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# Assessment of Fungal Involvement in the Production of Bioethanol from Agro-Waste

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**ABSTRACT**

The challenges of agricultural solid waste in the environment are on the rise in Nigeria and most developing countries. Fungi play a pivotal role in fermenting lignocellulosic biomass into bioethanol offering a renewable alternative to fossil fuels. Cassava peels, an agro-waste contains cellulose, which is quite high at 42.626% content; a potential a raw material for bioethanol production. This study investigated the involvement of fungi in the production of bioethanol from cassava peels. Fungi employed were *Saccharomyces cerevisiae* and *Aspergillus niger*. Cassava peels collected from a cassava-processing plant were prepared by washing to remove sand and dirt. Washed peels were then sun-dried for seven days to remove moisture and ground into flour. The flour was thereafter cooked to slurry, filtered and autoclaved at $121^0$C for 15 minutes. *Aspergillus niger* and *Saccharomyces cerevisiae* were separately used as inoculants for the fermentation of the sterilized slurry for three days. The fermented broth was thereafter distillated. Results showed that the total ethanol produced from the sample with *Aspergillus niger* was 41.7% while the total ethanol produced from the sample with *Saccharomyces cerevisiae* was 58.7%. Total ethanol yield by *Saccharomyces cerevisiae* was higher than that of *Aspergillus niger*. The characteristics of the bioethanol produced recorded a pH of 6.71, the distillation range was 78-100, and the flash point was obtained at $24^o$C. The results showed that *Aspergillus niger* and *Saccharomyces cerevisiae* were able to degrade and ferment starches in cassava peels to produce ethanol. Production of bioethanol from agrowaste will contribute to the economy of any nation while riding the environment of solid waste.

**Keywords:** Cassava peels, fermentation, bioethanol, microorganisms, distillation, *Aspergillus niger, Saccharomyces cerevisiae*

## Introduction

The cellulose from cassava and its parts for the production of bioethanol is one of the materials that can substitute the petroleum oil, and it is considered as a clean liquid fuel (Sukphisal, 2005). The production of petroleum and biofuels through microbial fermentation of agriculture waste could be an alternative way (Adeniji *et al*., 2007). Bioethanol is the most common source of biofuel, and about 90% of total biofuel usage is based on bioethanol production. Ethanol can be produced from various feed stocks, such as corn (maize), sugar beets, other cereal crops, sugar cane, cassava, sorghum, and potatoes (Microsoft ® Encarta 2009). Biofuels and bioethanol are considered as a safe energy source for the future compared with the toxic effects of organic solvents like alcohol methylene chloride and benzene in terms of their risk for human health and the environment; biofuels and bioethanol are accepted as green organic solvent ( U.S. Energy Department, 2008).

Bioethanol is a renewable biofuel because it is made from biomass. It is a clear, colourless alcohol made from a variety of biomass materials called feed stocks (the raw materials used to make a product). Ethanol has been made since ancient times by the fermentation of sugars. All beverage ethanol and more than half of industrial ethanol are still made by this process. Starch from potatoes, corn or other cereals can be the raw material (*Wikipedia*, 2012). The yeast enzyme zymase, changes the simple sugars into ethanol and carbon dioxide. The fermentation reaction, represented by the simple equation $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$, is actually very complex because impure cultures of yeast produce varying amounts of other substances, including fossil oil, glycerin, and various organic acids. The fermented liquid, containing 7 to 12 per cent ethanol, is concentrated to 95 per cent by a series of distillations (Microsoft ® Encarta ®, 2009). Biofuels can be synthesized from a variety of plants and grains. For example, soybeans and rapeseed can be processed into a diesel-like fuel.



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Corn and sugarcane can be fermented into alcohol (IITA, 2010). Organic matter such as wood, paper, and grass can also be synthesized into alcohol when certain fermentation-triggering fungi (organisms that decompose organic matter) are added. Biofuels can also be produced by many different types of substrates; among these, cassava (*Manihot esculenta Crantz*), a plant with high starch content, is considered a cheap, abundant and renewable resource for production of fermentable glucose syrups and dextrins. Moreover, it is easily produced in tropical and sub-tropical zones, mainly in Asia, South-America and South-Africa (Tonukari, 2004; Onitilo *et al*., 2007).

