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Isolation of microorganisms from cassava peels for the production of bioethanol

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Abstract

This study investigates the production of bioethanol from cassava peels using the inherent isolated microorganisms. Cassava peels were collected from cassava processing unit in selected towns in Ekiti State. Microorganisms were isolated from the cassava peels by using pour plate method; the isolates were identified by different biochemical tests and molecular characterization. The bacterial isolates include, *Sporosarcina terrae*, *Bacillus cereus*, *Proteus mirabilis and Pseudomonas taiwanensis*; while the fungal isolates include yeast *(Wickerhamomyces rabaulensis)*, *Rhizopus* and *Aspergillus niger.* The entire bacterial isolates and *A. niger* tested positive to degradative test, while only *W. rabaulensis* tested positive to fermentation test. Four bacterial species were detected using 16S rDNA nucleotide sequence. High yields of the bioethanol was obtained using the combination of different inoculants, however the combination of *B. cereus* and *W. rabaulensis* produced the highest yield of 19.3 g/cm^3 at a concentration of 38.6% and 17.7 g/cm^3 at a concentration of 34.4% from 100g of cassava peels when distilled at 14 days and 21 days respectively. Yields of 562.31g/cm^3 (67.0%) and 481.9g/cm^3 (65%) of bioethanol were obtained from 5 kilograms (Kg) of cassava peels with a combination of *B. cereus* and *W. rabaulensis* and a combination of *P. taiwanensis and W. rabaulensis* respectively. The results of this study confirmed that bioethanol can be produced from cassava peels using the inherent isolated microorganisms as it is simple to produce, environmental friendly. and reliable therefore, healthy environment, wealth and energy generation from wastes (cassava peels) is assured in Ekiti State.

Keywords: Bioethanol; Cassava peels; Environmental pollution; Microorganisms; Waste

1. Introduction

Cassava (*Manihot esculenta*) cultivated extensively as a food crop in Africa is the third largest source of carbohydrate in food for human consumption in the world [1]. About 10 million tons of cassava tubers are produced in Nigeria annually, cassava peels which is considered to be an agricultural waste is usually generated from the peeling of these cassava tubers in the processing of garri, fufu, lafun flour and starch in Nigeria [2]. It is a waste usually dumped indiscriminately and constitutes environmental pollution which endangers terrestrial life [3]. The peels easily got rotten after some days of disposal and produce foul odours considered to be environmental pollution due to microbial activities such as fungi, yeast and bacteria [4]. The indiscriminate and lack of control measure in the disposal of this waste has resulted in the release of obnoxious smell and health hazards.

Clean environment is the focus of world governments and agencies in the 21st century but cassava peels is a source of environmental pollution which is generated every day in Ekiti-State. In view of this, there should be a way of recovering resources from this waste in order to find beneficial use for it, therefore, waste like cassava peels can be suggested to be a potential source of raw materials for the production of bioethanol, since the peels can be conditioned to produce microorganisms capable of degrading cellulose into simple sugar which can be used as a raw material for the production of bioethanol [5]. Much work has been carried out regarding the utilization of cassava peels as substrates for microbial

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protein enrichment, increasing microorganism biomass and on their use as food additives [6]. Sulfahri *et al*. [7] carried out investigation on production of bioethanol from algae (*Spirogyra)* with fermentation by *Zymomonas mobilis* and *Saccharomyces cerevisiae*, Mustafa *et al*. [8] also carried out preliminary investigation on the production of bioethanol using acid hydrolysis and microorganisms from other sources, but the possibility of using cassava peels for the production of bioethanol using the inherent isolated microorganisms as inoculants has not been given much attention.

Bioethanol produced from cassava peels can be used as petrol substitute for vehicles [9], for the production of hand sanitizers, as a disinfectant in the laboratories, homes and hospitals, and in the industries. Cassava is one of the major farm products in Ekiti State, hence sustainable supply of cassava peels for bioethanol process is assured. Hence, healthy environment, wealth and energy generation from wastes (cassava peels) in Ekiti-State.

2. Material and methods

2.1. Collection of Samples

Cassava peels were collected from five selected towns within Ekiti-State, Nigeria.

2.2. Isolation of Microorganisms

The isolation of microorganisms was carried out using pour plate method as described by Sam [10]. The nutrient agar plates were incubated at 37 °C for 24 hours while the PDA plates were incubated at room temperature for 5 days, the colonies on the nutrient agar plates were sub cultured to get pure isolates.

