EFFECT OF GROUNDNUT CAKE AND SOYABEANS ON CITRIC ACID PRODUCTION BY *Aspergillus niger*

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Abstract: Citric acid (CA) is a valuable organic acid used in many pharmaceutical and industrial food products. The increased demand for its use has led to search for yielding fermentable strains of microorganisms to meet up with the demand. This study evaluated the potentials of groundnut cake and soyabeans as nitrogen supplements on production of CA with pawpaw peels (PP) and orange peels (OP) substrates using *Aspergillus niger*. Isolation of fungal strain from spoilt fruit sample was done with Potato Dextrose Agar. They were screened for CA production on Czapek-Dox Agar (CZA) and identified morphologically. Each substrate was oven dried at 60ºC for 2 h and analyzed for their proximate composition. Glucose and sucrose at 1, 3 and 5% concentrations were added to each of the substrates and inoculated with the isolate. The effect of groundnut cake and soybean flour was studied at 1, 1.5 and 2% in combination with the highest CA producing carbon source. Methanol was further added to the best carbon and nitrogen source to formulate the solid state fermentation (SSF) medium for CA production. Addition of 5% sucrose proved better than 5% glucose on each substrate with a yield of 73.17 g/kg of CA on PP and 41.27 g/kg on OP after fermentation. Similarly, addition of 2% groundnut cake to the media, gave a higher yield (104.36 g/kg) on PP than OP in the presence of 2% methanol. SSF media of PP containing 5% sucrose and 2% groundnut cake proved better for CA production when optimized with methanol.

Keywords: Glucose, groundnut cake, methanol, orange peel, pawpaw peel, sucrose

Introduction

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) was first produced in the 19th century using lemon as raw material subsequently many researchers have employed various raw materials for the production of citric acid (Ajiboye *et al*., 2015). Various microorganisms are being utilized for the synthesis of citric acid using solid state fermentation but the ability to synthesize high and better yield of citric acid from a variety of cheap raw material have been attributed to *Aspergillus niger* due to its ease of handling (Dutta *et al*., 2019). Therefore, it is superior to other microorganisms as an important citric acid factory.

Aspergillus niger is commonly found in warmer climates as bioaerosols and moulds in stored foods, thus isolated in decaying vegetation, soil, or plants. It is also known to be responsible for post-harvest decay of fresh fruits, which are utilized as substrates. These inexpensive agro-industrial by-products such as molasses, carob pod extract, rape seed oil, apple and grape pomace, kiwi-fruit peel and mandarine orange ultimately serve as carbon sources for the production of citric acid commercially by *A. niger* (Shetty *et al*., 2015).

Carica papaya L. used in this study as a carbon source is an herbaceous plant that grows in tropical and subtropical countries. At the early stage of its fruit development, glucose is the main sugar but the sucrose content increases during ripening and can reach up to 80% of the total sugars (Martial Didier *et al*., 2017). The fruit pulp as well as the peel and seed has good nutritional content as reported by Chukwuka *et al*. (2013). Due to the high carbohydrate content of the peel, it can be employed for citric acid production using microorganisms that would naturally ferment it most especially, fungi. However, according to Kareem *et al.* (2010), *A. niger* has been proven to be the best choice for citric acid production for its ability to ferment a variety of raw agro materials and give a high yield of citric acid.

Orange peels also contain soluble sugars and fibers as its main components as reported by Adewole *et al*. (2014) on the analysis of the proximate and phytochemical constituent of orange peels.

The concentration of carbon source is crucial for citric acid fermentation due to the fact that final yield of citric acid increases with initial sugar concentration in batch processes (Roukas, 2010). However, high nitrogen concentration increases the growth of fungi and the consumption of sugars but limits the production of citric acid (Auta *et al.*, 2014).

Owing to the need to optimize citric acid production with the use of less expensive substrates this study aimed to produce citric acid using *A. niger* from pawpaw peels and orange peels fortified with different concentrations of sucrose, glucose, nitrogen methanol in a solid state fermentation process.

Materials and Methods

Fungal isolation and screening

The fungi used for the study was derived from decayed fruits. Spoilt golden melon, pawpaw and oranges were collected from a fruit vendor. The spoilt parts of the fruits were removed with sterile knife and placed on Potato Dextrose Agar plates. Plates were incubated at 25ºC between 12 and 72 h. The plates were observed for the appearance of *A. niger* in mixed cultures. Axenic cultures of fungal isolates were obtained by sub culturing and screened for citric acid production

The fungal isolates were screened according to the method described by Kareem (2010). Using about 10 ml of cooled and solidified Czapek-Dox agar medium in sterile petri plates. The isolates were inoculated and incubated at 30ºC for 72 h. Citric acid production was detected by yellow colour zones around the mycelia growth. The zones of colouration were measured with rulers by taking two perpendicular measurement of diameter from the point of inoculation.

