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Ethanol Production From Cassava Wastes (Pulp And Peel) Using Alcohol Tolerant Yeast Isolated From Palm Wine

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Abstract

Introduction: The efficient ethanol production using Saccharomyces spp. isolated from palm wine in utilizing industrial wastes (cassava pulp and peel) was studied in the liquid state fermentation process.

Methods: The percentage alcohol in the cassava pulp and peel was obtained by digesting the cassava waste with three different commercial exogenous enzymes which include alpha amylase (Termamyl), Amyloglucosidase (AMG), β-glucanase t a-amylase (Cereflo) and fermenting with yeast (*Saccharomyces* spp.) isolated from palm wine. The combination of two enzymes in starch hydrolysis produced more sugars than individual enzyme usage and also yielded more of ethanol when compared to a single enzyme activity. It was also observed that AMG combined with Termamyl yielded 2.05% ethanol. Cereflo combined with Termamyl yielded 1.6% ethanol while Tennamyl alone yielded 1.26% ethanol from cassava pulp. However, it was also observed that AMG combined with Termamyl yielded 0.46% ethanol, Cereflo combined with Termamyl yielded 0.73% ethanol while Termamyl alone yielded 0.33% ethanol from cassava peel.

Results: This implies that ethanol produced from cassava pulp is higher than ethanol produced from cassava peel, since the cassava pulp contains high starch than the peel.

Conclusions: These digestions using these enzymes and subsequent ethanol production can go a long way in waste management for economic purposes.

INTRODUCTION

Cassava (*Manihot esculanta*) is a short lived erect perennial shrub, planted vegetatively from hard wood stem cutting. It is an important crop across a wide range of tropical environments and is a significant component of cropping systems [1]. Cassava peels and pulps derived from garri processing are normally discarded as wastes and allowed to rot in the open, thus resulting in health hazard. Cassava peels contain high level of hydrogen cyanide. This toxic compound is removed by drying the peels under the sun in order to make it suitable as animal feeds [2]. On the other hand, cassava pulp produced in large amount is a low cost solid by-product of starch manufacturing. It is a major biomass resource in south East Asia countries. It contains abundant starch (approximately 60%) and cellulose fiber (approximately 20%). In addition, cassava pulp has been proposed as a high potential ethanolic fermentation substrate due to its high residual starch level, low ash content and small particle size of lignocellulosic fibers [2]. Ethanol gotten from agricultural feedstock is called bioethanol. Bioethanol is a form of renewable energy obtained from conversion of carbon based feedstock. Agricultural feedstock are considered renewable because they utilize free solar energy using photosynthesis provided that all minerals required for growth such as nitrogen, and phosphorus are returned to the land. Ethanol can be produced from a variety of feedstock such as sugar cane, sorghum, cassava, sunflower, potatoes, barley, molasses, fruits, corn and wheat etc.

According to Amoa et al. [3], the sap of the oil palm tree (*Elaeis guinnensis*) serves as a rich substrate for various types of microorganisms to grow. The sap of the palm is tapped and allowed to undergo spontaneous fermentation which allows the proliferation of yeasts species to convert the sweet substrate into an alcoholic beverage. Several other studies have shown that the alcohol fermenting yeast *Saccharomyces cerevisiae* naturally colonizes palm sap [4-9] High alcohol tolerant yeast, *S. cerevisiae* dominated the yeast biota and was the only species isolated in the mature samples while *Lactobacillm plantarum* and *Leuconostoc mesenteroides* were the dominated lactic acid bacteria while acetic acid bacteria were

isolated only after the third day when the levels of alcohol had become substantial.

