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A comparative study on bacterial conversion of selected agricultural biomass to ethanol

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The aim of the study was to investigate the bioethanol production potentials by indigenous bacterial isolates screened for amylolytic and cellulolytic activities. Cassava peels were obtained from local 'garri' producers, while corn grains and corn cobs were gotten from retailers within the Erinfun farm settlement along Afe Babalola University Road, Ado-Ekiti, Ekiti State, Nigeria on June 4, 2015. The substrates (corn flour, corn cob and cassava peels) were sundried for 5 days and ground into fine powder using a hammer mill and analyzed for proximate nutrient composition and reconstituted to a 5% concentration in water before fermentation. The fermentation was set up for 20 days using single cultures of bacteria and co-cultures of bacteria and yeast selectively isolated and screened for amylolytic and cellulolytic activities. Parameters such as pH, total titratable acidity, reducing sugar and optical density were taken at intervals of 5 days and ethanol production was analyzed at the end of fermentation. Three bacterial isolates (*Bacillus macerans, Bacillus subtilis* and *Micrococcus varians*) yielded ethanol in all the feed stocks. The highest percentage ethanol yield was observed in the fermentation of corn flour and corn cobs with *B. macerans* which were 3.6 ± 0.009 and 3.5 ± 0.009, respectively. There was reduction in the pH and total titratable acidity and increase in the reducing sugar content and optical density. *B. macerans* exhibited the highest average ethanol production (3.5±0.07%). A proximate analysis of the feed stocks showed presence of nitrogen, protein, fat, carbohydrate and ash. This study investigated the potentials of wastes (cassava peels and corn cob) used as feed stocks for bio-ethanol production, in substantially replacing major food crops (corn) as a case study.

Key words: Fermentation, amylolytic activity, cellulolytic activity, corn flour, corn cob, cassava peels, feed stocks.

INTRODUCTION

Bioethanol as an alternative source of energy has received special attention worldwide due to the depletion of fossil fuels (Sanat et al., 2014). The world production of bioethanol increased from 50 million m^3 in 2007 to over 100 million m^3 in 2012. The United States and Brazil are known to produce approximately 80% of the total world

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supply derived from corn and sugarcane. In developing economies, food related feedstocks are preferably substituted with non-food raw materials. This is being achieved with farm products that are considered to be less edible (Qian et al., 2014). Interest in the production of biofuel from agricultural wastes is driven by several reasons such as global search for alternative source of energy and transporting fuel to replace the depleting fossil fuel. It is also as a result of several benefits derivable from the use of ethanol such as its use as solvents, germicides, antifreeze and intermediates for other organic chemicals (Kingsley, 2012).

Cassava production in Africa accounts for over 54% of the total world production, with Nigeria taking the global lead with a production of about 54.8 million metric tons in 2014. Most of the cassava harvested in Nigeria is processed into food to obtain 'garri', 'fufu' and 'lafun'. There is little processing of cassava into products such as ethanol, chips, syrups and starch (Sahel, 2016). The value of corn cobs as a feedstock for ethanol production could be attributed to the large amount of carbohydrates, specifically starch, present in corn and can be rather easily processed to break it down into simple sugars, which can then be used to biologically produce ethanol (Nathan, 2006). There is an emerging trend in the utilization of biomass conversion technologies on rice husk and sugarcane bagasse, and gasification of other agricultural residues. Biomass is still largely underutilized and left to decompose in the fields; this is a common phenomenon in developing countries that do not have strong regulatory instruments to control such polluting practice (UNEP, 2007). Ethanol producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of the yeasts presently used for the practical production of fuel alcohol and with the recent advances in biotechnology (Zuber and Anjani, 2013). The inability of yeast fermentation to utilize xyloses also hampers the yield of ethanol from lignocellulosic biomass (Qian et al., 2014). As a result, this study aimed to investigate bioethanol production potentials by indigenous bacterial isolates screened for amylolytic and cellulolytic activities.