Bioethanol is a microbiological way of converting simple sugar into ethanol and carbondioxide ($CO_2$) (Damaso *et al*., 2004). Ethanol has been produced in batch fermentation with fungi strains such as *Aspergillus niger*, *Mucor mucedo*, and *Saccharomyces cerevisiae* that can tolerate high concentration of ethanol (Ledward *et al.*, 2003; Seema *et al*., 2007; Oyeleke *et al.*, 2008). Diverse microorganisms, such as yeast and bacteria, are able to produce bioethanol on a large scale which can satisfy human's daily need with cheap and applicable methods. *Saccharomyces cerevisiae* and *Pichia stipitis* are two of the pioneer yeasts in ethanol production owing to their abilities to produce a high amount of ethanol (Wang *et al.*, 2014).

Yeasts have advantages over bacteria for commercial fermentation owing to the thickness of their cell wall, less stringent nutritional requirements, large sizes, utmost resistance to contamination, and better growth at acidic pH (Akin-Osanaiye *et al*., 2005). According to Dien *et al*. (2003) and Sulfahrib *et al*. (2011), *Saccharomyces cerevisiae* and *Pischia stipitis* are some of the most promising model organisms in microbiological fermentation as a result of their ability to produce high amount of ethanol. They are able to utilize diverse substrates that are full of carbohydrates through fermentation processes. Besides, they are resistant to high ethanol concentrations. To Ado *et al*. (2010) *Saccharomyces cerevisiae* remains the premier host for metabolic engineering of biofuel pathways owing to the availability of many systems and synthetic biological tools for genetic manipulation. When compared to other types of microorganisms, *Saccharomyces cerevisiae* is the most common microbe employed in ethanol production by virtue of its high ethanol productivity, high ethanol tolerance and ability of fermenting wide range of sugars.

Filamentous fungi are known to be able to produce a wide range of enzymes, enabling them to degrade complex substrates. This is why some filamentous fungi, such as *Aspergillus* sp. and *Saccharomyces cerevisiae*, are used for the production of fermented food, such as *oncom* (Nout & Aidoo, 2002; Shigechi *et al*., 2011).

Cassava peel is the back of cassava tuber, obtained by peeling the back of cassava tuber. Cassava peels can represent 5-15% of the root, 30% starch, and 10% cellulose fibre on a dry mass basis. They are got after the tubers have been washed and cleansed with water and peeled mechanically. They usually contain high amount of cyanogenic glycosides and higher protein content than any other tuber parts (Gu & Guozhen, 2002; Aro *et al*., 2010; Efeovbokhen *et al*., 2019).

According to Lucero *et al*. (2000) and Kiran *et al*. (2014), the continuous usage of fossil fuels to meet the demands of sufficient energy has given room to severe environmental problems, such as acid rain, air pollution, and global warming. All over the universe, humans always depend on energy derived from biological sources and carbon for survival and nutrition. In order to compete with the existing ways and means of petroleum production, the fermentation process needs to be more efficient, or comparable for maximum production of the targeted chemical. Therefore, the alternative way of production of petrochemical and biofuels is by means of microbial fermentation of agricultural wastes. The production of bioethanol by the utilization of sugarcane, starch, and microbial fermentation of sugar compounds is considered as the main key to dispensing with classical fuel, which causes noticeable pollution for this planet. Many researchers that are working on fermentation devote more of their attention to lignocellulose, because it is considered as an alternative and more promising way for bioethanol production in future research (Yu and Zhang, 2004; Xie, 2017).

Today, because of the increasing population and rate of consumption of energy, fossil-based energy sources are becoming exhausted at a great speed. This will cause major energy problems to humans in the near future (Pandey *et al.*, 2000; Adelekan, 2010; Olayide *et al*., 2015). To solve this problem in the near future, environment-friendly energy sources, such as biofuels, should be the alternative and must be given urgent attention.



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Biofuels will contribute greatly to the economy of many countries along with the conversion of natural sources (such as wind and solar) into energy (Scott *et al*., 2002; Robertson *et al.,* 2006; Akaracharanya, *et al*., 2011).