Identification of fungal strain for citric acid production

The identification of fungal isolates were carried out according to the method of Sukesh *et al*. (2013) and Ajiboye *et al.* (2015). Fungal isolates already screened for the production of citric acid were identified with morphological characteristics of the isolates based on colour and growth pattern on PDA and Sabouraud agar (SDA). Microscopic examination of the fungal isolates were carried out by preparing micro-slides to obtain detailed morphological features. A small portion of the fungal growth was gently removed from midway between the colony centre and edge with two dissecting needles and examined microscopically following the method of Alexopoulos *et al*. (1996).

Proximate analysis

Proximate analysis was carried out according to the method of Abdulrazak *et al*. (2014) and Adewole *et al*. (2014), moisture

content, protein, lipid, ash and fiber content were determined using standard methods (AOAC, 1995). Moisture content was determined by heating two grams of each sample to a constant weight in a crucible placed in an oven maintained at 103ºC for 16 h. The drying was repeated until a constant weight was obtained. The dry matter was used in the determination of the other parameters. Crude protein was estimated by the Kjeldahl method, using five gram of the dried sample. Crude fat was obtained by exhaustively extracting five grams of the sample in a Soxhlet apparatus using petroleum ether (boiling point range 40-600 C). The weight of the lipid obtained after evaporating off the petroleum ether from the extract gave the weight of the crude fat in the sample. Ash was determined by incinerating six grams in a muffle furnace and ashed at 550ºC for eight hours. Crude fiber was obtained by digesting four gram of each sample already extracted with petroleum ether and air dried with H_2SO_4 and NaOH. The residue was incinerated in a muffle furnace and maintained at 550ºC for five hours. Total carbohydrate content was determined by difference obtained after subtracting the values of organic protein, lipid, ash and fibre from the total dry weight.

Production of citric acid

The fermentation medium was prepared by adding 5 g of each substrate into two sterile 250 ml labelled Erlenmeyer conical flasks, moistened to 70% moisture content and incubated at 28ºC for 5 days. The effect of sucrose and glucose (1 – 5 g) and nitrogen sources (1 – 2 g) was studied on each substrate (Balogun-Abiola and Kareem, 2017).

The flask was cotton plugged and autoclaved at 121ºC for 15 min. After cooling at room temperature, the medium was inoculated with 0.2 ml of the *Aspergillus niger* suspension and incubated at ±28ºC in a rotary shaker incubator for 5 days. After fermentation, the solid substrates were weighed, diluted and assayed for citric acid content (Kareem and Rahman, 2013).

Effect of alcohol as stimulant on citric acid production

The effect of methanol and ethanol at 0.1 – 5% w/v on citric acid fermentation was varied and observed. The formulated media containing sucrose, glucose and the nitrogen sources were inoculated with 0.2 mls of the *Aspergillus niger* suspension and incubated at ±28ºC in a rotary shaker for 5 days to determine the effect of varying alcohols (Balogun-Abiola *et al*., 2017).

Citric acid determination by titration

The crude citric acid recovered from each of the media were used as the crude citric acid source for citric acid determination using the modified method of Ajiboye *et al*. (2015). Sodium Hydroxide solution (0.1M) was titrated against 10 ml of the diluted filtrate in a 250 ml Erlenmeyer conical flask using two drops of phenolphthalein as indicator.

$$\%CA = \frac{normality\ x\ volume\ of\ NaOH\ x\ Equiv.wt.of\ CA\ x\ dilution\ factor}{weigth\ of\ sample\ x\ 10}$$

Results and Discussion

Different widths of yellow colouration zones were formed around the mycelia of the four fungal isolates, namely; *A. fumigatus*, *A. flavus*, *Rhizopus sp.* and *Aspergillus niger*. The isolate with the highest zone of colouration was selected for citric acid production (Fig. 1). Similar screening has been reported earlier by Ajiboye *et al.* (2015) and Dutta *et al*. (2019) which showed the ability of other organisms apart from *A. niger* to produce citric acid.

