Production of Citric Acid from Solid State Fermentation of Sugarcane Waste using *Aspergillus Niger* and Indigenous Sugarcane Microflora

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Abstract

Aspergillus niger is the leading microorganism of choice for citric acid production. Sugarcane waste was used as substrate under solid state fermentation to comparatively evaluate the citric acid production capacity of *Aspergillus niger* isolates and the indigenous microflora in the sugarcane waste. Known optimal cultural parameters such as moisture content, particle size and sugar level were used. *Aspergillus niger* produced 30.54 g/L citric acid while the indigenous microflora produced 28.93 g/L citric acid after fermentation at 30°C for 8 days. The residual sugar concentration decreased from 11.35 g/L to 3.14 g/L in the *Aspergillus niger* fermentation medium and from 10.67 g/L to 5.99 g/L in the indigenous microflora fermentation medium.

Keywords: Citric acid production; sugarcane waste; indigenous micro flora; fungus-specific fermentation extract

Introduction

Sugarcane waste (bagasse) is the fibrous cellulosic material that remains after sugarcane stalks are crushed to extract their juice (Rani et al., 2012). Bagasse, the primary solid by-product of sugarcane is

considered as waste that goes to landfill or is allowed to decay in the environment that the waste was indiscriminately disposed. This adoption of end-of-pipe option of disposal without considerations for treatment and utilization of waste vivifies the poor status of our waste management systems. The management of sugarcane bagasse (and other agricultural by-products) offers substantial economic, environmental and strategic advantages (Shao et al., 2009).

Sugarcane bagasse represents a unique renewable carbon source. An estimated one ton of sugarcane generates 280 kg of bagasse (Aigbodion et al., 2010). Sugarcane bagasse is considered as waste that is allowed to decay in public open places, goes to landfill or dumped into water courses like canals or rivers. Improper disposal of this agro-waste causes a serious ecological threat by encouraging growth of microbial pathogens of various origins. The alternatives for utilisation of such cellulosic biomass is therefore of great importance.

Citric acid, a tricarboxylic acid, has been produced from a variety of raw materials which may have been considered as wastes. These wastes include orange peels, pineapple peels, bagasse, wheat bran, molasses, corn cob, beet pulp, soy residues, areca husk, apple pomace, grape pomace etc. (Kapoor et al., 2004; Kumar and Jain, 2008). This study focuses on the use of sugar cane bagasse (chewed sugarcane stalks), often considered as waste. The use of strain-specific microbial fermentation in the production of citric acid has been extensively studied (Kumar et al., 2002; Khalil et al., 2010; Nadeem et al., 2010). To this end, microorganisms like *Aspergillus niger, A. wentii, A. clavatus, Penicillium luteum, P. citrinum, Mucor piriformis, Candida guilliermondii, Saccharomycopsis lipolytica, Tirchoderma Viride, and Arthrobacter paraffineus* (Al-Khadir and Mohd, 2011) have been adopted. However, the use of the indigenous microflora toward such end has not been researched.

This study therefore, compared the citric acid production efficiency of *Aspergillus niger* (which is the world's leading choice microorganism for the synthesis of citric acid) and the indigenous microbial communinty in the sugarcane bagasse.

Materials and methods

Substrate

Source-segregated sugarcane wastes were collected from sugarcane sellers in Sabo and Mokola in Ibadan, Nigeria. The substrates were sun-dried to reduce the moisture content.

Isolation of fungal strain

The locally isolated fungal strain of *Aspergillus niger* was isolated from soil. The soil sample was collected from the topsoil where there was high moisture content and decaying debris. The soil sample was collected using a hand trowel which was swabbed with 70% ethanol solution. The hand trowel was applied perpendicular to the vertical surface of the profile. Then was placed in a sterilized beaker and covered with aluminium foil.

The soil fungi were isolated by serial dilution technique using the pour plate method. Soil sample (1 g) was weighed in 10 ml of sterile water in order to obtain suspension of their microbial content. The sample suspension was mixed thoroughly so as to have the microorganisms evenly distributed. A series of six McCartney bottles, each containing 9 ml of sterile water were labelled 1 to 6 respectively. A sterile 1 ml pipette was used to accurately transfer 1 ml of soil suspension to McCartney bottle 1 and mix thoroughly to give a 1 in 10 dilution. A fresh sterile pipette was used to transfer 1 ml sample from the 1 in 10 to McCartney bottle 2. Mix thoroughly again and this gives a dilution of 1 in 100. The process was then repeated for the remaining McCartney bottles so that the 6th bottle has a dilution of 1 in 1 million.
Potato Dextrose Agar (PDA) weghed 9.75 g was weighed and dissolved in 250 ml of distilled water (39 g/l). To inhibit the growth of bacteria, 30 mg/l of streptomycin was added to the medium. The culture medium was sterilized in the autoclave at 121°C for 15 minutes and allowed to cool partially to about 45°C.