MATERIALS AND METHODS

Sample collection and preparation

Cassava peels were obtained from local garri producers, while corn grains and corn cobs were gotten from retailers within the Erinfun farm settlement along Afe Babalola University Road, Ado-Ekiti, Ekiti State, Nigeria on June 4, 2015. They were sundried for 5 days and ground into fine powder using a hammer mill. The ground samples were then collected in clean polyethylene bags and stored in the laboratory at room temperature until use. The waste water from the fermentation of cassava was also obtained in sterile container and transported to the laboratory for storage at refrigerating conditions.

Isolation of bacteria

The bacteria were isolated from soil contaminated with the effluent discharged during cassava fermentation for 'garri' production. The soil was obtained using a hand trowel to collect some surface and sub-surface soil materials. Bacterial isolates were also obtained from the other samples (cassava peels, corn grains and corn cobs).

Identification of bacterial isolates

The bacterial isolates were identified according to the biochemical tests described by Bergey and John (2000) and Barrow and Feltham (1993).

Screening of isolates for starch hydrolysis

Ten grams each of the powdered samples (cassava peel flour, corn flour and corn cob) were weighed into separate 1000 ml conical flasks containing 500 ml of water and boiled at 100°C for 30 min. The preparation was allowed to cool at room temperature and the starchy liquid sieved with a muslin cloth. Two percent of the starch extract from the samples were used to enrich the nutrient agar and malt extract agar for the bacteria and yeast respectively. The preparation was sterilized at 121°C for 15 min and dispensed into 15 Petri dishes for 15 bacterial isolates and the sterilized starch enriched malt extract agar dispensed into 5 Petri dishes for the 5 yeast isolates. The media were allowed to solidify and the isolates streaked on the solidified media. The plates were incubated at 37°C for 24 h. Drops of Lugol's iodine were added to the bacterial growth observed on the plates after 24 h of incubation. Clear zones around the cultures indicated the ability of the isolate to hydrolyze starch while the absence of clear zones indicated the inability of the isolate to hydrolyze starch (Barrow and Feltham, 1993).

Inoculum preparation

Sterile 10 ml nutrient broth in test tubes was inoculated with 24 h cultures of bacterial isolates.

Preparation of sample for fermentation

Twenty-five grams each of the samples (cassava peel flour, corn flour and corn cob) were weighed in a 1000 ml conical flask each. Five hundred milligrams of distilled water was added and the preparation were sterilized at 121°C for 15 min. The samples were allowed to cool and dispensed into 1 L sterile flasks. 10 ml of the inoculum was introduced into the prepared samples for a 20-day fermentation trial.

Analysis of fermentation medium

Physiochemical parameters such as pH, total titratable acidity, optical density, determination of reducing sugar and analysis of ethanol concentration were carried out with the fermenting media according to AOAC (2000).

Enzyme assays

Cellulase activity was determined by the method of Camassola and Dillon (2012) and the amylase activity by the method of Naguib (1964).

Proximate analysis

The proximate analysis of the samples such as moisture content,

Table 1. Biochemical characteristics and identification of bacterial isolates.

Code/Isolate	Spore staining	Starch hydrolysis	Catalase	Voges/Proskauer test	Methyl Red test	Swollen test	6.5% NaCl	Acid from Arabinose	Gas from Glucose	Acid fast test	Citrate test	Probable organism
CFWI	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	ND	ND	*Bacillus macquariensis*
CFW2	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	ND	ND	*Bacillus subtilis*
CFW3	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	ND	ND	*Bacillus circulans*
CF1	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	ND	ND	*Bacillus macerans*
CP1	+ve	+ve	+ve	-ve	-ve	+ve	+ve	+ve	-ve	ND	ND	*Bacillus macquariensis*
CP2	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	ND	ND	*Bacillus circulans*
CC1	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	ND	ND	*Micrococcus varians*
CC2	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	ND	ND	*Bacillus circulans*
FCS.A	-ve	-ve	+ve	ND	ND	ND	ND	ND	ND	-ve	ND	*Corynebacterium xerosis*
FCS.B	-ve	-ve	+ve	ND	ND	ND	ND	ND	ND	ND	ND	*Corynebacterium xerosis*
FCS.C	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	*Bacillus azotoformans*
FCS.D	-ve	-ve	+ve	ND	ND	-ve	ND	ND	ND	-ve	+ve	*Bacillus insolitus*
FCS.E	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	*Bacillus azotoformans*
FCS.F	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	*Bacillus azotoformans*
FCS.G	+ve	-ve	-ve	ND	ND	-ve	ND	ND	ND	ND	+ve	*Bacillus azotoformans*