Furthermore, ethanol production from cassava waste (pulp and peel) can be performed in two steps namely; 1) enzymatic hydrolysis which converts cellulosic materials and starch to fermentable sugars 2) ethanol fermentation which converts fermentable sugars to ethanol by *S. cerevisiae* TISTR5596 [10]. What lead to this research is that cassava processing is generally considered to contribute significantly to the environmental pollution and aesthetic nuisance. About ten million tons of cassavas are processed for garri annually in Nigeria alone [11]. In the processing of cassava fermented products, the peels and pulps are regarded as wastes and usually discarded to rot. The wastes generated at present pose a disposal problem and would even be more problematic in the future with increased industrial production of cassava products such as cassava flour and dried cassava fufu. Products of fermentation of cassava peels and pulps from such heaps include foul odour and sometime poisonous and polluted air, which when inhaled by man or animals may result into infection and disease that may take a long time to manifest [12]. In the same vein, vegetation and soil around the heaps cassava peels are rendered unproductive and devastated due to biological and chemical reactions taking place between the continuously fermenting peels, soil and surrounding vegetation [13]. The production of ethanol from agricultural feedstock for use as alternative fuel has attracted worldwide attention because of the depleting fossil fuel sources and volatile petroleum prices in the international market [14].

This study was aimed at investigating the ethanol producing abilities of cassava peel and pulp using 3 different exogenous enzymes for liquefaction and saccharification from yeast isolated from palm wine for fermentations. Also to minimize the detrimental effects caused by cassava wastes (peel and pulp) and utilize them into the production of ethanol, which does not cause air pollution or any environmental hazard.

METHODS

Samples/Enzyme Collection

The raw material cassava waste (peel and pulp) were obtained from garri processing plant around police quarters Okwe, Asaba, Delta State Nigeria. Palm wine was also obtained from Okwe market, Asaba. While the enzymes used in this study were a-amylase. β-giucanase and amyloglucosidase which were obtained from the Department of Microbiology and Brewing laboratory of Nnamdi Azikiwe University, Awka. Nigeria. The samples were sorted and then washed under running tap water to remove sands and other dirt particles. Samples were sun dried for about two weeks and then milled into a powder form and stored in a polythene bag.

Microorganisms Isolation

The yeast used in this study was isolated from palm wine fermented for about 24 hours in a closed system at the Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka. SDA with different ethanol concentrations. The high ethanol tolerant yeast was used.

These were done in duplicate and addition of ethanol was done aseptically at lower temperature of about 50°C. With a sterile loop, inoculum was taken from the fermented palm wine into the different plates of SDA with different ethanol concentrations and cultured for about three to five days. The culture was selected from the plate containing the highest volume of ethanol.

Digestion of Cassava Wastes (Peel and Pulp)

Two grams of powdered cassava peels and pulps were weighed in six different conical flasks and 50 ml of distilled water was added to each. They were heated to boiling for 10 minutes. They were allowed to cool and 2 mL of enzymes (Termamyl, AMG. Cereflo) were added in each case and incubated at 50°C in water bath for two hours with stirring every 30 minutes. Then, the temperature was increased for 5 minutes and then filtered using filter paper. Whatman No. 1.

Estimation of Reducing Sugar

This was carried out according to the method of Miller [15]. One millilitre of DNS was added to 1 mL of each supernatant (filtrates) in test tubes labeled accordingly and mixtures heated in boiling water for ten minutes. The test tubes were cooled rapidly in tap water and the volume adjusted to 12 mL with distilled water. A blank containing 1 mL distilled and 1 mL DNS was prepared. The OD of the samples was read against the blank in a Spectrophotometer at 540 nm. The concentration of reducing sugar in the supernatant estimated from the glucose standard curve.

Growing of Yeast

One gram and 4 g of Mycological peptone and dextrose respectively were measured out using weighing balance. They were put into a conical flask and 100 mL distilled water was added. The broth was sterilized by autoclaving at 121°C for fifteen minutes and was allowed to cool and then with a sterile loop, three loopful of the organisms from the plates were inoculated into the broth and shaken for three days in a rotary shaker.

Fermentation of Cassava Waste with Yeast

Ten grams of cassava peel (ground) were measured out into a conical flask and 200ml of water added, the solution was heated to 90°C and maintained for ten minutes. The temperature was reduced to 70°C and 4ml of termamyl was added and allowed to stand for one hour followed by filtration of the solution using filter paper. Whatman No 1.

Ten grams of cassava pulp (ground) were measured out into a conical flask and 200ml of water added, the solution was heated to 90°C and maintained for ten minutes. The temperature was reduced to 70°C and 4ml of termamyl was added and allowed to stand for one hour followed by nitration of the solution using filter paper. Whatman No 1.

