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Screening of Agricultural Wastes for Substrates in Oxalic Acid Production Using *Aspergillus niger*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The disposal and attendant problems associated with agro-wastes have remained a challenge to the environment. Three agricultural wastes (cassava whey, banana peels and groundnut shells) were collected from the Choba and Yam zone markets in Rivers State, Nigeria and screened for their potential as substrates in the formulation of fermentation media to produce oxalic acid. The inoculum for the study was isolated from the banana peels and identified using the megablast search for highly similar sequences from the NCBI non-redundant nucleotide database. The microbial load and proximate composition of the substrates were determined, and the fermentation media formulated. The organism used for the study was identified as *Aspergillus niger* MW188538. The results showed a total bacterial count of $9.5x10^4$ cfu/ml, 1.87×10^5 cfu/ml, and 4.0×10^4 cfu/g for cassava whey, banana peels and groundnut shell respectively. The carbohydrates of the cassava whey, banana peels and groundnut shells were 67.74 %w/v, 53.24%w/v and 38.8% w/v respectively. After 12 days of fermentation, the substrates from cassava whey, banana peels, groundnut shells accumulated 2.5 ppm, 1.8 ppm and 1.3 ppm of oxalic acid respectively. The study hypothetically indicates that agro-wastes could be utilized as media components for production of industrial organics.

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1. INTRODUCTION

With over 998 million tonnes of agricultural wastes produced annually, an increased clamour for the commoditization of these wastes has become more imperative [1]. Agricultural wastes simply refer to unwanted products generated during the cultivation, harvesting and processing of agricultural produce [2]. Prior to this time, most of the biomass and agro-wastes generated were burnt or disposed indiscriminately into the environment. This practice has shown to be eco-harmful with the attendant release of toxic pollutants into the atmosphere, lands and water bodies.

Nigeria currently produces about 25 million tonnes of wastes annually [3]. Moreso, current efforts aimed at diversifying revenue streams for federation have seen increased activities in the agro-economy. Thus, there has been a consequent increase in agro-waste generation and concrete steps must be taken for the recycling of these wastes.

Currently, there is an increase in the search for suitable substrates to produce organics because of the unsustainability and eco-unfriendliness of the chemical processes used hitherto. Many researchers have reported the use of agricultural wastes to produce industrial-important organic acids. For instance, lactic acid has been reported to be produced from inexpensive carbon sources such as barley hydrolysates, oat, liquefied cassava starch bagasse, and red lentil flour [4,5,6]. Citric and itaconic acids have also been reported to be produced by *Aspergillus* spp. using cassava peels as substrate [7].

In this study, three substrates (groundnut shell, cassava whey and banana peels) were screened for their potential to serve as fermentation medium to synthesize oxalic acid using *Aspergillus niger* strains.

2. MATERIALS AND METHODS

2.1 Substrate Preparation

The cassava whey (CW) utilized for this study was collected from local cassava processing plants within Choba, banana peels (BP) from Choba market and groundnut shell (GS) from yam zone market at Rumuokoro, all within Port Harcourt, Rivers State, Nigeria. The samples (banana peels and groundnut shells) were air dried for two weeks before they were ground into powdery forms for media formulation. The cassava whey was filtered with a fine cloth to remove particle before use in the media formulation.

2.2 Enumeration of Total Bacterial and Fungal Counts in Substrates

The enumeration of total aerobic heterotrophic bacteria was done using a ten-fold serial dilution of the powdery substrates in physiological saline up to 10^{-4} and plating out 0.1 ml of dilutions 10^{-2} and 10^{-4} in duplicate sets of nutrient agar using the spread plate technique [8]. Afterwards, the plates were incubated at 37^{o}C for 24 hours and the colonies counted at the end of the incubation period. The fungal counts were determined using the same spread plate technique after serial dilution of the substrates on Potato Dextrose Agar and incubated at 28^{o}C for 5 days.

2.3 Inoculum Isolation

The *Aspergillus niger* used in this study was isolated from the dried banana peels sample. The mould was identified using the molecular technique of Raper and Fennell [9] and Klich and Pitt [10], using the internal transcribed spacer sequence (ITS) region of the nucleotide sequence. The *A. niger* species was identified based on similarity of 99-100 % in NCBI. The culture was then preserved on Potato Dextrose Agar slants and stored at 4^{o}C for further studies.