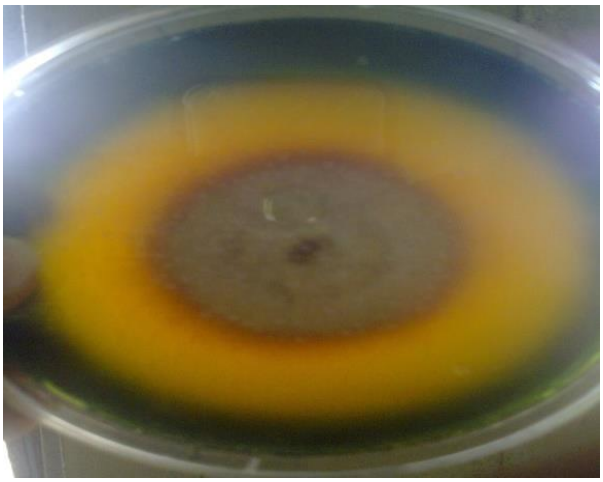

Fig. 1: Plate showing screening of the citric acid producing isolate on Czapek-dox agar

Table 1: Screening of fungal isolates for citric acid production

Isolate	Zones of inhibition (mm) in Czapeck-dox agar			
	12 hrs	24 hrs	36 hrs	72 hrs
A. niger	0.00±0.00^{a}	11.50±0.29^{c}	24.67±1.20^{d}	39.00±0.58^{d}
A. flavus	6.67±0.88^{b}	10.83±0.60^{bc}	20.83±0.60^{c}	27.33±1.43^{c}
Candida spp	0.00±0.00^{a}	9.83±0.44^{b}	14.50±0.76^{b}	21.00±0.58^{b}
A. fumigatus	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Values with the same letter along the column are not significantly different at P≤0.05; Mean of triplicate determinations

When screened in Czapeck dox agar (CZA) with bromocresol green only *A .flavus* produced citric acid at 12 h. However,*A.fumigatus* did not produce citric acid even at 72 h. *A. niger* produced the peak of citric acid at 72 h (Table 1). The choice of *A. niger* used throughout the study was determined by the width of colouration zones on Czapek dox agar medium which depicts *A. niger* as the highest citric acid producer.

In this study, *Aspergillus niger* was isolated and observed as black powdery spores with yellow edges (Table 2). Similar morphological features were observed by Gonu *et al*. (2015) who described *A. niger* as an initially yellow colony with dotted black conidia which gradually became jet black and powdery on potato dextrose agar (PDA). The morphological characteristics of each isolate such as the colour, texture and edges were unique. Microscopically, *A. niger* was observed to have septate hyphae and hyaline which was also reported by De *et al.* (2014); Gonu *et al*. (2015).

Table 2: Identification of fungal isolates for citric acid production

Isolate	Morphology on PDA	Microscopic appearance	Suspected organism
A	Brown-black and powdery colony with pale yellow edges	Large globose conidiophore and septate hyphae	*A. niger*
B	Olive-green and wrinkled colony with whitish edges	Thick-walled and colourless globose conidiophore	*A. flavus*
C	Green-black appearance with fluffy cottony mycelia	Non-septate and branched sporangiophores with globose sporangia	*Rhizopus spp*
D	Grey-green filaments with smooth colony	Branched septate conidiophore	*A. fumigatus*

In this study, the *Aspergillus flavus* appeared initially colourless or clear white on PDA and then became a rough textured green colony with a golden yellow colour on the reverse side.This morphological appearance correlates with the study of Thathana *et al*. (2017) who reported the colony of *A. flavus* as initially white with olive and dark green conidia, plain and flat at the edges with raised and wrinkled centre. However, it is slightly contrary to the report of Gonu *et al*. (2015) who reported *A. flavus* colony as yellowish green colony on PDA. *Rhizopus spp* was isolated in this study and was initially seen macroscopically as white cottony, then dark grey with powdery appearance. This is in agreement with the report of Chay *et al*. (2017) in which *Rhizopusoryzae* as described as cottony, non-septate with sporangiospores. The strain of *A. fumigatus* isolated in this study appears as a smooth colony with greyish-green colour and pale yellow on the reverse. This has the same morphological appearance with the report of *A. fumigatus* by Gautam and Bhadauria (2012) whose isolate is yellow-greyish green in colour but colourless to yellow on the reverse. Microscopically, hyphae were reported to have branched septate with globose conidiophore.