The aim of this study is to investigate the possibility of producing bioethanol using two different species of fungi (*Saccharomyces cerevisiae* and *Aspergillus niger*) with cassava peels, a waste product, and to make a comparison between the two microorganisms.

## Materials and Method

### Collection of Samples and Microbial Inoculants

Six hundred grams (600g) of cassava peels was collected from the cassava-processing plant at Mgbirichi, Owerri. The peels were collected aseptically in a sack and brought to the laboratory of the Department of Biology, Ignatius Ajuru University of Education and sun-dried for seven days before grinding. The microbial inoculants or organisms used for the fermentation were *Aspergillus niger* and *Saccharomyces cerevisiae* isolated from rotten tomatoes.

### Media and Sterilization

The glassware used for the experiments was properly washed, dried and sterilized in the oven at 160°c for one hour. The entire working surface was also disinfected with ethanol to reduce contamination. Potato Dextrose Agar was used for the isolations. Twenty grams (20 g) of the powder was weighed and poured into a conical flask and 500 ml of sterile distilled water was added into the conical flask. Further sterilization was performed by autoclaving at 121°c for 15 minutes. The medium was brought out of the autoclave and allowed to cool, after which it was poured into 35 plates, each sterile petri dishes containing the medium and allowed to solidify.

### Sample Preparation

The cassava peels samples after preparation were removed from the conical flask covered with aluminium foil and allowed to sediment. The different samples were inoculated into the agar plate aseptically using direct plating technique and incubated at 28°c for 3 days.

### Isolation of Pure Cultures

Two fungal species; *Aspergillus niger* and *Saccharomyces cerevisiae* were isolated from rotten tomatoes purchased from the Mile one market, in Port-Harcourt, Rivers State, Nigeria (Which?) and were subjected to morphological test, to identify the desired organisms for the inoculation. The isolated fungi were aseptically introduced into the flask in which the ethanol would be produced. The samples of rotten tomatoes were thoroughly washed and cut into small pieces, then mashed with a blender to make a pulp-like consistency. Thereafter, it was transferred into a sterilized 500cm$^3$ conical flask, was sealed loosely to allow the release of gas during fermentation. Tenfold serial dilution as described by Frazier and Westhoff (2006) was used. In this method, one milliliter (1ml) of the rotten tomatoes sample was aseptically pipetted into test tube containing 9mL of normal saline. After which a step wise dilution was made by transferring 1mL from the previous dilution into another test tube containing 9mL normal saline. This was done serially until a dilution of $10^{-6}$ was reached. After the serial dilutions, aliquots (0.1ml) of $10^{-2}$, $10^{-3}$ and $10^{-4}$ dilutions were plated into prepared Potato dextrose Agar (PDA) plates which were fortified with tetracycline antibiotics for inhibition of bacterial growth spread evenly with bent L shaped glass rod. Plates were spread evenly using sterile glass bent rod and the inoculated plates were incubated in an inverted position at 28$^0$C-30$^0$C for 3-7 days.

After incubation of the plates at 28°C-30$^0$C for 3-7 days, discrete colonies were isolated using an inoculating needle which was sterilized by flaming in the Bunsen burner. The isolated colonies were sub-cultured on freshly prepared Potato Dextrose Agar (PDA) in plates to obtain pure cultures. The pure cultures were transferred into broth medium and incubated on a shaker for 24 h at 28$^o$C, after which they were ready to be used as inoculums in the cassava hydrolysates.

### Identification of Isolates

Fungal isolates were identified based on their macroscopic, colony morphology and microscopic characteristics, as recommended by Frazier and Westhoff (2006).



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**Preparation of Substrate**

The cassava peels were washed to remove dirt, dust and other impurities and sun-dried for three days to remove moisture, and thereafter ground to flour. The flour was cooked to slurry to allow the activities of enzyme. The substrates were weighed and poured into different 500 $cm^3$ conical flasks; 300 grams in two separate conical flask. Sterile distilled water was added into the various flasks to make up to the mark and the flasks were covered with sterile cotton wool wrapped in aluminium foil to avoid contamination. The mixtures were sterilized in an autoclave at 121$^oC$ for 15 minutes, allowed to cool and sterile distilled water was aseptically added again to make up to mark.

