Original Research Article

PRODUCTION OF BIOETHANOL FROM SELECTED LIGNOCELLULOSIC AGROWASTES

ABSTRACT

This study evaluated the ability of cassava peels, banana peels, orange peels and corn cobs hydrolysates to produce bioethanol. Fibre fractions analysis was carried out using standard methods. The samples were pretreated with acid and base, followed by simultaneous saccharfication and fermentation (SSF) for bioethanol production. During fermentation, pH, total titratable acidity, reducing sugar, microbial load and bioethanol yield were determined. The reducing sugar yield for *Aspergillus niger* and *Bacillus cereus* were 30.28g and 13.35g for corn cobs. The pH was observed to decrease during fermentation period with orange peels having the lowest pH of 2.6 after 240 hours of fermentation using *A. Niger* and *S. cerevisiae,* when *B. cereus* and *S. Cerevisiae* were used the pH was observed to be 4.10. Total titratable acidity showed increase in all the substrates, with corn cobs having the highest when *B. cereus* and *S. Cerevisiae* were used (1.62), followed by cassava peels when *A. niger* and *S. cerevisiae* were used (1.52). Highest ethanol yield following simultenous saccharfication and fermentation with *A. niger* and *S. cerevisiae* was obtained in corn cobs with 17.43g/100g, while orange peels gave the lowest with 8.02g/100g, the ethanol yield from each substrates as well as the combined substrates were significantly different at $p \leq 0.05$. The combined substrates (1:1:1:1) gave the

highest ethanol yield of 12.44g/100g using *A. niger* and *S. cerevisiae.* This study therefore revealed that *A. niger* had the highest bioethanol yield using corn cobs as the carbon source, therefore it could be used for mass bioethanol production.

Key words: simultaneous saccharification, bioethanol, agrowastes, titratable acidity, reducing sugar

INTRODUCTION

Agricultural wastes have become an alternative raw material for bioethanol production, to prevent competition between food security and ethanol production that the initial use of food crops for bioethanol has caused. lignocellulosic biomass can easily be utilized for biofuel compared to food crops and holds the key to supplying society's basic needs for sustainable production of liquid transportation fuels without impacting the nation's food supply (Alexander *et al.,* 2012).

Lignocellulosic biomass is a major component of plants that provides them structure and is usually present in stalks, leaves and roots. Lignocellulosic biomass consists mainly of three types of polymers: Cellulose (30% - 60%), hemicelluloses (20% - 40%) and lignin (10% - 25%) which are interlinked to each other in a hetero-matrix (Nanda *et al.,* 2014). Approximately 90% of dry matter lignocellulosics consists of cellulose, hemicelluloses and lignin, whereas the rest comprises of ash and extractives (Rosa *et al.,* 2013). Composition of lignocellulosic

biomass is influenced by the plant's genetic and environmental factors that vary considerably; however, the relative abundance of cellulose, hemicelluloses and lignin depends on type of biomass and varies in different lignocellulosic biomass (Sanchez, 2009).

Cellulose $(C_6H_{10}O_5)_n$ is a homopolysaccharide composed of linear chains of β-D-glucose units linked by β-1, 4 glycosidic bond. These chains are linked by strong hydrogen bonding which forms the cellulose chains into microfibrils, making it crystalline in nature. These microfibrils are bundled together to form cellulose fibres. Cellulose is made up of crystalline structure which is resistant to degradation and amorphous region which is easy to degrade (Agbor *et al.,* 2011). The cellulose fibres are embedded in an amorphous matrix of hemicelluloses, lignin and pectin. Lignin and hemicellulose are present in the space between cellulose microfibrils in primary and secondary cell walls and middle lamellae (Van and Pletschke, 2012).

Hemicelluloses are the branched heteropolymers consisting of pentose sugars (D-xylose and L-arabinose) and hexose sugars (D-mannose, D-glucose and D-galactose) with xylose being most abundant (Juturu and Wu, 2012). Hemicelluloses are composed of xylan, mannan, arabinan and galactan as main heteropolymer (Beg *et al.,* 2001). Xylan is the major structural component of the plant hemicelluloses and it is the second most abundant renewable polysaccharide in nature after cellulose. Xylan represents approximately one-third of all the renewable organic carbon on earth (Collins *et al.,* 2005). Xylan is a complex polysaccharide consisting of a backbone of xylose residues connected by β-1, 4-glycosidic linkage along with traces of L-arabinose. The xylan layer with its covalent interaction to lignin and its non-covalent linkage with cellulose may be essential in maintaining the integrity of cellulose in situ and in protecting the cellulosic fibers against degradation to cellulases (Beg *et al.,* 2001).

Lignin is an aromatic polymer, consisting of phenyl propane units which are organized in to a large three dimensional network structure. Lignin acts as glue and fills up the gap between and around cellulose and hemicelluloses in lignocellulosic biomass which binds them tightly. Lignin is an amorphous heteropolymer which makes the cell wall impermeable, resistant against microbial and oxidative attack (Shahzadi *et al.,* 2014). The presence of lignin in lignocellulosic biomass makes difficult the release of monomer sugars from holocellulose (Nanda *et al.,* 2014).

Extractives are low molecular weight and non-structural components of lignocellulosic biomass which are soluble in neutral organic solvents or water. Extractives consist of biopolymers such as terpenoids, steroids, resin acids, lipids, waxes, fats, and phenolic constituents in the form of stilbenes, flavanoids, tannins, and lignans. Generally, percentage of extractives is higher in leaves, roots and bark compared to steam wood (Zhao *et al.,* 2012); (Nascimento *et al.,* 2013).

Bioconversion potential of lignocellulosic biomass from grasses, crop residues, forestry waste, and municipal solid waste into various value added biological and chemical products is very essential and achievable. Accumulation of lignocellulosic biomass in large quantities presents a disposal problem which results not only in deterioration of environment but also loss of valuable materials. This lignocellulosic biomass can be used in paper manufacture, animal feed, biomass fuel production, and composting (Sanchez, 2009). Biotechnological transformation of lignocellulosic biomass can make significant contribution for the production of organic chemicals. Over 75% of organic chemicals are synthesized from five primary base chemicals which are ethylene, propylene, toluene, xylene and benzene (Howard *et al.,* 2003). These lignocellulosic biomass resources can also be used to produce various organic chemicals such as ethanol (Oberoi *et al.,* 2010), acetone (Amiri *et al.,* 2014), butane (Al-Shorgan *et al.,* 2012), bio-methane (Song *et al.,* 2013) etc. Aromatic compounds might be produced from lignin whereas

the low molecular weight aliphatic compounds can be derived from ethanol produced by fermentation of sugars (glucose, mannose and xylose) generated from saccharification of lignocellulosic biomass (Howard *et al.,* 2003). Biotechnological conversion of lignocellulosic biomass in various industrial products is cost effective and environmentally sustainable.

Lignocellulosic biomass are recalcitrant against enzymatic attack therefore, a pretreatment step is required which makes lignocellulosic biomass suitable for fermentation. Lignocellulosic biomass-derived sugars are economically attractive feedstock for large scale fermentation of different chemicals. Sugars released after hydrolysis of cellulose and hemicelluloses are converted into different industrial products like ethanol, butanol, glycerol, organic acids.

MATERIALS AND METHODS

Collection of samples

One thousand (1,000) grams each of fresh orange peels, cassava peels, banana peels, and corn cob were collected from Federal University of Technology, Akure (FUTA) farm and Oba Market in Akure South Local Government, Ondo State, located in south-west Nigeria. Akure lies about 70°15 North of the equator and 50°15 East Meridian. The city has a population of 588,000 which is 0.305% of Nigeria population based on 2006 population census. The samples collected from these locations were then sundried for three days after which they were milled. The dried samples were divided into two portions; the first portion was pretreated while the second was not.

Pretreatment of Samples

A two - stage process which combines the dilute acid pre-hydrolysis (DAPH-100-121) and alkaline delignificaton using NAOH as described by Olugbenga and Ibileke (2011) was used. Dry samples were treated with dilute sulfuric acid which involved the use of 1.25% (w/v) H_2SO_4 solution in a 1: 8, g : g, solid : liquid ratio. The one step dilute acid pre-hydrolysis (DAPH-100-121) was performed in an autoclave at 121^0C for 17min, after which the solids were collected and drained. The solids were then treated with 2% (w/v) sodium hydroxide solution in a solid: liquid ratio of 1: 20, g: g, at 120^0C for 90 min. after that, the residual solid material (Cellulose pulp) separated by filtration was washed with water to remove the residual alkali, and was dried at 50 ± 5^{o}C for 24 hours.