The process was repeated using Cereflo plus Termamyl, 2 mL each but at the temperature of 55°C. Also, the process was repeated using AMG plus Termamyl, 2 mL each but at the temperature of 55°C. The filtrates were sterilized in an autoclave at 121 °C for fifteen minutes. Then 100ml of each cooled sample were taken and 15ml of inoculum was added into the samples and stored in refrigerator. From the remaining filtrate, 50ml in each case was stored in a freezer for fur-

ther analysis. Reducing sugar was estimated from the stored samples using the method of Miller [15].

Distillation Process

The distillation apparatus was set up for the distillation of fermented samples. The specific gravity of the distillates was determined by dividing the weight of distillate by the weight of equal volume of distilled water. Furthermore, the percentage ethanol in the distillates was determined from the Association of Official Analytical Chemist [16].
Physicochemical Properties and Proximate Analysis of Samples were determined according to AOAC [16].

Proximate Analysis

Determination of Ash

This was carried out according to the method of analysis of Association of Official Analytical Chemist, [16]. The sample (2 g) was weighed into a porcelain crucible. This was transferred into the muffle furnace set at 550°C and left for about four hours. About this time, it had turned to white ash, the crucible and its content were cooled to about 100°C in air, then room temperature in a dessicator and weighed. The percentage ash was calculated from the formula

Crude Fiber Determination

This was carried out according to the method of analysis of Association of Official Analytical Chemist [16] The sample (2 g) was weighed accurately into the fiber flask and 100ml of 0.2 NH_2SO_4 added. The mixture was heated under reflux for one hour with the heating mantle. The hot mixture was filtered through a fiber sieve cloth. The filtrate obtained was thrown off and fiber was returned to the fiber flask to which 100 mL of 0.31 N NaOH was added and heated under reflux for another one hour. The mixture was filtered through a fiber sieve cloth and 10ml of acetone added to dissolve any organic constituent. The residue was washed with about 50 mL hot water twice on the sieve cloth before it was finally transferred into the crucible. The crucible and the residue was oven dried at 105°C overnight to drive off moisture. The oven-dried crucible containing the residue was cooled in a dessicator and later weighed to obtain the weight Wl. The crucible with weight Wl was transferred to the muffle furnace for ashing at 550°C for four hours. The crucible containing white or grey ash (free of carbonaceous material) was cooled in the dessicator and weighed to obtain W2. The percentage fibre was obtained by the formula:

Determination of Protein

This was carried out according to the method of analysis of Association of Official Analytical Chemist [16].
The micro kjeldahl method for protein determination is employed for protein determination. This is based on three principles.
Digestion: $RNH_2 + 2H_2S0_4 \rightarrow (NH_4)_2S0_4 + C0_2 + H_20$
Distillation: $(NH_4)SO_4 + 2NaOH \rightarrow NH_3 + H_20 + NaS0_4$
Absorption: $3NH_4 + H3 + HBO_3 \rightarrow (NH_4)_{33}O_3$
Titration: $(NH_4)3BO_3 + HC1 \rightarrow H_3BO_3 + 3NH_4 - C1$

Method

The sample (0.5 g) was weighed into the micro Kjeldahl flask. To this were added one Kjeldahl catalyst tablet and 10 mL of concentrated H_2SO_4. This was set in the appropriate hole of the digestion block heaters in a fume cupboard. The digestion was left for four hours after which a clear colourless solution was left in the tube. The digestion was carefully transferred into 100ml volumetric flask, thoroughly rinsing the digestion tube with distilled water and the volume of the flask made up to the mark with distilled water. 5ml portion of the digest was pipette to kjedahl apparatus and 5 mL of 40 % (w/v) NaOH added.
The mixture was then steamed distilled and the liberated ammonia collected into a 50 mL conical flask containing 10 mL of 2% boric acid plus mixed indicator solution. The green colour solution was then titrated against 0.01 NHC1 solution. At the end point, the green colour turns to wine color, which indicates that all the nitrogen trapped as ammonium chloride. The percentage nitrogen was calculated by the formula Nitrogen, % = Titre value x atomic mass of nitrogen × normality of HCl used × 4 The crude protein is determined by multiplying nitrogen by a constant factor of 6.25.

