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Research Article

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Assessment of intrinsic physico-chemical fermentation conditions (pH and total titratable acidity) on liberated reducing sugar by bacteria and bacteria-yeast coupled fermentation of selected lignocellulosic biomass to ethanol

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Abstract

The study investigated the use of indigenous bacterial and yeast isolates to establish a single culture and a co-culture fermentation of selected lignocellulosic biomass (corn cobs and cassava peels) with bacteria and yeast. This was done to determine the effect of pH and total titratable acidity established by the activity these microorganisms on the liberation or reducing sugars available for bioethanol fermentation. The bacteria used in the study are: *Bacillus macerans, B. subtilis and Micrococcus varians*, while the yeast used is *Saccharomyces cerevisae*. The bacterial isolates were screened for amylase and cellulase activities and were found to express amylase relatively between 0.50-0.60 U/g and relatively between 0.040-0.0422 FPU for cellulase activity. The organisms were used in a single and co-culture fermentation trial for 20-days and intrinsic physic-chemical parameters (pH and Total titratable acidity) were examined at an interval of 5 days for 20 days. The pH of all the fermenting media were found to decrease progressively with time while the total titratable acidity and reducing sugar increased progressively with time. Highest reducing sugar was observed at pH values between 4.01 and 4.36 in both fermentation set up. At total titratable acidity between 0.133-0.146, the liberated reducing sugars were observed to reach a height of 45.46±0.00mg/g in the corn cob and cassava peels on the 20th day of fermentation with M. varians. At P≥ 0.05, there was statistical significant difference in the liberated reducing sugar by both fermentation set up of bacteria and bacteria-yeast couple. The study demonstrated the pH and total titratable acidity trend established by spontaneous fermentation of selected lignocellulosic biomass and how the gradual fermentation process increased the acidity overtime and as such increased reducing sugar concentration were observed at reduced pH and increased titratable acidity. Therefore fermentation processes in which there is interest in increased reducing sugar such as bioethanol fermentation should be consciously monitored to continue until appropriate levels of acidity are reached for optimum liberation of reducing sugars which are essential for increased conversion efficiency into desired end product.

Keywords: pH, total titratable acidity, reducing sugar, fermentation, amylase and cellulase activity.

Introduction

The microbial fermentation of sugars is the most prominent in the conversion of sugars inherent within biomass feedstocks into liquid fuels such as ethanol (Biofuels Joint Roadmap, 2006). Bioethanol can be produced from lignocellulosic feedstocks because a major problem with its production is the availability of raw materials needed for its production. Lignocellulosic biomass is the most promising

feedstock considering its availability and low cost. However, the large scale commercial production of fuel bioethanol from lignocellulosic materials has still not been implemented. The conversion technologies for producing bioethanol from lignocellulosic biomass resources such as: agricultural residues and urban wastes are still under development and have not yet been demonstrated commercially (Mustafa *et al.,* 2008).

However, there are intrinsic physico-chemical parameters such as the degree of acidity and alkalinity, total titratable acidity, temperature and reducing sugar that affect the metabolic activity of microorganisms involved in the fermentation process and this is the focus of the study. At lower reducing sugar concentrations, the amount of ethanol produced are at minimal values and vice-versa because the reducing sugars are liberated sugars required for bioconversion into metabolic end products such as ethanol (Tahir *et al.*, 2010). pH ranging from 4.0-6.0 are understood to be the most favourable for optimal bioethanol production and this values are observed during the progress of fermentation by Narendranath and Power (2005) and Fakruddin *et al.* (2012) to be responsible for optimum production of bioethanol. A decreasing trend in pH during fermentation and an increase in total titratable acidity is a consequential phenomenon resulting from the metabolic activities of microorganisms during fermentation as this demonstrates the progress of the process Wahab *et al.* (2005). This was described by Michelle (2011) as due to fermentation by-products resulting from the incomplete oxidation of sugar residues to organic acids such as acetic and formic acids.

The study is therefore concerned with an assessment of these parameters (pH, total titratable acidity and reducing sugar) during fermentation by bacteria and bacteria-yeast coupled fermentation of some lignocellulosic biomass such as cassava peels and corn cobs.

Materials and Methods

Sample collection and Preparation

Cassava peels were obtained in large quantities in a polythene bag from local garri producers while corn grains and corn cobs were gotten from retailers of corn within the Erinfun farm settlement along Afe Babalola University Road, Ado-Ekiti, Ekiti State on June 4, 2015. The waste water from the fermentation of cassava was also obtained into a covered plastic container and transported to the laboratory for storage at refrigerating conditions.

They were sundried for 5 days and ground into fine powder using a hammer mill. The samples were then bagged in clean polythene bags and stored at room temperature until use.

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. The bacterial isolates were also obtained from the samples.

Identification of Bacterial Isolates

The bacterial isolates used in this study were identified according to biochemical tests described by Bergey and John (2000) and Barrow and Feltham, 1993.

Identification of yeast isolates

Morphological studies of the various yeast cells were carried out using a light microscope and a simple staining technique. A high power objective (x1000) was used and staining was done on clean slides with crystal violet using a sterile inoculating loop. The staining technique was the same as that of the staining technique but the secondary stage of the Gram stain was excluded in this technique. Sugar fermentation tests were further carried out on the isolates. The yeast isolates were identified according to the methods Barnett *et al.* (2000).

