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Research Article

Effects of Pretreatments on the Production of Bioethanol from *Manihot esculenta* Crantz Peels Using *Zymomonas mobilis*

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Abstract

Waste management is a global issue which has become increasingly serious with population and economic growth, particularly in Nigeria. Bioethanol is a suitable substitute to hydrocarbon as it is produced by fermenting carbohydrate biomass derived from plants. This research investigation, the use of cassava peels serves as the main carbon source for production of bioethanol. Bioethanol production from the peels of cassava was examined using co-culture of *Aspergillus niger* and *Zymomonas mobilis* through simultaneous saccharification and fermentation process. 0.1 M sulphuric acid and sodium hydroxide was used for substrate pretreatment. *Aspergillus niger* and *Zymomonas mobilis* were added to ferment the peels at 28°C for 5 days during which the total bacterial and fungal count, mineral composition, cellulose content, reducing sugar, pH, temperature and the quantity of bioethanol produced were determined. The bacterial count (3.58 x 10^3), fungal count (2.81 x 10^3), mineral composition (15.64 mg/100g), cellulose content (66.08% and 69.29%), reducing sugar (6.98 ± 0.01 and 8.98 ± 0.00 g/l), pH (7.67), temperature was at 36°C at the initial stage of fermentation and later remain static at 28°C till 5th day of fermentation, the total quantity of bioethanol yield obtained was (17.08 g/ml) after the 5th day of fermentation. Fermented hydrolysate was distillated at 78°C. Therefore, *Aspergillus niger* and *Zymomonas mobilis* are proven effective for saccharification and fermentation of cassava peels for bioethanol production as an alternative fuel and energy source.

Keywords: Bioethanol; Cassava Peels; Pretreatment; Fermentation

Introduction

Biofuels are expected to reduce dependence on imported petroleum with associated political and economic liability, diminish greenhouse gas emissions and other pollutants, and invigorate the economy by increasing demand and prices for agricultural products [1]. There is an increasing demand for bio-ethanol as alternative source of energy and Nigeria currently depends on the importation of ethanol to meet its local demand.

Cassava is a very cheap source of carbohydrate and is the main carbohydrate source in the diet of the teeming population of the third world countries where it is largely grown. Banjoko., *et al.* [2] posited that cassava is a supplementary staple food of more than 200 million Africans aside from its uses as livestock feed particularly for monogastrics animals. Cassava is the most widely distributed major food crop with a high content of cyanogenic glycosides. It is also known as Manioc (*Manihot esculenta*), yuca tapioca, or guacamate. Other foods such as sweet potatoes, yams, maize, mil-

let, bamboo sugarcane, peas, beans, as well as kernel of almond, lemon, lime, apple, pear, cheery, apricot, prune and plum contains cyanide. Cassava contributes immensely to human nutrition and livelihood of the world [3] as varieties of products such as starch, garri, cassava flour, and fufu are commonly processed from cassava tubers. During the processing of cassava tubers into these essential products, an enormous quantity of cassava peels (about 30% of processed cassava tubers) are generated as waste [4] and only an insignificant proportion is usually fed to livestock such as goats. The remaining, usually heaps are thrown along roadsides in places where cultivation and processing of cassava tubers is a common livelihood activity. The peels and other wastes such as chaffs are being considered as an inconvenience rather than a potential resource in West Africa [5]. The potential of these peels to be used in the production of other products such as biogas, mushroom and improved animal feed has also been established by previous scholars [6-10]. However, most of the cassava processors and farmers are neither aware of the technologies that can be used to add value to cassava peels neither were they knowledgeable about the procedure of these different technologies. This informs why most cassava processors still throw away the peels instead of either converting it to usable forms or selling the peels as an additional source of income. Aside from not getting additional source of income from adding value to cassava peels, the environmental nuisance constituted by cassava peels on dumping sites "Akitan" cannot be overlooked.

