Production of Citric Acid by Local Strains of *Aspergillus niger* using Pineapple Peels as Substrate

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Abstract: This study was conducted to screen for citric acid production by local strains of *Aspergillus niger* isolated from soils of four locations within Ahmadu Bello University, Zaria, Nigeria namely: botanical garden, refuse dumpsite, flower bed and sheep pen sites. Proximate compositions of the pineapple peel were determined using standard procedures described by the Association of Official Analytical Chemists (AOAC). A total of sixteen (16) soil samples were collected from the different locations and stock suspensions were prepared before being separately diluted serially from 10^{-1} to 10^{4}. Aliquots of each suspension were separately inoculated onto Potato Dextrose Agar (PDA) and incubated at room temperature (25°C) for seven (7) days for the isolation of *Aspergillus niger*. Colonies suspected to be *Aspergillus niger* were characterized macroscopically and then microscopically using lactophenol cotton blue-staining preparations. The seven isolates identified were then screened on a Czapek-Dox agar medium for potential citric acid production. The isolates were further subjected to citric acid production by submerged fermentation using pineapple peels as the substrate. The isolates confirmed to be *Aspergillus niger* had percentage occurrences of 25%, 100% and 50% from sheep pen, flower bed and botanical garden sites respectively. No *Aspergillus niger* was isolated from refuse dumpsite soil. Isolate BGS3 (from botanical garden soil) produced the highest yellow zone of citric acid production (78.5mm) during screening, whereas, isolate SPS (from sheep pen soil) showed the lowest (41.5mm) potential. During production, an overall yield of 0.76g/100ml was obtained using pineapple peel as substrate. *Aspergillus niger* can be easily isolated from various soil types with highest frequency in soils from sheep pen. The research revealed the potential of various A*spergillus niger* isolates from different soil to produced citric acid using pineapple peels (agricultural waste) as substrate.

Keywords: *Aspergillus niger*, Citric acid, Isolation, Pineapple peels, Screening, Soil

INTRODUCTION

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) is a weak acid belonging to the family of carboxylic acids, present naturally in citrus fruits like limes, lemons, oranges berries, tangerines and grape fruits and in many animal tissues and fluids (Vidya *et al.,* 2018). It has found a wide variety of applications in the food, pharmaceutical, cosmetics, agricultural, diary and biochemical industries among others (Dhillon *et al.,* 2011; Makut and Ade-ibijola, 2012). Owing to its unique taste and being water-soluble, citric acid is mainly used as a food additive. Citric acid is widely considered safe for consumption the world-over (Pandey *et al*., 2001). More than 10 % of the total citric acid produced worldwide is used in the pharmaceutical and cosmetic industries and the rest is used for other purposes such as chelation, metal finishing, animal feeds production and in making plasticizers (Bauweleers *et al.,* 2014).

Citric acid is an important commercial product which in present day could be produced by fungal fermentation (Yigitoglu, 1992).

Citric acid could also be produced chemically, however, this is relatively more expensive than fungal fermentation and not eco-friendly (Dhandayuthapani, 2009). Various microbial strains are known to accumulate citric acid in their culture media, these include strains of molds like *A. niger, A. carbonarius, A. awamori, A. foetidus, Penicillium janthinellum, Trichoderma viride* and yeasts such as *Candida tropicalis*. However, *Aspergillus niger* is mostly chosen for the production of citric acid due to its high variability for substrate utilization owing to its well-developed enzymatic machineries (Munshi *et al.,* 2013).