Sterilization, Preparation of Culture Media and Isolation

All glass wares (Petri dishes, beakers, conical flasks) were washed thoroughly, air dried, sterilized in hot oven around 180^{o}C for 2 hours. Nutrient agar (NA) and Potato dextrose agar (PDA) were prepared according to manufacturer's specifications and autoclaved at 121^{o}C for 15 minutes and allowed to cool to 45^{o}C before pour plating.

Six fold serial dilutions was carried out on collected agro waste samples and pour plated with molten nutrient agar and the potato dextrose agar media, cooled to 45^{o}C. Nutrient agar plates were incubated at 37^{o}C for 24 hours for bacteria and 28^{o}C for 3 to 5 days for fungi on

potato dextrose agar plates respectively in triplicate before examination for microbial growth. The bacterial isolates were purified by streaking on fresh sterile nutrient agar before sub culturing. Fungal isolates were also sub cultured to obtain pure isolates. The pure isolates were stored temporarily on slants and kept at 4^{o}C for further use (Fawole and Oso, 2012). Colony count was carried out on plates (in triplicates) by using colony counter and expressed as colony forming unit for bacteria and spore forming unit for fungi respectively.

Starch hydrolysis test

This test was used to detect the ability of bacterial isolates to produce starch degrading enzymes. It was performed for fungi isolates also. Nutrient agar and potato dextrose agar were both prepared with 1% soluble starch for bacteria and fungi respectively. The media was sterilized, poured into sterile petri-dishes and allowed to solidify. Bacterial isolates were inoculated onto the surface by streaking after which incubation at 37^{o}C for 24 hours, while fungi isolates were inoculated by stabbing followed by incubation at ambient temperature for 3 days. After incubation, the plates were flooded with iodine; positive results were indicated by a clear zone around the colony which implies that starch was hydrolyzed, while a blue black coloration indicated a negative result (Fawole and Oso, 2001).

Determination of cellulose, Hemicellulose and lignin

The method of AOAC (2012) was used as described by Ververis *et al*. (2002). The substrates were analyzed for cellulose, hemicellulose and acid insoluble lignin which were done before and after pretreatment. Cellulose was determined using a colorimetric method with the

anthrone reagent. Ground samples were treated and boiled at 100^0C with a mixture of nitric/acetic acid (1: 8, v/v) for 1 hr to remove lignin, hemicelluloses and xylosans after successive cetrifugations, and diluted with 67% H_2SO_4 (v/v). Cellulose was then determined at 620nm using cold anthrone reagent.

Hemicellulose and lignin contents of the substrates were determined as follows; the residue from above containing Hemicellulose and lignin was then boiled with 5 ml of 72% (w/w) H_2SO_4 solution for 4.5 hours in order to hydrolyze the hemicellulose. The suspension remaining after the above treatment was filtered through a crucible and the solid residue dried at 105^0C for 24 hours and weighed (W1). The residue was then transferred to a preweighed dry porcelain crucible and heated at 600^0C for 5 hours. After cooling down, it was weighed (W2). Acid insoluble lignin was then calculated by the difference (W1-W2).

The filtrate from the H_2SO_4 treatment that contained the sugars released from hemicellulose was thoroughly stirred and homogenized. Glucose (C1) and reducing sugar (C2) concentrations in the filtrate were determined. Following these measurements, the hemicellulose content was then calculated from the following equation:

$$\%\left(\frac{w}{w}\right) hemicellulose = \left\{\frac{W}{S}\right\} \times (C2 - C1) X \left\{\frac{V}{M}\right\} X\ 100 \quad \text{..... Equation 1}$$

Where; W= molecular weight ratio of the polymer and monomer pentose, S= saccharification yield, C2= determined reducing sugars concentration (g/L), C1= glucose concentration (g/L), V= total volume of sugar solution (L), M = dry weight of the sample (g).

Microbial hydrolysis

One hundred (100) grams of each pretreated substrates was weighed in duplicates into 1000ml conical flasks and made up to mark with distilled water, corked and sterilized at 121^0C for 15 min. sterile distilled water was added to the flasks to final volume 1 liter and the flasks plugged with sterile cotton wool. After cooling, the medium was inoculated with 50ml of 36 hours culture of *Aspergillus niger* and *Bacillus cereus* separately; the pH of the medium was then adjusted to 5.0. Hydrolysis was carried out at room temperature for three days. A second un-inoculated flask served as control. Samples were taken at the end of three days for reducing sugar determination (Abdullahi, 2013).

Determination of reducing sugar

The method of Olugbenga and Ibileke (2011) was used. Two mls of the hydrolyzed sample was placed in a test- tube and 1g of activated charcoal was added. The mixture was shaken thoroughly. The mixture was then filtered with filter paper until a colorless filtrate was obtained. One ml of filtrate was placed in a test-tube and two drops of alkaline DNS reagent were added and the tube was placed in boiling water for 5 min. the mixture was allowed to cool and the absorbance was measured at 540nm. This measurement was taken after three days. A standard curve of glucose was prepared and used to calculate the percentage reducing sugar.

Physicochemical analysis

The following physicochemical properties of each fermenting substrate were measured;

Determination of pH

The pH of each fermenting substrate was measured at 24 hours interval for seven days using a digital pH meter, standardized with buffer of 7.0 the pH was then determined by inserting the electrode bulb into a sample from each fermenting substrate.

Total titratable acid

This was determined using the method of Lyumugabe et al. (2010) 10ml of the fermenting medium was transferred into a beaker, followed by the addition of 3 drops of phenolphthalein indicator. The sample was then titrated against 0.1M NaOH to an end point of a definite pink colour. The volume of NaOH used was noted and the titratable acid percentage was calculated using the following formula;

TTA (%) = V x 0.15 .....equation 2

Where; V = Volume of NaOH.

155 **Preparation of inoculum**

156 *Aspegillus niger, Bacillus cereus* and *Saccharomyces cerevisiae* inocula were prepared by introducing slant cultures to 150ml of sterile
157 growth media contained in 500ml conical flasks. The flasks were incubated on a rotary shaker at 30^0C for 96 hours (Ado *et al.,* 2009).

158 **Standardization of inoculum (McFarland Turbidity standard)**

159 Method modified by Cheesbrough (2006), was used to prepare the McFarland 0.5 turbidity standard which was used to measure the
160 density of microbial cells. In this method, fifty milliliter (50ml) of a 1.175% (wt/vol) dehydrates Barium chloride ($BaCl_2.2H_2O$) solution was
161 added to 99.4ml of 1% (vol/vol) sulfuric acid. McFarland standard tube was then sealed with Paraffin to prevent evaporation and stored in the
162 dark at room temperature. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer with a 1cm
163 light path. The 0.5 McFarland standards were vigorously agitated before use

164 **Fermentation**

165 Five sets of liquid state fermentation were carried out using the pretreated hydrolyzed samples. The hydrolysates from the above were
166 transferred into another set of conical flasks and labeled correctly, covered, autoclaved at 121^oC for 15 minutes and allowed to cool. The flasks
167 were inoculated with *Saccharomyces cerevisiae* to carry out fermentation for ten days. The fermentation was then monitored from day 1, the pH
168 of the hydrolysate containing *Saccharomyces cerevisiae* was adjusted to 5.0 and fermentation carried out at 30^oC in a rotary shaker. The ethanol

yield was determined at 24 hours interval during fermentation. The fermentate was separated by centrifugation at 9000 rpm to separate the waste from the supernatant (Abdullahi, 2013). All procedures were carried out in triplicates.

Distillation

It was carried out using a set up distillation apparatus. The fermented liquid was transferred into round bottom flask and placed on a heating mantle fixed to a distillation column enclosed in a running tap water. Another flask was fixed to the other end of the distillation column to collect the distillate at 78^{o}C (standard temperature for ethanol production). Ethanol yield was then determined by obtaining the mass of the distillate in grams. Percentage ethanol was then determined by obtaining the specific gravity of the ethanol produced and using it to calculate the percentage (v/v) ethanol produced (Abdullahi, 2013)

Statistical analysis

Data are presented as mean ± standard error (SE). Significance of difference between different treatment groups was tested using one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Science) version 20 software. For all tests, the significance was determined at the level of $P \leq 0.05$.