Sterile Petri dishes labelled with dilution numbers were set out for pour plating. 1 ml of each dilution was discharged into the centre of

the appropriate Petri dish. A clean, sterile, dry fresh pipette was used for each dilution. About 10 ml of molten potato dextrose agar (cooled to 45°C) was poured into each Petri dish. The Petri dishes were swirled (by a combination of to-and-fro and circular movements) rapidly but carefully to mix the medium and inoculum for about ten seconds. Care was taken not to allow the medium to spill or get on the lid of the Petri dish. The plates were allowed to set and incubated inverted at 30°C (4-6 days) for fungal development.

From the resulting fungal colonies, the fungal species of interest, *Aspergillus niger* was identified both macroscopically and microscopically. It was then purified by subculturing into fresh PDA plates under sterile conditions. Subculturing was repeated until a pure culture of *Aspergillus niger* was obtained.

Screening of citric acid producing strain

Citric acid producing strain of *Aspergillus niger* was identified by the use of Czepak-Dox Agar with Bromocresol green as indicator as described by Al-Khadir and Mohd., 2011. Czepak-Dox agar (11.375 g) was dissolved in 250 ml of distilled water according to label instruction (45.5g/L) and Bromocresol green was added as indicator. Streptomycin was also added to inhibit bacterial growth. The agar was then sterilized in an autoclave at 121°C for 15 minutes. The blue coloured agar was allowed to cool partially, poured into sterile Petri dishes and allowed to set. The agar medium was then inoculated with pure isolates of *Aspergillus niger* obtained from soil using a flamed wire loop. The plates were incubated upside down at 30°C for 4 days. The plates were checked for colour change from blue to yellow as an indication of organic acid production. Positive plates were subcultured and maintained on agar slants.

The agar slants were prepared by mixing PDA according to label instruction and homogenizing its content by subjecting to partial boiling. The agar was poured into McCartney bottles and sterilized at 121°C for 15 minutes. The bottles were allowed to cool in a slanted position until the molten agar medium solidifies. The surface of the slope was streaked aseptically with the pure acid-producing strain of *Aspergillus niger* and incubated at 30°C for 5 days when reasonable

growth was obtained. The agar slants were stored in the refrigerator at 4°C.

Citric acid production medium

Sugarcane bagasse collected was sun-dried (and oven dried), cut into small pieces, grounded and screened to collect a particle size of about 1.2-1.6 mm. The two different fermentation basis utilized in this study were initiated during the substrate preparation. The two substrate preparations were:

- *Aspergillus niger* fermentation (sterilization and inoculation)
- Natural/ crude fermentation (No sterilization or inoculation)

The *Aspergillus niger* fermentation medium was autoclaved (sterilized at 121°C for 60 min to provide proper cooking of the substrate and to increase its susceptibility to microbial attack). The crude fermentation medium was however not sterilized. This will help preserve the indigenous microbial populations present in the sugarcane waste (bagasse). This concept is based on the premise that sterilization is done for two purposes (i) eradication of microbial contamination and (ii) for mild hydrolysis (Al-Khadir and Mohd., 2011).

Inoculum

Citric acid producing isolates of *Aspergillus niger* were used. The isolates were grown on slants of potato dextrose agar and stored at 4°C. Spore suspension was prepared by adding sterile normal saline (0.9%) solution to slant culture of *A. niger* having profuse conidial growth on its surface. A sterile wire-loop was gently used to break the conidial clumps and shaken vigorously to make a homogeneous suspension. Spore suspension prepared from 8 to 10-day-old cultures of *A. niger* was used as inoculum in each experiment for fungal fermentation. One (1) ml of this suspension was used as the inoculum. However, no inoculation was carried out on the sugarcane bagasse for natural/crude fermentation. Therefore the substrate (sugarcane bagasse) still retains its indigenous microflora which is needed for crude fermentation.