**Inoculation of the Inoculums**

Isolated pure colonies of *Aspergillus niger* was inoculated into one set of the substrates (300 g) under aseptic condition. The flasks were covered and later incubated at room temperature (28$^oC$) for three days. At different intervals of 10 minutes, the flasks were shaken to produce a homogenous solution. Later, the mixtures were separately filtered after three days using No 1 Whatman filter paper. Supernatant were collected and poured into another sets of conical flasks, covered and autoclaved at 121$^oC$ for 15 minutes and allowed to cool. Also, isolated pure colonies of *Saccharomyces cerevisiae* were inoculated into the other set of substrate (300 g) for fermentation process. The flasks were corked using cotton wool, shaken and incubated at room temperature (28$^oC$ ±2$^oC$) for three days. At intervals of 10 minutes, the flasks were shaken to produce a homogenous solution and even distribution of the organisms in the substrates mixture.

**Production of Ethanol**

Bioethanol was produced from the hydrolysed starch of the cassava peels by the method described by Olayide *et al*. (2015). The following steps, enzyme hydrolysis, filtration, fermentation, and distillation were used.

**Determination of Sugar Consumption**

After fermentation, the amount of sugar consumed was determined as described by Olayide *et al.* (2015). The amount of sugar before (initial) and the amount of sugar after (final) fermentation was recorded and was used to calculate the amount of sugar consumed.

**Distillation Process**

The hydrolysate was filtered through No 1 Whatman filter paper, and the yeast inoculum was added to the filtrate before anaerobic incubation at 28$^oC$ and fermentation broth was filtered and the filtrate passed through the distillation unit twice at 90$^oC$. After that, the distillate was collected at 78$^0C$ (standard temperature for ethanol production), which was measured using a measuring cylinder and expressed as quantity of ethanol produced in g/l. The tests carried out to characterize the ethanol produced included: Appearance; pH; flash point; and ethanol yield using various techniques and tools. The fermented mixture was heated using a thermostatic heater and distilled through simple double binary distillation to obtain ethanol biofuel from the cassava peels.

**Characterization of Ethanol**

To determine the characteristics of the ethanol produced, various parameters were used: Appearance; the pH test; flash point; and ethanol yield. The distillate was observed visually, using visual test method. The pH probe was inserted first into a buffer solution to standardize the apparatus then placed into the sample (ethanol) and the readings were recorded.

**Determination of Flash Point**

The flash point was determined with the aid of Pensky Martens flash point apparatus. Fifty millilitres (50 ml) of each ethanol produced was transferred into the flash point cup and was fixed in the apparatus assembled with a thermometer. The apparatus was switched on; steady stirring of the sample was used to control the heat to maintain a uniform temperature while passing a small flame across the material every three seconds. The first flash of a blue flame indicated the flash point of the sample and was recorded. The cup was washed and dried after each test before the subsequent test was carried out.

**Determination of Ethanol Yield (U.S.E.D, 2008)**

The refractometer was used to determine the percentage total soluble sugar solids of the cassava hydrolysate after hydrolysis. This was done by placing a drop of cassava hydrolysate on the graduated hand refractometer glass slide and expressing the brix reading (%) $\text{Ethanol Prod} = \frac{\text{Ethanol yield}}{\text{Sugar consumed}} \text{x}100$



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## Results

The result of the sugar conversion and ethanol yield of the hydrolysates of starch with the inoculums during fermentation is presented in Table 1. The initial sugar concentration before fermentation of cassava peels hydrolysate inoculated with *Aspergillus niger* was 15.8mg, sugar content after fermentation was 18mg, final sugar content was 8.4mg, ethanol yield was 3.5mg and total ethanol produced was 41.7mg, while that of cassava peels hydrolysate inoculated with *Saccharomyces cerevisiae* was: 20.3mg; 25mg; 12.6mg; 7.4mg and 58.7mg respectively.