RESULTS

Effect of acid pretreatment on cellulose, hemicellulose and lignin of the agricultural wastes.

Table 1 shows the effect of pretreatment on the cellulose; hemicellulose and lignin components of cassava peels, orange peels, banana peels and corn cobs. The result indicates that there was significant difference ($p \leq 0.05$) in the effect of acid pretreatments of the substrates. There was high increase in cellulose content of corn cobs from 39.39% to 59.21%, while cassava peels showed an increase from 12.66% to 20.66%, orange peels also showed cellulose content increment after pretreatment from 13.64% to 17.06% and banana peels which had the lowest showed an increase from 2.09% to 9.43%. Hemicellulose content on the other hand decreased after pretreatment in cassava peels from 8.28% to 3.11%, in banana peels from 11.46% to 1.33%, in orange peel from 6.29% to 4.23% and in corn cob from 43.34% to 16.95%. Lignin content of corn cobs reduced drastically from 16.3% to 6.23%, similar decrease was also recorded for the lignin content of cassava peels, banana peels and orange peels.

Reducing sugar produced by each substrates after 3 days of hydrolysis using *Aspergillus niger* and *Bacillus cereus*.

The reducing sugar produced by each substrate as well as the combinations of the substrates in ratio 1: 1: 1: 1 after three days of hydrolysis using *Aspergillus niger* is given in Figure 1. The result revealed that highest reducing sugar yield was obtained in corn cobs with 30.28g, followed by cassava peels with a yield of 26.36g, combinations of all the substrates (OCBC) gave a yield of 21.62g, and banana peels also gave a reducing sugar yield of 20.32g, while orange peels had the lowest with 16.23g.

Furthermore, figure 1 also shows the reducing sugar yield of each substrates and combinations of the substrates in ratio 1: 1: 1: 1 after three days of hydrolysis using *Bacillus cereus*. However, the yield was considerably lower than what was obtained using *Aspergillus niger*.

Corn cobs gave the highest reducing sugar yield with 13.35g, followed by cassava peels with 11.14g, combinations of all the substrates (OCBC) gave a yield of 9.34g, and banana peels also gave a reducing sugar yield of 8.44g, while orange peels had the lowest with 5.88g.

Table 1: Effect of acid pretreatment on cellulose, hemicellulose and lignin of the agricultural wastes

Parameter	**CpB (%)**	**CpA (%)**	**BpB (%)**	**BpA (%)**	**OpB (%)**	**OpA (%)**	**CcB (%)**	**CcA (%)**
Lignin	9.34 ± 0.04^{b}	4.18 ± 0.02^{c}	12.23 ± 0.02^{c}	2.35 ± 0.01^{b}	2.25 ± 0.02^{a}	$1.19\pm.03^{a}$	16.34 ± 0.01^{d}	6.23 ± 0.02^{d}
Hemicellulose	8.28 ± 0.04^{b}	3.11 ± 0.00^{b}	11.46 ± 0.04^{c}	1.33 ± 0.03^{a}	6.29 ± 0.13^{a}	4.23 ± 0.02^{c}	43.34 ± 0.06^{d}	16.95 ± 0.0^{d}
Cellulose	12.66 ± 0.01^{b}	20.66 ± 0.30^{c}	2.09 ± 0.03^{a}	9.43 ± 0.022^{a}	13.64 ± 0.01^{c}	17.0600 ± 0.03^{b}	39.39 ± 0.08^{d}	59.21 ± 0.02^{d}

Key: **CpB (%)** = Cassava peels before pre-treatment, **OpB (%)** = Orange peels before pre-treatment, **BpB (%)** = Banana peels before pre-treatment

CcB (%) = Corn cob before pre-treatment, **CpA (%)** = Cassava peels after pre-treatment, **OpA (%)** = Orange peels after pre-treatment, **BpA (%)** = Banana peels after pre-treatment, **CcA (%)** = Corn cob after pre-treatment.

Values are means ± Standard error of agricultural wastes. Values in the same row carrying the same superscript are not significantly different at ($p \leq 0.05$) using Duncan's New Multiple Range test.

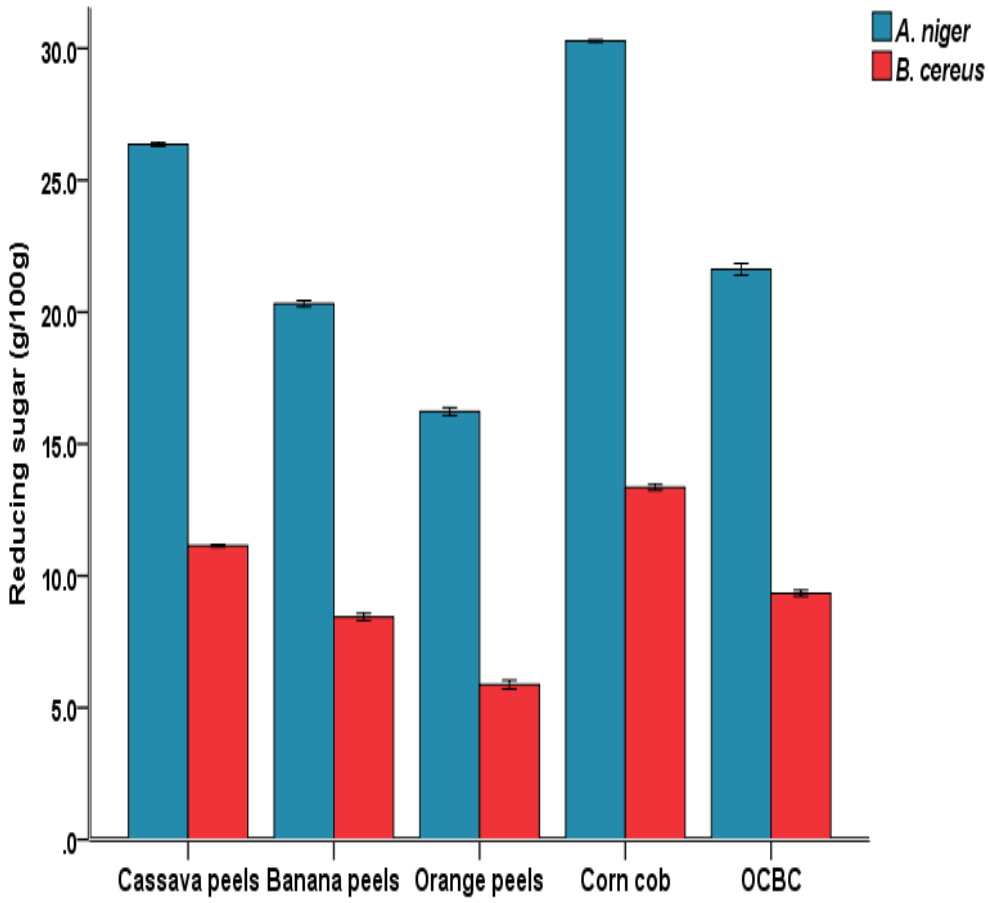

Fig 1: Reducing sugar produced by each substrates after 3 days of hydrolysis using *A. niger* and *B. cereus* respectively.

Bars represent reducing sugar (g/100g) ± standard error, significant difference were taken at ($P \leq 0.05$) according to Duncan's New Multiple Range tests.

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

Changes in pH during fermentation of different agricultural wastes using *A. niger* and *S. cerevisiae.*

The changes in pH during the fermentation of cassava peels, banana peels, orange peels, corn cobs and the combinations of all the substrates in ratio 1: 1: 1: 1(OCBC) using *A. niger* and *S. cerevisiae* are represented in Figure 2. A general decrease in the pH was observed from the initial standardized pH of 5.0 as fermentation proceeded. Fermentation of orange peels showed a decrease, with a pH of 3.0 after 7 days, cassava peels with a final pH of 4.0, banana peels with a pH of 4.0 after 7 days, and corn cobs with a final pH of 3.6. The combinations of all the substrates in ratio 1:1:1:1 (OCBC) showed a decrease from the initial pH of 5.0 to 3.0 after 7 days of fermentation.