2.4 Proximate Composition of Substrate

2.4.1 Moisture content

The amount of moisture in the banana peels and groundnut shells used as substrates were determined as described by AOAC [11]. The sample weights were initially taken and dried in a hot air oven at 101^{o}C for about 10 hours until constant weights were obtained. The difference between the initial and dry weights of the samples were used to determine the moisture content.

2.4.2 Ash content

The ash content of the three substrates was determined by heating the pre-weighed samples

in a furnace at 550^{o}C for six hours until constant weight was achieved. The cooled ashed samples were weighed and the difference in weights were used to determine the ash contents in the samples [11].

2.4.3 Crude lipids

The Soxhlet technique as described by AOAC [11] was utilized in determining the fat content in substrates. The fat content was extracted using petroleum ether boiled over the samples for 4 hours and the crude lipids left after evaporation of the solvent was calculated.

2.4.4 Crude protein content

The Kjeldahl method [11] was employed to determine the protein content. The percentage of protein was calculated using the conversion factor of 6.25 to convert total nitrogen to protein content.

2.4.5 Crude carbohydrates

The crude carbohydrates were determined by subtracting the sum of the fat content, protein content, ash content and moisture content from 100 [12].

2.5 Medium Formulation for Oxalic Acid Production

The media formulation adopted for the study was described by Strasser et al. [13] with slight modifications of the carbon source. Following aseptic techniques, the media is composed of carbon source (cassava whey, groundnut shells, banana peel, sucrose), $NaNO_3$ (1.5 g/l); KH_2PO_4 (0.5 g/l); $MgSO_4.7H_2O$ (0.025 g/l); KCl (0.025 g/l); yeast extract (1.6 g/l), [13] maintained with an agitation speed at 200 rpm.

2.6 Submerged Fermentation

Following the method of Adesina et al. [14] One hundred milliliters of the cassava whey were measured and transferred into a 250 ml conical flask and other micronutrients added as listed in the section above. Following the procedure of Singh et al., (2014), four grams of the banana peel and groundnut shell flour were weighed and transferred into a 250 ml flask containing 100 ml of distilled water and the micro nutrients earlier listed respectively. The pH of each medium was maintained at pH 6.0 using 1 N of HCl and 2 M of NaOH when necessary. The inoculum (5.0 % v/v) was aseptically inoculated into the flasks. The setups were incubated in a shaker incubator (GYROMAX Benchtop at 200 150 rpm) at 28^{o}C for 14 days and 10ml of samples were withdrawn every 48 hours for analysis.

2.7 Analytical Techniques

The oxalic acid concentration in the fermentation medium was determined through the catalytic kinetic spectrophotometric method described by Jiang et al. [15]. The principle is based on the catalytic effect of the redox reaction between dichromate and rhodamine B in sulphuric acid which was measured at wavelength of 555 nm. About 10 ml of the sample was withdrawn from the fermentation medium, filtered with Whatman No 1 filter paper. Subsequently, 1 ml of the filtrate was diluted with 100 ml of distilled water and the resulting solution was used for oxalic acid analysis. The analysis was done under condition of 0.05 mol $l^{-1}$of sulphuric acid, 0.03 mol l^{-1} potassium dichromate and $3.28x10^{-6}$ of rhodamine B at 90 ^{o}C for 8 min after which the calibration graph of oxalic acid was obtained.

The method of Saqib and Whitney [16] was adopted in the estimation of the reducing sugar content of the media at an interval of 2 days during fermentation. About 10 ml of the medium was withdrawn and centrifuged at 10,000 rpm at 23 ± 2 ^{0}C to separate the cells from broth. The supernatant after centrifugation was used to determine glucose contained in the remaining media using dinitrosalicylic acid (DNS) method with glucose as the standard. For the fungal biomass determination, dry cell weight was determined after re-suspending the cells in small amount of water and drying till constant mass at 65^{0}C [17].

3. RESULTS

The ITS sequence from the fungal inoculum produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database for *Aspergillus niger* (Figs.1-2).