Screening of Isolates for starch hydrolysis

Ten grams each of the samples (cassava peel flour, corn flour and corn cob) were weighed into separate 500ml conical flasks and boiled at 100^{o}C for 30 minutes the preparation was allowed to cool at room temperature and the starchy liquid sieved with a muslin cloth. Two percent of starch extract from the samples were used to enrich the nutrient agar and malt extract agar for the bacteria and yeast respectively. This was prepared from the sieved starchy liquid, the nutrient and malt extract agar. The preparation was sterilized at 121^{o}C for 15 minutes and dispensed into15 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 hours. Drops of Lugol's iodine were added to the bacterial growths observed on the plates after 24 hours 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).

Enzyme Assays

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

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. 500 ml distilled water were added and the preparation was sterilized at 121°C for 15 minutes. The samples were allowed to cool and dispensed into flasks which had been previously sterilized with 50 ml 3% sodium hypochlorite and rinsed vigorously with sterile distilled water.

Inoculum Preparation

One hundred millilitre of nutrient broth was prepared and dispensed at 10 ml into each of 10 test tubes for the bacterial isolates. The media were sterilized at 121°C for 15 minutes, allowed to cool and subsequently inoculated with 24-hour cultures of the bacterial and yeast isolates respectively.

Analysis of Fermentation Medium

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

Analysis of Data

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

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 identified the following organisms: *Bacillus substilis, 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 watter gotten from cassava fermentation, two isolates (*M. varians* and *B. circulans*) were gotten from corn cob, *B. macquariensis* was gotten from cassava peels while three other isolates (*C. xerosis, B. azotoformans* and *B. insolitus*) were gotten from the soil sample. This is represented in Table 1.

Biochemical characteristics of the Yeast isolates

The result of the biochemical tests carried out on the yeast isolates is as shown in table 2.0 identifying the yeast isolates as: *Saccharomyces cerevisiae.*

Sample starch hydrolysis test

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

The 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 was observed in corn flour by *Bacillus subtilis* (CFW2) in the corn flour (C.F) to be 0.61 U/mg while the lowest was observed in corn cob (C.C) by the *Bacillus macerans* isolate to be 0.50 U/mg. 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 *Bacillus macerans* (CF1) in the cassava peels (C.P) to be 0.0470 FPU while the lowest was observed in corn flour (C.F) by the *Micrococcus varians* to be 0.0301 FPU.

This is as presented in Figures 1and 2 .

Table 1.0: Biochemically characteristics and identification of bacterial isolates

Isolate Code	Spore staining	Starch hydrolysis	Catalase	Voges Proskauer test	Methyl Red test	Swollen cell	6.5% NaCl	Acid from arabinose	Gas from glucose	Acid fast test	Citrate test	Identified isolate
CFW1	+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	-ve	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*

Key: ND: Not Determined +ve: Positive -ve: Negative
CFW: Cassava Fermentation Water, CC: Corn Cob, CP: Cassava Peels, FCS.A-FCS.G: FCS stands for Fermented Cassava in soil, A-G- bacterial isolates obtained from the soil region containing fermented cassava effluents

Table 2.0: Biochemical tests carried out on the Yeast isolates

Isolate code	Glucose	Mannitol	Sucrose	Maltose	Lactose	Identified organisms
AY	+	-	+	-	-	*Saccharomyces cerevisiae*
BY	+	-	-	-	-	*Saccharomyces cerevisiae*

Key: +: Positive
-: Negative

Table 3: Sample 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

Key: ND: Not Determined +ve: Positive -ve: Negative
CFW: Cassava Fermentation Water, CC: Corn Cob, CP: Cassava Peels, FCS.A-FCS.G: FCS stands for Fermented Cassava in soil, A-G- bacterial isolates obtained from the soil region containing fermented cassava effluents