Fermentation technique

Solid-state fermentation technique was used as described by Mahin et al., 2008. Bagasse (3 g) was taken in 250-ml Erlenmeyer flasks and moistened with 10ml of 14% sucrose to set the desired moisture level. Each flask containing citric acid production medium was inoculated with 1 ml of spore suspension followed by mixing and incubation at 30°C in the a humidity-controlled incubator for 12 days for the estimation of citric acid produced and sugar consumed.

Fermentation of extracts

In all cases, the fermented materials were extracted with distilled water. Fermented sample extracts were collected every 48 hours for estimation of sugar and citric acid for a period of 12 days. Sugar concentration and citric acid produced by fermentation were estimated by the DNS method (Miller, 1959) and pyridine acetic anhydride method (Marrier and Boulet, 1958) respectively from 2 – 12 days. Total titratable acidity (TTA) was also estimated. In addition to these, dry weight of medium after fermentation was also carried out.The yield of citric acid was expressed as: weight of acid produced/weight of glucose used (Riviere, 1977).

Preparation of dinitrosalycyclic acid (DNS) solution

In the preparation of this solution 1416 ml of distilled water, 10.6 g of 3.5 DNS and 19.5 g of NaOH were dissolved gently in water bath at 80°C until a clear solution was obtained. Then 300 g Rochelle salt (sodium potassium tartrate), 7.5 ml of Phenol (melted at 60°C) and 8.3g of Sodium metabisulphate were added. After dissolving the above ingredients, the solution was filtered through a large coarse sintered glass filter and stored at room temperature in an amber coloured bottle to avoid photo oxidation.

Estimation of sucrose

Concentration of sucrose was estimated by DNS method. A single beam UV scanning spectrophotometer (Systronic made and model-117) was used for measuring colour intensity. One drop of

concentrated HCl solution was added to 1 ml of the sucrose solution in a flask. The flask was heated to 90°C for 5 minutes to allow hydrolysis. Three drops of 5 N KOH solution was added to neutralize the acid, because the DNS method must be applied in an alkaline condition to develop the red brown colour which represents the presence of reducing sugars. Then the DNS reagent was added and the colour intensity was measured at 540 nm using sucrose solution as a standard reference. The colour intensity is proportional to the concentration of sugar.

Sucrose salt was dissolved in appropriate quantity of distilled water to make various concentrations (1mM, 2mM, 4mM, 6mM, 8mM and 10mM) of sucrose solution. The DNS sucrose determination method was used and a blank was run in parallel replacing the sample solution with distilled water. The absorbance was measured at 540nm on a spectrophotometer. A graph of absorbance against the sugar concentration was plotted.

Estimation of citric acid

Citric acid is estimated gravimetrically, using pyridine– acetic anhydride method as reported by Marrier and Boulet (1958). One ml of the diluted culture filtrate along with 1.30 ml of pyridine was added in the test tube and swirled briskly. Then 5.70 ml of acetic anhydride was added in the test tube. The test tube was placed in a water bath at 32°C for 30 min. The absorbance was measured on a spectrophotometer (405 nm) and citric acid contents of the sample were estimated with reference (run parallel, replacing 1.0 ml of the culture filtrate with distilled water) to the standard which was set at 0 absorbance (100% transmission). Citric acid concentration was determined by referring to a standard curve for citric acid. Concentrations were expressed as grams per litre (g/L).

Standard curve for citric acid

Citric acid salt was dissolved in appropriate quantity of distilled water to make various concentrations of citric acid solution. The absorbance (measured at 405nm) for each concentration of the citric acid

solution was determined using the pyridine-acetic anhydride method. A graph of the absorbance and citric acid concentration was plotted.

Estimation of biomass (dry cell mass)

The dry cell mass was determined by filtering the fermentation media (which has been previously extracted with distilled water) through a pre-weighed filter paper. After filtration it was dried in an oven at 105°C for 2 hours, cooled in a desiccator and then the final weight was measured. The difference between initial and final weights was the biomass.

Results and discussion

Physico-chemical properties of sugarcane wastes

The physicochemical properties of the sugarcane wastes are reported in Table 1. The moisture content of the sugarcane wastes was 11.46%, crude fibre was 41.87% and the reducing sugar content was 13.67%.