2.3. Identification of Microorganisms

The bacterial isolates were identified by morphological characteristics and different biochemical tests such as catalase test, coagulase test, fermentation test, starch hydrolysis test and growth at 55^{o}C with 6.5% sodium chloride while the fungal isolates were identified based on their appearances and staining with lacto- phenol cotton blue stain as described by Fawole and Oso [11].

2.4. Screening of the Isolates for Degradative Ability

Screening of the isolates for degradative ability was done by inoculating the isolates on a medium containing Carboxyl methyl cellulose (CMC) according the method of Kiri and Fuji [12]. The plates were flooded with Congo red solution for 15mins after 96 hours of incubation period and later flooded with 1 molar sodium chloride solution for another 15 minutes to check for the presence of hydrolytic zone.

2.5. Screening of the Isolates for fermentative Ability

A drop of phenol red was added to each sugar fermentation broth, these were sterilized, inoculated separately with the fungal isolate from each sample. The inoculated broths were incubated at 35^{o}C for 48 hours and observed for colour change [10].

2.6. Molecular Characterization of the isolates

One fungus and six bacterial isolates were selected for molecular characterization; this was carried out by PCR using 16S RNA gene amplification for bacteria and ITS gene amplification for fungal isolates according to Amoon *et al*. [13]. The DNA was isolated by the Chelex method and used as templates for PCR amplification of the 16S rRNA and ITS genes. 27F-AGA GTT TGA TCM TGG CTC AG and 1492R- TAC GGY TAC CTT GTT ACG ACT T primer was used to amplify the 16S rRNA genes while ITS1-TCCGTAGGTGAACCTGCGG and ITS4- TCCTCCGCTTATTGATATGC was used to amplify the ITS gene by a conventional PCR technique, the amplified PCR products were sequenced and the sequences were viewed by Finch TV program version 1.4.0. The identity and similarity of the nucleotide sequence of the isolated strains was detected by comparing them with published sequences using BLAST

2.7. Production of Bioethanol from cassava peels

Production of bioethanol from cassava peels using the inherent isolated microorganisms involves hydrolysis, fermentation and distillation process, bioethanol was produced by method of Maki and Kazuhiro [14] which employs simultaneous saccharification and fermentation of any of the isolates that showed positive degradative test (*Bacillus cereus* and *Proteus mirabilis*, *Pseudomonas taiwanensis*, *Sporosarcina terrae* and *Aspergillus niger*) and yeast (*Wickerhamomyces rabaulensis*) to the cassava peels slurry in different proportions inside the fermentation vessels.

Water was added to 20 g, 40 g, 60 g, 80 g and 100 g of the powdery cassava peels flour to turn them into slurries in separate fermentation vessel up to 500 cm^3 mark, this was done in pent duplicate and sterilized. Each vessel was inoculated with one of the five isolates tested positive to degradative test (*B. cereus, P. mirabilis, P. taiwanensis*, *S. terrae and A. niger*), a yeast *W. rabaulensis was added* to each flask separately to aid fermentation. The set up was done in duplicate, the first set were left for 14 days, while the second set were left for 21 days under anaerobic condition in order to compare the yields from the two sets. At the end of the fermentation period, the culture broth was qualitatively tested for alcohol production using dichromate ($K_2Cr_2O_7$) reagent test. The content of the fermentation vessels was distilled at 78 ℃ (standard temperature for ethanol distillation). A series of tests was carried out on the product (The pH was measured, the distillation range was observed and the flash point was also determined) in order to compare the bio-ethanol produced with the standard ethanol. The combination of the isolates that produced the highest yield of bioethanol was selected for the production of bioethanol in large quantity.

2.8. Statistical Analysis

Data obtained from this study were analyzed by descriptive statistical method and one-way analysis of variance (ANOVA) at 95% confidence level.

3. Results and discussion

A total of fourteen (14) bacterial isolates belonging to four genera were obtained from the cassava peels samples (Table 1), this includes; *Bacillus cereus, Sporosarcina terrace, Proteus mirabilis and Pseudomonas taiwanensis.* This was confirmed by nucleotide sequence analysis of the 16S rDNA gene, yielding the expected amplicon of 1500 bp in product size (Figure 1), however, the presence of bacteria does not indicate that all are pathogenic. This result corroborates the work of Elijah *et al*. [15] that isolated *Bacillus cereus*, *Staphylococcus xylosus*, *Lactobacillus manihotivorans* and *Bacillus subtilis* from cassava wastes waters.