Determination of Fat

This was carried out according to the method of analysis of Association of Official Analytical Chemist [16] 250 mL boiling flask was dried in an oven at 105°C-110°C for about thirty minutes after which it was transferred into a desiccator and allowed to cool. 2 g of the sample was weighed accurately into labelled thimble. The boiling flask was filled with about 300 mL of petroleum ether (boiling point 40-60°C). The extraction thimble was pugged lightly with cotton wool. Then soxhlet apparatus was assembled and allowed to reflux for about six hours. The thimble was removed with care and petroleum ether collected in the top container of the set up and drained into a container for re-use. As soon as the flask was free of petroleum ether, it was removed and dried at 105-110°C for one hour after which was cooled into a desiccator and then weighed. The percentage fat was calculated by the formula

The Phenol-Sulphuric Acid Method of Starch Determination

This was carried out according to the method of analysis of Association of Official Analytical Chemist [16] Six test tubes were labelled accordingly and carbohydrate standard dispensed. Distilled water was then added to make it up to 0.5 mL, then 0.5 mL of 5% phenol solution was added and mixed thoroughly. 2.5 mL of concentrated sulphuric acid was carefully dispensed and mixed thoroughly and then allowed to stand for twenty minutes and read in a spectrophotometer at 470 mm. Unknown samples were treated equally as standard in duplicates and concentrations extrapolated from the calibration curve.

Yeast Identification (Lactophenol Blue Stain)

On a clean slide, a drop of lactophenol blue was placed. Then using strong sterile straight wire, a piece fungal culture was removed and teased out in the lactophenol stain carefully and

slowly using two straight wires. It was covered with cover slip, taking care to remove air bubbles, and this was achieved by using the straight wire to slowly lower the cover slip. Then excess of stain flowing out the cover slip was removed with the help of a blotting or filter paper. The preparation was first examine under the low power of the microscope and then the high power. The morphological characteristics were noted [17].

RESULTS

Cultural, Morphological and Biochemical Characteristics of Yeast Identification

On Sabouraud's Dextrose Agar plate, colonies grow rapidly and mature in three days at room temperature. Colonies are white to cream in colour, flat, smooth, moist, glistering, glabrous, and yeast-like in appearance. While microscopic morphology shows large globose to ellipsoidal budding yeast-like cells or blastoconidia.

Table 1 shows the results of the estimation of reducing sugar in digested samples and percentage alcohol recovered from fermented samples using yeast isolated from palm wine are as follows .

Estimation of reducing sugar in digested samples in first trial shows that the amount of reducing sugar (glucose mg/mL) obtained by digesting cassava pulp at 50°C with Cereflo. AMG, Termamyl and no enzyme yielded 0.4149, 0.1875, 0.1785 and 0.0012 respectively, while that of cassava peel yielded 2.1875, 1.6831, 0.2855 and 0.0010 respectively with cassava peel digested at 50°C with Cereflo giving the highest value and cassava peel digested at 50°C without enzyme giving the lowest.

Estimation of reducing sugar in digested samples in second trial shows that the amount of reducing sugar (glucose mg/mL) obtained by digesting cassava pulp at 50°C with Cereflo, AMG Termamyl and no enzyme yielded 0.4149, 0.1875, 0.1785 and 0.0010 respectively, while that of cassava peel yielded 2.1875, 1.6831, 0.2855 and 0.0010 respectively with cassava peel digested at 50°C with Cereflo giving the highest value and cassava pulp digested at 50°C with no enzyme giving the lowest value.

Estimation of reducing sugar in digested samples in third trial shows that the amount of reducing sugar (glucose mg/mL) obtained by digesting cassava pulp at 50°C with Cereflo, AMG, Termamyl and no enzyme yielded 0.2916, 0.1032, 0.0607 and 0.0010 respectively, while that of cassava peel yielded 0.4036, 0.3506, 0.3619 and 0.0013 respectively with cassava peel digested at 50°C with Cereflo giving the highest value and cassava peel digested at 50°C with no enzyme giving the lowest value.