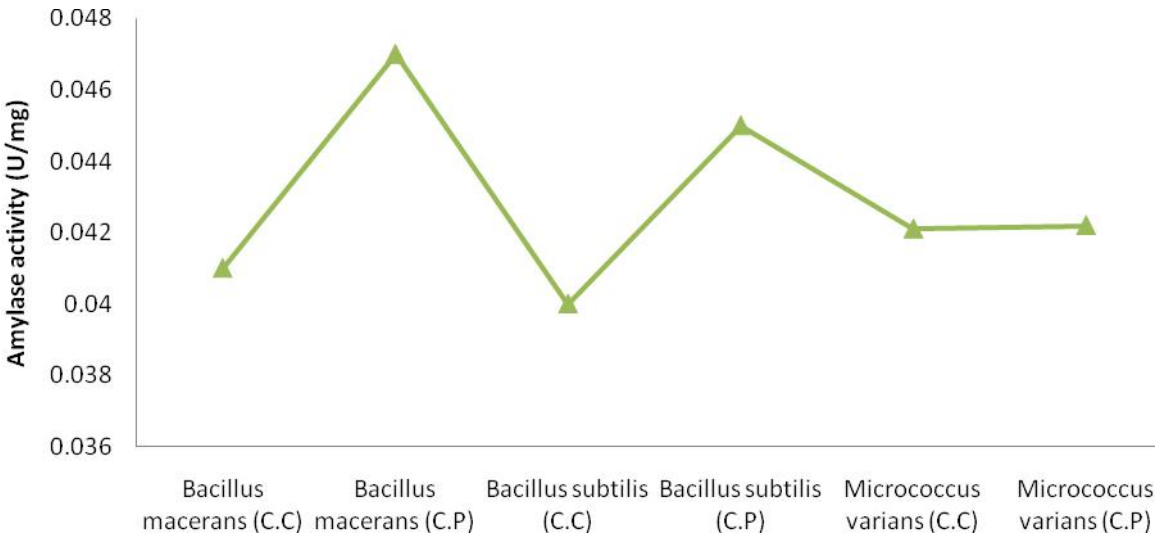

Figure 1: The amylase activity of the bacterial isolates

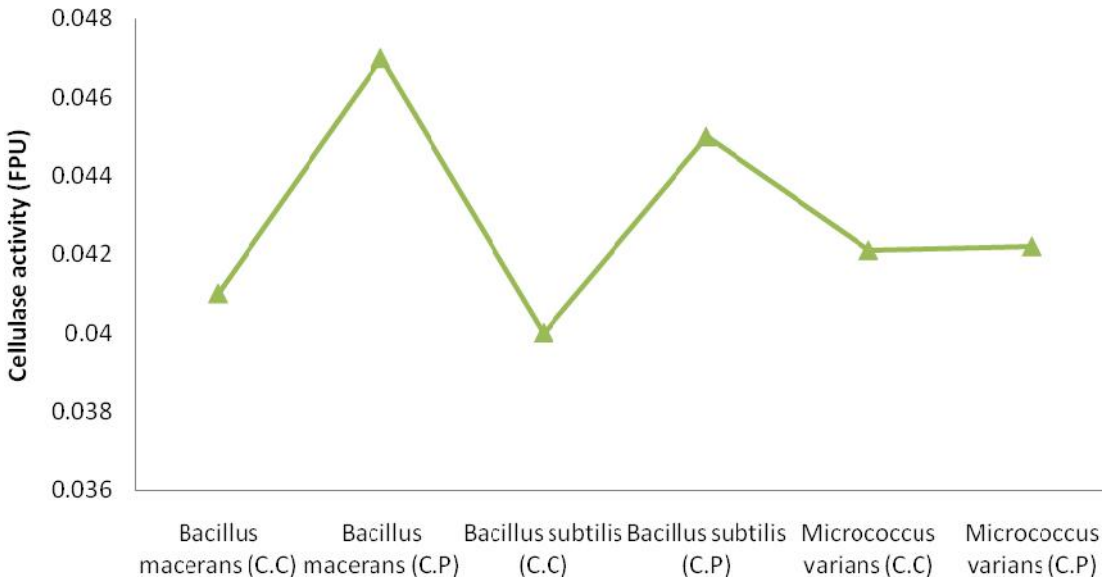

Figure 2: The cellulase activity of the bacterial isolates

pH of Samples inoculated with bacterial isolates

The pH of the samples after screening during fermentation by selected pure cultures is as shown in Figure 3. There was a progressive decrease with time. The fermentation carried out with *Bacillus macerans* on the corn cob was observed to have a decreasing trend for 20 days. The pH on day zero was 6.80, 5.52 the 5th day, 4.75 on the 10th day, 4.41 on the 15th day and 4.10 on the 20th day. With *Micrococcus varians,* the following pH trend was observed in the fermented cassava peels: 7.04 on day zero, 5.52 on the 5th day, 4.85 on the 10th day, 4.35 on the 15th day and 4.20 on the 20th day. Overall the highest pH was observed to be 7.10 in the corn flour on the 1st day of fermentation while the lowest recorded was observed in the corn flour and corn flour on the 20th day to be 4.1. At P≤0.05 there was statistical significant difference between the pH values observes in all the samples for the number of days.