Generally, pretreatment improves the predisposition of polysaccharides to hydrolysis of cassava peels which influences the structural and compositional constituents of Cassava peels [11]. A reduced cost of pretreatment would have a noteworthy bearing on the inclusive cost of ethanol production. However, a major impediment in bioethanol production from cassava peels is the removal of bioactive fibre component which is exceedingly impervious to solubilization and is also a key inhibitor for hydrolysis of cellulose and hemicellulose in the molecular structure of cassava [11]. Some of the major factors are the recalcitrance of the cassava plant cell wall due to essential fundamental complexity of fibre-related compounds [12]. Precious little studies have been conducted on the efficacy of pretreatments on the synthesis of bioethanol from cassava peels utilizing facultative anaerobes *Zymomonas mobilis.* Therefore, the effects of pretreatments on the production of bioethanol from *Manihot esculenta* Crantz peels using *Zymomonas mobilis.* The aim of this study is to ascertain the effect of pretreatments on the production of bioethanol from cassava peels using *Aspergillus niger* and *Zymomonas mobilis*.

Materials and Methods

Samples collection

Cassava peels (CP) and rotten oranges were collected from a local garri processing industry and local vendors located at Shagari village and Southgate, Akure, Ondo State. The samples were aseptically collected into a polythene bag and taken to the laboratory for further analyses. The cassava peels were washed thoroughly with distilled water to eliminate adhering soil and dust. The peels were sun-dried and then grinded into powdered form. The grinded powder was then sieved through muslin cloth to standardize the particle range of 1 mm. The samples were kept in a tightly close container at room temperature.

Biochemical characterization of *Aspergillus niger* and *Zymomonas mobilis* from cassava peels and rotten oranges

Aspergillus niger was isolated from the cassava peels while *Zymomonas mobilis* was isolated from rotten sweet oranges using standard microbiological methods as described by Hemraj., *et al*. [13]. The cultures were characterized and identified using morphological and biochemical methods described by Fawole and Oso [14].

Bioethanol production from Manihot esculenta peels

The pretreatment phase of the bioethanol production was conducted utilizing the protocol of Saha and Cotta [15] with trivial alterations. 20g each of the cassava peels was weighed and poured in a 500 ml conical flask, then distilled water, and 0.1M of sulphuric acid (H_2SO_4) and sodium hydroxide (NaOH) were added separately to each conical flasks. Sterile distilled water was added to make up to 200 ml, they were autoclaved at 121°C for 15 minutes and were allowed to cool down and filtered using a sterile muslin cloth. The pH of the medium was adjusted to 6.0 using H_2SO_4 normal and NaOH. The residual samples were washed with sterile distilled water to obtain a neutral pH for all treatment. The residual samples were oven-dried at 90°C overnight and the hydrolysates obtained were subjected to further analyses. The fermentation of the cassava peel hydrolysates was conducted with concurrent saccharification and fermentation process (SSF), as described by [16].

The hydrolyzed samples were autoclaved at 121°C for 15 minutes and allowed to cool at room temperature. Co-cultures of *Aspergillus niger* and *Zymomonas mobilis* was aseptically inoculated into each flasks containing the hydrolyzed samples while the untreated cassava peels serve as control. The flasks were incubated at room temperature (28°C) for five days. The flasks were shaken at interval for homogenicity with even distribution of the organisms in the hydrolyzed samples achieved. The fermented liquid was transferred into a round bottom flask and placed on a heating mantle fixed to a distillation column embedded to a running tap water. Another flask was fixed to the other end of d column to extract the distillate at 78°C which is the standard temperature for the production of ethanol as depicted by Oyeleke., *et al*. [17]. pH meter (pH meter (pH 7110) (Inolab, Xylem Analytics, Bellingham Stanley, UK) was first calibrated and inserted into each filtrate. The readings were recorded according to the method used by Ademiluyi., *et al*. [18].

Determination of quantity of bioethanol produced

The distillates collected from the fermented broth were measured using a measuring cylinder and expressed as quantity of ethanol produced in g/l by multiplying the volume of the distillate by the density of ethanol (0.8033 g/ml) [19].