Nowadays, the substrates and media used in the production of microbial metabolites are getting more expensive and this ultimately affects the costs of the final products (Vidya *et al.,* 2018). Molasses is the substrate of choice for industrial production of citric acid but due to increase in demand, molasses is not readily available and therefore becoming expensive to procure. Therefore, alternative source of carbon is needed to meet demands (Iqbal *et al.,* 2014). Large amount of organic or agricultural wastes such as wheat bran, sweet lime peel, sweet orange peel, apple pomace, pineapple peel, cassava bagasse and lot more are found to accumulate in the environment which results in pollution of the environment and also leads to loss of potential valuable materials which can be channelled for processing to yield a number of valuable products such as fuel, food and variety of chemicals including citric acid (Makut and Ade-ibijola, 2012). Similarly, synthetic production of citric acid from chemicals e.g. glycerol rather than microbial fermentation is expensive, and this may lead to high purchasing cost (Kareem *et al.,* 2010). Generally, synthetically produced citric acid is associated with potential health problems (Iliana and Bryan, 2018) and therefore considered unsafe for human consumption.

MATERIALS AND METHODS

Collection of Pineapple peels

A total of 1000 g of pineapple peel samples were collected from local traders in Samaru, Sabon Gari Local Government Area of Kaduna State, Nigeria. The peels were packaged in a clean polyethene bag, labelled appropriately and transported to the Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for analyses. The peels were first dried under shed (in the absence of direct contact with sunlight) for one week prior to pre-treatment.

Collection of Soil samples

Four samples each were collected from different locations namely: botanical garden, refuse dumpsites around the campus, flower beds within the Department of Microbiology and sheep pen sites at the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria; giving a total of fourteen (14) samples. Ten (10) g of each sample was separately collected from the locations from a depth of 5-10cm using a hand shovel and packaged into clean polythene bags. The soil samples were labelled appropriately and transported to the Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for analyses.

Acid Pre-treatment of the Pineapple Peels

A modified method of Rakesh *et al*. (2013) was adopted. Exactly 25g of the dried pulverized pineapple peels sample was added to a 1000mL flask and 225mL of 1.0M sulphuric acid added. The mixture was autoclaved at 121°C for 15 min and the treated material obtained after treatment was then filtered through muslin cloth and washed several times under running tap water until no color is visible in the wash water and the pH adjusted to physiological value (7.2) following drop-wise addition of 1.0M calcium hydroxide (over-liming). The neutralized residue was then pressed manually to remove excess water before drying. A small portion of the treated biomass was dried in an oven at 70ºC for 24 h and was ground to fine particle size in a laboratory mill for the proximate analysis (Rakesh *et al*., 2013).

Proximate Analyses of the Pineapple Peels

Ten (10) g of the acid-treated dried unfermented pineapple peels samples were used to carry out proximate analyses at the Food Science Unit, Institute of Agricultural Research, Ahmadu Bello University, Zaria. Parameters determined include carbohydrate, crude protein, crude fat, crude fibre, ash content and moisture as the percentage compositions of the substrate according to AOAC methods as also adopted by Ahmed *et al*. (2013) with some modifications.

Isolation and Characterization of the Fungus

Potato Dextrose agar (PDA) and Czapek–Dox agar used in this study were of analytical grade and were prepared according to manufacturers' instructions.

Ten (10) g of each soil samples were separately suspended in 90ml distilled water. The samples were serially diluted to a dilution of 10^{-4}. Aliquots of 0.1ml each from the 10^{-1} and 10^{-3} dilutions were separately inoculated on the freshly prepared potato dextrose agar plates by spread plating technique. The inoculated plates were incubated at room temperature (25°C) for seven (7) days and observed for presence of colonies. The isolates exhibiting cultural characteristics of *Aspergillus niger* were sub-cultured onto fresh PDA plates to obtain pure isolates and then preserved on PDA slant in refrigerator at 4°C until required for further analyses.

Identification of the Fungal Isolates

The identification of *Aspergillus niger* was primarily based on observation of distinct cultural and morphological properties as described by Fawole (2004). Cultural characteristics were observed visually on the PDA plate, whereas, wet mount and slide culture technique were employed according to standard procedure for morphological identification of the isolates (Ajiboye *et al.,* 2015).

