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Citric Acid Production Potential of *Aspergillus niger* Using *Chrysophyllum albidum* Peel

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Abstract

The production of citric acid using *Chrysophyllum albidum* an indigenous under-utilized fruit waste peel and genetically characterized strains of *Aspergillus niger* was carried out. The *Chrysophyllum albidum* peel was dried, sieved to remove dirt, dry milled and the powder used as substrate for citric acid production. Thirteen fungal isolates were obtained from soil samples and decayed agricultural waste by spread plate technique and screened for citric acid producing capabilities on Czapek dox agar. Citric acid producing capability of the isolates revealed a wide yellow zone around the inoculated colonies. Two (F1 and F3) out of the thirteen isolates exhibited positive reactions and were identified based on their cultural, morphological and molecular characteristics. The fungal species were identified using PCR as *Aspergillus niger* DTO: 133-E8 and *Aspergillus niger* DTO: 131-H5. Their cultural/growth optimal conditions were determined through Solid State Fermentation of the substrate using two species of the test organism. The effects of fermentation period examined revealed, *Aspergillus niger* DTO: 133-E8 which produced the highest amount of citric acid 15.7 ± 0.08 g/l, lower reducing sugar and final pH of 2.1 and 121.5 ± 0.31 g/l respectively after 192 h of growth at 30˚C. *Aspergillus niger* DTO: 131-H5 showed highest amount of citric acid 10.2 ± 0.22 g/l, lower reducing sugar and final pH of 2.4 and 128.5 ± 0.15 g/l respectively after 192 h of growth at 30˚C. Maximum concentration of citric acid ranging between 16.3 ± 0.30 g/l and 12.6 ± 0.11 g/l with reducing sugar 125.4 ± 0.11 g/l and 127.2 ± 0.03 g/l was achieved at an initial pH of 5.5. Methanol was used to stimulate citric acid production (0% - 3% (v/v)) and was found to be effective at 2% (v/v) level with 21.2 ± 0.20 g/l of citric acid produced with residual sucrose concentration of 129.5 ± 0.44 g/l. The effect of trace element on citric acid production showed that Cu^{2+} and Fe^{2+} stimulated citric acid production; while other ions reduced citric acid production. There was a statistically significant difference ($P > 0.05$) between the citric acid produced with the various parameters investigated in this research. From the

study, it has been found that *Chrysophlum albidum* is suitable for the cultivation of *Aspergillus niger* and has great potential for citric acid production and could be exploited to promote certain industrial applications of underutilized fruits in Nigeria. This can be an application of waste to wealth as well as a way of minimizing agro-based environmental pollution for a cleaner environment.

Keywords

Aspergillus niger, Citric Acid, *Chrysophyllum albidum*, Substrate, Solid State Fermentation (SSF)

1. Introduction

The increased demand of citric acid by industries coupled with the search for a cheap raw material from agricultural waste residue for its production is of great concern. Citric acid is a tribasic acid which has tremendous application and high commercial value in food, beverages, and other industries [1].

Although many potent microorganisms have been assessed for citric acid production [2] [3], the fungus *Aspergillus niger* is a more preferable microorganism due to the ease in handling, ability to utilize variety of substrate and produce large amount of citric acid [4] [5].

Significant attention has been directed towards the proficient use of waste and its management. A number of value-addition to agricultural waste has been evaluated for this purpose with an overview of environmental littering reduction, pollution control and the concept of recycling upheld. Common agricultural waste residues such as cassava, potato residue, banana peel, bagasse, pineapple waste, apple pomace, soybean, wheat bran, kiwi fruit peel, okaro, carob pods, corncobs, sugar cane have been exploited as raw material for the synthesis of citric acid. Using inexpensive and cheap raw materials as carbon source from agricultural waste residues 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 [6].