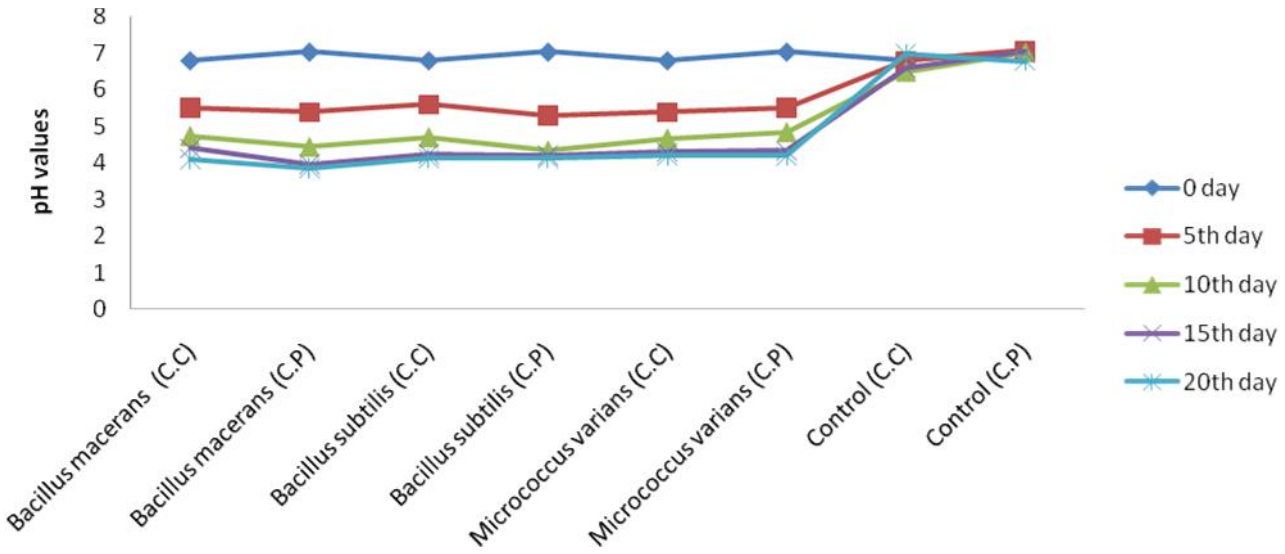

Figure 3: pH values of samples inoculated with bacterial isolates

Total titratable acidity of samples inoculated with the bacterial isolates

The total titratable acidity of the samples during fermentation by selected bacteria pure cultures is as shown in Figure 4. There was a progressive increase with time. The fermentation carried out with *Bacillus macerans* on the corn cob was observed to have an increasing trend for 20 days. The total titratable acidity on day zero was 0.00, 0.050 the 5th day, 0.055 on the 10th day, 0.081 on the 15th day and 0.120 moles on the 20th day. With *Micrococcus varians*, the following trend was observed in the total titratable acidity of the fermented cassava peels: 0.00 on day zero, 0.055 on the 5th day, 0.064 on the 10th day, 0.088 on the 15th day and 0.130 moles on the 20th day. The highest titratable being 0.1800 moles observed in the corn cob on the 20th day of fermentation while the lowest recorded was observed in all samples on the 1st day of fermentation to be 0.0000 moles. At P≤0.05 there was statistical significant difference between the total titratable acidity observed in all the samples for the number of days.

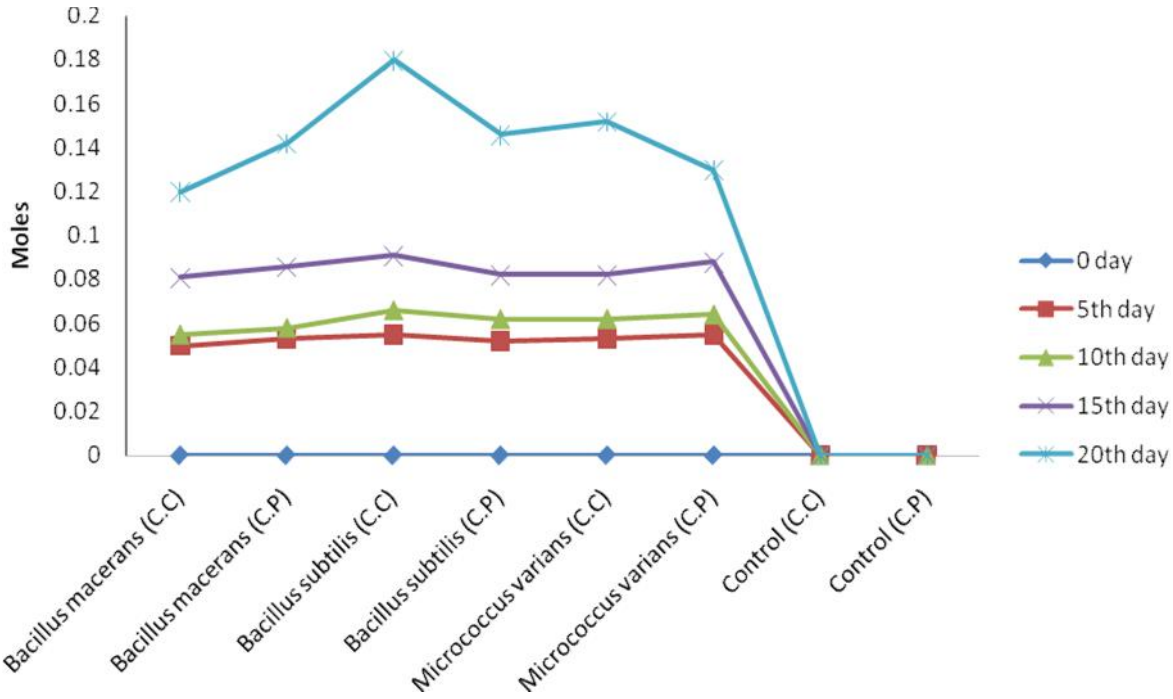

Figure 4: Total titratable acidity of samples inoculated with bacterial isolates

pH of Samples inoculated with co-cultures of bacterial and yeast isolates

The pH of all the samples during fermentation by selected pure cultures of bacteria and yeast are shown in Figure 5. There was a progressive decrease with time. The fermentation carried out with *Bacillus macerans* and *Saccharomyces cerevisae* on the corn cob was observed to have a decreasing trend for 20 days. The pH on day zero was 6.80, 5.52 the 5th day, 4.75 on the 10th day, 4.41 on the 15th day and 4.20 on the 20th day. While the following pH trend was observed using *Bacillus subtilis* and *Saccharomyces cerevisae* to ferment cassava peels: 7.04 on day zero, 5.73 on the 5th day, 4.75 on the 10th day, 4.36 on the 15th day and 4.20 on the 20th day. With *Micrococcus varians and Saccharomyces cerevisae*, the following pH trend was observed in the fermented cassava peels: 6.80 on day zero, 5.76 on the 5th day, 4.91 on the 10th day, 4.62 on the 15th day and 4.27 on the 20th day. Overall the highest pH was observed to be 7.04 in the cassava peels on the 1st day of fermentation while the lowest recorded was observed in the cassava peels on the 20th day to be 4.10. At P≤0.05 there was statistical significant difference between the pH values in all the samples for the number of days.