Table 1: Physico-chemical properties of sugarcane wastes

Parameter	Values (%)
Moisture	11.46
Crude fibre	41.87
Crude fat	0.89
Crude protein	5.38
Ash	1.97
Nitrogen	0.861
Carbohydrate	72.85
Reducing sugar	13.67

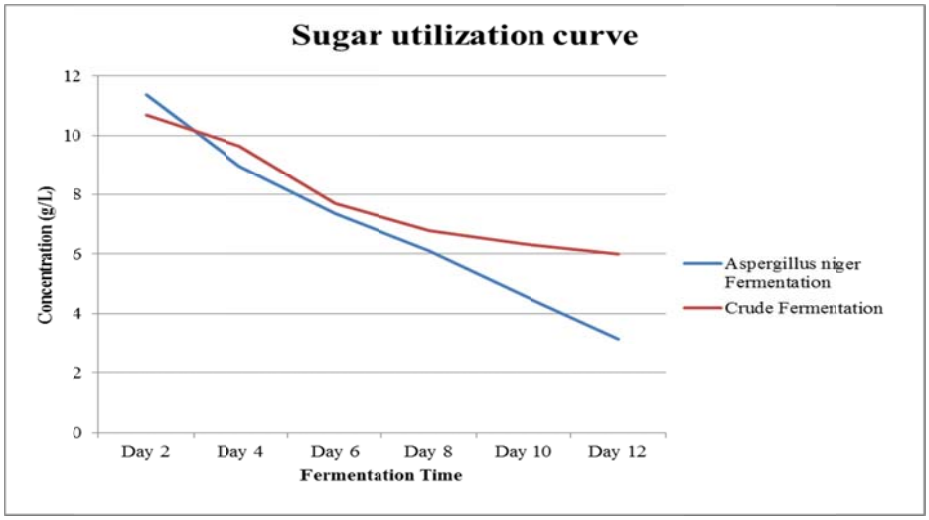

Figure 1: Sugar utilization at different days of fermentation

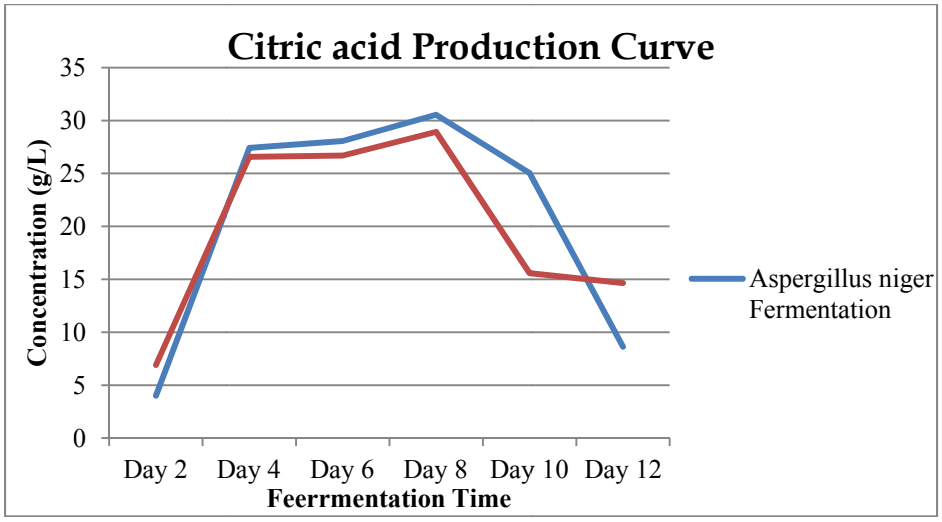

Figure 2: Citric acid production at different days of fermentation

Table 2: Weight of biomass at different days of fermentation during citric acid production

Day	Biomass weight (g) at different period of fermentation	
	Aspergillus niger fermentation	Crude fermentation
Day 2	3.44	3.28
Day 4	3.53	4.03
Day 6	3.71	3.72
Day 8	3.53	3.39
Day 10	3.50	3.26
Day 12	3.42	3.07

Analysis of fermentation extracts

Estimation of sugar: The concentration of sugar in the *Aspergillus niger* fermentation extract decreased from 11.35 g/L on Day 2 to 3.14 g/L on Day 12. The sugar concentration in the crude fermentation extract however, decreased from 10.67 g/L on Day 2 to 5.99 g/L on Day 12 Estimation of citric acid: The concentration of citric acid in the *Aspergillus niger* fermentation extract increased from 4 g/L to 30.54 g/L on Day 8 and then decreased to 8.63 g/L. in the crude fermentation extract, the concentration of citric acid increased from 6.91 g/L on Day 2 to 28.93 g/L on Day 8 and the decreased to 14.64 g/L.