Table 1 Bacterial Isolates from the samples

Samples	Bacterial isolates
1	*Bacillus cereus, Sporosarcina terrace , Proteus mirabilis*
2	*Bacillus cereus, Proteus mirabilis, Pseudomonas taiwanensis*
3	*Bacillus cereus, Proteus mirabilis*
4	*Bacillus cereus, Proteus mirabilis, Pseudomonas taiwanensis*
5	*Sporosarcina terrace*, *Proteus mirabilis, Bacillus cereus*

The fungi isolated is shown in table 2, this includes; *Aspergillus niger*, *Rhizopus nigrican* and yeast (*Wickerhamomyces rabaulensis* CBS 6797). ITS gene amplification of the yeast produced an expected PCR amplification product of size 500-600-bp fragment (Figure 2). This yeast was isolated in all the samples, it was the only fungal isolate that had fermentative ability (Table 4), this ability is due to the possession of enzyme zymase which catalyzes the *fermentation* of sugar into ethanol and carbon dioxide, this implies that the isolated yeast can be used to ferment the cassava peels slurry into bioethanol. This result is in line with Balarabe *et al*. [16].

Table 2 Fungal Isolates from the samples

Samples	Fungal isolates
1	*Wickerhamomyces rabaulensis*, *Aspergillus niger*
2	*Rhizopus nigrican, Wickerhamomyces rabaulensis*
3	*Wickerhamomyces rabaulensis*, *Rhizopus nigrican*
4	*Aspergillus niger*, *Wickerhamomyces rabaulensis*
5	*Wickerhamomyces rabaulensis*

Table 3 shows the degradative abilities of the bacterial isolates, all the isolates tested positive to the test, this implies that any of these isolates can be used to hydrolyze the lignocellulose complex present in the cassava peels slurry, the fungus, *Aspergillus niger* also show positive degradative test. This ability of the isolates to degrade the lignocellulose materials may be due to their highly efficient enzymatic systems namely, the hydrolytic and ligninolytic systems. The hydrolytic system produces hydrolases that are responsible for polysacchide degradation and production of sugars while the ligninolytic system degrades lignin component and opens phenyl rings [17]. Consequently, reducing sugars are further converted into ethanol production using different fermentative microorganisms. This result is in tandem with the findings of Pratima *et al*. [18] that isolated cellulolytic microorganisms from the gut of four different invertebrates (termite, snail, caterpillar, and bookworm).

Table 3 Degradative ability of the isolates

Organisms	Degradative ability
Bacillus cereus (S_1)	+
Sporosarcina terrae (S_1)	+
Proteus mirabilis (S_1)	+
Bacillus cereus (S_2)	+
Proteus mirabilis (S_2)	+
Pseudomonas taiwanensis (S_2)	+
Proteus mirabilis (S_3)	+
Bacillus cereus (S_3)	+
Pseudomonas taiwanensis (S_4)	+
Proteus mirabilis (S_4)	+
Bacillus (S_4)	+
Bacillus cereus (S_5)	+
Sporosarcina terrace, (S_5)	+
Proteus mirabilis (S_5)	+
Aspergillus niger (S2)	+
Aspergillus niger (S2)	+

S_1 = sample 1; S_2 = sample 2; S_3 = sample 3; S_4 = sample 4; S_5 = sample 5; + = Positive

Table 4 Fermentative ability of the fungal isolates

Fungi	Fermentative ability
Yeast (*Wickerhamomyces rabaulensis*) (S_1)	+
Aspergillus niger (S_1)	-
Rhizopus nigrican (S_2)	-
Wickerhamomyces rabaulensis (S_2)	+
Rhizopus nigrican (S_3)	-
Wickerhamomyces rabaulensis (S_3)	+
Aspergillus niger (S_4)	-
Wickerhamomyces rabaulensis (S_4)	+
Wickerhamomyces rabaulensis (S_5)	+

S_1 = sample 1; S_2 = sample 2; S_3 = sample 3; S_4 = sample 4; S_5 = sample 5; + = Positive