Changes in pH during fermentation of different agricultural wastes using *B. cereus* and *S. cerevisiae.*

Figure 3 showsthe changes in pH during the fermentation of cassava peels, banana peels, orange peels, corn cobs and the combinations of all the substrates in ratio 1: 1: 1: 1(OCBC) using *B. cereus* and *S. cerevisiae*. A decrease in the pH was observed from the initial standardized pH of 6.0 as fermentation proceeded. Fermentation of corn cobs showed a decrease with a final pH of 4.8 after 8 days, cassava peels recorded a decrease with a final pH of 4.2, with the combinations of all the substrates in ratio 1:1:1:1 (OCBC) having a decrease from the initial pH of 6.0 to 4.2, while orange peels had the lowest final pH of 4.0. However, a slight fluctuation was observed from day 4.

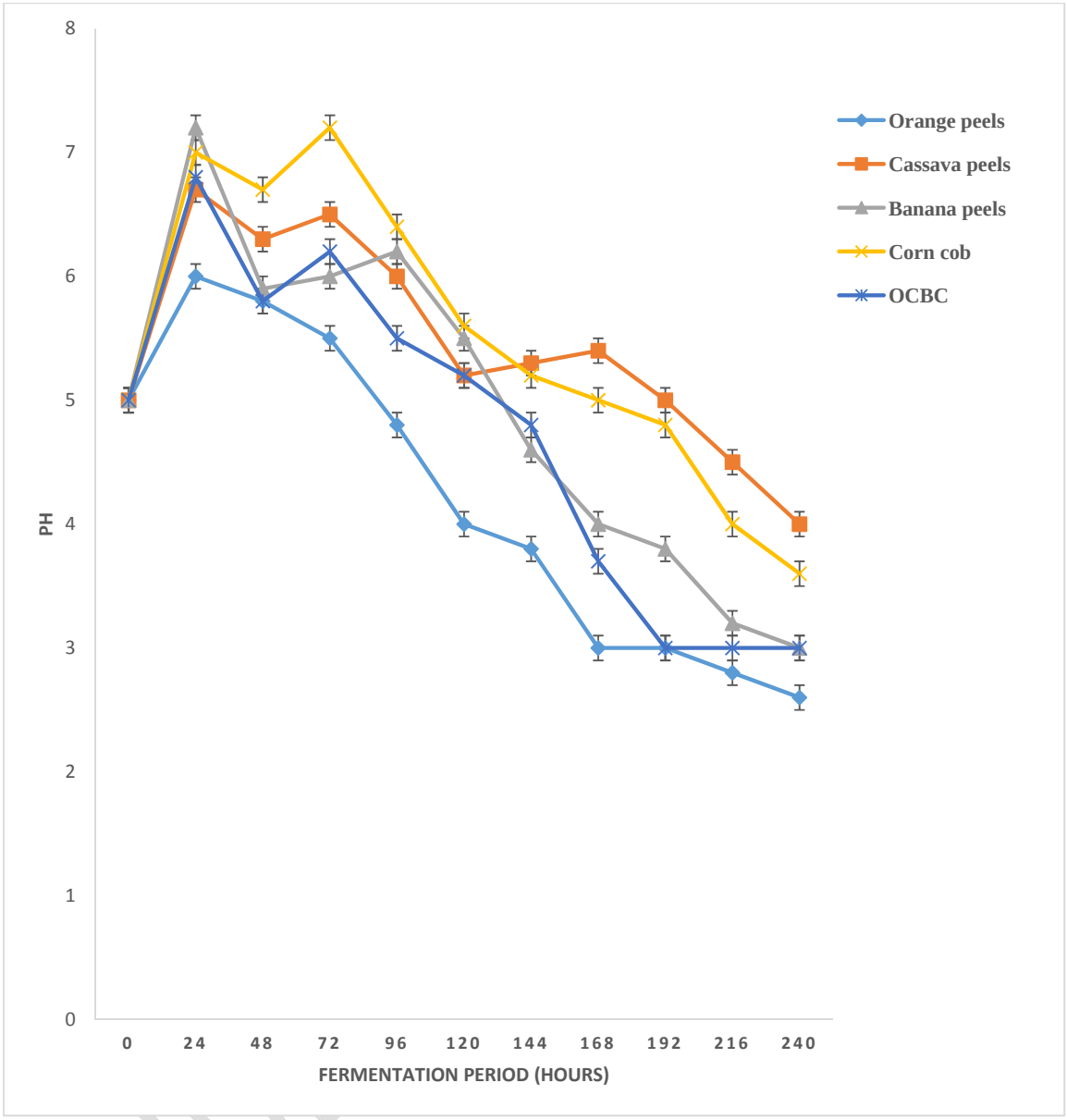

Fig 2: Changes in pH during fermentation of different agricultural wastes using *A. niger* and *S. cerevisiae.*

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

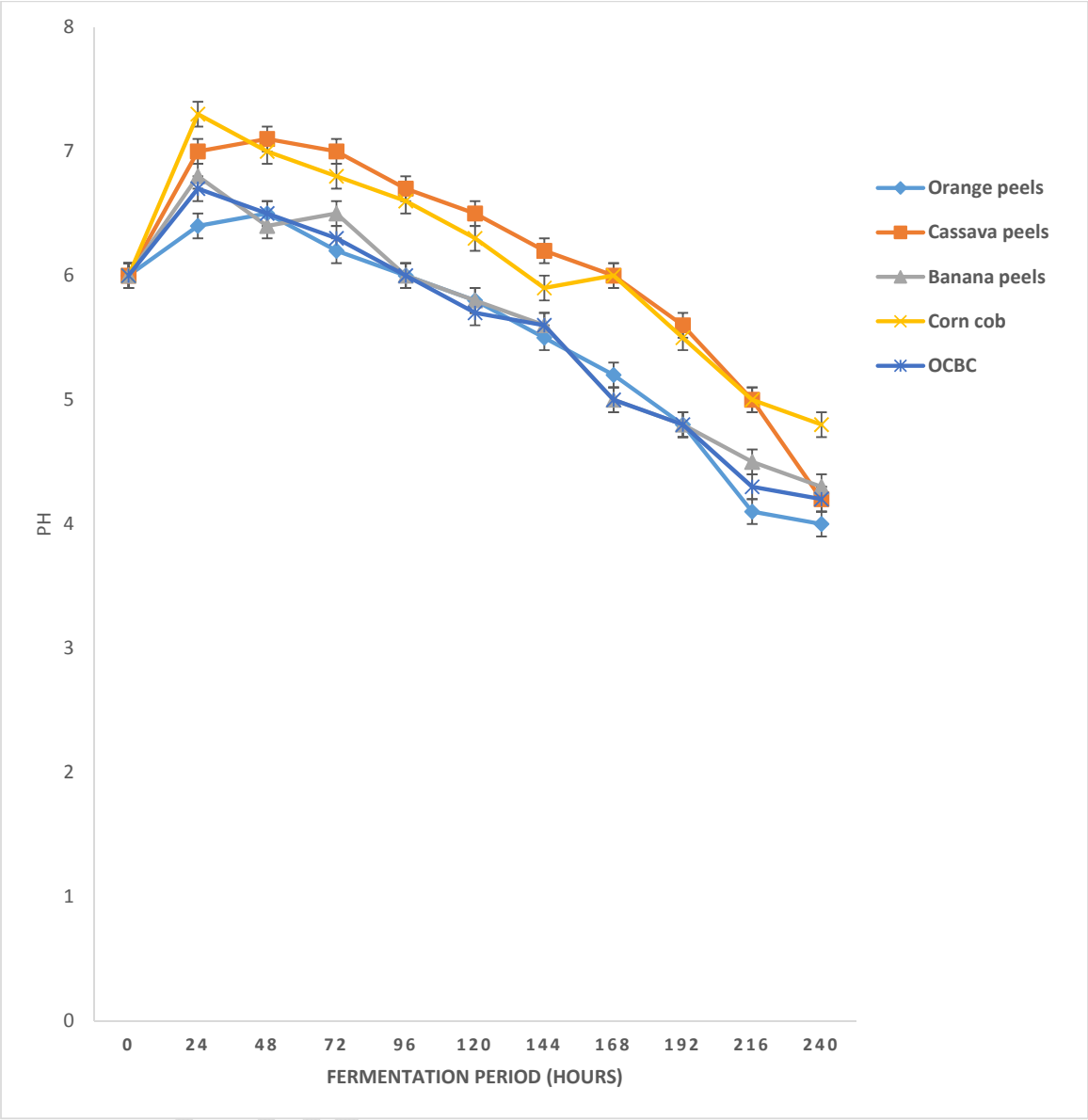

Fig 3: Changes in pH during fermentation of different agricultural wastes using *B. cereus* and *S. cerevisiae*

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1)in grams.