The total ethanol produced after distillation, the cassava peels hydrolysate inoculated with *Aspergillus niger* recorded 3.5g ethanol yield and 41.7g ethanol produced. While cassava peels hydrolysate with *Saccharomyces cerevisiae* recorded 7.4g ethanol yield and 58.7g ethanol produced.

Table 2 shows the result of the distillation range of ethanol produced from the substrate. *Aspergillus niger* after 24hr recorded 120g, 48 hr 125g, and 72 hr recorded 105g with the distillation range of 78-100. While the substrate with *Saccharomyces cerevisiae* after 24 hr recorded 230g, 48hr, 250g; and 72hr, 201g.

**Table 1: Conversion of Sugar, Fermentation and Ethanol Yield from Cassava Peel Hydrolysate**

| Inoculant | Initial sugar Concentration Before Fermentation | Sugar content after Fermentation | Final sugar content | Ethanol yield | Ethanol produced |
|---|---|---|---|---|---|
| *Aspergillus niger* | 15.8mg | 18mg | 8.4mg | 3.5g | 41.7g |
| *Saccharomyces cerevisiae* | 20.3mg | 25mg | 12.6mg | 7.4g | 58.7g |

**Table 2: Distillation Range of Ethanol Produced from the Cassava Hydrolysates (g/ml)**

| Inoculant | Quantity of Ethanol produced (g/ml) | | | Distillation range |
|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | |
| *Aspergillus niger* | 120 | 125 | 105 | 78-100 |
| *Saccharomyces cerevisiae* | 230 | 250 | 201 | 78-100 |

Figure 1 shows the result of the Sugar concentration in hydrolysates of starch with *Aspergillus niger* and *Saccharomyces cerevisiae* during the fermentation period.

Figure 2 shows the result of the percentage ethanol yield after the duration of fermentation; the cassava peels hydrolysate inoculated with *Aspergillus niger* recorded 15% after 24 hrs, 18.4% after 48 hrs, and 8% after 72 hrs. While the cassava peels hydrolysate inoculated with *Saccharomyces cerevisiae* recorded 17%. 25.7% and 16% respectively.



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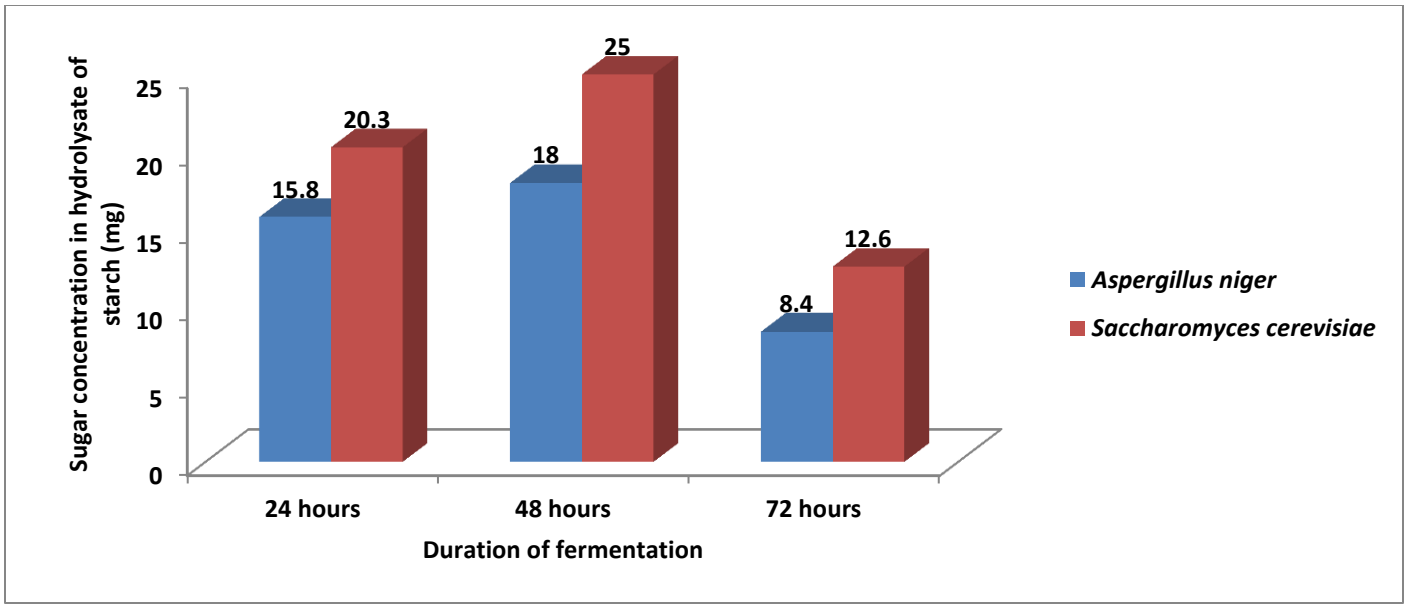