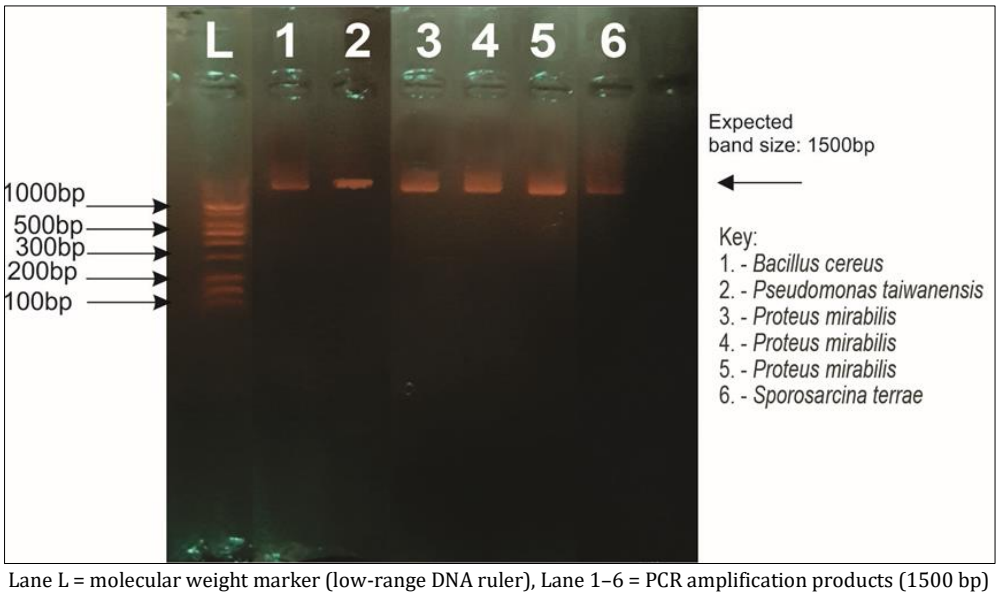
The 16S rRNA gene of the bacterial isolates were amplified by Polymerase Chain Reaction (PCR), sequences were identified by aligning with sequences in the GenBank (Table 5). Four bacterial species were detected, this includes; *Proteus mirabilis* strain ATCC 29906 (3 species), *Pseudomonas taiwanensis* strain BCRC 19751, *Bacillus cereus* strain IAM 12005 and *Sporosarcina terrae* strain L22, *Proteus mirabilis* strain ATCC 29906 were the dominant species, the bacterial isolates also yielded the expected amplicon of 1500 bp (Figure 1). ITS gene sequencing of the yeast detected *Wickerhamomyces rabaulensis* CBS 6797 strain with a product size of 500- 600bp (Figure 2). The result is in line with Ayansina et *al*. [19] who detected *Bacillus olivae* BRB18, *B. licheniformis* HT-26-B1, *B. cereus* H17, *B. safensis* MS40, *B. pumilus* 07 from peels and soil from two cassava dumpsites.

Table 5 Molecular characterization of the bacterial isolates using 16S rRNA

Bacteria identity	Accession number	Percentage identity (%)	Total score
Proteus mirabilis strain ATCC 29906	NR114419.1	88.69	881
Proteus mirabilis strain ATCC 29906	NR11419.1	86,16	518
Pseudomonas taiwanensis DMS21145 strain BCRC 19751	NR116172.1	89.45	929
Proteus mirabilis strain ATCC 29906	NR114419.1	88.82	929
Bacillus cereus strain IAM 12005	NR115526.1	76	909
Sporosarcina terrae strain L22	NR157634	85	139

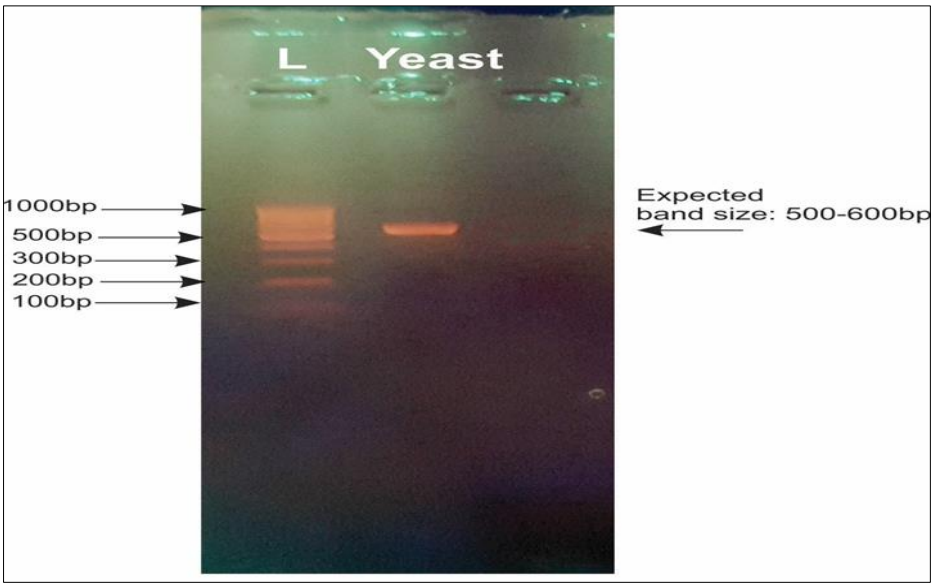
Table 6 Molecular characterization of the fungal isolate using ITS genes