ND, Not determined; +ve, positive; –ve, negative; CFW, cassava fermentation water; CC, corn cob; CP, cassava peels; CF, corn flour; FCS, soil containing fermented cassava effluent.

crude protein, crude fibre, crude fat, ash content and the determination of the carbohydrate content were carried out as described by AOAC (2000).

Analysis of data

The results are presented as the mean standard values of three replicates. A one-way analysis of variance (ANOVA) was carried out using SPSS 16.0. Significant difference was at P≤0.05.

RESULTS

Morphological and biochemical characteristics of the bacterial isolates

The bacterial isolates were mostly Gram positive rods. The biochemical tests carried out on the bacterial isolates showed the following organisms: *Bacillus subtilis, Bacillus macerans, Bacilus macquariensis, Micrococcus varians, Corynebaterium xerosis, Bacillus azotoformans* and *Bacillus insolitus*. Three isolates (*B. macquariensis, B. subtilis* and *B. circulans*) were isolated from effluent obtained from cassava fermentation, two isolates (*M. varians* and *B. circulans*) were got from corn cob, *B. macquariensis* from cassava peels while three other isolates (*C. xerosis, B. azotoformans* and *B. insolitus*) were obtained from the soil sample (Table 1).

Sample starch hydrolysis test

Six of the bacterial isolates (*B. macquariensis, B. subtilis, B. circulans, M. varians, B. macerans* and *B. azotoformans*) from the soil samples were observed to be capable of starch hydrolysis (Table 2).

Amylase and cellulase activity

The amylase activity of the bacterial isolates was examined in all samples on the 20th day of fermentation. The highest activity of 0.61 U/mg was observed in corn flour by *B. subtilis,* while the lowest (0.50 U/mg) was observed in corn cob (C.C) by *B. macerans*. The cellulase activity of the bacterial isolates was examined in all samples on the 20th day of fermentation. The highest activity was observed in corn flour by *B. macerans* (CF1) in the cassava peels (C.P) to be 0.0470 FPU

Table 2. Result of starch hydrolysis test.

Isolates/code	Reaction
Bacillus macquariensis	+ve
Bacillus subtilis	+ve
Bacillus circulans	+ve
Micrococcus varians	+ve
Bacillus circulans	+ve
Bacillus macerans	+ve
Bacillus macquariensis	+ve
Bacillus circulans	+ve
Corynebacterium xerosis	-ve
Corynebacterium xerosis	-ve
Bacillus azotoformans	-ve
Bacillus insolitus	-ve
Bacillus azotoformans	-ve
Bacillus azotoformans	-ve
Bacillus azotoformans	-ve