**Fig. 1: Sugar Concentration in Hydrolysates of Starch with *A. niger* and *S. cerevisiae* during Fermentation**

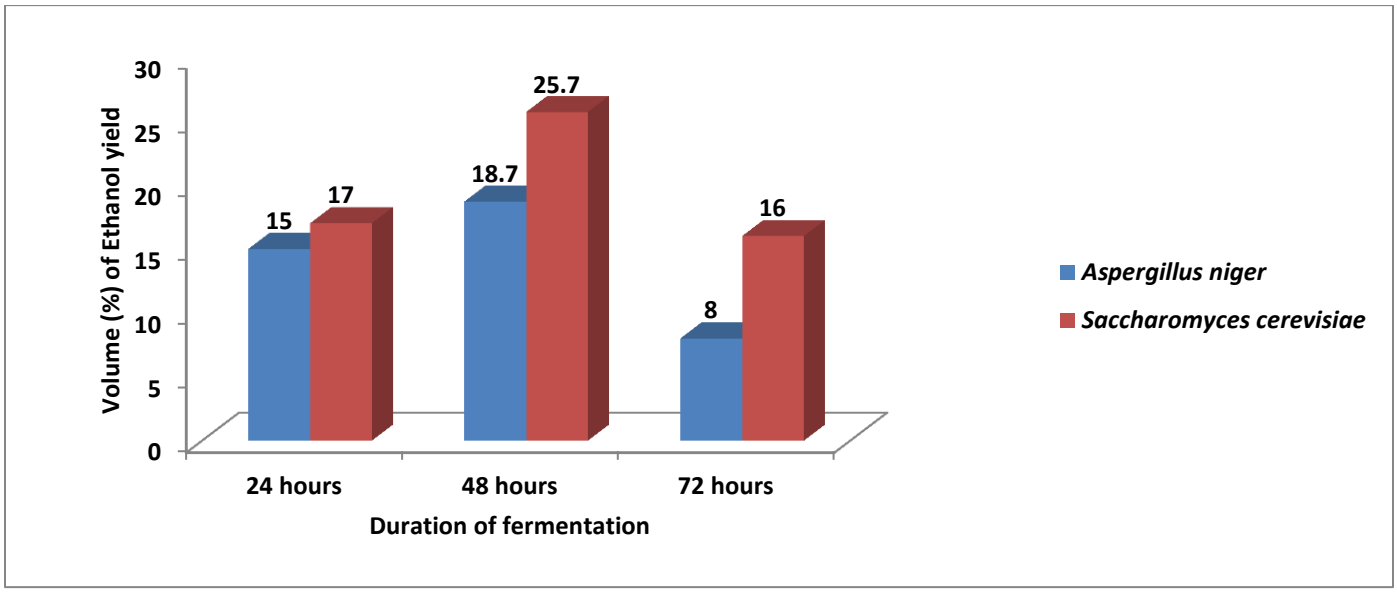


**Fig. 2: Volume of Ethanol Yield of the Hydrolysates with the Inoculants after Distillation**

## Discussion

Various yeast strains from different sources have been employed by previous researchers in the production of ethanol (Lucero *et al.*, 2000). *Saccharomyces* species are generally tolerant of alcohol and can grow in the presence of 8-12% v/v alcohol, surviving exposure to up to 15% alcohol (Olayide *et al*., 2015).