Chrysophyllum albidum (African star apple) is an indigenous tropical fruit borne by a wild plant from the family *Sapotaceae* and its origin traceable to the low-lands of Central America and West Indies [7]. The plant grows well in Nigeria bearing fruits during the dry season especially during the months of December through April, when most ingredients for feed formulation are usually scarce [8]. *Chrysophyllum albidum* is locally known in Nigeria as "Udara" "Agwaluma" or "Agbalumo". The fruit usefulness lies on its production of sweet fleshy fruits which had been reported as a rich source of vitamin C. Previous studies have indicated that they contain invaluable raw materials for the production of many cherished consumable items such as, desserts, confectionary, syrup and soft drinks, while the leaves and seeds are used in the pharmaceutical as an anticoagulants in blood banks [9] [10].

The ripe fruit is highly perishable and has a very short life span and deteriorate within 5 - 7 days of harvest [8]. The fruit short life span, lack of preservative necessitated the search for an alternative use for efficient utilization [10]. This stemmed the quest for its use in citric acid production using solid state fermentation technique. The study was aimed at the screening of the two fungal isolates to ascertain the best citric acid producers, and study the effect of various fermentation parameters on the production condition.

2. Materials and Methods

2.1. Substrate Collection and Processing

The substrates *Chrysophyllum albidum* peels used for this research were collected from Rumuokoro market, Mile 1 market all in Port Harcourt, Rivers State, Nigeria. The fruits were washed with distilled water, skin peeled and the pulp (edible portion) was removed and dried at 28˚C ± 2˚C for 3 days. The dried samples were milled filtered with 60 Mesh (0.250 mm) sieve to obtain fine particles, then packed in a sterile air tight container, labelled and stored under dry conditions until when needed for laboratory analyses.

2.2. Physico-Chemical Analyses

Chrysophyllum albidum peel was examined in terms of physical and chemical content. The physico-chemical properties of this substrate were taken.

2.3. Isolation and Identification of Fungi Strains

Species of *Aspergillus niger* were isolated from soil and onion samples by serial dilution technique. The dilutions were inoculated by the spread plate technique on potato dextrose agar (PDA) and incubated at 28˚C ± 2˚C for 5 - 7 days. Based on predominance and distinct morphological properties three fungal isolates were selected and purified by repeated sub culturing. The isolated colonies obtained were examined by lacto phenol cotton blue technique and the colonies where identified as *Aspergillus niger* [11].

2.4. Screening of Isolates

Czapeck-dox Agar medium containing Bromocresol green as an indicator using Plate method was employed for the screening of citric acid producers. The surface of the medium was point inoculated with the spores of the individual fungal isolate and incubated at 28˚C ± 2˚C for 5 - 7 days. The colonies which showed yellow colour zones around them were selected as citric acid producers [12].

2.5. Molecular Identification

Extraction of DNA from the fungal culture was performed using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. The DNA fragment of the ITS region was amplified with a pair of primers ITSIF-5'-CTT GGT CAT TTA GAG GAA GTA A-3', and ITS4 5'-TCC TCC

GCT TAT TGA TAT GC-3'.

By the reaction system and thermocycler parameters, the assay of the PCR products was performed. The amplified products were also purified using DNA clean and concentrator (DCC) kit (Zymo research institute, South Africa) and were ready for sequencing. DNA sequencing was carried out at Inqaba Biotechnical Industries, South Africa using Sanger method of sequencing with 3500 ABI genetic analyser.

2.6. 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 sterilize conditions and then shaken thoroughly to prepare a homogenized spore suspension [13].

2.7. Determination of Cell Number of the Isolate

The number of spores was counted using a Neubauer's Counting Chamber following after Grigoryey [14]. One ml of spore suspension was used as the inoculum to carry out solid state fermentation.

2.8. Citric Acid Production by Solid State Fermentation

Twenty-five gram of milled *Chrysophyllum albidum* peel, (% w/v); was taken in an Erlenmeyer flask (250 ml) and moistened with 25 ml of sucrose medium (g/l) (Sucrose 150, KH_2PO_4 2.5, $MgSO_4{\cdot}7H_2O$ 20.3, NH_4N0_3 3.0, pH 3.5) and autoclaved at 121˚C for 15 minutes. After cooling to room temperature, the flasks were inoculated with one ml of fungal spore suspension, following incubation at 28˚C ± 2˚C for 10 days. Methanol (1% - 3% v/v) was added to the medium before inoculation.