Fungi identity	Accession number	Percentage identity (%)	Total score
Wickerhamomyces rabaulensis CBS 6797 ITS region	NR138207	98.69	950



Lane L = molecular weight marker (low-range DNA ruler), Lane 1–6 = PCR amplification products (1500 bp)

Figure 1 PCR amplification of bacterial 16S rDNA gene sequence



Lane L = molecular weight marker (low-range DNA ruler), Lane 1= PCR amplification products (500 – 600 bp)

Figure 2 PCR amplification of the yeast (*Wickerhamomyces rabaulensis*) ITS gene sequence

Figure 3 shows the yields of bioethanol obtained from cassava peels using different combinations of microorganisms after fourteen (14) days of simultaneous saccharification and fermentation. The highest bioethanol yield of 19.3 g/cm^3 at a concentration 38.6% was obtained from 100g of cassava peels with *B. cereus* IAM 12005 and *W. rabaulensis* CBS 6797. A yield of 17.7 g/cm^3 (34.4%) was also obtained from a combination of *Pseudomonas taiwanensis* strain DMS 21145 and *W. rabaunensis* CBS 6797 from the same quantity of cassava peels. The microorganisms produced amylolytic enzymes which acted on the cassava peels, this could be attributed to the presence of more carbohydrates from cassava peels which was fermented to ethanol in the presence of the amylolytic microorganism (*B. cereus and P. taiwanensis*) and the length of the saccharification and the fermentation period. This result is in line with the work of Mustafa *et al*. [8] that gave high yields because of the presence of cassava peel substrate and good pH conditions. The present result is higher than that obtained by Asif *et al*. [20], who obtained 9.3 (v/v) and 8.3% of ethanol from sugarcane molasses using *Zygmomonas mobilis* and *Saccharomyces cerevisiae* respectively.

Other microbial combinations such as 20 g of cassava peels + *P. taiwanensis* + *W. rabaulensis* and 20g of cassava peels + *A. niger* + *W. rabaulensis* also gave relatively high yields of 7.23 g/cm^3 and 6.43 g/cm^3 respectively. This is slightly higher than the report of Oyeleke *et al*. [21], whose study gave 6.75 g/cm^3 when *Gleophyllum sepiarium* and *S. cerevisiae* were used to produce bioethanol from 20g of cassava peels. The high yields can be ascribed to the enzyme content of *B. cereus*, *P. taiwanensis* and *A. niger*, these microorganisms are known to contain enzymes such as α-amylase, glucoamylase and cellulase necessary for the breakdown of the complex cellulose composition of cassava peels [17].

The average percentage concentration of ethanol in the present study is relatively high as compared to the average yield reported by Agulejika *et al*. [22], who reported an average ethanol concentration yield of 16%, this is likely to be due to the presence of more carbohydrate content in cassava peels than in cocoyam peels.

3.1. Determination of the quantity of Bioethanol produced

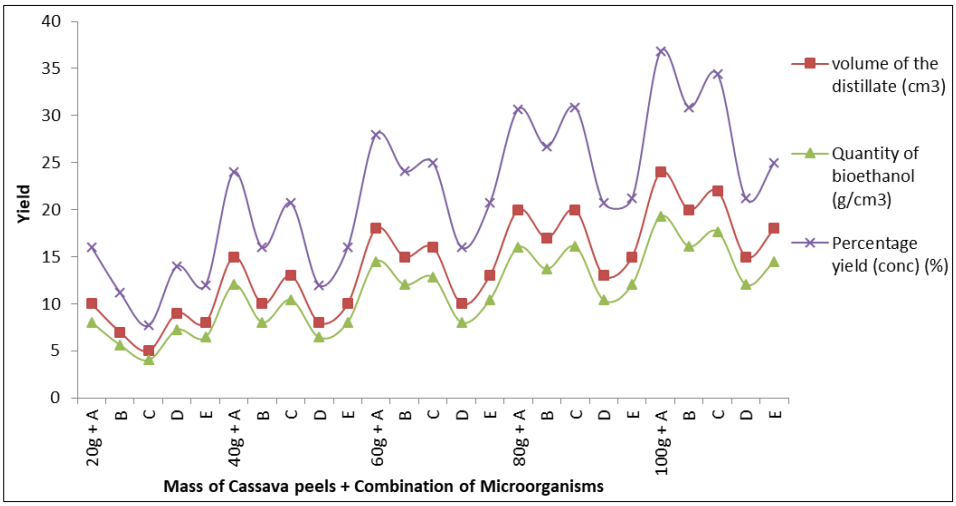
The quantity of the bioethanol produced was determined by measuring the volume of the distillate and expressed it as quantity of bioethanol produced in g/cm^3 by multiplying the volume by the density of ethanol (0.8033 g/cm^3) [8].