This study evaluated the involvement of microorganisms in the production of ethanol using cassava peels. The organisms used were *Aspergillus niger* and *Saccharomyces cerevisiae* isolated from rotten tomatoes. The results showed that these organisms had great potential for ethanol production from cassava peels, although more research is required to improve the efficiency of the process used.



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After hydrolysis, the amounts of reducing sugars in the hydrolysates during fermentation were monitored. There was a gradual decline in per cent brix for both hydrolysates (Table 1). The initial sugar content increased from 15.8 to 18.0 at the second day and dropped slightly to 8.4 on the third day for the substrate with *Aspergillus niger;* and that of *Saccharomyces cerevisiae* from 20.3 first day, 25.0 second day and third day 12.6. Nevertheless, the amounts of sugars consumed during fermentation by *Saccharomyces cerevisiae* were higher.

The ethanol conversion values and amounts of ethanol produced were also higher than those of the substrate with *Aspergillus niger* (Table 3*). S. cerevisiae* was able to produce more ethanol than *A. niger* and was able to utilize more substrate for ethanol conversion during the fermentation than *A. niger*. There was also gradual decline of ethanol production by 72 h. fermentation time. *A. niger* produced ethanol volume of 15% at 24 hour, 18.7% at 48 hour and 8% at 72 hour; while *S. cerevisiae* produced 17%; 25.7%; and 16% respectively. This implies that the ethanol produced from the sample with *Saccharomyces cerevisiae* is higher than the ethanol produced from the sample with *Aspergillus niger*. The differences in ethanol yield are attributed to differences in the ability of the organism to degrade and utilize sugar to convert to ethanol.

Agricultural wastes are potential sources for commercial production of biofuels because of their availability and low market price. In this work, a performance of comparison toward ethanol production between *A. niger* and *S. cerevisiae* was carried out, as indicated in the Tables. Higher ethanol production was achieved when *S. cerevisiae* was used as the fermenting agent. *S. cerevisiae* produced 58.7% of ethanol, while *A, niger* produced 41.7% ethanol.

Such difference should be related to the higher enzymatic capability of *S. cerevisiae* to consume and convert glucose and xylose-based saccharides present in the substrate (cassava peel). The performance of *S. cerevisiae* in ethanol production was clearly superior to that of *A. niger*, indicating the significant potential of using this species of yeast in existing industrial process of ethanol production.

This research pointed out the important role of microorganisms for reduction of organic matter (agricultural waste) to produce bioethanol. The hydrolysis of cassava peels was possible for the production of ethanol since polysaccharide must be broken down into fermentable sugar which can be utilized by microorganisms. Many studies have been conducted on the utilization of cassava peel for the production of bioethanol (Shigechi *et al*., 2004; Saha *et al.*, 2014). Their findings correlate with those of this work, indicating the ability of some species of yeast in fermenting sugar to produce bioethanol.

Quantity of ethanol production depends on the quantity of the substrates and that of the organisms involved. In this case, 600 g of cassava peels was used, and it gave rise to a total of 100.4 litres.

Although the two yeast species used in this study were able to convert fermentable sugars into ethanol effectively, Ado *et al*. (2010) and Akaracharanya *et al.*, (2011) have observed that yeasts isolated from natural sources, such as palm wine, possess very high levels of ethanol and sucrose tolerance and may grow well in various substrates. A major contribution of this study to knowledge s is the establishment of the feasibility of using microorganisms for effective bioethanol production with agricultural waste and more consideration of using *Saccharomyces cerevisiae* in the production of bioethanol.

In conclusion, this study used agricultural waste (cassava peels) and produced bioethanol with *Aspergillus niger* and *Saccharomyces cerevisiae*. Based on the results, it was concluded that a high rate of ethanol was produced from the two species of yeast. But the highest yield was obtained in the reaction medium prepared with *Saccharomyces cerevisiae*. Therefore, bioethanol production was achieved with high yield using agricultural waste with microorganisms (yeast).Based on the findings, it is recommended that, owing to the performance of *Saccharomyces cerevisiae* in ethanol production, this species of yeast should be used more in the production of bioethanol and biofuel. Utilization of cassava peels for the production of bioethanol should be embraced and more research is required to improve the efficiency of the process used.



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