2.9. Extraction of Citric Acid

After fermentation, the contents of the flasks were harvested at regular intervals (48 hrs) by adding 100 ml of sterile distilled water followed by filtration and then centrifugation. The resultant clear extract was used for estimation.

2.10. Determination of Citric Acid

Citric acid was determined titrimetrically using 0.1 NaOH and phenolphthalein as indicator [15] as given by [16] [17]. In this procedure, 1 ml of culture filtrate was taken into a flask and then 2 - 3 drops of phenolphthalein indicator was added and filtrated with 0.1 N NaOH. The end point was noted when the filtrate changes from colourless to pink colour then it was calculated using the formula:

$$\text{Citric acid} = \frac{\text{Titre} \times 100 \times 10 \times 0.0064}{10\ \text{ml}\left(\text{Volume of sample}\right)} = 0.64 \times \text{Titre}$$

Citric acid equivalent acid factor is 0.0064 g/l citric acid. Total citric acid was

reported in g/l.

2.11. Effect of Fermentation Period on Citric Acid Production

Effect of fermentation period on citric acid production was examined by varying the incubation time of the culture medium from 2 - 10 days. Each of the culture broth was inoculated with 1 ml of the prepared inoculum suspension obtained from the different fungi isolates and incubated at 28˚C ± 2˚C.

2.12. Effect of Different pH on Citric Acid Production

The effect of different pH on citric acid production was investigated by using the pH range 3.5 - 6.5 separately and 1 ml of the prepared spore suspension was inoculated and incubated for 10 days at 28˚C ± 2˚C.

2.13. Impact of Methanol on Citric Acid Production

The impact of different concentration of methanol was determined by using concentrations between 0% - 3% v/v, 1ml of the prepared inoculum suspension was added and incubated for 10 days at 28˚C ± 2˚C.

2.14. Impact of Trace Elements on Citric Acid Production

The impact of different trace elements (copper, iron, manganese and zinc ion) on citric acid production was determined by supplementing each of the culture medium with the different trace elements and 1 ml of the prepared inoculum suspension was added and incubated for 10 days at 28˚C ± 2˚C.

2.15. Determination of pH, Residual Sugar and Biomass

The pH of the filtrate was measured using a pH meter (HANNA® products, US). The amount of reducing sugar was estimated by 3, 5-Dinitrosalicyclic acid (DNS) method and sucrose as standard [18]. A UV spectrophotometer (Model: Spectrum Lab 752S) was used to read the colour development at 546 nm.

2.16. Statistical Analysis

Effect of each parameter was studied in triplicate and the corresponding data graphically represented as the mean ± S.D. of triplicates (n = 3). The differences within the means were expressed using one way analysis of variance (SPSS version 22.0, 2013). Post hoc test (Duncan) was used to determine the different p-values < 0.05 that were considered significant with a confidence limit of 95%.

3. Results and Discussion

The potential of using *Chrysophyllum albidum* peel as a substrate for citric acid production was investigated for 10 days. The proximate analysis of *Chrysophyllum albidum* peels (**Table 1**). The result revealed high amount of carbohydrate content (21.6 ± 0.60) and crude fibre (33.2 ± 0.10), which serves as a good source of nutrient. Carbohydrates, which are present in the substrate are quickly taken

Table 1. Proximate analysis of *Chrysophyllum albidum.*

Parameters	*Chrysophyllum albidum* (%)
Crude protein	5.0
Crude fat	23.7
Crude fibre	33.2
Carbohydrate	21.6
Ash	5.6
Moisture content	10.9

up by microorganisms, are necessary for good citric acid synthesis [19].