3.2. Determination of ethanol concentration

Ethanol concentration (v/v) was determined by extrapolation using the absorbance of ethanol obtained from the standard ethanol concentration curve. The standard ethanol curve was obtained according to the methods of Oyeleke and Jubril [1].

Figure 4 shows the yields of bioethanol produced by the different combination of microorganisms when 40 g of cassava peels was used, this was done in order to identify the combination of microorganisms with the highest yield of bioethanol. *B. cereus + W. rabaunensis* had the highest yield, this is followed by the combination of *P. taiwanensis + W.*

rabaunensis and *A. niger* + *W. rabaunensis* respectively. This study showed that cassava peels was readily degraded and transformed to simple reducing sugar by these three organisms (*B. cereus*, *P. taiwanensis and A. niger*), this may be attributed to their amylolytic nature. In addition, the hydrolysis of cassava peels by these three organisms to yield simple reducing sugars was sufficient to allow the yeast (*W. rabaulensis*) to produce bioethanol by fermentation process. This result corroborates the result of Olayide *et al*. [3] that observed hydrolysis of cassava peels by *Aspergillus niger and* other microorganisms.



A - Bacillus cereus + Wickerhamomyces rabaulensis; B - Proteus mirabilis + W. rabaulensis; C - Pseudomonas taiwanensis + W. rabaulensis; D - Sporosacina terrae + W. rabaulensis; E - Aspergillus niger + W. rabaulensis

Figure 3 Yields of bioethanol obtained at 14 days' distillation

The quantity of bioethanol produced by the different combination of microorganisms at 21 days is shown in Table 8, the highest yield of 17.7 g/cm3 was obtained with 100g of cassava peels using a combination of B. cereus and W. rabaulensis. There was a decrease in the yield (19.3 g/cm3 produced by the combination of the organisms when distilled at the 14th day using the same quantity of cassava peels (Figure 5). The lowest yield obtained from 14 days' and 21 days' distillation is 4.02 g/cm3 and 3.2g/cm3 respectively from 20g of cassava peels + Sporosarcina terrae+ W. rabaunensis). The decrease in the yields may be due to decrease in the metabolic activities of the organisms which may be due to starvation, exhaustion of the available nutrient and death of microorganisms. This result is in line with the result of Mustafa et al. [8] that observe a decrease in the yield of bioethanol from cassava peels using different inoculants. This shows that the maximum day for the simultaneous saccharification and fermentation of cassava peels slurry to produce bioethanol should not be more than 14 days.

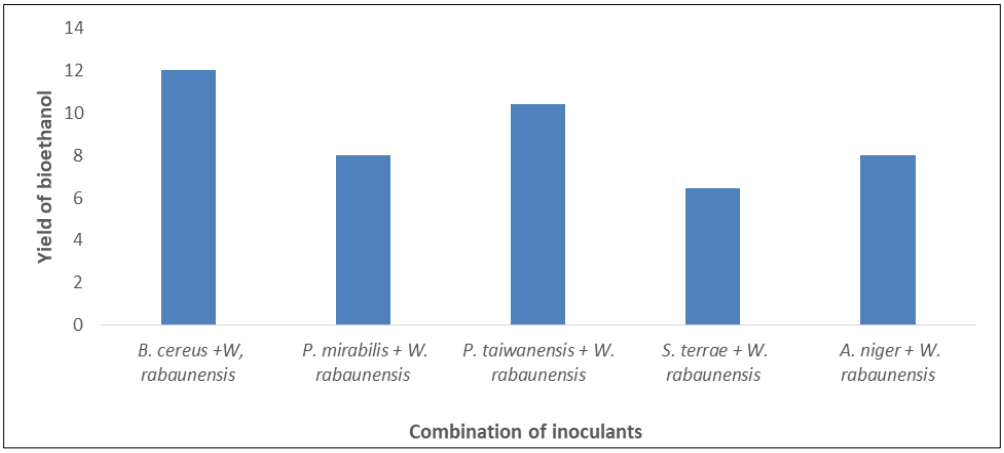
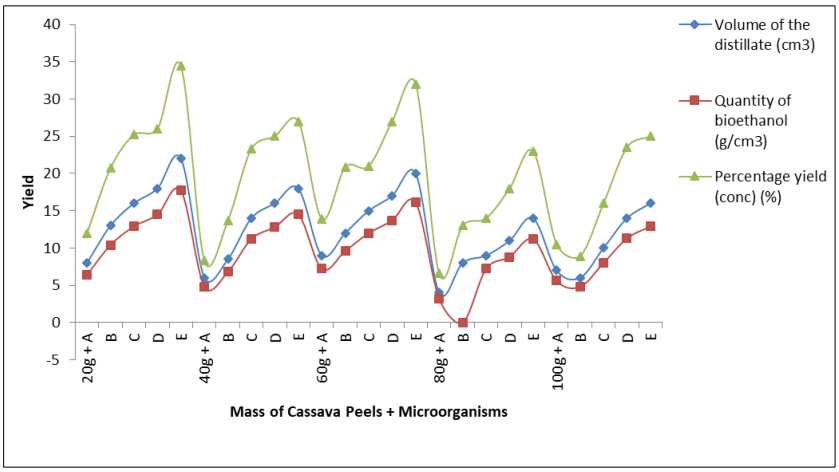