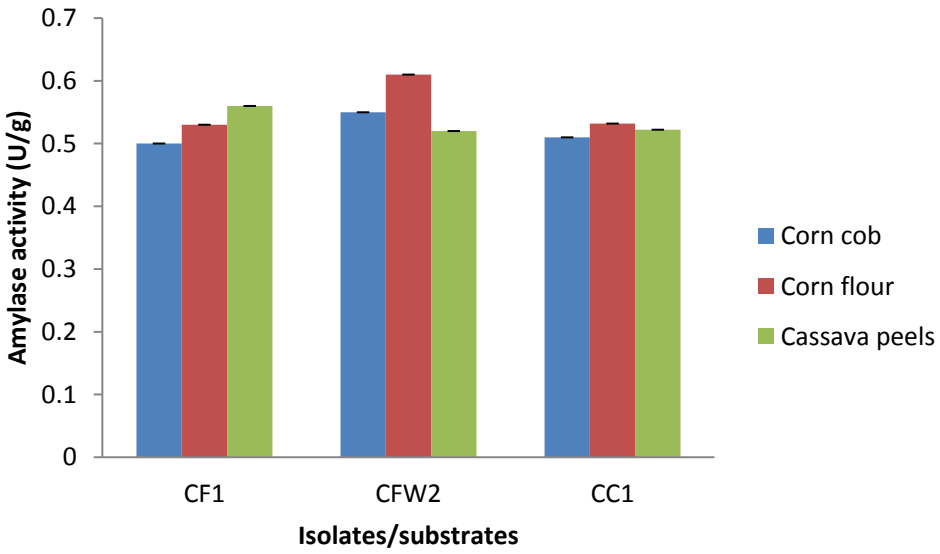

Figure 1. Amylase activity of the bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis.*

while the lowest was observed in corn flour (C.F) by the *M. varians* to be 0.0301 FPU (Figures 1 and 2).

Ethanol yield of the different isolates using corn cob, corn flour and cassava peels

Most of the isolates and substrates used for fermentation gave ethanol yield of between 3.0 and 3.6%. The lowest ethanol yield was recorded for cassava peels (2.0±0.07%) by *B. subtilis* while the highest percentage ethanol yield was observed in corn flour (3.6±0.07%) by *B. macerans* (Figure 3). At P≤0.05, there was statistical significant difference in the percentage ethanol yield of the samples after 20 days of fermentation.

pH of samples inoculated with bacterial isolates

There was a decrease in the pH of the samples inoculated with the bacterial isolates as the fermentation period increased. *B. marcerans* with corn cob had an initial pH on day 0 of 6.80 and deceased to 4.10 on day 20. The lowest pH on day 20 was recorded for *B.*

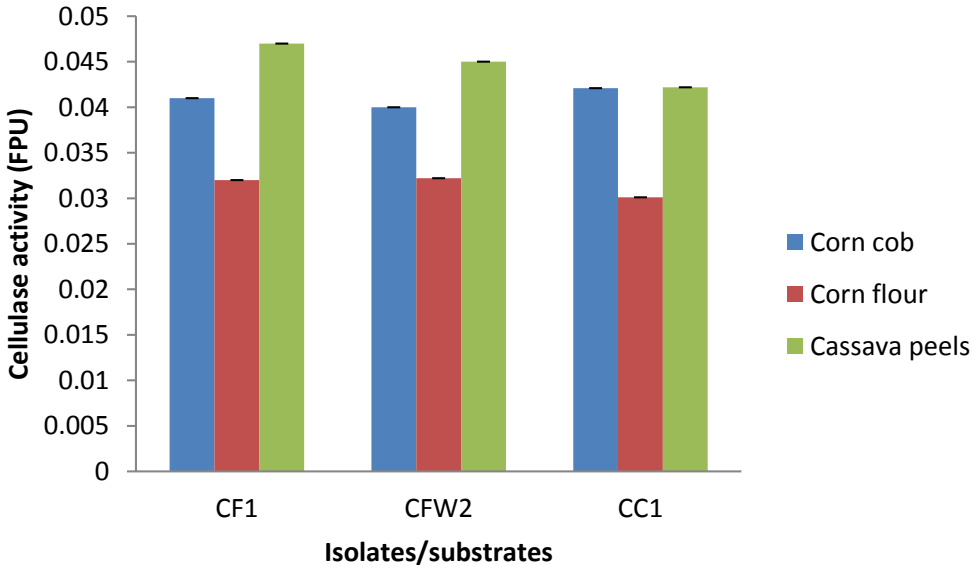

Figure 2. Cellulase activity of the bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis.*