Proximate analysis of pawpaw peel and orange peel

The result of the proximate analysis showed oven dried pawpaw peel to contain appreciable amounts of ash content (2.25%), crude fibre (5.23%), lipid (7.17%), crude protein (16.2%), and carbohydrate (69.14%) (Fig. 2). The pawpaw peel is adequately rich in carbohydrate and protein content for production of citric acid. Similar result has been reported by Martial-Didier *et al*. (2017) whose proximate composition of pawpaw peel showed the dry weight to contain carbohydrate (47.33±0.08%), proteins (11.67±0.04%) and ash (5.98±0.03%). The higher content of carbohydrate in this research compared to Martial-Didier *et al*.(2017) may be due to some content of ripened fruit present in the pawpaw peel sample used for the proximate analysis. For the second substrate, the ash contents, crude fibre, lipid, crude protein and total carbohydrate of the orange peel dry weight basis were determined as 3, 7, 12, 14 and 64%, respectively (Fig. 3). The result obtained here is slightly lower to the result obtained by Adewole *et al*. (2014) who reported the dry matter of orange peel to contain protein (16.40±0.39%), ash content (5.51±0.02%) and crude fibre (12.47± 0.54). The difference in result should be the extent of ripening of the orange peel used. Determination of principal substrate out of the two agricultural waste materials used in the production of citric acid as done in this study was reported by Kudzai *et al*. (2015) who used potato and rice extract as media for the production of citric acid and compared the variation in the proximate analysis for the two.

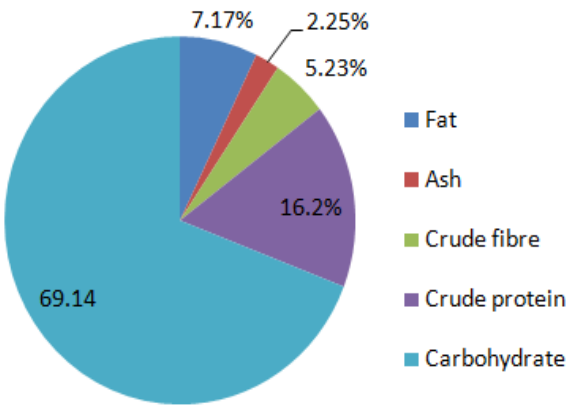

Fig. 2: Proximate composition of pawpaw peel

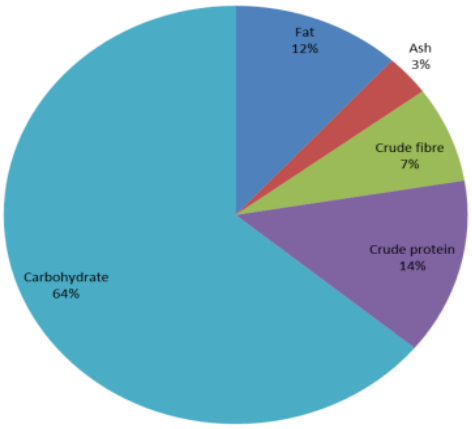

Fig. 3: Proximate composition of orange peel

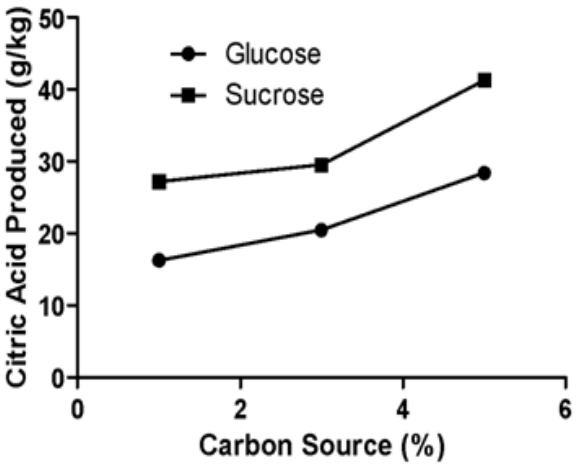

Fig. 4: Effect of carbon sources on orange peel

Production of citric acid

Citric acid production by *A. niger*, from orange peel (OP) increased with increase in concentration of the two carbon sources. There was a greater increase in production of citric acid with 5 g sucrose which peaked with a citric acid value of 41.6 g/kg while a peak value of 28.8 g/kg was obtained with 5 g glucose (Fig. 4). The addition of carbon sources to the orange peels (OP) and pawpaw peels (PP) for solid state fermentation media is in conformity with the research of El-Holi and Delaimy (2003) who reported a lower value of citric acid production from whey alone but had enhanced citric acid production with addition of different sugars.