Determination of reducing sugar produced

The reducing sugar was determined by titrimetric methods. Thirty (30) ml of fermentation broth was weighed into the burette. Ten ml of mixed Fehling's solution was pipetted into a conical flask and 4 drops of 1% methylene blue indicator was added. The solution was heated and while boiling, the broth in the burette was titrated against the solution in the conical flask until the colour disappeared as described by Srichuwong and Jane [20].

The reducing sugar was calculated as follows:

% Reducing sugar = $47.5 \times 300/T \times W$

T = Titre value

W = Weight of the peel sample (in grams).

Determination of temperature during fermentation

The temperature was obtained using a calibrated thermometer. The thermometer was inserted into the fermented broth and readings were then taken.

Results and Discussion

The effects of pretreatment on bioethanol production from *Manihot esculenta* Crantz peels using *Zymomonas mobilis* was assessed in this study. Findings from this research experiment (Table 1) have found *Bacillus cereus* and *Bacillus anthracis* to be evidently prevalent in Cassava peels (*Manihot esculenta*). This is attributed to the nature of the environment from which the samples were collected which can be proficient consortium for other microorganisms to survive. *Bacillus* are heterogenous and they are very versatile in their adaptability to the environment. In fact, in a related research, displayed the activity of bacillus in cassava to be 17.0 units/ml at pH 4.0 which is acidic [21]. Consequent to this, can pose unheard biohazards to ruminants as well as Zoonotic impression towards man. These impressions are vastly occurring as gastrointestinal symptoms such as stomach pain, nausea, diarrhea etc and in worst cases, death. The presence of *Bacillus anthracis* could cause food poisoning and blood clotting to animals during consumption of the peels thereby leading to the death of the animal.

Table 2 shows the morphological and microscopy characterization of the fungi isolated from the peels of cassava which shows that *Aspergillus flavus* and *Aspergillus niger* were found present in the peels. They are ubiquitous in nature and for some time, have become an increasing cause of life threatening opportunistic diseases for animals during consumption of the peels [22]. The reason for the presence of *Aspergillus* species in *Manihot esculenta* peels in this study could be attributed to fungi being a major mycoflora of various agricultural commodities [23,24].

Table 3 shows the morphological and biochemical characteristics of microbial species isolated from rotten orange shows that the organisms are anaerobic short rod, gram negative, catalase positive, non-motile and ferment glucose and sucrose with evolution of gas. Hence, *Zymomonas mobilis* was pertinent for the fermentation of cassava peel in this study. Findings were compared with Bergey's manual of determinative bacteriology who stated that *Zymomonas mobilis* are anaerobic gram negative rods with single polar flagellum that ferments fructose, sucrose and glucose with evolution of carbon dioxide. The result also conforms with the work of Rabah., *et al*. [25] that reported the isolation of *Zymomonas mobilis* from rotten sweet orange and stated that the organism can thrive

Cell shape	Oxidase	Motility	H_2S	Citrate	Urease	Indole	Glucose	Sucrose	Lactose	Starch hydrolysis	Catalase	Manittol	Arabinose	Xylose	Galactose	MR	VP	Probable bacteria
Rod	-	+	-	+	-	-	+	+	+	+	+	-	-	-	-	+	-	*Bacillus coagulans*
Rod	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	*Xenorhabdus japonica*
Rod	-	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	*Bacillus mycoides*
Rod	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-	*Bacillus anthracis*
Rod	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-:	-	*Bacillus barbaricus*
Rod	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-	-	-	*Bacillus cereus*

Table 1: Biochemical characterization of the bacterial isolates from *Manihot esculenta* peel.

Keys: - = negative to the test, + = positive to the test, MR = methyl red, VP = Voges proskauer, H_2S = Hydrogen Sulphide gas production.