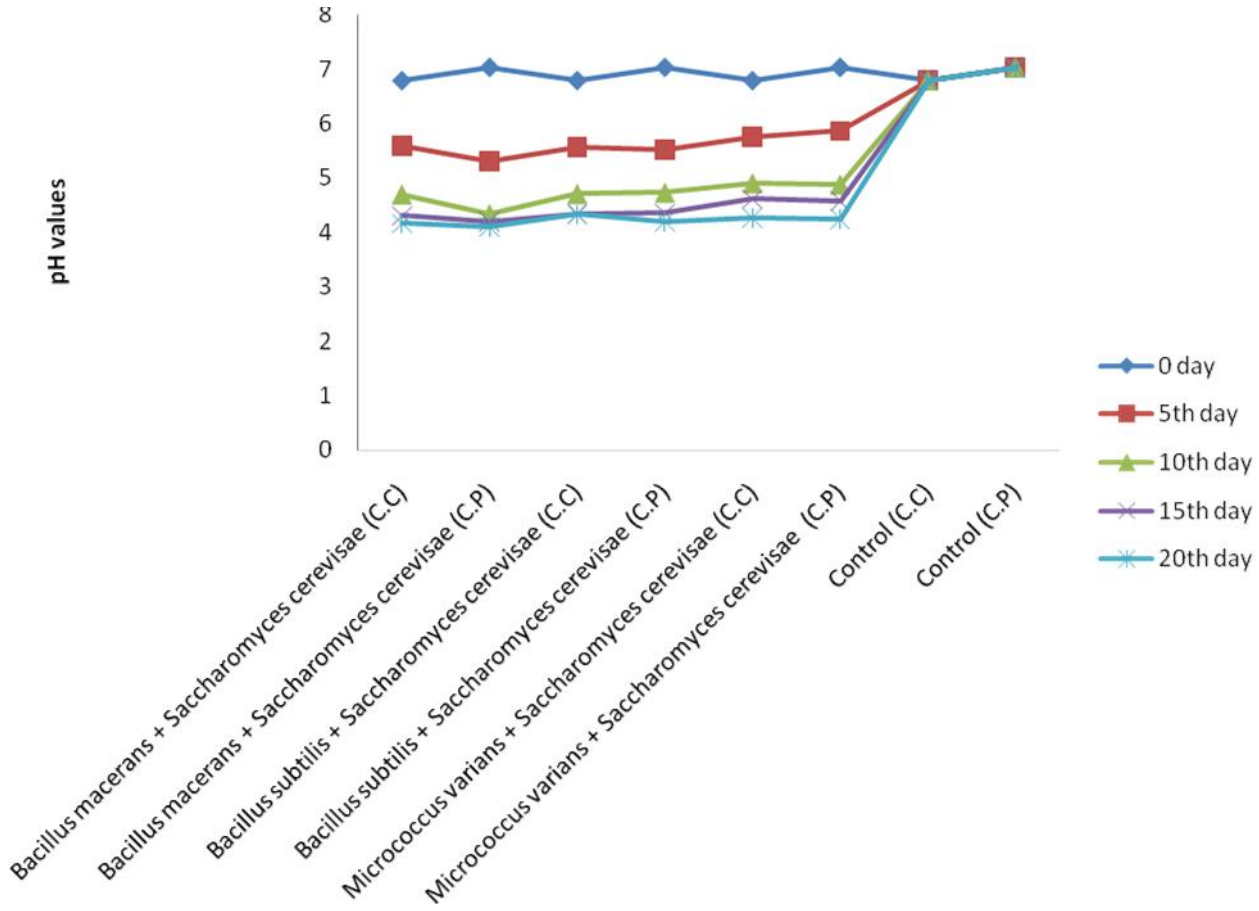

Figure 5: pH values of samples inoculated with co-culture of bacterial and yeast isolates

Total titratable acidity of samples inoculated with co-cultures of bacterial and yeast isolates

The total titratable acidity of all the samples during fermentation by selected pure cultures of bacteria and yeast is shown in Figure 6. There was a progressive increase with time. The fermentation carried out with *Bacillus macerans* and *Saccharomyces cerevisae* on the corn cob was observed to have an increasing trend for 20 days. The total titratable acidity on day zero was 0.00, 0.053 the 5th day, 0.068 on the 10th day, 0.080 on the 15th day and 0.146 moles on the 20th day. While the following trend was observed in the total titratable acidity using *Bacillus subtilis* and *Saccharomyces cerevisiae* to ferment cassava peels: 0.00 on day zero, 0.056 on the 5th day, 0.064 on the 10th day, 0.086 on the 15th day and 0.133 moles on the 20th day. With *Micrococcus varians and Saccharomyces cerevisae*, the following trend was observed in the total titratable acidity of the fermented cassava peels: 0.00 on day zero, 0.054 on the 5th day, 0.066 on the 10th day, 0.081 on the 15th day and 0.133 moles on the 20th day. Overall the highest titratable acidity was observed to be 0.150 moles in the corn cob on the 20th day of fermentation while the lowest recorded was observed in the samples on the 20th day to be 0.0000 moles. At P≤0.05 there was statistical significant difference between the total titratable acidity in all the samples for the number of days.