The effect of carbon sources on citric acid production by *A. niger* on pawpaw peel medium (medium P) showed that citric acid production accelerated with increase in concentration of the two carbon sources. There was a greater increase in production of citric acid with 5 g sucrose which peaked with a citric acid value of 73.6 g/kg and a rate of 46.4 g/kg with 5 g glucose (Fig. 5). The remarkable increase observed in the citric acid production with sucrose at 5% on both media is similar to the increase observed by Kareem *et al.* (2010) whose addition of 15% sucrose to the fermenting medium enhanced citric acid production more, compared to the production on addition of 15% glucose. In addition, the general increase in CA production on the two media when supplemented with sucrose is in conformity with Shetty *et al*. (2015) who produced the highest citric acid in sucrose supplemented medium.

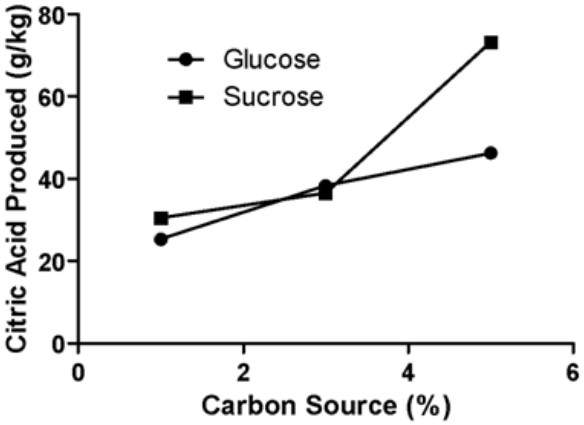

Fig. 5: Effect of carbon sources on pawpaw peel

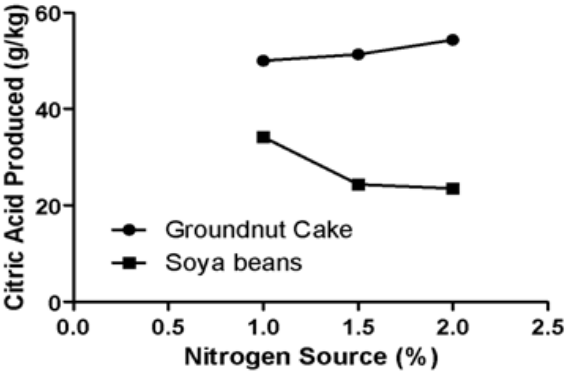

Fig. 6: Effect of nitrogen on orange peel with sucrose (OS) medium

The citric acid produced by *A. niger* on orange peel supplemented with sucrose (OS) medium shows increased production with increase in concentration of groundnut cake with a peak value of 54.0 g/kg at 2%, but decreased with the increase in concentration of soya beans with a peak production of 34.0 g/kg at 1% (Fig. 6).

The effect of groundnut cake and soya bean as nitrogen sources on citric acid production using *A. niger* on medium PS (pawpaw peel with sucrose) showed that the production of citric acid increased as the concentration of groundnut cake increased with a production of 89.6 g/kg at 2% as the highest value. However, production of citric acid declined as the concentration of soya beans in the medium increased, with the highest production of 69.0 g/kg at 1% concentration of soya beans (Fig. 7). This contradicts the report of Makut and Ekeleme (2018) whose result showed that addition of soya beans to potato peel as a substrate increased the production of citric acid. However, the increased citric acid production with groundnut cake in this study can be attributed to the lower percentage of crude protein in groundnut – 25.20% (Ingale and Shrivastava, 2011). Cystein as a major content of soya beans depicts that the decline in citric acid production observed in the media with soya beans is as a result of the relatively higher percentage of crude protein in soyabeans which is estimated to be 40% according to ElShemmy, (2011). However, this is in contrary to the report of Ekeleme and Makut (2018) who had enhanced citric production with soya bean cake and reduced citric acid production with groundnut cake.