Table 2 shows the estimation of reducing sugar in digested samples with different enzyme combinations shows that the amount of reducing sugar (glucose mg/mL) obtained by digesting cassava pulp at 70°C with Termamyl yielded 1.7838, and at 55°C with enzyme combination of Celeflo plus Termamyl and AMG plus Termamyl yielded 2.2361 and 2.1892 respectively, while that of cassava peel at 70°C with Termamyl yielded 1.9088 and at 55°C with enzyme combination of Celeflo plus Termamyl and AMG plus Termamyl yielded 2.0156 and l.9244 respectively with cassava pulp digested at 55°C with AMG plus Termamyl giving the highest value and cassava peel digested at 70°C with no enzyme giving the lowest value.

Table 1: Estimation of Reducing Sugar in Digested and Undigested Samples

Enzyme Used for Digestion (2nd)	Temperature, ˚C	Reducing Sugar, mg/mL Glucose			Average
		1 Trial	2 TRIAL	3 TRIAL	
Cassava Pulp					
No Enzyme	50	0.0012	0.0010	0.0013	0.0011
Cereflo (β-gluconase + α-amylase)	50	0.4149	0.4601	0.2916	0.3888
AMG (Amyloglucosidase)	50	0.1875	0.2491	0.1032	0.1799
Termamyl (Amylase)	50	0.1785	0.0182	0.0607	0.0858
Cassava Peel					
No Enzyme	50	0.0010	0.0010	0.0012	0.0010
Cereflo	50	2.1875	0.6796	0.4036	1.0902
AMG	50	1.6831	0.5234	0.3506	0.8523
Termamyl	50	0.2855	0.3603	0.3619	0.3359

Table 2: Estimation of Reducing Sugar in Digested Samples with Different Enzyme Combinations

Enzyme Combination for Digestion, 4 mL	Temperature, ˚C	Reducing Sugar, mg/mL Glucose
Cassava Pulp		
No enzyme	70	0.0015
Termamyl	70	1.7838
Cereflo + Termamyl	55	2.2361
AMG + Termamyl	55	2.1892
Cassava Peel		
No Ezyme	70	0.0014
Termamyl	70	1.9088
Cereflo + Termamyl	55	2.0156
AMG + Termamyl	55	1.9244

Fig 1 shows, the quantity of alcohol produced after fermentation from cassava pulp digested with AMG plus tennainyl, cereflo plus termamyl and termamyl alone yielded 2.05%, 1.66% and 1.26% respectively while that of cassava peel digested with AMG plus termamyl, cerello plus termamyl and termamyl alone yielded 0.46%, 0.73% and 0.33% respectively, with cassava pulp digested at 55°C with AMG plus termamyl giving the highest value and cassava pulp digested at 70°C with termamyl giving the lowest value.

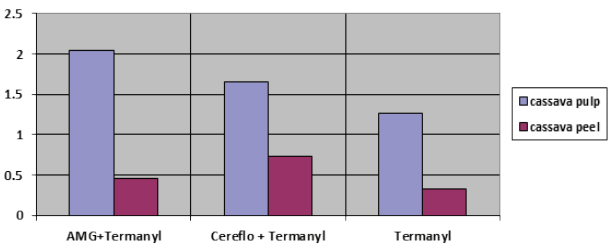

Figure 1: Quantity of Alcohol Produced After Fermentation

From the results obtained in Fig 2, it shows that cassava peel had 12.6% protein, 15% fat, 3.5% ash, and 15% fibre and 0.025 mg/mL starch while cassava pulp had 8.2% protein, 5% fat, 2% ash, 8.5% fibre. With cassava peel giving the highest values of 12.6% protein, 15% fat, 3.5% ash, 15% fibre and cassava pulp giving the lowest values of 8.2% protein, 5% fat, 2% ash, 8.5% fibre.