Thirteen different fungi were isolated from the different soil samples and agro wastes and differentiated on the basis of colony morphology. After screening on Czapeck-dox Agar medium containing Bromocresol green as an indicator, two fungal isolates designated as F1 and F3 were selected based on the wide yellow zone and zone of clearance which they showed in the screening tests, and were subsequently identified to the specie level. PCR assays were carried out using primer ITS1F and ITS4. The 18S rRNA of *Aspergillus niger* and its region were amplified, obtained an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database (**Figure 1**). After amplification, the 18S rRNA of the isolate showed a percentage similarity to other species at 99% as shown in **Figure 2**. The evolutionary distances computed using the Jukes-Cantor method was in agreement with the phylogenetic placement of the 18S rDNA of the isolates within the *Aspergillus*. The fungi isolate F1 and F3 used in the study were classified as *Aspergillus niger* DTO: 133-E8 and *Aspergillus niger* DTO: 131-H5. The DNA sequence of the ITS-5.8S region of the examined strain was deposited in the GenBank database under the accession number: KY941099 and KY941101.

The safety of using black mold (*Aspergillus niger*) fermentation in industries is "generally recognized as safe" (GRAS) by the Food and Drug Administration [20].

Incubation period plays an important role in substrate utilization and citric acid production. **Figures 3-5** illustrates the impact of fermentation period, final pH and residue sucrose on citric acid production after 10 days of incubation at 28˚C ± 2˚C. At 0 hrs there was no lag phase and no citric acid was produced but a further increase in fermentation period up to 192 hrs enhanced citric acid synthesis. During the fermentation period 192 hrs was best for both *Aspergillus niger* DTO: 133-E8 (15.7 ± 0.08 g/l) and *Aspergillus niger* DTO: 131-H5 (10.2 ± 0.22 g/l) in solid state fermentation (**Figure 3**) with a final PH of 2.1 and 2.4 (**Figure 4**) and the amount of residual sucrose are 121.5 ± 0.31 g/l and 128.0 ± 0.44 g/l (**Figure 5**) respectively for both strain Significant difference ($P < 0.05$) was observed in citric acid synthesis by both strains. The best time of incubation for

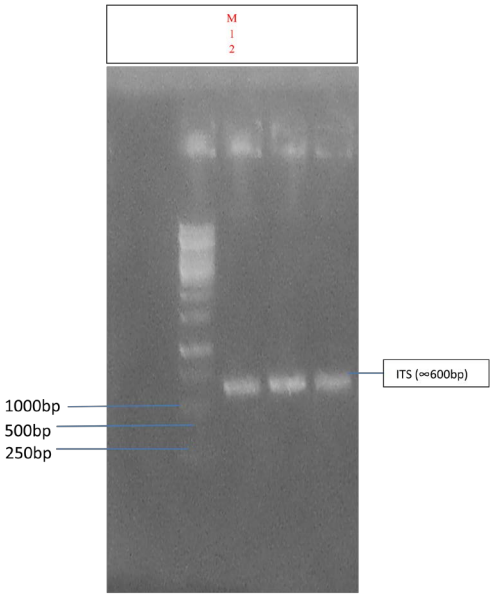

Figure 1. PCR amplification images of the 18S rRNA gene bands of the fungi used in the study. Lane (M): Ladder; Lane 1 & 2: 18S rRNA (ribosomal RNA) of F1 & F3 isolates.