Screening of the Fungal Isolates for Citric Acid Production

All the *Aspergillus niger* isolates obtained were screened for citric acid production using plate method according to standard procedure as described by (Patil and Patil, 2014). The isolates were screened for citrate production on Czapek-Dox Agar medium supplemented with 1% bromocresol green. Spores of each isolate were separately inoculated on the freshly prepared Czapek-Dox medium and incubated at room temperature (25°C) for 144 hours.

Citric acid producers were identified as being surrounded with characteristic yellow zones (Patil and Patil, 2014). The diameters of the yellow coloured zones were measured using a ruler in millimetre (mm). The screening was carried out in duplicate and results are presented in arithmetic mean.

Citric Acid Production by Submerged Fermentation

Preparation of Inoculum

Spore suspension was counted using a Neubauer's Counting Chamber to obtain a concentration of 1.0 $x10^6$ spore/ml (Kareem *et al*., 2010). A spore suspension of the selected isolate was prepared by adding 10ml of sterile distilled water containing two drops of 0.1% Tween 80 to the sporulated five (5) days old slant cultures of *Aspergillus niger*. Then suspension was shaken vigorously to obtain a homogenous spore suspension (Pandey, 1992).

Preparation of Medium for Submerged Fermentation

Ten (10) g of the pineapple peels milled using mortar and pestle were weighted into a 250mL Erlenmeyer flask containing 90mL of mineral salt solution which comprised of the following in g/L: $NaNO_3$, 2g; KCl, 0.35g; $MgS0_4.7H_2O$, 0.2g; K_2HPO_4, 0.7g; $FeSO_4.7H_2O$, 0.02g. The pH of the medium was then adjusted to 5.0 prior to autoclaving at 121°C for 15 minutes. After cooling at room temperature (25°C), content of the flask was inoculated with 10mL of *Aspergillus niger* spore inoculum, followed by incubation at room temperature (25°C) for six (6) days on a shaker incubator set at 180 rpm (4 g). Five (5) g of glucose as exogenous source of carbon was supplemented to one of the flasks to serve as inducer and used as control.

Extraction and Assay for Citric Acid Produced

After fermentation, the contents of the flask were initially filtered through a clean muslin cloth. The filtrate was then centrifuged at 4000rpm (1800 g) for 15 minutes and the supernatant was carefully decanted and used to determine the amount of citric acid produced (Dienye *et al.,* 2018).

Titration of the supernatant was carried out using 0.01M NaOH to estimate the amount of citric acid produced using phenolphthalein indicator (AOAC, 1995). In the procedure, 2-3 drops of the phenolphthalein indicator were added into an Erlenmeyer flask containing 10mL of the supernatant. The mixture was then titrated against the freshly prepared solution of 0.01M NaOH. The end point was then determined and the citric acid concentration in the supernatant was estimated using the formula;

$$\text{Citric acid} = \frac{\text{Titre} \times \text{dilution factor} \times \text{citric acid equivalent}}{\text{Volume of the sample (mL)}} \times 100$$

The Citric acid equivalent factor = 0.0064 g/l; Dilution factor =10

Total citric acid was reported in g/100ml (Dienye *et al.,* 2018).

RESULTS

The results of the compositional analyses of the pineapple peels are shown on Table 1. The treated pineapple peels were highly rich in carbohydrates (81.56 %) but with limited ash content of just 4.30 %. While the carbohydrates serve as the source of sugar which the fungus, *Aspergillus niger* metabolizes for growth and to produce the citric acid, proteins serve as the source of amino acids. The ash content serves as source of mineral nutrients such as Na^+ and K^+ which play roles as co-factors during enzymatic activities.

Table 1: Proximate Composition of Acid-treated Pineapple Peels

Parameters	Proximate Compositions (%) *
Moisture	4.70 ± 0.15
Ash	4.30 ± 0.29
Crude Protein	5.24 ± 0.14
Crude Fat	4.20 ± 0.01
Crude Fibre	18.68 ± 0.25
Carbohydrate	81.56 ± 0.30

** = values are mean ± SD*

The morphological Characteristics of the *Aspergillus* Isolates obtained are presented on Table 2 below. Isolates which formed black (grey), compacted colonies with long and smooth conidiophores were identified as *Aspergillus niger. Aspergillus niger* was isolated in all the various soil samples analysed except the refuse dumpsite soil.