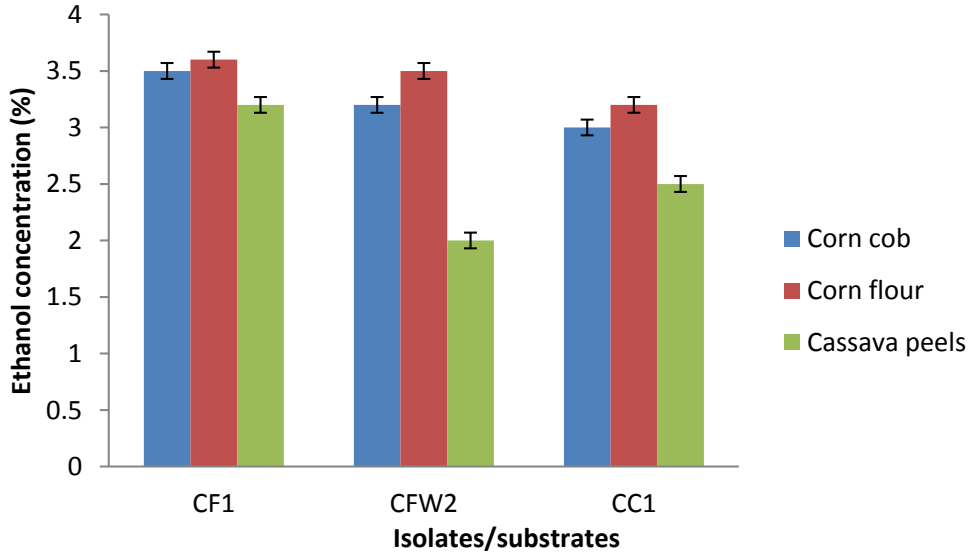

Figure 3. The ethanol yield of isolates using different substrates after 20 days of fermentation. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis.*

marcerans with cassava peels which was 3.85 from the initial value of 7.04 (Table 3).

Total titratable acidity of samples inoculated with bacterial isolates

The total titratable acidity of the samples during fermentation by selected bacteria are presented in Table 4. There was a progressive increase with time. The highest Total titratable acidity (TTA) at day 20 (0.180 moles) was observed for fermentation with *B. subtilis* on the corn cob, while the least value of 0.120 moles was obtained for *B. marcerans* with corn cob.

Reducing sugar values of samples inoculated with ethanol yielding bacterial isolates

The reducing sugar of the samples during fermentation by bacterial isolates is shown in Figure 4. There was a

Table 3. pH of samples inoculated with bacterial isolates during fermentation.

Isolate/substrate	Number of days				
	0	5	10	15	20
CF1 (C.C)	6.80	5.52	4.75	4.41	4.10
CF1(C.F)	7.10	5.67	4.62	4.32	4.11
CF1(C.P)	7.04	5.41	4.45	3.97	3.85
CFW2 (C.C)	6.80	5.61	4.71	4.25	4.12
CFW2(C.F)	7.10	5.76	4.62	4.31	4.20
CFW2(C.P)	7.04	5.31	4.35	4.22	4.12
CC1 (C.C)	6.80	5.41	4.68	4.31	4.20
CC1(C.F)	7.10	5.71	4.65	4.22	4.10
CC1(C.P)	7.04	5.52	4.85	4.35	4.20

Table 4. Total titratable acidity of samples inoculated with bacterial isolates

Isolate/substrate	Number of days				
	0	5	10	15	20
CF1 (C.C)	0.00	0.050	0.055	0.081	0.120
CF1(C.F)	0.00	0.053	0.056	0.083	0.122
CF1(C.P)	0.00	0.056	0.058	0.086	0.142
CFW2 (C.C)	0.00	0.055	0.066	0.091	0.180
CFW2(C.F)	0.00	0.061	0.065	0.088	0.151
CFW2(C.P)	0.00	0.052	0.062	0.082	0.146
CC1 (C.C)	0.00	0.053	0.062	0.082	0.152
CC1(C.F)	0.00	0.052	0.063	0.084	0.144
CC1(C.P)	0.00	0.055	0.064	0.088	0.130