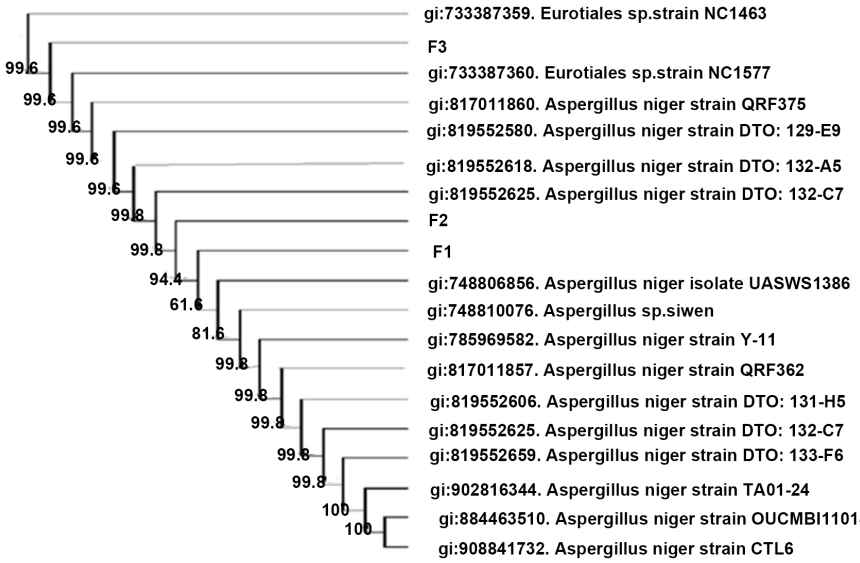

Figure 2. Neighbour-joining phylogenetic tree of isolate F1 and F3 (Tamara *et al.,* 2013). Bootstrap values of >50% (based on 500 replicates) are given in the nodes of the tree. NCBI accession number is given in parenthesis.

maximum yield of citric acid depends on the fermenting organisms and conditions of fermentation [21] [22].

This observation corroborates with Alvarez [23] and Nadeem [1] in which high citric acid yield occurred after 192 hrs of fermentation. Similarly, Maharani [5] reported the production of citric acid by solid state fermentation (SSF) employing an *Aspergillus sp* isolated from spoiled coconut. It was found that citric

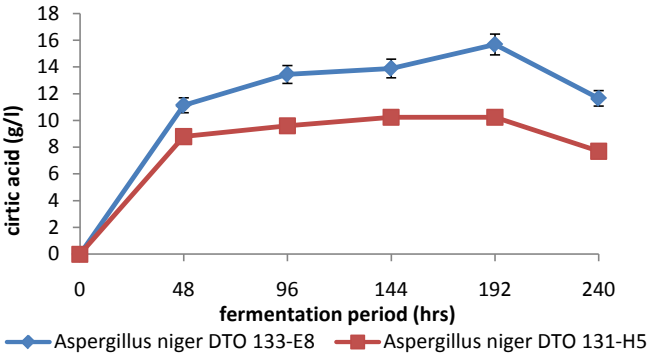

Figure 3. Effect of fermentation period on citric acid production.

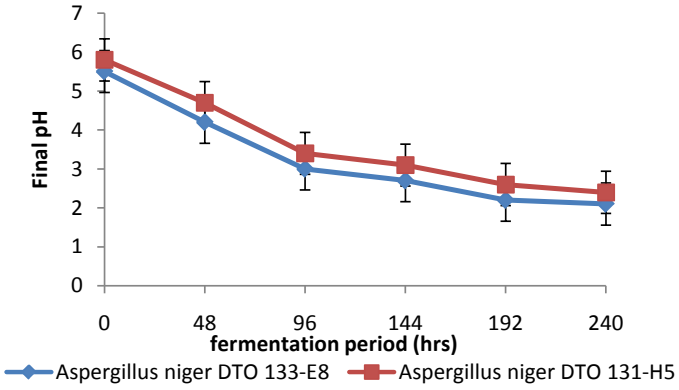

Figure 4. Effect of fermentation period and pH on citric acid production.

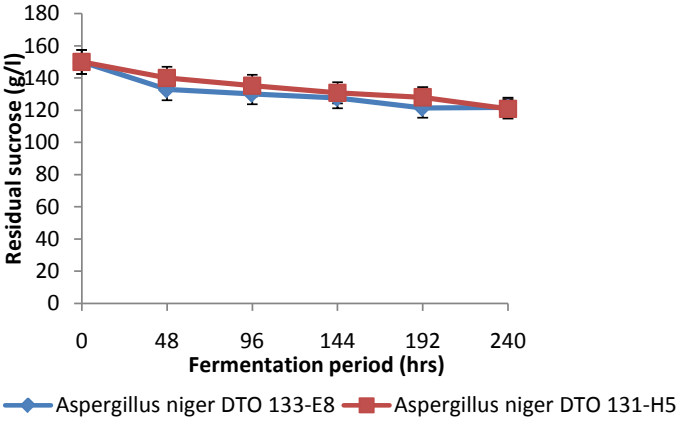

Figure 5. Effect of fermentation period and residual sucrose on citric acid production.