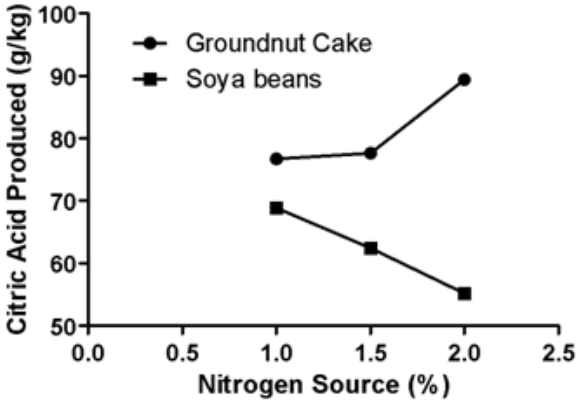

Fig. 7: Effect of nitrogen on pawpaw peel with sucrose (PS) medium

Effect of alcohol on citric acid production

The citric acid production from the *A. niger* on orange peel medium supplemented with sucrose and groundnut cake (OSG) and pawpaw peel medium supplemented with sucrose and groundnut cake (PSG) mediumrise as the concentration of methanol increased. The maximum production was at 2.0% with a value of 80.2 g/kg on OSG and 104.3 g/kg on PSG medium (Fig. 8).

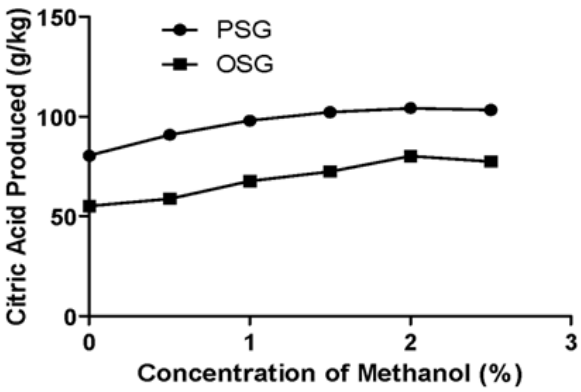

Fig 8: Effect of methanol on orange peel with sucrose and groundnut cake (OSG) and pawpaw peel with sucrose and groundnut cake (PSG) media

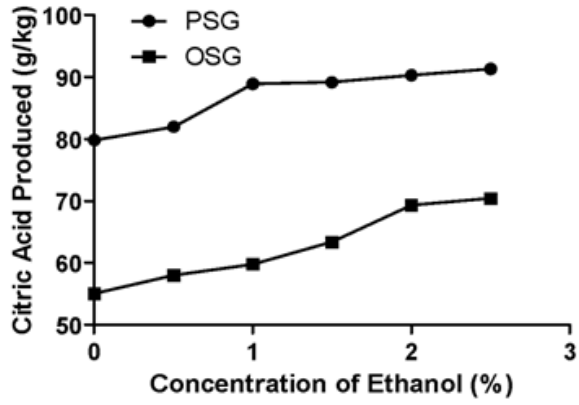

Fig. 9: Effect of ethanol on orange peel with sucrose and groundnut cake (OSG) and pawpaw peel with sucrose and groundnut cake (PSG) media

Citric acid production with the *A. niger* on orange peel medium supplemented with sucrose and groundnut cake (OSG) peaked with a value of 77.5 g/kg at 2.5% concentration of ethanol (Fig. 9). The production by the *A. niger* on both OSG and pawpaw peel medium supplemented with sucrose and groundnut cake (PSG) increased as the concentration of ethanol increased. Highest production was obtained at 2.5% with a value of 94.3 g/kg on PSG. Studies have shown that alcohols stimulate citric acid production, 2% concentration of methanol which improved the citric acid production in this study conforms with result of Shetty (2015) in which 2% methanol also gave the maximum citric acid production. However, the decline in citric acid production was observed after 2% methanol and 2.5% for ethanol has earlier been reported by Showy *et al.* (2015) that higher concentration of alcohols cause decline in citric acid production. Similarly, El-holi and Al-Delaimy (2003) reported the production of lower citric acid value from whey media containing 15% sucrose on addition of higher methanol concentration of about 5% concentration.

Conclusion

This study has presented pawpaw peels and orange peels as potential substrate for the production of citric acid. Sucrose proved better than glucose as a carbon source when added to pawpaw peel and orange peel substrate. It has also been shown that groundnut cake supported the citric acid production better as a suitable nitrogen supplement on both substrates when supplemented with sucrose. The citric acid produced on addition of methanol to OSG and PSG media was higher than the citrate produced with the addition of ethanol to both media at the same temperature. Furthermore, it indicated that pawpaw peel is a better substrate for citric acid production with *Aspergillus niger* compared to orange peel.

Conflict of Interest

The authors declare that there is no conflict of interest.

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