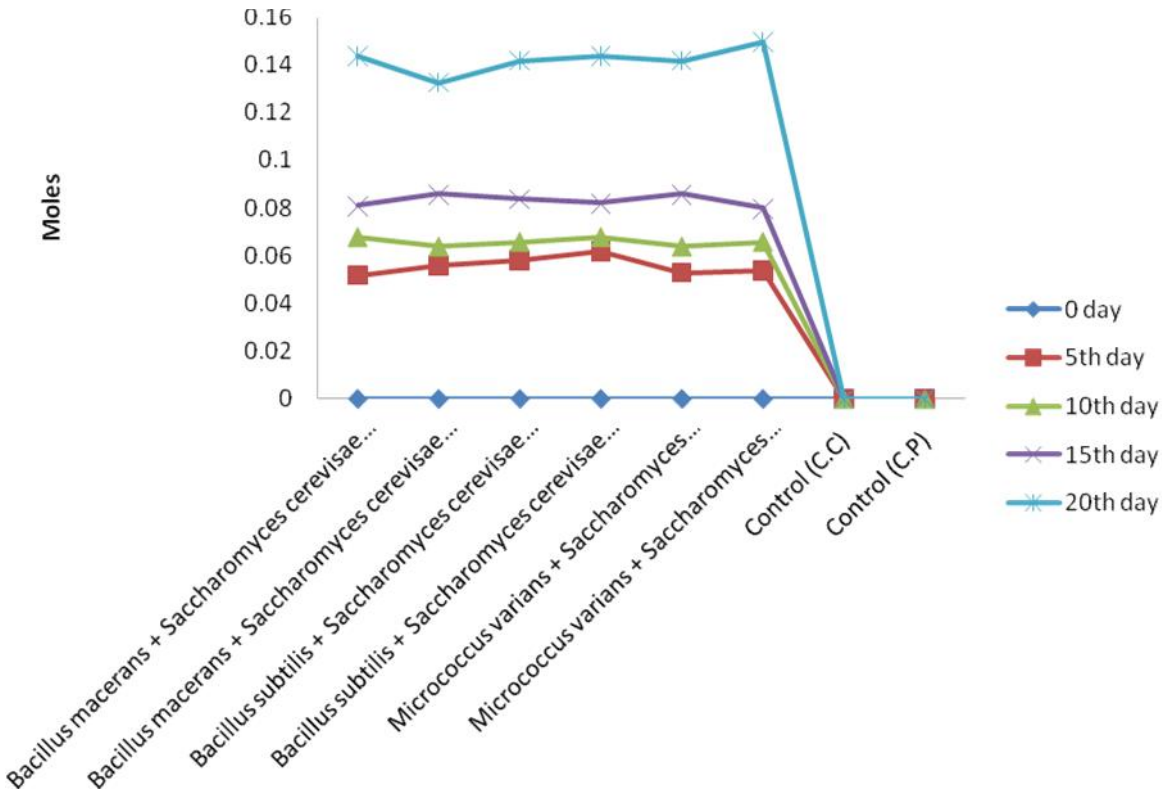

Figure 6: Total titratable acidity of samples inoculated with co-culture of bacterial and yeast isolates

Reducing sugar values of samples inoculated with bacterial isolates

The result of the reducing sugar of the samples during fermentation by selected bacteria pure cultures is as shown in figure 7. There was a progressive increase with time. There was a progressive increase with time. The fermentation carried out with *Bacillus macerans* on the corn cob was observed to have an increasing trend for 20 days. The reducing sugar on day zero was 25.10±0.00, 30.40±0.00 the 5th day, 35.50±0.00 on the 10th day, 40.50±0.00 on the 15th day and 43.86±0.00 mg/g on the 20th day. With *Micrococcus varians*, the following trend was observed in the reducing sugar values of the fermented cassava peels: 28.70 on day zero, 33.44 on the 5th day, 40.46 on the 10th day, 42.65 on the 15th day and 45.46 mg/g on the 20th day. The highest reducing sugar value being 45.46±0.00 mg/g in the corn cob and cassava peels on the 20th day of fermentation with *Micrococcus varians* while the lowest recorded was observed on day zero in the corn cob to be 25.10±0.00 mg/g with *Bacillus macerans* and with *Bacillus subtilis*. At P≤0.05 there was statistical significant difference between the reducing sugar yields in all the samples.

Reducing Sugar values of samples co-inoculated bacterial and yeast isolates

The result of the reducing sugar of all the samples during fermentation by selected pure cultures of bacteria and yeast is shown in Figure 8. There was a progressive increase with time. The fermentation carried out with *Bacillus macerans* and *Saccharomyces cerevisae* on the corn cob was observed to have an increasing trend for 20 days. The reducing sugar on day zero was 25.10±0.00, 30.50±0.00 the 5th day, 38.55±0.00 on the 10th day, 44.62±0.00 on the 15th day and 48.86±0.00 mg/g on the 20th day. While the following trend was observed in the reducing sugar using *Bacillus subtilis* and *Saccharomyces cerevisiae* to ferment cassava peels: 28.70±0.00 on day zero, 32.25±0.00 on the 5th day, 36.20±0.00 on the 10th day, 40.20±0.00 on the 15th day and 45.60±0.00 mg/g on the 20th day. With *Micrococcus varians* and *Saccharomyces cerevisae* the following trend was observed in the reducing sugar values of the fermented cassava peels: 28.70±0.00 on day zero, 29.6±0.00 on the 5th day, 39.52±0.00 on the 10th day, 42.66±0.00 on the 15th day and 48.50±0.00 mg/g on the 20th day. Overall the highest was observed to be 50.56±0.00 mg/g in the corn cob on the 20th day

of fermentation with *Micrococcus varians* and *Saccharomyces exiguous* while the lowest recorded was observed in the samples on the 5th day to be 25.10±0.00 mg/g in the corn cob on day zero of fermentation with *Bacillus macerans* and *Candida humilis*. At P≤0.05 there was statistical significant difference between the reducing sugar yields in all the samples.