acid production (85.18 gl^{-1}) was highest on 192 hrs of fermentation period at 28˚C ± 2˚C. However, these findings differ from the results reported by Nwoba [24] in a similar study which stated that citric acid production increased steadily during the process of fermentation reaching a peak value of 144 hrs after inoculation and followed by a steady decline in citric acid concentration as fermentation period increases.

Figures 6-8 depicts the impact of different initial pH (3.5 to 6.5) of the medium on citric acid synthesis. After 192 hrs of incubation at 28˚C ± 2˚C, maximum yield of citric acid was observed with increasing initial pH value in the medium up to 5.5. Similar trend was observed in the initial pH of both strains (Figure 6). *Aspergillus niger* DTO: 133-E8 clearly gave the highest amount of citric acid maximum 16.3 ± 0.30 g/l (Figure 6) with reducing sugar of 125.4 ± 0.11 g/l (Figure 7) and final pH value of 2.3 (Figure 8) when initial pH of the medium was adjusted at 5.5. Further increase in the initial pH of the diluent resulted into decrease in citric acid yield. pH is very important for maximum yield, growth and metabolic activities of the microorganisms. From the result, with increase in pH value from 3.5 to 5.5, citric acid reached to the maximum followed by a gradual decrease thereafter. pH of 5.5 was found to be best for citric acid by using *Chrysophyllum albidum* peel.

Notably our findings corroborates with Khosravi-Darani and Zoghi [17], who similarly reported initial pH 5.5 for the maximum amount of citric acid by *Aspergillus niger.* Similarly, Al-Shehri and Mostafa [25], in a study on citric acid synthesis using *Aspergillus niger* immobilized on date-syrup, reported a high yield of citric acid at pH 5.5.

Fungal strains seem to thrive best in acidic medium ranging from 3 to 6 [26]. The pH range of 2 to 6 is frequently utilized for submerged and solid state fermentation [27] [28].

The result of the impact of methanol concentration, final pH and residue sucrose on citric acid production by *Aspergillus niger* are shown in Figures 9-11. Figure 9 shows that with 2% (v/v) of methanol (21.2 ± 0.20 g/l) and (13.4. ± 0.20 g/l) amount of citric acid was recorded for *Aspergillus niger* DTO: 133-E8 and *Aspergillus niger* DTO: 131-H5 with a final pH value of 2.2 and 2.4 (Figure 10) and residual sucrose of 129.5 ± 0.44 g/l and 130.0 ± 0.20 g/l (Figure 11) was left over in the medium by both strains respectively.

2% (v/v) methanol was found to be best suitable for maximal yield of citric acid further increased in methanol concentration, caused a decreased in citric acid production. This could be due to the fact that the substrate may contain sugars which inhibit citric acid production at higher concentrations. Similarly, Kareem [29] reported that supplementation of 2% (v/v) methanol to medium

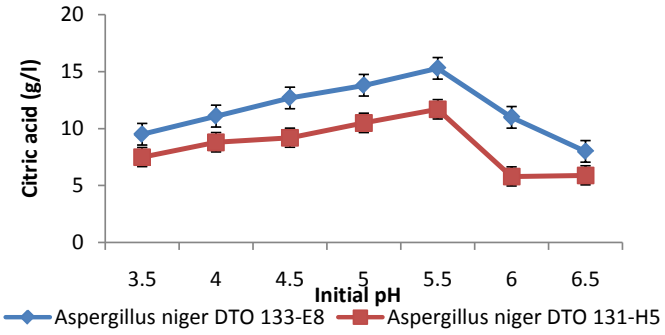

Figure 6. Effect of initial pH on citric acid production.

