Production of Bioethanol from Cassava Peels Using Isolate from Palm Wine

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Abstract: Bioethanol, an alcohol derived from microbial fermentation of carbohydrates found in various plants, has garnered significant attention as a renewable fuel source. This study aims to investigate bioethanol production from cassava peels using bacteria isolated from palm wine. The cassava peels were sourced from a local farm in Ubahumonum Okija, while fresh palm wine was obtained from Obele Nkwo, Ubahumonum Okija, both in Anambra state. These raw materials were processed in a microbiology laboratory at Legacy University Okija, where the cassava peels were washed, sundried, and milled into powdered form using crushing mortar. A standard media and biochemical test isolated the bacterium from the freshly tapped palm wine. Its morphological characteristic shows a bright-creamed-coloured colony and biochemical properties revealed that the isolate was rod-shaped, Gram-negative, and could ferment glucose, fructose and sucrose, also the isolate was urease-negative, indole-negative and oxidase-negative. The suspected bacterium was *Zymomonas spp*. Bioethanol production from cassava peels involved sugar fermentation using a conical flask and *Zymomonas spp* was used to ferment the substrate at 28°C for four days. The distillation was carried out using a soxhlet extractor apparatus to distil the fermented cassava water, these showed a high ethanol yield of 97%, the distillation range was at 78°C and the flash point was 24°C. Furthermore, a confirmatory test was done using the iodoform test and a yellow precipitate formed, indicating the presence of ethanol. Integrating *Zymomonas spp* isolated from palm wine in bioethanol production from cassava peels holds promise for developing sustainable and environmentally friendly biofuels. This study lays the groundwork for further research to optimize fermentation conditions and scale up the process for commercial applications, thereby contributing to sustainable energy solutions and waste reduction.

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Introduction

Cassava (*Manihot esculanta*) is a short-lived erect perennial shrub, planted vegetatively from hardwood stem cutting. It is an important crop and a significant cropping system component across many tropical environments. Cassava peels and pulps derived from garri processing are normally discarded as wastes and allowed to rot in the open, thus resulting in health hazards. Cassava peels contain high levels of hydrogen cyanide. This toxic compound is removed by drying the peels under the sun to make them suitable as animal feeds (Archibong *et al.*, 2016). In some developing countries, efforts are being directed towards transforming organic wastes into wealth by employing biotechnology approaches in the biotransformation of environmental wastes to reduce pollution problems. Microbial activities can facilitate the biotransformation of organic pollutants in converting these wastes into useful products. This technology still suffers a setback in Nigeria due to a lack of essential infrastructural tools and farmer's awareness of the potential and economic relevance of cassava waste in bioethanol production (Adegbehingbe *et al.*, 2021).

Bioethanol, a colourless liquid, is the most widely produced biofuel in the world, with Brazil and the United States being the leading producers. Bioethanol is mostly obtained by fermentation of sugar beet, sugarcane, corn, barley, wheat, woody biomass, or black liquor (Karimi and Chisti 2024). Bioethanol is mostly obtained by fermentation of sugars converted into carbon dioxide and ethanol by microorganisms; *Saccharomyces cerevisiae* (yeast) is the most frequently used in this bioprocess. Another promising microorganism for the production of bioethanol is *Zymomonas mobilis*. This is a bacterium belonging to the genus *Zymomonas*. It is notable for its bioethanol-producing capabilities, which surpass yeast in some aspects. It was originally isolated from alcoholic beverages like the African palm wine, and the Mexican pulque, and also as a contaminant of cider and beer in European countries (Migap *et al.*, 2023).

Bioethanol has been produced using a batch fermentation process in an anaerobic generation plant inoculated with fungi strains, such as *Aspergillus niger*, *Mucor mucedo*, and *Saccharomyces cerevisiae* (Adegbehingbe *et al.*, 2021). Production is generally in large-scale facilities. Most bioethanol production today is based on feedstocks from food crops (Karimi and Chisti 2024). The basic procedural steps involved in bioethanol production include: (i) hydrolysis i.e. the conversion of cellulosic materials of the biomass into fermentable sugars, (ii) fermentation i.e. activities of microorganisms in the conversion of sugar into alcohol, and (iii) distillation i.e. recovery of bioethanol from the fermentable substrates. Bioethanol production in a fermentation medium can be charged with free or immobilized cells. Cell mobilization in the fermentation medium can be employed in enhancing bioethanol yield due to the ease of separation of cell mass from the bulk liquid, reduced risk of contamination, optimization of operational stability, and cell viability throughout the operation system. Immobilized cells are easily entrapped within the polymeric matrices, such as agar-agar, calcium alginate, gelatin, and k-carrageenan. The most suitable carriers for cell immobilization are entrapment in calcium alginate beads due to simple technology, cost-effectiveness, and non-toxicity. Improving the bioethanol yields from fermentable substrates can be achieved by single or combined microbial isolates under controlled fermentation by optimization of process parameters and adjustment where necessary by supplying adequate nutrients to the fermenting microorganisms. Therefore, fermentation of pretreated cassava peels with *S. cerevisiae* and *Z. mobilis* for bioethanol production was performed.