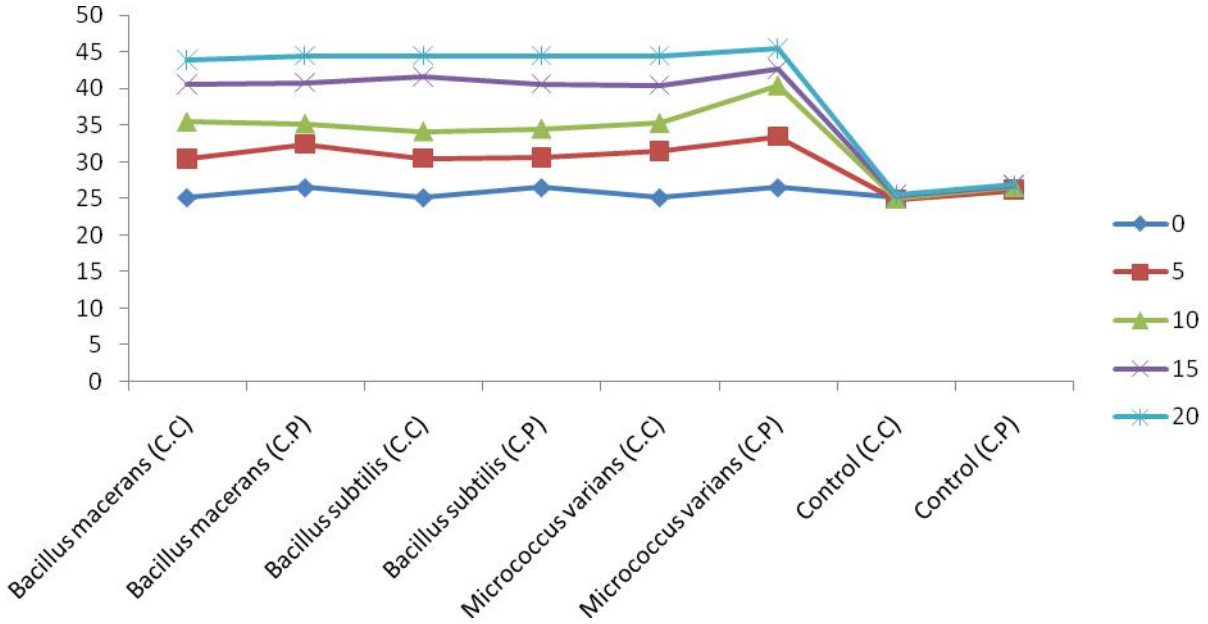

Figure 7: Reducing sugar values of samples inoculated with bacterial isolates

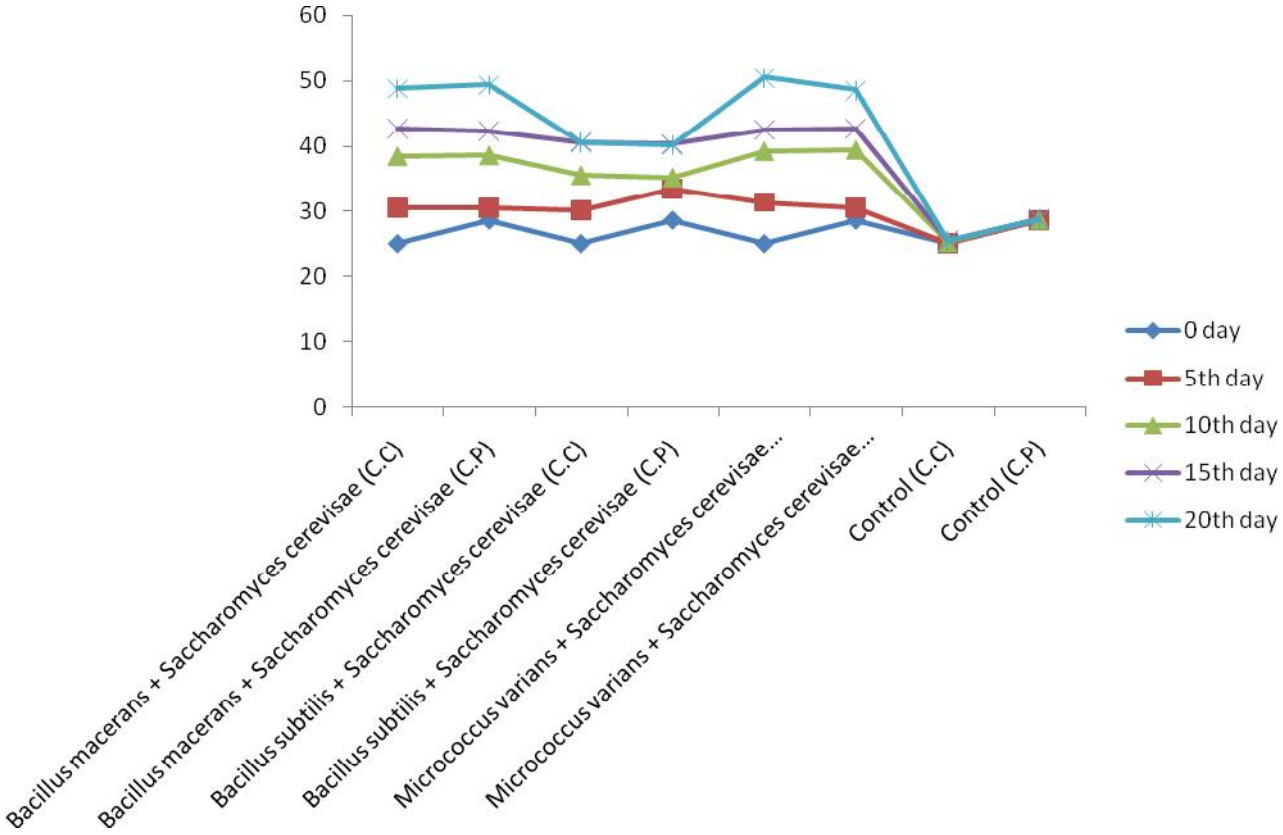

Figure 8: Reducing sugar values of samples inoculated with co-culture of bacterial and yeast isolates

Discussion

The characterization of the indigenous microorganisms isolated from the samples (corn cob and cassava peels) and water from fermented cassava showed the morphological and biochemical characteristics of the isolates. The findings from this study was compared with the Bergey's manual of determinative bacteriology and the bacteria isolates were identified as: *Bacillus subtilis, B. macquariensis, Micrococcus varians, B. macerans* and *B.circulans* while the yeast isolates were identified according to Barnett *et al.* (1993) as: *Saccharomyces cerevisae*. Some non-starch hydrolyzing isolates were also identified as *Corynebacterium xerosis, B. azotoformans* and *B. insolitus.* These were not used for the fermentation process as they were not capable of the initial break down of the starch present in the compounded samples used as sources of starch.

The bacteria identified in this study were similar to those obtained from the study carried out by Olusola (2012) who isolated *Bacillus subtilis* in a submerged fermentation of cassava. *B. macquariensis, B. macerans* and *B. subtilis* have also been shown to be present in fermenting media (Sarka *et al.*, 2002; Zuber and Anjani, 2013). A specie of *Micrococcus* commonly discussed for its prominent role in fermentation is *M. luteus* (Achi, 2007). However this research established the isolation of *M. varians* and its ability to liberate ethanol from the biomass used. 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 Ajani, 2013).

The isolated yeasts in this study can be linked to the liberation of sugars from complex starch containing substrates by the bacteria isolates present in the fermenting substrates. The liberated sugars are enough to encourage yeast succession as reported by Joanita *et al.* (2014). Among several yeasts, *Saccharomyces species* are the most important species present during the fermentation process. (Pretorius, 2000; Querol and Fleet, 2006). The report of this study on amylase activity is similar to the observations of Harikrishna *et al.* (2012) and Adeyemi (1990), who reported amylase production in *B. subtilis, B. macerans and Micrococcus varians* in the fermentation of cassava peels and corn. The report of this study on cellulase production is supported by the works of Nisha *et al.* (2014) and Saraswati *et al.