Morphological and microscopy characterization	Fungal isolates
Colonies are white to cream, smooth, glabrous, pseudo-hyphae are formed with large globose to ellipsoidal cells with multilateral budding.	*Saccharomyces cerevisae*
Mycelium growing fast, with black conidia, the reverse of the plate is creamy. Conidiophores is smooth, black, long and coarse	*Aspergillus niger*
Yellowish green colony, present of metular, biseration, sclerotia, smooth surface	*Aspergillus flavus*
Rapidly growing, consist of green dense form of conidiophores on Potato Dextrose Agar. Conidiophores are hyaline and rough walled. Phialides are flask-shaped, consisting of a cylindrical basal part and a distinct neck. Conidia are cylindrically arranged in chain	*Penicillum notatum*
Dark colony colour, rough surface, no sclerotia, brown-green conidia with velvety surface. Conidiophores irregularly branched, consisting of short stripes. Conidia are cylindrical an smooth walled	*Penicillum digitatum*

Table 2: Morphological and Microscopy Characterization of Fungal Isolates from *Manihot esculenta* peel.

in sweet mediums because they love sugar and receive available juices that they utilize as their growth factor.

Isolated organism/tests	Results
Gram reaction	-
Motility	-
Catalase	+
Glucose	+
Sucrose	+
Lactose	-
Maltose	-
Fructose	-
Urease	-
Oxidase	-
Citrate	-
Indole	-
Starch hydrolysis	-
Arabinose	-
Organism	*Zymomonas mobilis*

Table 3: Morphological and biochemical characterization of *Zymomonas mobilis* from rotten orange (*Citrus aurantium*).

Keys: +: fermentation/positive; -: no fermentation/negative.

The result in Table 4 showed the percentage content of carbohydrate in *Manihot esculenta* is higher with 67.84 ± 0.44%. The carbohydrate content almost conforms to 72.50 ± 0.02% according to the findings of Okpako., *et al*. [26] for fermented cassava peels. Lower than values of 193.1% carbohydrate yield reported by Adamafio., *et al*. [27] for the treatment of groundnut shell with potash. They also stated that treatment improve biodegradability of lignocellulosic biomass with increased amount of carbohydrate. This generally shows that *Manihot esculenta* peels are rich in sugar, which is a serious source of vitality in the animal feed stuff.

The result of the mineral composition (Table 5) showed that the magnesium content of *Manihot esculenta* was 15.64 mg/100g. Values obtained showed the magnesium content were lower as compared to report by Charles., *et al*. [28] with values of 30 mg/100g and 43 mg/100g. Table 6 showed that the compositional property of the un-treated *Manihot esculenta* peels samples showed a lignin composition of 15.86%, hemicellulose- 21.43%, cellulose- 62.05%. In a related experiment, analysis of the untreated *Manihot esculenta* peel, recorded hemicellulose composition of 29.81%, cellulose- 3.05% and lignin- 2.65% according to Onyelucheya., *et al*. [29]. Results by other workers obtained cellulose- 5.40%, hemicellulose- 21.65% and lignin- 4.81%. The differences in the compositional character may be due to *Manihot esculenta* variety being analyzed, age of *Manihot esculenta*, as well as duration of storage before analysis.

Parameters	Dry *Manihot esculenta* (%)
Moisture	11.33 ± 0.15
Ash	2.39 ± 0.07
Crude fat	1.39 ± 0.09
Fibre	11.68 ± 0.21
Protein	5.44 ± 0.08
Carbohydrate	67.84 ± 0.44

Table 4: Proximate composition of *Manihot esculenta* peels.

Each value is a mean ± standard deviation of three replicates.

The moderately high cellulose content i.e. 66.08% and 69.29%, derived from *Manihot esculenta* peels was actualized by catabolic effect of NaOH and H_2SO_4 (Table 6 and 7). This claim is supported by Aripin., *et al*. [30] who experimented on the production of bio-

S/N	Parameters	*Manihot esculenta*
1	Magnesium (Mg)	15.64
2	Calcium (Ca)	8.84
3	Iron (Fe)	0.81
4	Zinc (Zn)	1.15
5	Phosphorus (P)	3.05

Table 5: Mineral composition of *Manihot esculenta* peels.