METHODOLOGY

- **Source and Collection of Sample**

Matured cassava tubers were harvested from a farm in Ubahumonum Okija, Anambra State. The cassava tubers were peeled and collected using a sterile bag and were transferred to the Microbiology Laboratory at Legacy University Okija, Anambra State for further microbiological and chemical analyses.

- **Preparation and Pretreatment of Cassava Peels**

Cassava peels were washed thoroughly under running tap water to remove the dirt; the surface was sterilized with 3% hypochlorite and later washed with sterile distilled water. The samples were air-dried and milled into powder before pretreatment by pasteurization in a hot water bath at 72^{o}C for 30 minutes and then ready for fermentation (Adegbehingbe *et al.*, 2021).

- **Preparation of Standard Media**

The isolation medium was prepared by combining the following reagents. Glucose – 10g, Yeast extract – 3g, Ethanol – 30ml, Malt extract – 5g. These reagents were then mixed with 1 litre of water and 5ml of actidione was added and autoclaved for 15 minutes. The isolation medium was composed of Glucose – 15g, Malt extract – 5g Yeast extract – 5g, Ethanol – 30ml. The compounded components were then put in a conical flask, 1 litre of distilled water was added to it and 5ml of actidione – an antibiotic was added to it and then it was autoclaved for 15 minutes after being covered with cotton wool and a wrapping sheet. After autoclaving, the medium was spread into Petri dishes and stored in the refrigerator.

- **Collection of Palm Wine**

Palm wine was collected by the method of inflorescence tapping from Ubahumonum Okija, Anambra State. It was collected immediately and placed in a container with ice to slow down the fermentation process. It was then taken to the lab within 2 hours of tapping and then introduced into the prepared media and stored in an anaerobic jar for 24 hours, after which it was observed for the growth of the microorganism.

- **Isolation of the Microorganism from Palm Wine**

Freshly collected palm wine was introduced into 50 ml bottles with Durham tubes inserted into those bottles to monitor the displacement of gas. The bottles were then transferred into an anaerobic jar and incubated at 30^{o}C for 24 hours.

- **Isolation of microorganism**

Samples were collected from the anaerobic jar by the use of a wire loop having observed displacement of gas in the Durham tubes. The sample was then introduced into the spread plates and incubated in an anaerobic jar for 2 days at 30^{o}C. Colonies suspected to be those of *Zymomonas spp* were then isolated and purified by streaking on freshly prepared media and incubated for another two days in the anaerobic jar. Isolates from the plate were then subjected to the following biochemical test for the characterization of the isolates; Gram stain, oxidase, catalase and carbohydrate fermentation abilities.

Gram Stain

A smear was prepared with a microscopic slide. The slide was marked and distilled water from a glass was used to smear the glass as it was being sterilized at the same time with a Bunsen burner. A sterilized wire loop was then used to transfer to the slide a small portion of

the growth to be examined and then it was emulsified in the slope of water until a thin homogenous film was produced, it was then allowed to dry, fix and stain. A solution of crystal violet was applied for 30 seconds and washed. Crystal violet was then replaced with a lugol's iodine solution allowed to act for 30 – 60 seconds and washed. Absolute ethanol was then used to rinse until no more colour appeared to flow from the preparation. It was then washed with water. Safranin was then added for 3 minutes, rinsed with water and dried with gentle heat. It was then monitored using a microscope (Agbo, 2022).

Catalase Activity

Transfer a small amount of bacterial colony to the surface of a clean, dry glass slide using a loop or sterile wooden stick.

A drop of 10% V/V hydrogen peroxide was added to a clean dry glass slide containing a growing organism. The glass slide was then examined immediately for the production of bubbles gas which indicates catalase activity (Agbo, 2022).

Oxidase Activity

2-3 drops of oxidase reagents were placed on a piece of filter paper and then colonies of the suspected microorganism were smeared across the same area. A positive oxidase reaction turned the reagent a dark purple colour within 10 seconds (Agbo, 2022).