* (2012) who reported cellulase activity in *M. varians*, and *B. subtilis*. In this study *B. macerans* was also found to be capable of cellulase production in fermenting media. In this study, physicochemical parameters such as pH, total acidity, and reducing sugar concentration were carried out 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 acid and formic acid 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 the results obtained in this study as the fermentation proceeded for 20day and the optimum yield at pH not less than 4.0. The total titratable acidity during fermentation of all the substrates were observed to progressively increase just as the pH decreased and this corresponds to the report of Obueh and Ikenebomeh (2014). There was increase in the amount of reducing sugar as the fermentation progressed and the highest reducing sugars were observed on the last day of fermentation. It would have been expected that there would be a decline in the reducing sugar but as reported by the work of Zakpaa *et al.* (2009), enzyme activities are improved at suitable pH. Therefore the pH of the media during fermentation which lied between 4.10-4.36 resulted in improved reducing sugar concentration. The work of Zakpaa *et al.* (2009), recorded highest saccharification at pH 4.0, so it can be inferred that the considerable increase on the last day was as a result of the pH of the media. At $P \geq 0.05$, there was no statistical significant difference in the outcome of the bacteria fermentation and the bacteria-yeast coupled fermentation. According to Tapan *et al.* (2005), there was continuous amylase activity between pH 3.5 and 8.5, therefore the acidic nature of the media as reported in this study will not inhibit enzyme activity as fermentation progressed. Ruby *et al.* (2012) reported an increase in cellulase activity from pH 3.0 and a decline at pH 6.0. This means that above the pH 5.0, there is reduced cellulase activity and this supports the report of this study as the reducing sugar liberated from the lignocellulosic polymers increased at lower pH.

Conclusion

Conclusively, the study was able to demonstrate the relationship between the pH, total titratable acidity and reducing sugar liberation and it was observed that the activity of the enzymes produced by the

microorganisms was more active while the pH decreased and the total titratable acidity increased. It can therefore be said that microbial fermentations which involve considerable reducing sugar for desired end product, should be examined to ensure that the media in which the fermentation of the microorganisms are taking place reaches the appropriate degree of acidity since the activity of enzymes are observed to be more effective at such conditions of specific levels of acidity.

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