Figure 4 Yields of bioethanol obtained at 14 days' distillation from the Co-culture of microorganisms with 40 grams of cassava peels



A - Bacillus cereus + Wickerhamomyces rabaulensis; B - Proteus mirabilis + W. rabaulensis; C - Pseudomonas taiwanensis + W. rabaulensis; D - Sporosarcina terrae + W. rabaulensis; E - Aspergillus niger + W. rabaulensis

Figure 5 Yields of bioethanol at 21 days' distillation

Figure 6 shows the yields of bioethanol at 21 days' distillation by the different combination of microorganisms when 40g of cassava peels was used, this was done in order to identify the combination of microorganisms with the highest yields of bioethanol. *B. cereus+ W. rabaunensis* had the highest yield, this is followed by the combination of *Pseudomonas taiwanensis* + *W. rabaunensis* and *Sporosarcina terrae* + *W. rabaunensis* respectively, *A. niger* had the lowest yield. This study showed that cassava peels was readily degraded and transformed to simple reducing sugar by these microorganisms. Therefore, the combination of *B. cereus* + *W. rabaunensis*, and *P. taiwanensis* + *W. rabaulensis* can be used to produce large quantity of bioethanol.

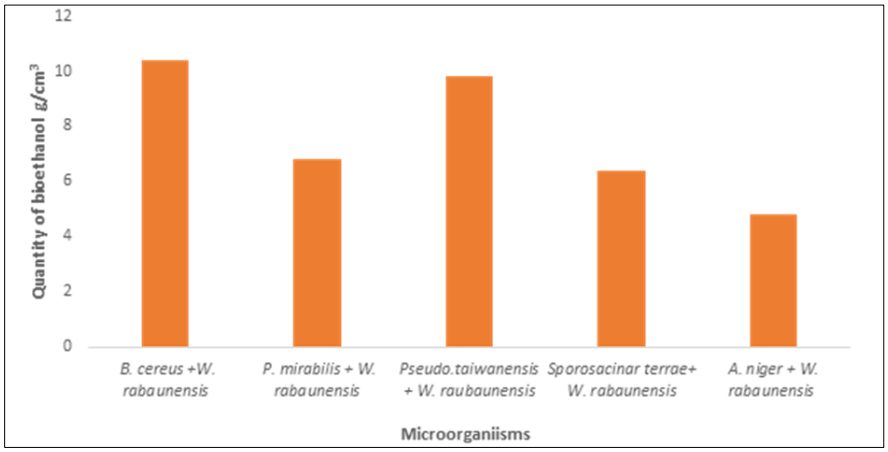
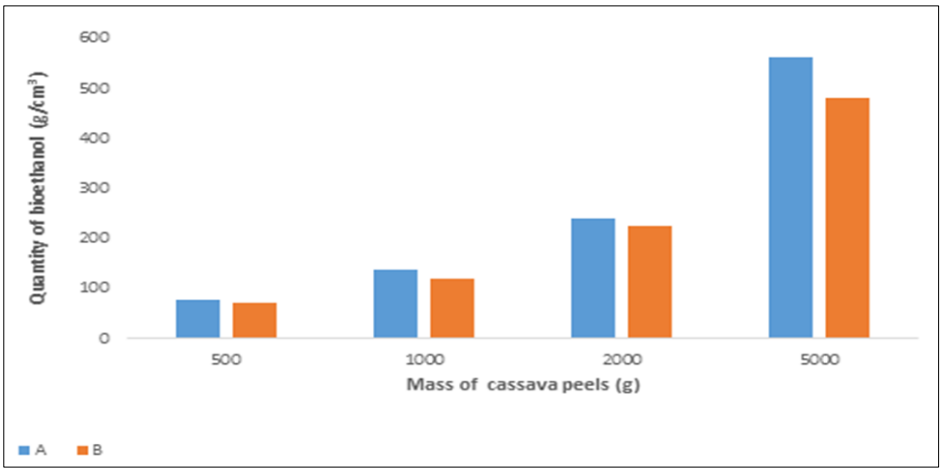