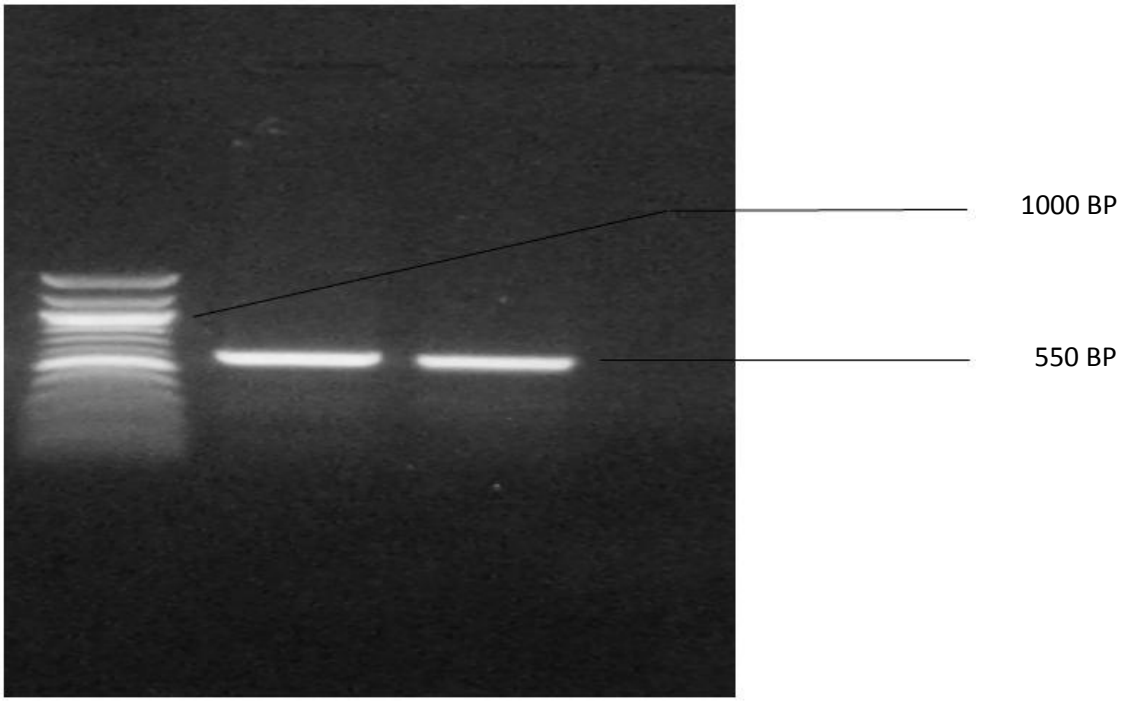

Fig. 1. Agarose gel electrophoresis of ITS gene of suspected *Aspergillus* sp isolate

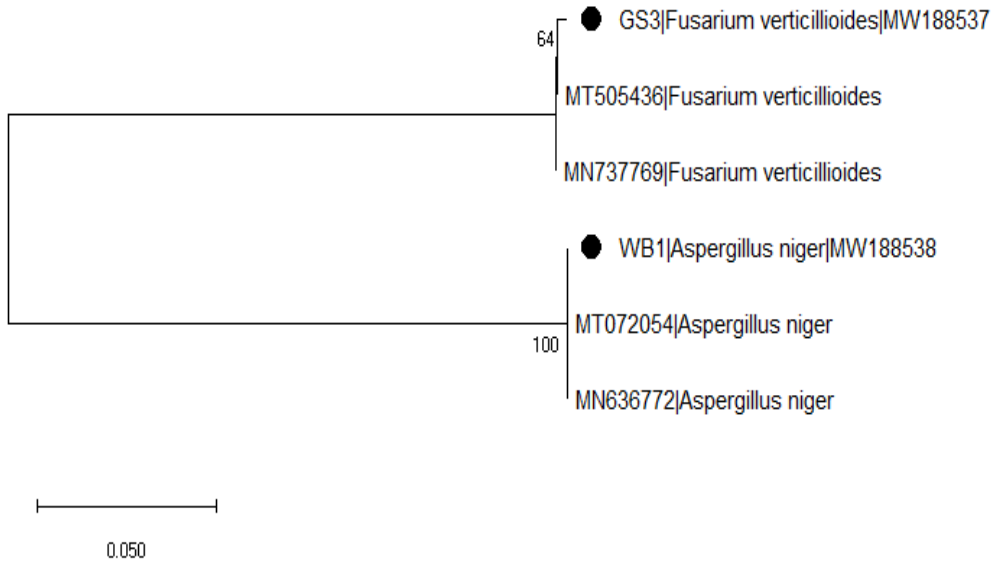

Fig. 2. Phylogenetic tree of isolated *Aspergillus niger*

The production of oxalic acid by *A. niger* using three agricultural wastes was investigated and studied for 14 days. The nutrient matrix of the wastes was initially studied by determining their proximate composition as shown in Table 1. The cassava whey (CW) was shown to have the highest amount of carbohydrates (67.74 %) followed by the banana peels (53.24 %) and the groundnut shells (38.8%). Conversely, the cassava whey reported the least amount of crude protein (1.18%) while the banana peels reported the highest amount (3.94%). Table 2 describes the total aerobic heterotrophic bacterial and fungal population of the substrates. While the cassava whey recorded bacterial and fungal counts of $9.5x10^4$ cfu/ml and $2.5x10^3$ cfu/ml respectively; the groundnut shells (GS) had $4.0x10^4$ cfu/g and $3.1x\ 10^2$ cfu/g for the bacterial and fungal counts respectively. The dynamics in the change of cell dry weight during the 2-weeks