- **Preparation of *Zymomonas spp***

The detection medium was prepared and broth was poured into test tubes. Isolates of *Zymomonas spp* were then incubated anaerobically in an incubator at 30^{o}C. It was then ready for use for fermentation.

- **Fermentation Process**

Ten millilitres (10 mL) of microbial suspensions each (*S. cerevisiae* and *Zymomonas spp*) were inoculated into separate fermenters containing 500 mL pre-treated cassava peels mixture while un-inoculated fermenters served as control. The content of each fermenter was allowed to stand for 7 days, while samples were withdrawn at intervals of 24, 72, 120, 168, and 216 hours for further analyses.

- **Production of bioethanol.**

Distillation was carried out using a soxhlet extraction apparatus setup, the fermented liquid was transferred into the round bottom flask and placed on a heating mantle fixed to a distillation column enclosed to a running tap. Another flask was fixed to the other end of the distillation column to collect the distillate at 78^{0}C (Standard temperature for ethanol production). This was done for each of the bottles containing the fermented liquid according to the method described by (Oyeleke *et al*, 2012).

- **Production and Determination of Bioethanol.**

The soxhlet extraction method was used for the determination of bioethanol production. The distillate obtained was re-distilled and the percentage of ethanol was estimated using the method of Dias, *et al.*,(2011)

$$\% \text{ Ethanol (v/v)} = \frac{\text{Volume of distillate} \times 100}{\text{Volume of the fermentation mixture}}$$

- **Confirmatory Test for Bioethanol Production from Cassava Peels.**

Using the iodoform test, 1 ml of the distillate was displaced in a test tube. Another 1 ml of 1% iodine solution was added to the test tube. Drops of dilute sodium hydroxide solution were added until the brown colour of iodine was discharged. The mixture was heated gently in a water bath. The formation of a yellow precipitate indicates the presence of ethanol.

Results

Table 1. Colony Morphological characterization

Characteristics	**Appearance**
Colour	**bright-creamed-coloured colony**
Shape and margin	**Convex, regular with entire edged, 1–2 mm in diameter after 2–4 days at 30 °C**

Table 2. Biochemical Test

Isolate	Gram stain	Motility	Catalase	Glucose	Urease	Oxidase	Lactose
Zymomonas spp	**Rod, Negative**	**Positive**	**Positive**	**Positive**	**Negative**	**Negative**	**Negative**

Table 3. Bioethanol produced from cassava peels

Sample fermented with	Initial volume (ml)	Final volume (ml)	Ethanol volume (ml)	Percentage yield (%)	Distillation range (°C)	Flashpoint (°C	Confirmatory test
Zynomonas spp	230	193	37	16	78-100	24	Formation of yellow precipitate colour.
S. cerevisiae	230	180	50	21	78-100	24	Formation of yellow precipitate colour.

Discussion

The result of Bioethanol produced from cassava peels shows a relatively high yield of ethanol (17%) with an ethanol volume of 37 mL recovered from the fermented sample inoculated with *Zynomonas spp*, while the result of ferment cassava inoculated with *S. cerevisiae* shows a high yield of ethanol (37%) with an ethanol volume of 50mL recovered from fermented sample. There were variations in the final volume of ethanol produced, while a flash point of 24°C was recorded for both samples inoculated with *Zynomonas spp* and S. *cerevisiae*. According to Adegbehingbe *et al.,* (2021). A high yield of ethanol (30%) with an ethanol volume of 45 mL was recovered from the fermented sample inoculated with *S. cerevisiae.* There were variations in the final volume of ethanol produced, while a flash point of 24°C was recorded for both samples inoculated with *S. cerevisiae* and *Z. mobilis*. We suggest that the high concentration of ethanol and glucose associated with increased sugar concentration may limit the expected performance of *Zymomonas mobilis*. A reduction in the percentage theoretical yield at 37^{0}C can be attributed to *Zymomonas mobilis* losing its enzyme activity above 35°C (Ona *et al.*, 2018). Furthermore, according to Adegbehingbe *et al.*, (2021), inoculation of cell-free cassava peel hydrolysate with *S.cerevisiae* and *Z. mobilis* yielded the utmost ethanol production after incubation for seven days, with sample inoculated with *S. cerevisiae* produced a high ethanol concentration of 30%.

Conclusion

Integrating *Zymomonas spp* isolated from palm wine in bioethanol production from cassava peels holds promise for developing sustainable and environmentally friendly biofuels. This study lays the groundwork for further research to optimize fermentation conditions and scale up the process for commercial applications, thereby contributing to sustainable energy solutions and waste reduction.

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