Figure 6 Yields at 21 days' distillation with 40 grams of Cassava peels

The bacterial isolates that produced high yields of bioethanol at 14 days' distillation were selected for the production of bioethanol in large quantity. In Figure 3, a combination of *Bacillus cereus* and *W. rabaulensis* produced the highest yield of 19.3 g/cm^3 at a concentration of 36.8%, while another combination of *Pseudomonas taiwanensis and W. rabaulensis* produced the highest yield of 17.7g/cm^3 at a concentration of 34.4%, these two combinations also produced high yields in Figure 5. The two combinations were selected to produce bioethanol using 500g, 1kg and 5kg of cassava peels. After the simultaneous saccharification and fermentation of the substrate (cassava peels) for 14 days, the filtrates were distilled to obtain various yields of bioethanol (Figure 7). Yields of 5 62.31g/cm^3 (67.0%) and 481.9g/cm^3 (65%) of bioethanol were obtained from 5 kilograms (Kg) of cassava peels when a combination of *B. cereus* and *W. rabaulensis* and a combination of *Pseudomonas taiwanensis and W. rabaulensis* were used respectively, but the combination of *B. cereus* and *W. rabaulensis* produced the highest yield. This shows that the three microorganisms can be used to produce bioethanol in large quantities. This result is in accordance with Titiladunayo *et al*. [24] that obtained high yields of bioethanol from cassava peels.



A = Bacillus cereus + Wickerhamomyces rabaulensis; B = Pseudomonas taiwanensis + Wickerhamomyces rabaulensis

Figure 7 Yields of bioethanol produced by *B. cereus* and *W. rabaulensis* and *Pseudomonas taiwanensis* and *Wickerhamomyces rabaulensis*

4. Conclusion

Four bacterial species were detected on the basis of 16S rDNA nucleotide sequence, this includes; *Proteus mirabilis* strain ATCC 29906 (3 species), *Pseudomonas taiwanensis* strain BCRC 19751, *Bacillus cereus* strain IAM 12005 and *Sporosarcina terrae* strain with the product size of 1500 bp. The fungi isolated includes; *Aspergillus niger*, *Rhizopus nigrican* and yeast (*Wickerhamomyces rabaulensis* CBS 6797). PCR amplification of ITS gene of the yeast produced a product of size 500-600-bp. All the bacterial isolates and *Aspergillus niger* tested positive to degradative test, while only the yeast (*W. rabaulensis*) tested positive to fermentation test. The highest bioethanol yield of 19.3 g/cm^3 (38.6%) was obtained from 100g of cassava peels *with B. cereus* IAM 12005 and *W. rabaulensis* CBS 6797 strain at 14 days' distillation, while the highest yield of 17.7 g/cm^3 was obtained with 100g of cassava peels using a combination of the same microorganisms at 21 days' distillation, showing a decrease in the yields of bioethanol at different distillation time.

There was also a decrease in the yield produced by a combination of *Pseudomonas taiwanensis* strain BCRC 19751 and *W. rabaulensis* from 17.7 to 16.1 g/cm when the cassava peels slurry was distilled at 21 days. This shows that the maximum day for the simultaneous saccharification and fermentation of cassava peels slurry to produce bioethanol should not be more than 14 days and the combination of *B. cereus* + *W. rabaulensis* and *P. taiwanensis* + *W. rabaulensis* can be used to produce large quantity of bioethanol. One-way analysis of variance shows that there is significant effect of the mass of cassava peels and the different combination of microorganisms on the yield of bioethanol produced at 14 and 21 days' distillation ($P < 0.05$). Hence, the isolated microorganisms can be used to produce bioethanol from cassava peels (waste) to generate wealth and energy in Ekiti State, Nigeria.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

There is no conflict of interest.

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