fermentation is depicted in Fig. 3. The 12th day of fermentation recorded the highest cell dry weight amongst the substrate with the cassava whey as highest (1.06 mg/10ml), followed by the banana peels substrate (0.73 mg/10ml) and the groundnut shell with the least weight (0.13 mg/10ml). The change in the total sugar available in the substrates during fermentation are shown in Fig. 4. The cassava whey had the greatest steep in total sugar concentration during the fermentation decreasing from 61.2 ppm to 13.2 ppm. The GS substrate had the least decline in sugar available as it reduced from 4.75 ppm to 0.29 ppm. The oxalic acid produced during the fermentation was highest on the 12th day of the fermentation before a decline set in. The CW substrate accumulated the most oxalate (2.5 ppm) while the GS substrate had the least oxalate concentration (1.26 ppm) as shown in Fig. 5.

Table 1. Proximate composition of substrates

Samples	Cassava whey	Groundnut shells	Banana peels
Ash (%)	2.97	2.03	7.28
Moisture content (%)	25.04	7.43	5.28
Crude lipid (%)	0.94	0.79	17.21
Crude fibre (%)	2.13	48.56	13.05
Crude protein (%)	1.18	2.39	3.94
Carbohydrates (%)	67.74	38.8	53.24

Table 2. Total aerobic heterotrophic bacterial and fungal counts in substrates

Sample	Total bacterial count cfu/ml*,g#	Total fungal count cfu/ml*,g#
Cassava whey*	9.5×10^4	2.5×10^3
Banana peel#	1.87×10^5	1.9×10^2
Groundnut shell#	4.0×10^4	3.1×10^2