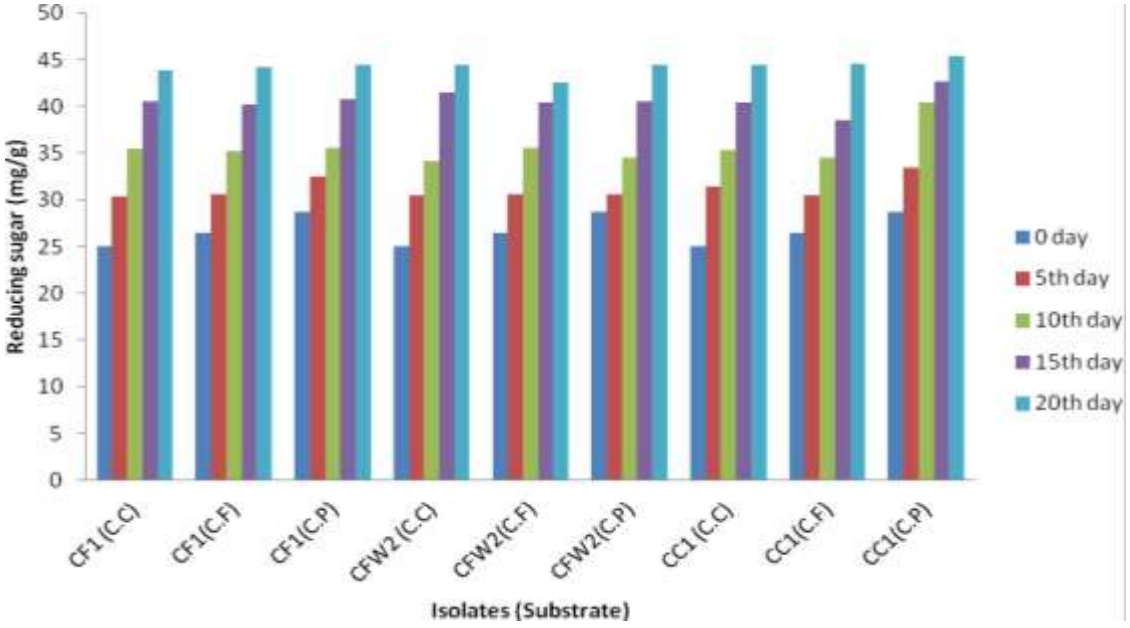

Figure 4. Reducing sugar values of samples inoculated with ethanol yielding bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis.*