Total titratable acidity during fermentation of different agricultural wastes using *A. niger* and *S. cerevisiae*

The total titratable acidity during fermentation of each susbtrate using *A. niger* and *S. cerevisiae* is shown in Figure 4. An increase in the TTA was observed from the initial TTA as fermentation proceeded. Fermentation of corn cobs showed an increase in TTA, from an initial TTA of 0.12% to 1.27% after 168 hours; banana peels showed an increase from 0.1% initial to a final TTA of 0.9%, cassava peels also showed a very high TTA from 0.14% initial to a highest of 1.5%. The combinations of all the substrates in ratio 1:1:1:1 (OCBC) showed an increase in TTA from 0.09% to a highest of 1.23%.

Total Titratable acidity during Fermentation of different Agricultural Wastes using *B. cereus* and *S. cerevisiae*

Figure 5 shows the total titratable acidity during fermentation of different agricultural wastes using *B. cereus* and *S. cerevisiae.* The result revealed that, as fermentation proceeded from day zero to day seven, increase in the TTA was observed, corn cobs TTA was conspicuously higher than the rest from an initial TTA of 0.1% to 1.7%, followed by combinations of all the substrates in ratio 1:1:1:1 (OCBC) from initial TTA of 0.09% to 0.84%, while the lowest TTA was recorded for orange peels from 0.07% to 0.38%

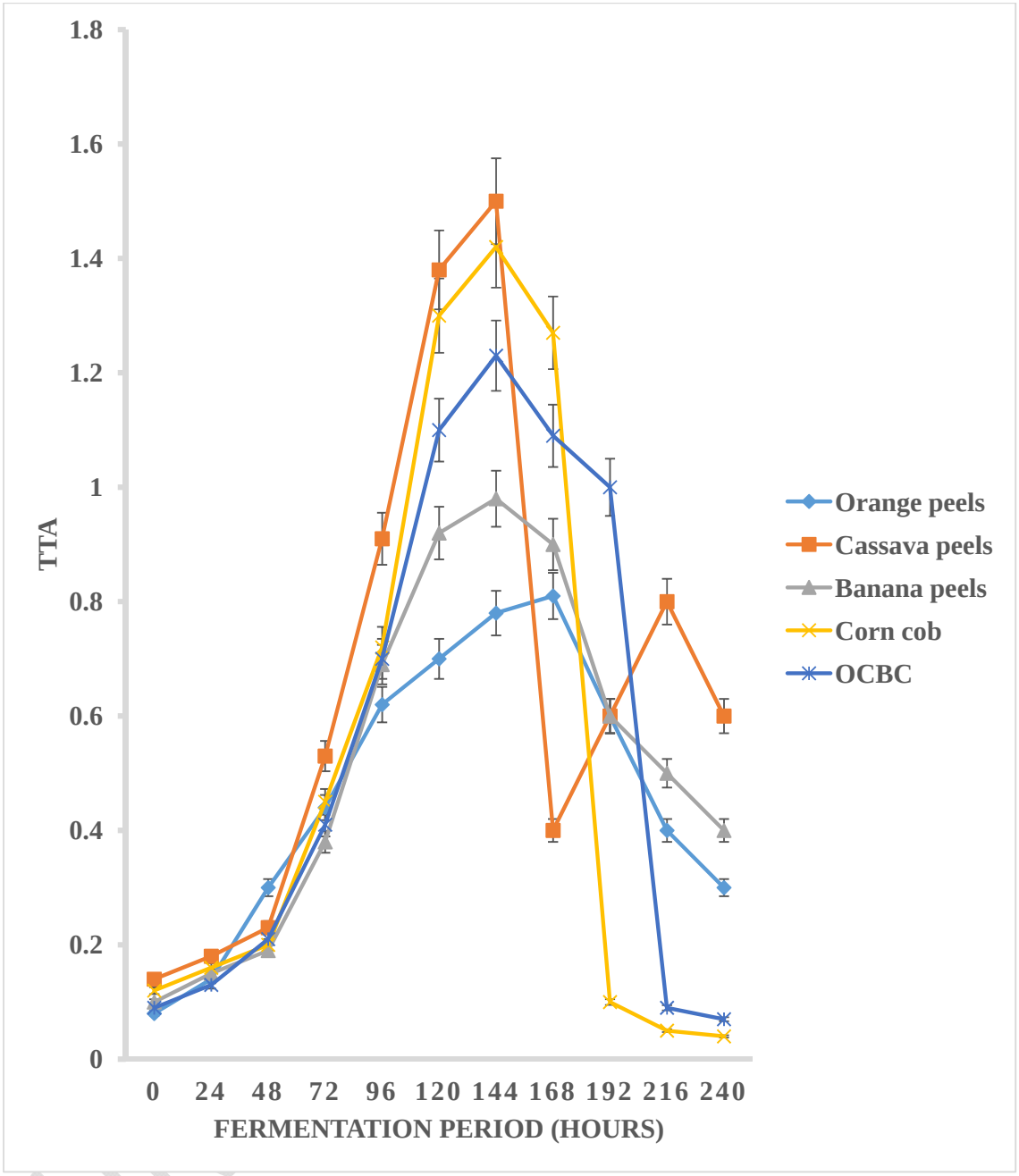

Fig 4: Total titratable acidity during fermentation of different agricultural wastes using *A. niger* and *S. cerevisiae*

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

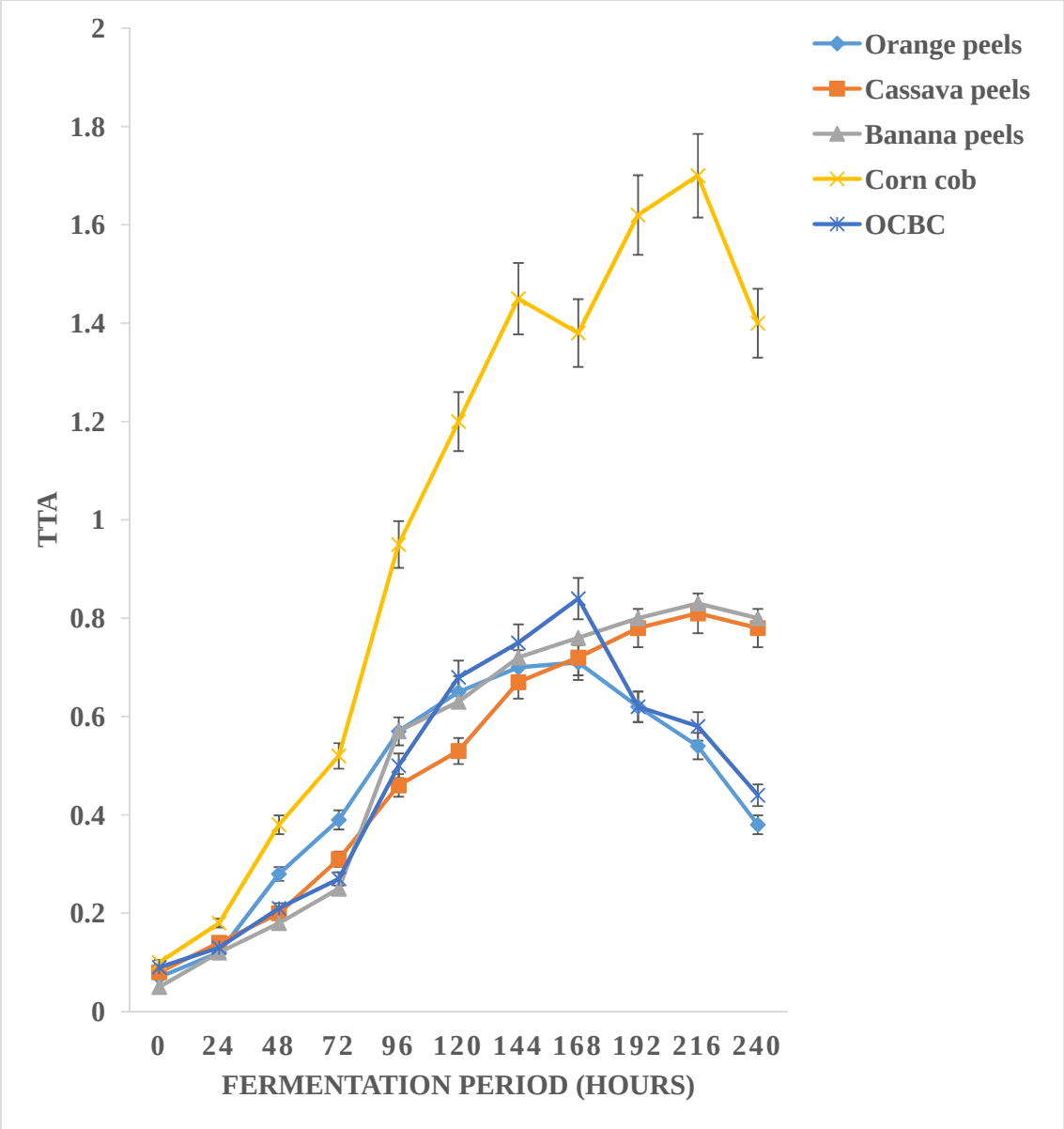

Fig 5: Total Titratable acidity during Fermentation of different agricultural wastes using *B. cereus* and *S. cerevisiae*