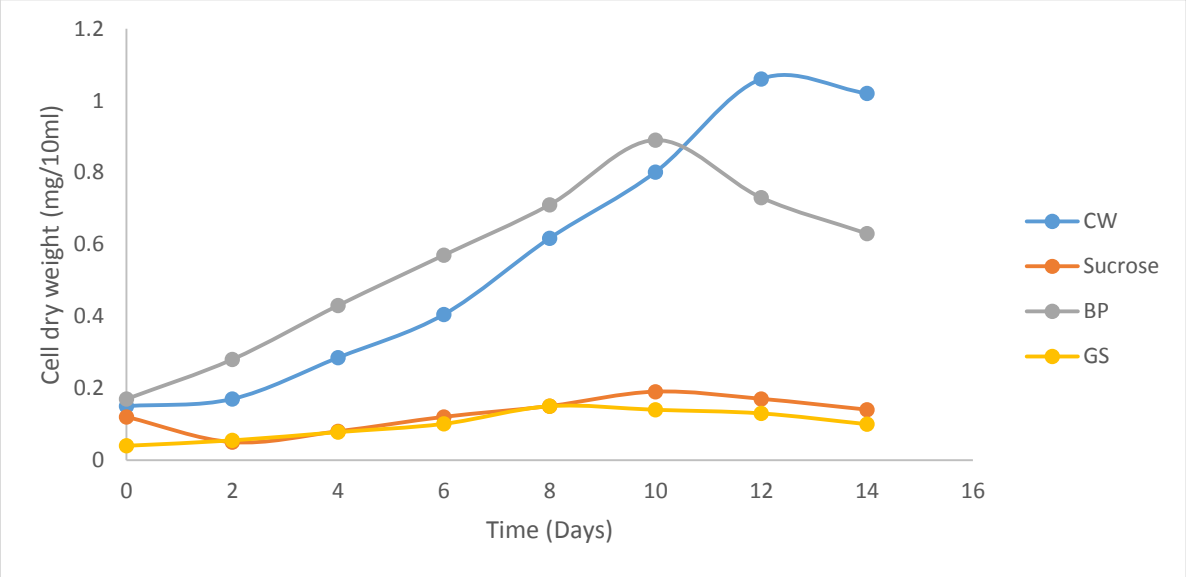

Fig. 3. Cell dry weight during fermentation

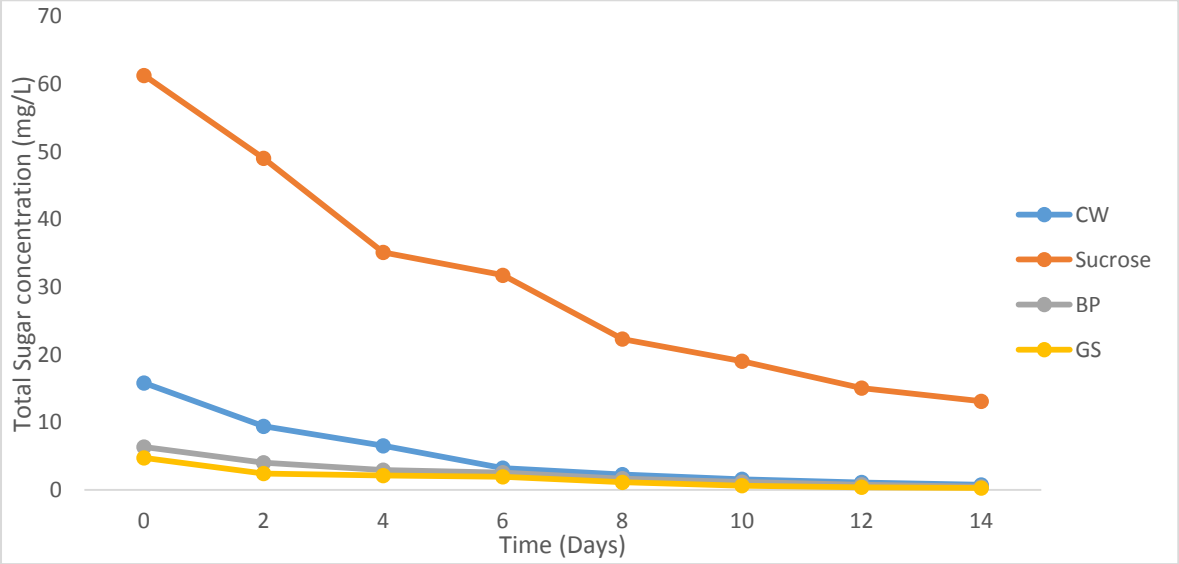

Fig. 4. Total sugar concentration during fermentation

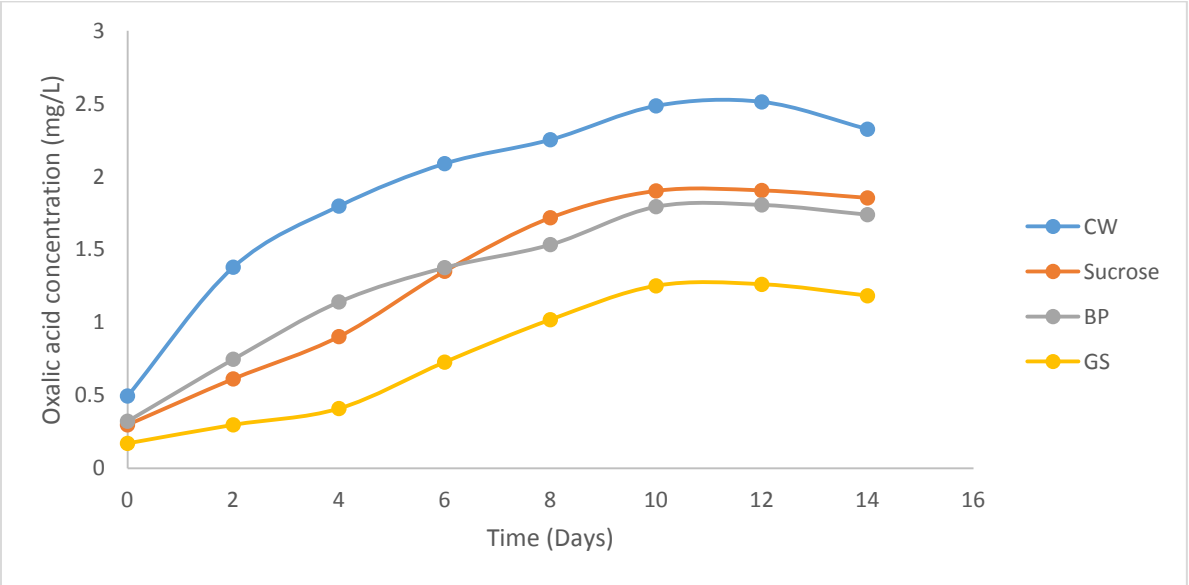

Fig. 5. Oxalic acid concentration during fermentation

4. DISCUSSION

The isolation of the fungus from the banana peels indicates the ubiquitous nature of *Aspergillus niger* in the environment especially in plant residues and soil [18]. The macroscopic and microscopic identification of the organism was based on colony pigmentation and the structure of the conidial head as described by Verweji et al., (2007) who reported that colonies of *A. niger* are carbon black with a dark globular conidial head. In addition to the conventional identification, the evolutionary distance computed using the Jukes-Cantor method agreed with the phylogenetic placement of ITS of the isolate within the *Aspergillus* sp and closely related to *Aspergillus niger* (MW188538).

The proximate composition of the banana peels agrees with the reports of Dhanasekaran et al., [19] and Sanker et al. [20] that banana peels contain substantial amounts of carbohydrates thus, can be a medium to produce inorganics. However, the carbohydrates content of the dried peels reported in this work (53.24%) differ from the reports of Hassan et al. [21] and Abubakar et

al. [22] who reported carbohydrate contents of 11.82% and 32.39% respectively. The difference could be attributed to the nature of the peels before the analysis was carried out. Asif-Ul-Alam et al. [23] reported slight difference in carbohydrate contents of oven dried and freeze-dried banana peels. In addition, the crude protein content (3.94 %) reported in the banana peel is similar to the oven dried banana peels (3.19 %) as documented by Asif-Ul-Alam et al. [23].

The nutritional value of the cassava whey shows some potentials to be used as fermentation medium and livestock feed [24], fertilizer [25]. The reported crude protein and ash contents in this research (Table 1) were higher than that of Aro et al. [24] with 2.46 % and 1.88 % respectively. The high carbohydrate present in the whey makes it a suitable source of energy for microorganism thus its use to produce industry important chemicals like ethanol [26].

The nutrient matrix of the groundnut shells as shown in Table 1 above reinforces its potential reuse in the environment. The shells have been used as medium for producing bioethanol [27,28]; enzyme production [29,30] and single cell protein [31]. The moisture and ash contents of the shell (Table 1) reported in this study is similar to Abdulrazak et al. [32] who reported 8% and 2.5% respectively. The carbohydrate and protein contents of the groundnut shells used in this study is lower than 63.52% and 12.14 % respectively documented by Omogbai et al. [33]. This difference may be due to the difference in varieties of the groundnut plants used for the studies [34].