Table 2: Cultural and Microscopic Characteristics of the Fungal Isolates

Isolate's Code	Macroscopic Characteristics			Microscopic Characteristics		Tentative Identity
	Colour Change of Colony	Colony Texture	Colour of Reverse	Conidiophore	Phialides	
BGS3	White-Black	Floc/compacted	White-Yellow	Long smooth	Biseriate cover entire vesicle	*A. niger*
SPS1	White-Black	Floc/compacted	White-Yellow	Long smooth	Biseriate cover entire vesicle	*A. niger*
SPS2	White-Black	Floc/compacted	White-Yellow	Long smooth	Biseriate cover entire vesicle	*A. niger*
SPS3	White-Black	Floc/compacted	White-Yellow	Long smooth	Biseriate cover entire vesicle	*A. niger*
SPS4	White-Black	Floc/compacted	White-Yellow	Long smooth	Biseriate cover entire vesicle	*A. niger*
FBS1	White-Black	Floc/compacted	White-Yellow	Long smooth	Biseriate cover entire vesicle	*A. niger*
FBS4	White-Black	Floc/compacted	White-Yellow	Long smooth	Biseriate cover entire vesicle	*A. niger*

Key: BGS botanical garden sample, SPS sheep pen sample, FBS flower bed sample

Table 3 below shows the percentage occurrence of *Aspergillus niger* isolated from the various soil samples analysed. The sheep pen had the highest occurrence of 100 % whereas no *Aspergillus niger* was isolated from any of the soil samples collected from the refuse dumpsite. This might be due to the richness of the soil from sheep pen in organic matter. However, only 50 % and 25 % of the soil samples from flower bed and botanical garden respectively were positive for *Aspergillus niger*.

Table 3: Occurrence of *Aspergillus niger* in Various Soil Samples Analysed

Sample Location	Number of Samples Collected	Number of Positive (%)
Botanical garden	4	1 (25)
Sheep pen	4	4 (100)
Refuse dumpsite	4	0 (0)
Flower bed	4	2 (50)

The results of the screening for citric acid production by the *Aspergillus niger* isolates are presented on Table 4 below. Despite the low (25 %) occurrence of *Aspergillus niger* in soil samples of the botanical garden, the BGS3 was the most potent in citric acid production with 78.5 mm of hydrolysis during screening. Interestingly, isolates from the sheep pen showed relatively low potential for citric acid production.

Table 4: Citric Acid Produced by *Aspergillus niger* Isolates using plate method

Isolate's Code	Diameter of Zone of Hydrolysis for Citric Acid Production (mm)
BGS3	78.5
SPS1	67
SPS2	41.5
SPS3	59
SPS4	72
FBS1	76.5
FBS4	45

Key: BGS botanical garden sample, SPS sheep pen sample, FBS flower bed sample

The results for citric acid production by the isolate of *Aspergillus niger* BGS3 under submerged fermentation are shown in Figure 1. The isolate produced 0.76 g/L of citric acid from the treated pineapple peels without supplementation. However, a 22 % increase (0.96 g /L) in citric acid production was obtained after addition of glucose as inducer.