		Before			After	
Biomass	**Lignin (%)**	**Hemicellulose%**	**Cellulose %**	**Lignin %**	**Hemicellulose %**	**Cellulose %**
Manihot esculenta	15.86 ± 0.15	21.43 ± 0.10	62.05 ± 0.27	14.13 ± 0.11	24.73 ± 0.15	66.08 ± 0.04

Table 6: Fibre contents of *Manihot esculenta* before and after pretreatment with NaOH.

Each value is a mean ± standard deviation of three replicates.

ethanol from cassava peels. They proved that 1% NaOH was potent to pretreat *Manihot esculenta* peels, as 66% of cellulose content was recovered from *Manihot esculenta* peels. Table 8 showed the pattern of the reducing sugar during the fermentation period. The reducing sugar in cassava peel showed that the neutralized hydrolysate produced a higher sugar of 6.98 ± 0.01 and 8.98 ± 0.00 g/l respectively. This could be due to the utilization of the sugar as carbon source for the growth of the microorganisms. This is in line with the findings made by Yoonan and kongkiattikajorn [31] with their evidence gathered that high reducing sugar were produced from *Manihot esculenta* peels, 2.64% to be exact.

		Before			After	
Biomass	**Lignin %**	**Hemicellulose %**	**Cellulose %**	**Lignin %**	**Hemicellulose %**	**Cellulose %**
Manihot esculenta	31.55 ± 0.02	24.86 ± 0.03	69.24 ± 0.10	31.602 ± 0.02	24.915 ± 0.03	69.29 ± 0.15

Table 7: Fibre contents of *Manihot esculenta* before and after pretreatment with H_2SO_4.

Each value is a mean ± standard deviation of three replicates.

Period	H_2SO_4	NaOH	Neutral	Control
24	2.30^{c} ± 0.10	2.00^{b} ± 0.00	3.48^{d} ± 0.02	1.48^{a} ± 0.00
48	2.46^{c} ± 0.00	1.96^{b} ± 0.01	3.47^{d} ± 0.00	1.48^{a} ± 0.00
72	2.50^{b} ± 0.00	1.00^{a} ± 0.00	8.99^{d} ± 0.01	2.98^{c} ± 0.00
96	2.97^{b} ± 0.01	0.98^{a} ± 0.00	8.98^{b} ± 0.00	3.85^{c} ± 0.01
120	2.99^{b} ± 0.01	2.00^{a} ± 0.00	7.98^{c} ± 0.01	3.00^{b} ± 0.00

Table 8: Reducing sugar produced by *Manihot esculenta* peels hydrolysates.

Key: Control is the sample without pretreatment.

Neutral is the sample acidified with H_2SO_4 and neutralized with NaOH.

Figure 1 showed the pH of *Manihot esculenta* peels during the production of bioethanol. In this study, the pH was varied between 4.44 to 7.67 and the results obtained as presented indicate that the bioethanol yield increases with increase in pH. The highest pH of 7.67 was observed in day 1 in neutralized pretreated cassava peels while the lowest pH of 4.44 was observed in day 2 in pretreated NaOH sample. This result closely agrees with the results reported by Muhammad., *et al*. [32] and Akpan., *et al*. [33] who reported the highest pH of 5 and 5.12 respectively.

The result in figure 2 revealed that the temperature of the fermented *Manihot esculenta* peels. During the period of fermentation, the temperature of *Manihot esculenta* peels pretreated with

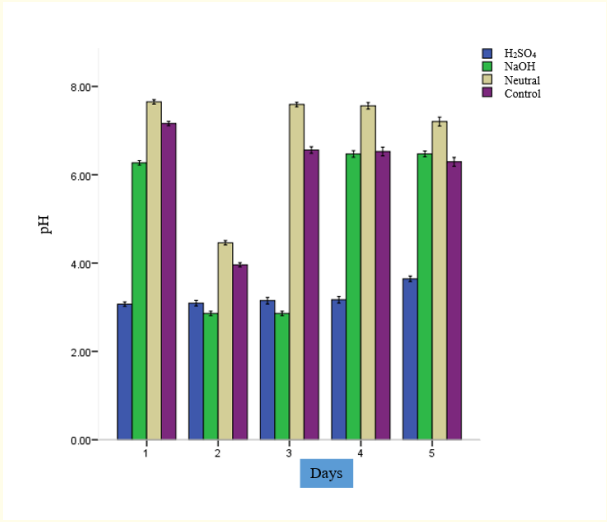

Figure 1: pH of *Manihot esculenta* during fermentation.