The high microbial load reported in the substrates reaffirms the ubiquity of microbes in every environment. The largest heterotrophic bacterial count was reported in the banana peels waste and this could be attributed to increased handling by prospective buyers and the sellers of the fruit.

The steady increment of cell dry weight (Fig. 3) of the inoculum attests to the capacity of the inoculum to utilize the substrate as nutrients for growth (Abd el Rahim et al., 2016). The study shows that cassava whey is the cheapest medium amongst the substrates for growing *A. niger*. On the 12th day of the fermentation period, the cassava whey medium had a cell dry weight that is six times that of the sucrose (Fig. 3). Also, the steep reduction in the utilization of glucose during fermentation shows the ability of the organism to utilize the media for growth. After the first 4 days of fermentation, more than 50 percent of the reducing sugars present in the substrate have been utilized by the inoculum (Fig. 4). This report agrees with Favela-Torres et al. [35] who posited that about 50% of glucose was utilized by *A. niger* after 80 hours in a submerged fermentation. The oxalate accumulation in the substrates as shown in Fig. 5 above describes their potential as media for oxalate production. The following is the order of the production by the substrates in descending order: CW>S>BP>GS. The reported value of the highest substrate (2.5 mg/L) differs with the report of Adesina et al. [14] and Betiku et al., [36]. While the former reported a yield of 103.26 g/L from sweet potato starch hydrolysate, the latter reported an oxalate yield of 122.68 g/L from cashew apple juice. The significant difference could be attributed to the difference in substrates of choice, the strain of the organism utilized and the optimization of the processes. Pre-treatment of substrates prior to fermentation have been reported to increase yield. It is desirable to pretreat raw substrates as well; this would increase the bioavailability of essential nutrients [37,38].

5. CONCLUSION

The results from this study showed the potential of agro-wastes (cassava whey, groundnut shells, banana peels) to serve as potential feedstock for the production of inorganics. Furthermore, the study indicates the ability of *Aspergillus niger* to serve as inoculum for synthesis of oxalic acid.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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