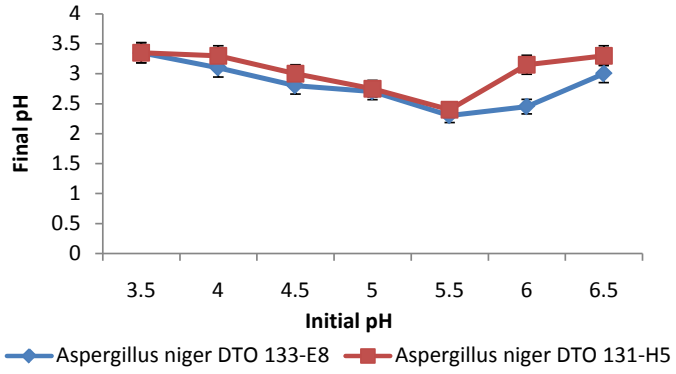

Figure 7. Effect of initial pH and final pH on citric acid production.

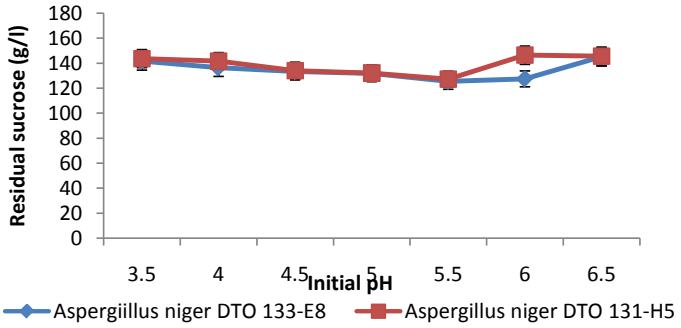

Figure 8. Effect of initial pH and residual sugar on citric acid production.

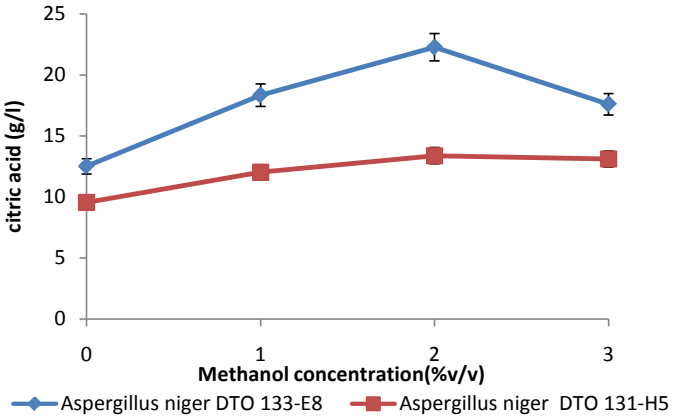

Figure 9. Effect of methanol concentrations on citric acid production.

effectively increased citric acid production by *B. subtilis* in solid state fermentation. This implies that the concentration of 2% methanol could support citric acid production but an increase from 2% would result in higher levels of certain sugars present in the peel that are inhibitory to citric acid production. Most study indicated that High citric acid yield is obtained by adding methanol to most fungal fermentation between concentrations of 1% - 4% v/v citric acid production (140 u/g) with copper but at a level of 1% (w/v).

The different trace elements used affected the yield of citric acid production. The results presented in **Figure 12** showed that copper gave the best yield

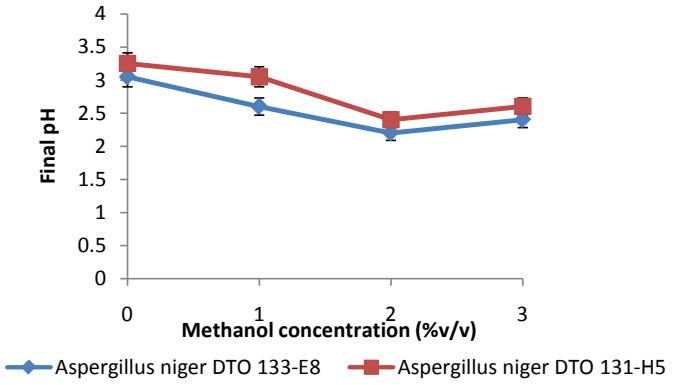

Figure 10. Effect of methanol concentration and final pH on citric acid production.