H_2SO_4, NaOH, neutralized (H_2SO_4 neutralized with NaOH) and control was at 36 °C at the initial stage and remain static at 28°C daily till 120 hours respectively. The static temperature as observed in the hydrolysates in the course of fermentation of the peels could be due to the ambient temperature of the experimental environment as well as the temperature of the fermentation vessels. The outcome of the findings in this study is in contrast of the observation of Bagus and Bondan [34], Brooks, [35], Kadambini and Anoop,

[36], Michelle., *et al*. [37], Mohammed., *et al*. [38] and Oyeleke., *et al*. [39] who observed that temperature of 30-37°C is the optimum temperature used for the production of bioethanol from *Manihot esculenta* peels.

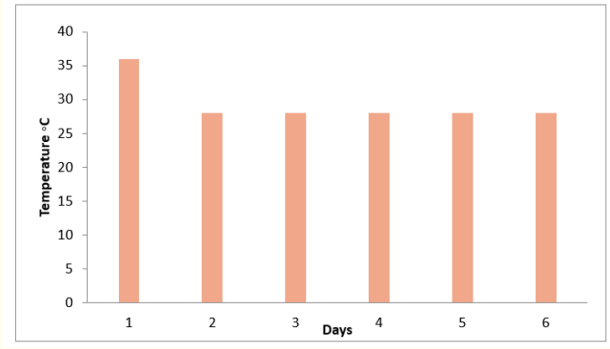

Figure 2: Temperature of *Manihot esculenta* during fermentation.

Findings from figure 3 showed that high bioethanol content were produced from *Manihot esculenta* peels by the pretreatment of H_2SO_4 at 17.08 g/ml. Thus, H_2SO_4 is a potent oxidizing agent and also improve enzymatic hydrolysis for the production of bioethanol. This is supported by the research carried out by Ouyang., *et al*. [40] who developed two step pretreatment of Chinese fir sawdust using dilute sulphuric acid. Under optimal condition, they quantified 76.8% yield of bioethanol which depict the efficacy of sulphuric acid.

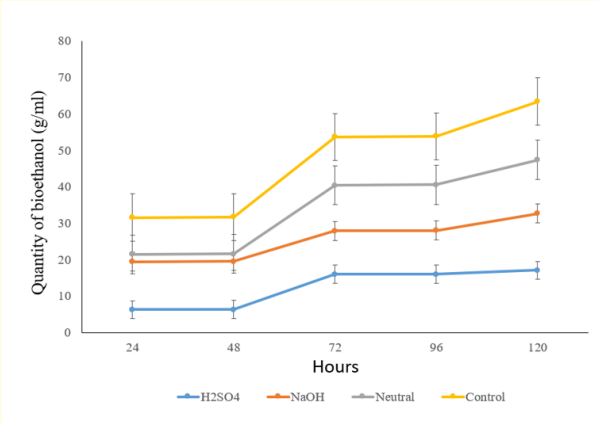

Figure 3: Quantity of bioethanol contents produced from *Manihot esculenta* peels.

Conclusion

This study established that bioethanol can be produced from *Manihot esculenta* peels and this serves as cheap alternative source of biofuel and bioenergy generation. Bioethanol was successfully produced from *Manihot esculenta* peels through simultaneous saccharification and fermentation of their hydrolysates with *Aspergillus niger* and *Zymomonas mobilis* in 120 hours fermentation period. From the results gotten it can be resolved that the 0.1M of H_2S0_4 is the most effective concentrations for pretreating the peels of cassava.

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Volume 4 Issue 11 November 2021