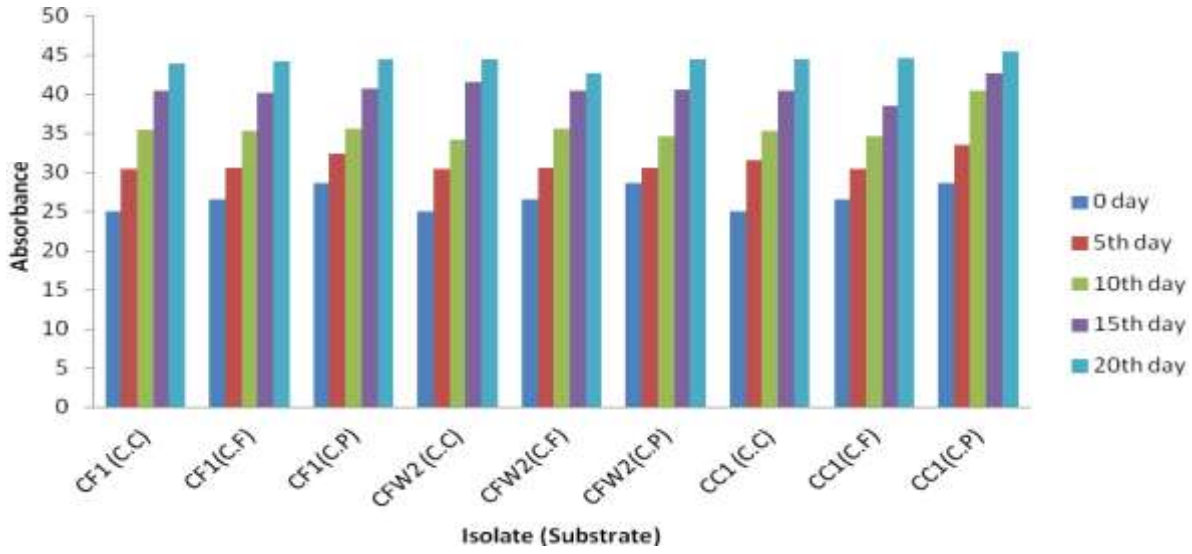

Figure 5. Optical density of samples inoculated with ethanol yielding bacterial isolates. CF1, *Bacillus macerans*; CC1, *Micrococcus varians*; CFW2, *Bacillus subtilis.*

Table 5. Proximate analysis of selected samples for fermentation.

Parameters (%)	Samples		
	Cassava peel	Corn cob	Corn flour
Moisture	28.06±0.007^{c}	27.69±0.007^{b}	31.00±0.007^{a}
Ash	1.94±0.007^{c}	1.99±0.007^{b}	2.1±0.007^{a}
Nitrogen	0.021±0.0007^{c}	0.028±0.0007^{b}	0.063±0.0007^{a}
Protein	0.13±0.0014^{c}	0.18±0.0014^{b}	0.39±0.0014^{a}
Crude Fibre	0.80±0.007^{c}	0.77±0.007^{b}	1.30±0.0007^{a}
Crude fat	0.58±0.007^{c}	0.65±0.007^{b}	1.80±0.0007^{a}
Carbohydrate	68.47±0.0014^{b}	68.70±0.007^{a}	63.34±0.0007^{c}

a, b and c, Means on the same row with different superscripts are statistically significant (P≤ 0.05).

progressive increase with time. *M. varians* had the highest value of 45.46±0.00 mg/g when used on cassava peels. At P≤0.05, there was statistical significant difference between the reducing sugar yields in all the samples.

Optical density of samples inoculated with ethanol yielding bacterial isolates

The optical density of the samples is shown in Figure 5. The highest optical density of 4.04 was observed in cassava peels with *B. macerans* after fermentation for 20 days.

Proximate composition of selected sample for fermentation

The proximate analysis of the sample showed the percentage composition of moisture, ash, nitrogen, protein, crude fibre, crude fat and carbohydrate of the samples (cassava peels, corn cob and corn flour) (Table 5). The highest value of moisture content was recorded for corn flour (31.00±0.007^{a}) and was significantly different from the others. The corn cob had the highest carbohydrate value of 68.70±0.007^{a}, while the highest value for crude fibre (1.80±0.0007^{a}) was recorded for corn flour. At P≤0.05, all the analyses carried out on the samples were statistically significant.

DISCUSSION

The aim of this study was to investigate the potential of using indigenous bacterial isolates for the sole hydrolysis and conversion of cellulosic and starch containing biomass to ethanol. The characterization of the indigenous microorganisms isolated from the samples (corn cob and corn flour) and water from fermented

cassava showed the morphological and biochemical characteristics of the isolates. Bacterial isolates were identified as: *B. subtilis, B. macquariensis, M. varians, B. macerans, B. circulans, C. xerosis, B. azotoformans* and *B. insolitus.* Some bacteria identified in this study were similar to those obtained from study carried out by Olusola (2010) who isolated *B. subtilis* in a submerged fermentation of cassava. *B. macquariensis, B. macerans* and *B. subtilis* have also been shown to be present in fermenting media (Sarkar et al., 2002). This research established the isolation of *M. varians* and its ability to produce ethanol from the biomass used.

In addition, starch hydrolysis was carried out compounding the substrates as sources of starch and some of the bacterial isolates were able to hydrolyze starch. The isolates capable of hydrolyzing the starch compounded from the samples were used for the fermentation trials. This according to Mohammed (2009) is due to the inability of the organisms to produce the enzyme amylase which is a starch digesting enzyme. Such organisms lacking starch hydrolyzing enzymes were therefore screened out.