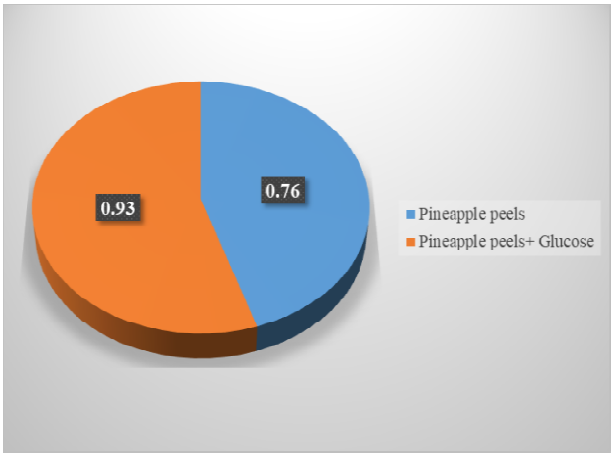

Fig. 1: Citric Acid Produced (g/100mL) from Pineapple peels by *Aspergillus niger* (BGS3) Isolate via Submerged Fermentation

DISCUSSION

The result of the proximate analysis of the pineapple peels revealed the percentage composition of carbohydrate (81.56%) and crude fat (4.20%) (Table 1). The carbohydrate present in the pineapple peels indicates the presence of enough fermentable sugars required for growth and production of citric acid by *Aspergillus niger.*

Of all the sixteen (16) soil samples analysed for the isolation of *Aspergillus niger*, seven (7) isolates were found to be *Aspergillus niger* based on their macroscopic and microscopic characteristics observed as described by Fawole (2004) which are shown on Table 2. Out of the sixteen (16) samples collected, percentage occurrences of 100%, 50% and 25% were obtained from soils of sheep pen, flower bed and botanical garden respectively while soil sample from refuse dumpsite had 0 % percentage occurrence (Table 3). *Aspergillus niger* are known to be ubiquitous in the soil, but no organism was isolated from the refuse dumpsite. This agrees with the findings of Rakesh *et al.* (2014) who isolated different fungal isolates with varying occurring percentages in which *Aspergillus niger* had the highest percentage (18.46%) while *Aspergillus oryzae* had the lowest percentage (1.53%).

All the seven fungal isolates of *Aspergillus niger* were screened on Czapek-Dox agar medium for citric acid production, the isolate from botanical garden (BGS3) had the highest yellow zone of hydrolysis for citric acid production (78.5mm) and least zone of hydrolysis (41.5mm) was observed with isolate SPS3 from sheep pen after 6 days of incubation (Table 4). This observation might be due to varying metabolic ability of the different fungal isolates, which might not be unconnected with nature of the soil from which they were previously isolated. The isolate with the highest yellow zone of hydrolysis for citric acid production was used for subsequent production.

Citric acid produced by microorganisms could be influenced by incubation period which determines the rate of substrate utilization (Dienye *et al.,* 2018). The highest yield of citric acid (0.93 g/L) produced by the selected isolate, BGS3 was observed in the flask containing pineapple peels with glucose supplementation (Figure 2). This might be because glucose is readily utilized by the organism which results in increased accumulation of citric acid in the organism leading to high yield of citric acid. The result of this finding lends credence to the work carried out by El-Holi and Al-Delamy (2003) who reported that there was a significant positive correlation between substrate utilization and citric acid production. Additionally, the crude protein showed the potential of the pineapple peels as a nitrogen source required for the growth of the organism, while the ash content represents the richness of minerals which are essential in citric acid production. This accounts for the citric acid produced (0.76g/100mL) and the use of additional exogenous carbon source (glucose) showed an increase in citric acid produced (0.93g/100mL), indicating that the availability of simple sugars like glucose are easily taken up and utilized which results in an increased accumulation of citric acid in the organism (Soccol *et al*., 2006). Thus, it can be concluded that glucose might have played an inducer role.

CONCLUSION

In conclusion, pineapple peels contain potential nutrients in significant quantities to support the growth of *Aspergillus niger* for citric acid production. Also, local strains of *Aspergillus niger* are ubiquitous in the soil and can be readily isolated from different soil types with the highest occurrence of 100% in soil samples from sheep pen. The seven (7) isolates of *Aspergillus niger* from three soil sources demonstrated the capability to produce citric acid.

A yield of 0.76 g/100mL and 0.93g/100mL of citric acid using pineapple peels and pineapple peels with glucose supplementation as fermentation substrate was obtained respectively.

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