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

Ethanol yield from different agricultural wastes using *A. niger* and *S. cerevisiae*

Figure 6 shows the ethanol yield of the various substrates and their combination during days of fermentation using *A. niger* and *S. cerevisiae*.. The ethanol yield was observed to increase as the fermentation continued. Corn cobs had the highest initial yield of 3.22g after 48 hours; followed by banana peels which had an initial yield of 2.21g, cassava peels had 2.07g, while orange peels recorded the lowest with 1.30g.The combinations of all the substrates in ratio 1:1:1:1 (OCBC) also had ethanol yield of 1.90g after 48 hours of fermentation, it was observed that corn cobs had the highest final ethanol yield of 17.43g, followed by cassava peels which gave a yield of 15.1g, while combinations of all the substrates in ratio 1:1:1:1 (OCBC) gave a yield of 12.44g. Orange peels on the other hand recorded the least ethanol yield of 8.03g

Ethanol yield from different agricultural wastes using *B. cereus* and *S. cerevisiae*

The ethanol yield of the various substrates and their combination during days of fermentation using *B. cereus* and *S. cerevisiae*are presented in Figure 7. The ethanol yield was observed to increase as the fermentation proceeded, however it can be observed that the ethanol produced was considerably lower than that produced by *A. niger* and *S. cerevisiae.* The combinations of all the substrates in ratio 1:1:1:1 (OCBC) had the highest initial yield of 2.46g after 24 hours, followed by corn cobs which had an initial yield of 2.16g. Cassava peels also had 1.91g, followed by banana peels with 1.41g, while orange peels had the lowest initial yield of 0.82g after 24 hours. After 8 days of fermentation, corn cobs were shown to have the highest final ethanol yield of 9.39g, followed by the combinations of all the substrates in ratio 1:1:1:1 (OCBC) which gave a yield of 9.14g. However, it can be observed that orange peels recorded the lowest ethanol yield of 5.50g after 7 days of fermentation.

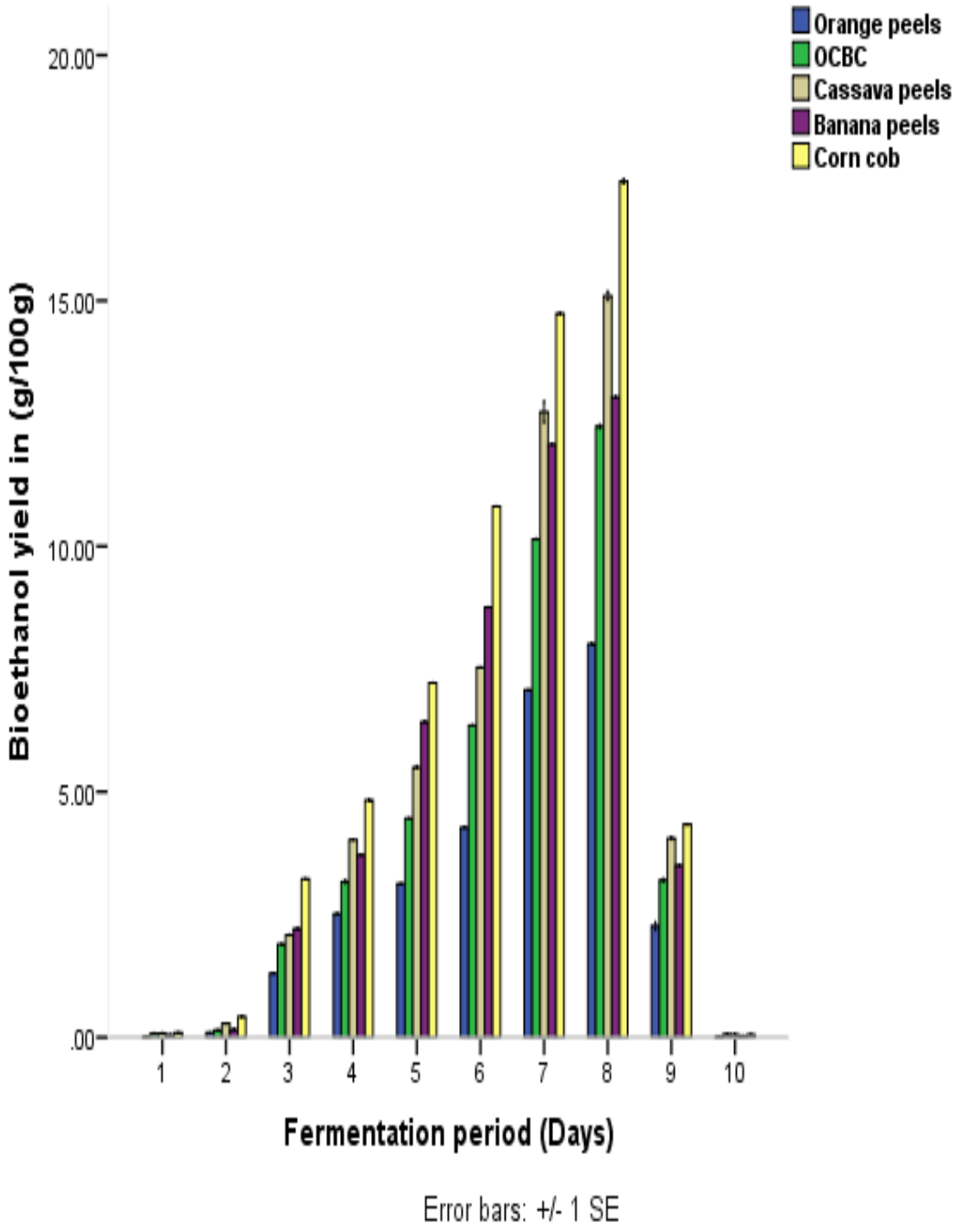

Fig 6: Ethanol yield from different agricultural wastes using *A. niger* and *S. cerevisiae*

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams.

