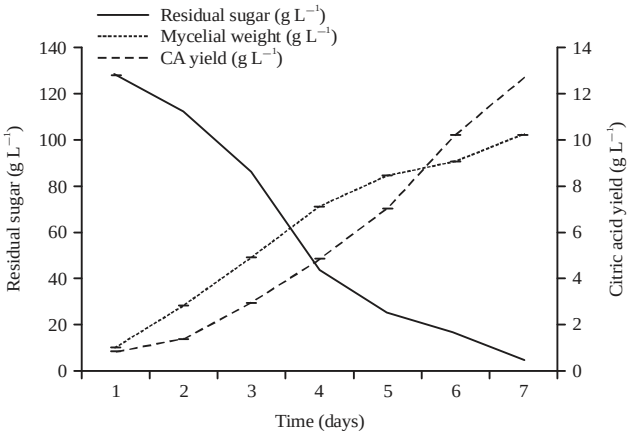

Fig. 6: Effect of residual sugars and mycelial weight on citric acid yield

Means with different letter superscript on each parameter differ significantly ($p<0.05$)

Effect of citric acid yield on residual sugar and mycelial weight: Effect of citric acid yield on residual sugar and mycelial weight is shown in Fig. 6. There was a statistically significant ($p<0.05$) decrease in the residual sugar concentration. The least concentration was recorded on day 7 (5.06 ± 0.02^{g} g L^{-1}) while the highest concentration was obtained on day 1 (128.42 ± 0.01^{a} g L^{-1}). Mycelial weight increased significantly ($p<0.05$) with the highest value obtained on day 7 (10.24 ± 0.01^{g} g L^{-1}) of fermentation while the lowest weight was obtained on day 1 (1.05 ± 0.01^{a} g L^{-1}). Day 1 of fermentation gave the lowest yield of citric acid (0.85 ± 0.02^{a}) which statistically differed ($p<0.05$) from the highest yield recorded on day 7 (12.67 ± 0.01^{g} g L^{-1}).

DISCUSSION

The proximate analysis of the corn stalk provides information on its nutritive value as a suitable substrate for the growth of *A. niger.* The high percentage carbohydrate composition revealed in this study implied that the corn stalk could provide in part nutrients needed for the growth of the organism. The low moisture and ash contents revealed in this study conform to previous observations that corn stalk contains low moisture, protein and ash contents[25-27].

The isolation of *A. niger* from soil revealed the ubiquitous nature of the organisms as they are universally found where environmental conditions are conducive especially in the soil[28]. The cultural and morphological characterisation of *A. niger* agreed with the findings of Verweji and Brandt[24] which describes colonies of *A. niger* as carbon black with a dark globular conidial head.

The addition of sucrose to corn stalk powder enhanced citric acid production than glucose. Shankar and Sivakumar[29] obtained a maximum citric acid yield of 319.0 g L^{-1} when the substrate was supplemented with sucrose. Similarly, maximum citric acid was produced from whey with 15% Sucrose[30]. It was suggested that the mycelial growth produces an enzyme which breaks the sucrose, producing energy exactly at the level in which the increase in citric acid production was observed[31].

Any increase or decrease other than 1.0 g of ammonium nitrate resulted in the disturbance of citric acid production. This report agreed with previous reports[32,33] which stated that fermentation media for citric acid biosynthesis should consist of substrates necessary for the growth of micro-organism primarily the carbon, nitrogen and phosphorus sources.

In this study, it was discovered that a decrease in pH below pH 5 caused a reduction in citric acid production. This may be due to the fact that ferrocyanide ions were toxic to the fungal mycelia at low pH[34]. Fungal strains seem to thrive best in acidic medium ranging[8,35] from 3-6. The pH range of 2-6 is frequently utilised for submerged and solid state fermentation[36,37].

The optimum temperature for high citric acid yield was 30°C. According to Munshi *et al.*[33], incubation temperature should be in the range of 28-32°C. Kishore *et al.*[38] also reported that maximum citric acid production was recorded at 30°C using *Aspergillus niger* NCIM 705. When the temperature of a medium is increased above 30°C, the biosynthesis of citric acid is decreased. This might be due to the accumulation of by-products such as oxalic acid[39].

The optimum incubation period for maximum citric acid yield was day 7 (168 h). Usually, the production begins to increase after a lag phase of approximately 2-3 days and reaches maximum at the stationery phase[40]. This observation corroborated with Shankar and Sivakumar[29] in which high citric acid yield occurred after 7 days of fermentation but differs with the findings of Alvarez-Vasquez *et al.*[10] and Nadeem *et al.*[41] in which high citric acid yield occurred after 192 h of fermentation.

An increase in methanol concentration caused a decrease in citric acid production. This finding accord with that of Ganne *et al.*[42], who reported that the addition of methanol significantly increased citric acid yield. In contrast, Kareem *et al.*[43] reported that addition of 2% (v/v) methanol to the medium effectively increased citric acid production by *B. subtilis* in solid state fermentation. The decrease in CA yield may be due to low tolerance to higher concentrations of methanol[43].

The fungal biomass increased steadily with a decrease in the residual sugar concentration thus showing the ability of the fungi to utilize the sugars in the production of citric acid. This agreed with the findings of Dashen *et al.*[2] as it revealed a steady increase in mycelial weight and CA yield with a proportional decrease in residual sugars.

Further studies on manipulating the genes of micro-organisms for efficient citric acid production and utilisation of other substrates that do not compete with the global supply of foods and feeds should be considered.

CONCLUSION

The result of this study concluded that *A. niger* showed a high potential of producing citric acid using corn stalk as a substrate. Due to its mass production and currently its position as waste, corn stalk could serve as a cheaper source for the production of citric acid. The efficient utilization of low-cost carbon sources (corn stalk) will bring significant economic benefits in CA fermentation industry.

SIGNIFICANCE STATEMENT

At the moment in Nigeria, little effort is being made to produce citric acid using micro-organisms. The best promising solution considering cost effectiveness may be the utilization of the indigenous micro-organisms and cheaper agricultural wastes (such as corn stalk) as substrate for the production of this valuable organic acid.

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