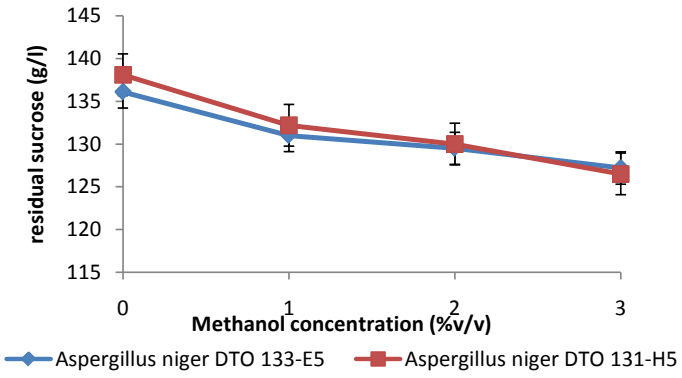

Figure 11. Effect of methanol concentration and residual sucrose on citric acid production.

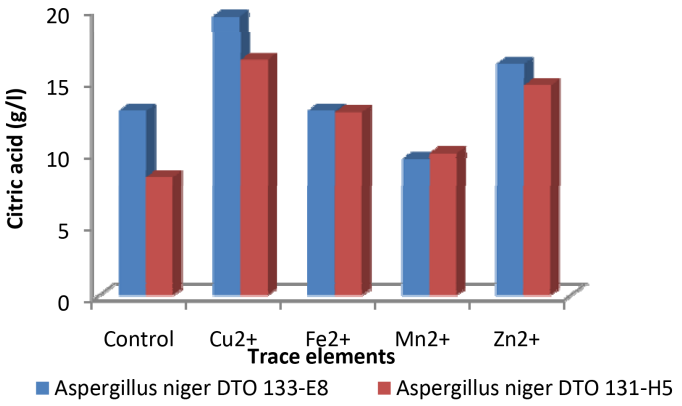

Figure 12. Impact of trace elements on citric acid production.

followed by Zinc and iron. *Aspergillus niger* DTO: 133-E8 and *Aspergillus niger* DTO: 131-H5, used the copper and improved citric acid yield (19.5 ± 0.00 g/l and 16.5 ± 0.10 g/l) respectively as compared to control which produced 12.9 ± 0.10 g/l and 8.2 ± 0.06 g/l of citric acid, respectively (**Figure 12**). The citric acid yield however was strongly repressed in the presence of manganese, showing only 9.5 ± 0.10 g/l and 9.9± 0.10 g/l of citric acid, respectively.

Hang [30]reported that trace elements have a significant impact on citric acid accumulation by *Aspergillus niger*. Highest amount of citric acid was obtained with copper, followed by iron, and zinc. The citric acid production however was strongly repressed in the presence of manganese. Kareem and Rahman [3] reported that copper and zinc as a trace element encourages maximum yield of citric acid. Kareem and Rahman [3] also obtained maximum citric acid production (140 u/g) with copper but at a level of 1% (w/v).

4. Conclusion

This study has shown the citric acid production potentials of using *Aspergillus niger* strains (DTO: 133-E8 and DTO: 131-H5). The steady state substrate (*Chrysophyllum albidum* peels) fermentation studies further revealed the impact of certain physicochemical parameters such as initial pH; incubation time, methanol concentration, and trace element on the citric acid production kinetic. The optimized cultural conditions for maximum yield of citric acid were therefore, pH (5.5), incubation time (8 days), methanol concentration (2% v/v), and trace element (copper and iron). Hence the present work, therefore suggests that solid state fermentation of an under-utilized food crop such as *Chrysophyllum albidum* peels for citric acid production is feasible. This could be of help to solve the problem of wastage during its season by adding value to the peel and converting waste to wealth.

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