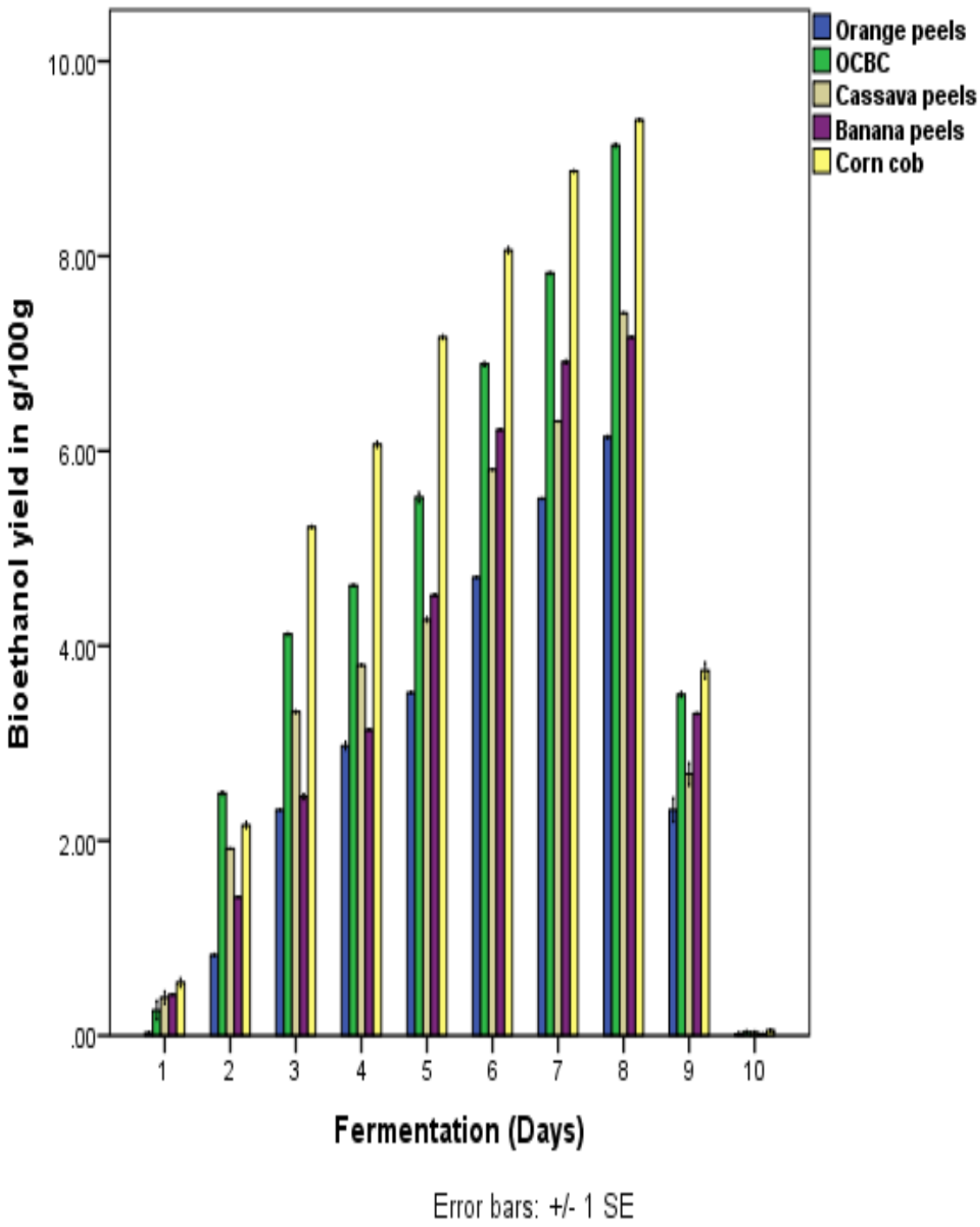

Fig 7: Ethanol yield from different agricultural wastes using *B. cereus* and *S. cerevisiae*

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

Bacterial counts in Cfu/mL during fermentation of the agricultural wastes

The result of bacterial counts observed on nutrient agar from fermentation of orange peels, cassava peels, banana peels, corn cobs and combinations of all the substrates in ratio 1:1:1:1(OCBC) is presented in Table 2.The results showed that cassava peels had the highest initial count of 5.10 x 10^6Cfu/mL, while orange peels had the lowest of 1.8 x 10^6 Cfu/mL. The combinations of all the substrates in ratio 1:1:1:1(OCBC) had the highest microbial load on Nutrient agar of 56.4 x 10^6Cfu/mL after 6 days of fermentation, while orange peels was observed to have the lowest with 10.08 x 10^6Cfu/mL after 9 days.

Fungal counts in Sfu/mL during the fermentation of the agricultural wastes

Table 3 shows Fungal Counts in Sfu/mL on PDA during the fermentation of the agricultural wastes, the result revealed that, banana peels had the initial highest count of 6. 7 x 10^5 Sfu/mL, while orange peels had the lowest of 2.1 x 10^5 Sfu/mL. After seven days of fermentation, the combinations of all the substrates in ratio 1:1:1:1(OCBC) had the highest fungal load of 5.2 x 10^5Sfu/mL, followed by banana peel with 4.1 x 10^5 Sfu/mL, while orange peels recorded the lowest overall after several days of fermentation with 1.1 x 10^5 Sfu/mL

Table 2: Bacterial counts in Cfu/mL during fermentation of the agricultural wastes

FERMENTATION DAYS	Orange peels Cfu/mL x 10^6	OCBC Cfu/mL x10^6	Cassava peels Cfu/mL x 10^6	Banana peelsCfu/mL x 10^6	Corn cob Cfu/mL x 10^6
0	1.8±0.12^{g}	4.90 ±0.21^{ab}	5.10 ±0.10^{b}	3.70 ±0.30^{c}	2.10 ±0.10^{d}
1	2.2 ±0.10^{bc}	5.20 ±0.00^{f}	5.50 ±0.10^{e}	3.90 ±0.20^{ab}	2.70 ±0.30^{gh}
2	2.8±0.40^{ef}	5.80 ±0.10^{c}	6.10 ±0.00^{ab}	4.00 ±0.30^{cd}	25.05±0.20^{a}
3	3.2 ±0.08^{a}	2.61 ±0.30^{bc}	11.22 ±0.06^{ef}	12.13 ±0.10^{e}	29.02 ±0.17^{f}
4	11.0 ±0.00^{d}	50.8 ±0.17^{de}	14.13±0.40^{bc}	25.05 ±0.15^{ef}	35 .08±0.10^{g}
5	21.2 ±0.09^{b}	50.6 ±0.00^{a}	27.08 ±0.17^{f}	29.18±0.10^{b}	48.17 ±0.27^{ab}
6	26.01±0.12^{e}	56.4 ±0.00^{ef}	31.05 ±0.14^{b}	31.10±0.13^{d}	50.30±0.10^{c}
7	29.12±0.10^{h}	52.2±0.26^{g}	34.21 ±0.06^{a}	41.09 ±0.27^{bc}	52.22 ±0.17^{cd}
8	33.42 ±0.00^{c}	48.31±0.11^{d}	25.11±0.26^{g}	52.03 ±0.23^{de}	53.10±0.10^{h}
9	22 .15±0.02^{ab}	36.12±0.00^{cd}	19.06±0.15^{h}	40.20 ±0.23^{gh}	42.12 ±0.20^{de}
10	10.08±0.14^{bc}	16.10 ±0.00^{ab}	14.10±0.00^{d}	21.00 ±0.20^{a}	12.01±0.13^{b}

Values are means ± Standard error of agricultural wastes. Values in the same column carrying the same superscript are not significantly different at ($p \leq 0.05$) using Duncan's New Multiple Range test.

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

Table 3: Fungal counts in Sfu/mL during the fermentation of the agricultural wastes

FERMENTATION DAYS	ORANGE PEELS Sfu/mL x 10^5	OCBC Sfu/mL x 10^5	CASSAVA PEELS Sfu/mL x 10^5	BANANA PEELS Sfu/mL x 10^5	CORN COB Sfu/mL x 10^5
0	2.1 ±0.14^{bc}	2.90 ±0.27^{b}	5.10 ±0.20^{h}	6.70 ±0.10^{d}	3.60 ±0.22^{e}
1	3.2 ±0.20^{c}	5.10 ±0.00^{ab}	5.40 ±0.12^{b}	7.20 ±0.23^{ef}	4.20 ±0.15^{cd}
2	3.8±0.30^{f}	5.80 ±0.10^{gh}	6.00 ±0.00^{e}	8.00 ±0.20^{cd}	4.50 ±0.30^{a}
3	4.2 ±0.16^{ab}	2.1 ±0.20^{a}	1.10 ±0.10^{ef}	1.90 ±0.10^{g}	1.20 ±0.12^{b}
4	1.10 ±0.10^{f}	3.0 ±0.15^{e}	1.50 ±0.21^{g}	2.6±0.33^{a}	1.3 ±0.16^{d}
5	2.3±0.20^{d}	4.0 ±0.30^{ef}	2.10 ±0.10^{a}	2.8±0.12^{e}	1.8 ±0.18^{c}
6	2.80 ±0.27^{g}	4.8±0.00^{d}	2.90±0.15^{cd}	3.20 ±0.00^{ab}	2.2 ±0.20^{gh}
7	2.9 ±0.37^{h}	5.2 ±0.20^{ab}	3.45 ±0.00^{de}	4.10 ±0.20^{b}	2.70±0.12^{g}
8	3.00 ±0.10^{a}	4.9±0.20^{bc}	2.7±0.28^{f}	5.30±0.23^{c}	1.72 ±0.30^{h}
9	2.1±0.12^{bc}	3.2±0.00^{ab}	2.0±0.11^{g}	4.20±0.20^{f}	1.52±0.20^{g}
10	1.10±0.14^{f}	1.8 ±0.00^{d}	1.3±0.00^{ef}	2.2 ±0.10^{ab}	1.4 ±0.00^{cd}

Values are means ± Standard error of agricultural wastes. Values in the same column carrying the same superscript are not significantly different at ($p \leq 0.05$) using Duncan's New Multiple Range test.