Physicochemical properties of sugarcane wastes

The moisture determined represents physically bound water which may be lost during sun-drying or oven-drying. The high moisture content of the sugarcane was indicative of susceptibility to microbial attack which may diminish their value for citric acid production. Therefore, source segregated sugarcane waste should be subjected to drying to improve its shelf life. Sugarcane waste is a rich source of carbohydrate, fibres and minerals. Crude fibre measures the cellulose, hemicellulose and lignin content of the waste. Cellulose and hemicellulose (which are precursors to glucose) can be enzymatically hydrolysed and fermented. Thus, sugarcane waste (bagasse) can be a good substrate for fermentation in citric acid production (Joshi and Attri, 2006). The high reducing sugar content (13.67%) of the waste was also an indication of its suitability for use in citric acid production.

Development of organic acid

The sugar concentration in the substrate decreased gradually with fermentation time. The decrease in sugar concentration was accompanied by increasing citric acid production until the 8th day of fermentation. Despite similar trend of sugar utilization in both fermentation media, the sugar utilization rate was different in each media during citric acid production with *Aspergillus niger* and indigenous microorganisms in the bagasse. With the increase of fermentation time, the sugar concentration in the media was reduced and maximum reduction was found on day 12.

Aspergillus niger fermented sugarcane bagasse substrate showed greater sugar utilization rate during the fermentation period than in the crude fermentation. The residual sugar concentration of *Aspergillus niger* fermented sugarcane bagasse was 3.14 g/L while the crudely fermented sugarcane bagasse had a sugar concentration of 5.99 g/L on day 12 of fermentation (Figure 1).

Aspergillus niger fermentation had a slightly higher citric acid production than crude fermentation. The concentration of citric acid increased with increasing fermentation time until eight days (Figure 2). The maximum citric acid production was 30.54 g/L for *Aspergillus niger* fermentation and 28.93 g/L for crude fermentation on the 8th day of fermentation. This finding was in agreement with those of Lofty *et al*., (2007) who reported that maximum productivity of citric acid was obtained after 8 days of fermentation period.

Citric acid, an intermediate product in the tricarboxylic acid is the most important metabolic product produced commercially by fermentation with specific moulds but *Aspergillus niger* remains the organism of choice for citric acid production (Ali *et al*., 2001). Result of citric acid production shows a similar acid production capacity of the strain-specific *Aspergillus niger* and the indigenous microflora from the fermentation of sugarcane bagasse. Therefore the use of crude fermentation is a cost effective option for the production of citric acid.
Initial dry weight of sugarcane bagasse used as the substrate for citric acid production was 3 g. The biomass increased up to a peak and then

decreased. The increase and subsequent decrease of the biomass during fermentation seemed to follow a similar trend as that of the citric acid concentration produced. The maximum biomass however for both *Aspergillus niger* fermentation and crude fermentation was recorded on Day 6. The biomass content was highest (4.03g) in the Crude fermentation media.

The biomass increase of substrate of solid-state fermentation for citric acid production is mainly due to production of mycelial body and their sporulation (Mahin et al., 2008). Biomass is a fundamental parameter in the characterisation of microbial growth. Its measurement is essential for kinetic studies on SSF. Direct determination of biomass in SSF is very difficult due to the problem of separating the microbial biomass from the substrate. This is especially true for SSF processes involving fungi, because the fungal hyphae penetrate into and bind tightly to the substrate. On the other hand, for the calculation of growth rates and yields, it is the absolute amount of biomass which is important (Raimbault, 1998).

Different raw materials have been used for the production of citric acid, but emphasis is mainly focused on sugary substrates as the organisms producing citric acid utilize simple sugars efficiently. (John et al., 2012).

Conclusion

The comparism of the citric acid production efficiency of *Aspergillus niger* and the indigenous microbial communinty in the sugarcane bagasse involved considering the variations in the amount of citric acid produced, amount of sugar utilised, total titratable acidity and biomass weight in both fermentation media. *Aspergillus niger* fermentation had a slightly higher citric acid production and faster sugar utilisation rate while crude fermentation had a higher total titratable acidity and peak biomass production.

Aspergillus niger fermentation had a 1.61 g/L higher citric acid production compared to the crude fermentation which shows its wide acceptance and suitability as reported by several previous studies. The high cost and technical skill however, required for the isolation of

fungus, screening of citric acid producing strain and fermenation of sugarcane waste (bagasse) is considered a limitation.

Therefore, the cheaply available substrate for fermentation and the low cost of the fermentation process in obtaining the crude fermentation extract using the indigenous microflora potentially makes its use more economically acceptable.

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