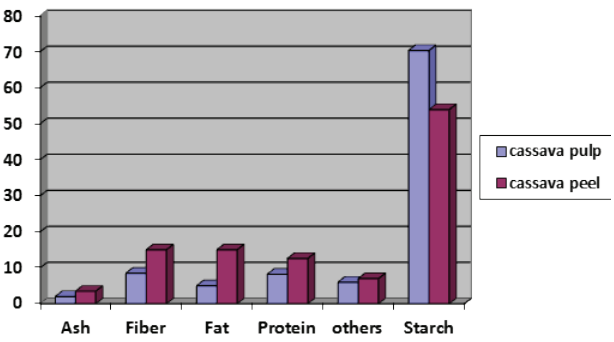

Figure 2: Showing Percentage of Proximate Content

DISCUSSION

The proximate values, however, tails within the ranges earlier reported by Aro and Aletor [18]; Singgih [19]. According to Aro and Aletor [18], cassava pulp had 1.12% protein, 19.2% crude fiber. 2.74% ash, 2.03% fat while cassava peel had 5.30% protein, 38.4% crude fiber, 4.86% fat and 6.12% ash. While Singgih [19] reported that cassava peel had 4.9% protein, 16.6% fiber, 5.9% ash, and 1.3% fat and cassava pulp had 2.3% protein, 3.4% fiber, 2.5% ash, and 1.4% fat. From the results obtained, cassava peel had 12.6% protein, 15% fat 3.5% ash, and 15% fibre while cassava pulp had 8.2% protein, 5% fat, 2% ash, 8.5% fibre. The variability in the values show above may be due to differences in climatology, soil fertility, age of harvest and methods of processing etc.

From the results obtained, the exogenous enzymes successfully hydrolyzed the starch and fiber content of cassava peel and pulp and this is attributed to their amylolytic nature. This observation is in agreement with the work of Adesanya et al. [20] In addition, the use of enzyme combination in hydrolyzing cassava pulp and peel provided better experimental results than the single enzyme. The hydrolysis of cassava pulp and peel by the combination of enzymes provided higher amount of reducing sugar. In cassava peel, combination of AMG with termamyl yielded more of reducing sugar. This implies that AMG hydrolyses alpha 1, 4 and 1, 6 linkages releasing much more of glucose unit while termamyl alone can only break down alpha 1, 4 linkages. Again, cereflo plus termamyl yielded more of reducing sugar when compared to termamyl alone, this is because cereflo hydrolyses beta glucose which is a monomer unit in cellulose and this lead to the release of more glucose unit and it also contains alpha amylase which can also break down alpha 1, 4 linkages.

Furthermore, inoculation of cell free cassava peel and pulp hydrolysate with *Saccharomyces* results in ethanol production after fermentation for three days, albeit that the concentration of ethanol produced was rather low. Since yeast were unable to synthesize amylase, the results obtained in this study indicated that hydrolysis of cassava peel and pulp by three different exogenous enzymes to yield simple sugar was sufficient to allow *Saccharomyces* spp to produce ethanol by fermentation.

Ameh and Okagbue [21]; Ezeogu and Emeruwa [22] also observed that yeasts isolated from natural sources such as palm wine possess a very high level of ethanol and sucrose tolerance that enables them to grow well in various sub-

strates. Despite the low concentration of ethanol produced results are comparable with data from Adesanya et. al. [20] which reported that cassava peel hydrolysate prepared enzymatically yielded 1.05% ethanol.

In general, enzyme combination provided higher alcohol concentration than the single enzyme; this may be as a result of their combined activities. Therefore, AMG plus termamyl yielded 2.05% ethanol because cassava pulp comprises of about 60% starch and AMG plus termamyl have the ability to hydrolyze alpha 1,4 and 1,6 linkages.

The use of enzymes combination and *Saccharomyces cerevisiae* from palm wine that are readily available in Nigeria, as in production of ethanol is being revealed by this study, will not only convert them to useful end products such as ethanol and as substrates for microbial protein enrichment rather than allowing these waste to become solid municipal waste. This research work has also revealed the possibility of obtaining higher alcohol from cassava waste using enzyme combination.

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None declared.

CONFLICTS OF INTEREST

There is no conflict of interest.

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