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

Comparison of commercial ethanol and bioethanol produced from different substrates

The Comparison of conventional ethanol commercially available and bioethanol produced from different agro wastes substratesis presented in Table 4, all the ethanol produced and commercial ethanol appeared colourless, burns with blue flame and have refractive index of 1.36. Other properties such as relative density, boiling point, melting point, viscosity, and flash point showed little discrepancies.

452 **Table 4: Comparison of commercial ethanol and bioethanol produced from different substrates**

Ethanol Properties	**Bioethaol From cassava peels**	**Bioethaol From Banana peels**	**Bioethaol From Orange peels**	**Bioethaol From Corn cob**	**Bioethaol From OCBC**	**Commercial Ethanol**
Appearance	Colourless	Colourless	colourless	colourless	Colourless	Colourless
Relative Density (g/cm^3)	0.756	0.773	0.777	0.782	0.774	0.789
Melting point (^0C)	-112	-114	-113	-112	-113	-114
Boiling point(^0C)	78.40	78.36	78.38	78.37	78.40	78.37
Viscosity	0.0092	0.0122	0.0119	0.0060	0.0114	0.0012 pa s at 20^0C
Burning characteristics	Burns with blue flame	Burns with blue flame	Burns with blue flame	Burns with blue flame	Burns with blue flame	Burns with blue flame
Refractive index	1.36	1.36	1.36	1.36	1.36	1.36
Flash point(^0C)	11	12	12	11	12	13-14

453 Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

Key: **OCBC** = Combinations of Orange peels /Cassava peels /Banana peels /Corn cob (Ratio 1:1:1:1) in grams

DISCUSSION

The result of the acid pretreatment of the substrates was highly effective after the application of NaOH. The result showed a drastic increase in the cellulose composition of the agro wastes with corn cob having the highest amount of cellulose, and a subsequent decrease in the hemicellulose and lignin content. This is a direct implication of the acid treatment that solubilized the hemi cellulosic fraction and increased the diffusion of sodium hydroxide into the lignocellulosic structure, thus enhancing soda pulping and liberating the cellulose fibres from lignin thereby causing the washing away of hemicellulose and lignin during the filtration hence obtaining a solid residue with high content (Abo- State *et al.,* 2014). The results obtained in this study are in agreement with the findings of Chen *et al.* (2010) who reported similar increase in cellulose and decrease in the hemicellulose and lignin contents of acid pretreated lignocellulosic substrates, and in contrast to that of Abo- State *et al.* (2014) who reported a decrease in all three components, probably due to simultaneous pretreatment and hydrolysis. The high cellulose content and decreased hemicellulose and lignin contents would allow for the enhancement of microbial saccharification (Jeya *et al.,* 2009).

It was observed in this study that the reducing sugar yield of *A. niger* was higher than *B. cereus* yield. This was in agreement with Elsayed (2011) who showed a great difference between the cellulase activity of *Trichodema sp* and *Bacillus sp* using rice straw residues as lignocellulosic substrate.This could be attributed to the ability of *Aspergillus niger* to produce all components of cellulase complex, endoglucanase, exoglucanase, and β- glucosidase in good proportions as well as production of other enzymes such as xylanases or laccases in comparison to other enzyme producers (Arantes and Saddler 2010). Since the main part of the reducing sugar originated from the cellulose fraction, the difference in reducing sugar yield observed for each substrate combination is invariably proportional to the initial cellulose contained by each substrate after pretreatment (Taherzadeh *et al.,* 2007). It could therefore be inferred from the findings that the amount reducing sugar generated by hydrolysis was a function of how effective the pretreatment stage was.

There was significant decrease in the pH of the fermenting media. This may be due to the release of various organic acids from the utilization of the substrates. It was observed that the combinations of all the substrates in ratio 1:1:1:1 (OCBC) showed the lowest pH in all the five fermentation sets after 7 days of fermentation. This could be the result of better nutrient composition which favoured the growth of the microorganisms and hence the production of metabolites. There was increase in total titratable acidity; this could be as a result of utilization of free sugars by yeast and *Bacillus* (Akinyele *et al.,* 2014). The result however showed no direct relationship between the pH and TTA and this can be attributed to the production of other metabolites by the microorganisms (Rajkovic *et al.,* 2007). The observed variation in both pH and TTA values for each substrate combination is a direct result of nutrient variation and hence metabolism of the microorganisms.

The fermentation of the substrates using *Saccharomyces cerevisiae* showed that the yield of ethanol is proportional to fermentation time, where the yield increased with increase in fermentation time, this correlation exist as a result of continuous utilization of the sugar by yeast, and this is in agreement with the findings of Chen *et al.*(2010). It was also revealed that the combination of *A. niger and S. cerevisiae* gave considerably gave higher ethanol yield in all the substrates as well as the substrates combination (OCBC), 100g of corn cob for instance gave an ethanol yield of 17.43g using *A. niger and S. cerevisiae,* and 9.39g using *B. cereus* and *S. cerevisiae* . Cassava peel also recorded high ethanol yield of 15.1g, this was higher than what was reported by Witantri *et al.* (2016) who produced bioethanol by utilizing cassava peels. This may be due to the efficiency of the microorganisms employed during the hydrolysis stage. However, the relatively low yield observed during the fermentation of orange peel may be as a result of antimicrobial activity of the peels that have been reported (Shetty *et al.,* 2016), which slowed down the efficiency of the microorganisms involved in hydrolysis and fermentation respectively, it could also be as a result of lignin which prevented the free access of cellulose by the microorganisms (Subramanian, 2010). The combination of all the substrates gave maximum ethanol yield of 12.44 less than 17.43 reported for corn cobs in this study, this in contrast with the work of Elsayad (2013) who stated that the ethanol yield of each substrate is

508 directly proportional to its cellulose content. This could be attributed to a number of factors including
509 nutrient variation of the substrates.

510 Bacteria counts obtained from the fermentation of cassava peels, banana peels, orange peels and corn
511 cobs showed that cassava peel had the highest initial count on nutrient agar, while the combinations of all the
512 substrates in ratio 1:1:1:1(OCBC) had the highest microbial load on nutrient agar after 6 days of
513 fermentation, this was probably due to the fact that the combined substrates may contain varieties of
514 components, thus serving as a better source of nutrients for microbial growth than individual substrate.
515 These findings conform to the work of Lyumugabe *et al.* (2010) and Ibeabuchi *et al.* (2014) that reported
516 significant bacterial counts on nutrient agar for fermented products. The fungal counts of each substrate
517 during fermentation on PDA in this study showed that banana peels had the highest initial count, while
518 orange peel had the lowest, this could be attributed to the fact that, banana peels has been described as a
519 mycological medium (Essien *et al.,* 2008). In addition it has the highest percentage of dietary fibres from
520 this study, while orange peel possibly has antimicrobial property as reported by Shetty *et al.*(2016) which
521 invariably have adverse effect on fungal growth in the fermentation medium.

522 The comparison between the properties of cassava peels, banana peels, orange peels, corn cob and
523 combinations of all the substrates in ratio 1:1:1:1(OCBC) with those of the conventional ethanol showed
524 that, the flash point of the conventional ethanol ranges between 13^0C and 14^0C, slightly higher than 12^0C
525 noted for the correlation of both banana peels and orange peels, the properties of the alcohols shows that
526 bioethanol derived from plant sources can serve similar purpose as their conventional counterparts.

527

528 **Conclusion**

529 This study established the efficiency of cassava peels, banana peels, orange peels, and corn cobs for
530 bioethanol production, as well as the efficiency of selected cellulolytic microorganisms in the production
531 process. *Aspergillus niger* was found to be more effective in cellulose hydrolysis than *Bacillus cereus,*

thereby generating higher reducing sugar in each substrate and their respective combinations. Furthermore, it was also observed that among the four substrates utilized, corn cob was found to be the most efficient substrate for bioethanol production.

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