The result of this study on amylase activity was similar to that of Harihrishna et al. (2012) and Adeyemi (1990), who reported amylase production by *B. subtilis, B. macerans* and *M. varians* in the fermentation of cassava peels and corn. Production of cellulase by some microorgamisms in this study was supported by studies carried out by Nisha et al. (2014) who reported cellulase activity in *M. varians* and *B. subtilis*.

At P≥0.05, there was no statistical significant difference between the ethanol yields of all bacterial isolates. It was also observed that at P≤0.05, there was statistical significant difference between the ethanol yield of the bacterial isolates in the corn cob, corn flour and cassava peels. Orji et al. (2016) reported 9% yield in ethanol using the corn cob as feed stock at a 10% substrate concentration. This study is comparable with that of Orji et al. (2016). The study recorded an average of 3.0% ethanol yield from corn cob at a 5% substrate concentration. Mohammed (2009) reported higher ethanol yields using corn starch with different organisms. The highest ethanol yield (3.20±0.00%) obtained from the cassava peels fermentation was lower than 17.6% reported in the study of Oyeleke et al. (2012).

In this study, physicochemical parameters such as pH, total acidity, reducing sugar concentration, absorbance (optical density) and ethanol concentration were evaluated during and after the fermentation processes. A steady reduction was observed in the pH of the samples as fermentation proceeded for 20 days. This was attributed to fermentation by-products from the incomplete oxidation of glucose residues to organic acids such as acetic and formic acids according to Michelle (2011). Noe et al. (2009) and Michelle (2011) reported pH 4.0 as the optimum pH for ethanol production. The report of these two authors is in conformity with that obtained in this study as the fermentation proceeded for 20 days after which, the ethanol concentration was determined and the optimum yield was less than 4.0.

No titratable acidity was reported on day zero before fermentation proceeded. The lowest titratable acidity observed in the cassava peels was between 0.05 and 0.061 moles in the bacterial fermentations on the 5th day of fermentation. It reached 0.13 moles on the 20th day of fermentation with the corn cob as substrate. At P≤0.05, there was statistical significant difference between the reducing sugar yields in all the samples using the bacterial isolates. Continuous increase in reducing sugar yield when the organisms were used for hydrolysis was reported by Mohammed (2009) to be due to substrate specificity of the enzymes. An increasing trend was observed in the optical density of the media while the bacteria fermented the media. This is similar to the report of Clark et al. (2013) who described this in a study on ethanol production as due to increase in reducing sugars and cell mass.

The samples (corn cob, corn flour and cassava peels) which served as substrates were analyzed for percentage proximate nutrient composition and at p≤0.05, the differences between the average values of moisture, ash, fat, protein, crude fibre and total carbohydrate of the samples were statistically significant. The highest percentage moisture content was observed in the corn cob while the lowest was found in the cassava peels. The percentage ash, nitrogen and protein content also showed similar trend. The highest crude fibre was found in the corn cob and the lowest in the cassava peels. The highest crude fat was found in the corn flour with the lowest in the cassava peels. The report on crude fat is similar to that of Ikram et al. (2012), while the reports on moisture, ash, fat, protein, crude fibre and total carbohydrate are similar to that of Ikram et al. (2012), Pointner et al. (2014), Ikeen et al. (2002), Christopher et al. (2016) and Ogbonna and Adewale (1993). The percentage ash and nitrogen, reported in the work of Pointner et al. (2014) are higher in the cassava peels when compared with the corn cob, while the percentage moisture, protein and carbohydrate were higher in the corn flour than in the other substrates. However, this study established that the percentage protein and ash content of the corn cob was higher than that of cassava peels.

Conclusion

This study investigated the potentials of wastes (cassava peels and corn cob) used as feed stocks for bio-ethanol production, in substantially replacing major food crops (corn) as a case study. The bio-conversion process of wastes shows that it is useful to mankind. This study also demonstrated the isolation of indigenous microflora as a successful approach to spontaneously carrying out fermentation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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