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BIOETHANOL PRODUCTION FROM CASSAVA PEELS USING (*Candida tropicalis* AND *Pichia caribbica)*

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Abstract

The studies were conducted on the production f ethanol, isolation and identification of two yeast strain from *Cola acuminata* and *Zea mays*, using spread plate technique. Physiological, morphological and cultural characteristic of *C. tropicalis* and *P. caribbica* were carried out such as sugar assimilation and fermentation test resulting in the discovery of the yeast species. Two isolates belonging to different genera which include *Candida tropicalis* and *Pichia caribbica* were used for the fermentation of a particular substrate (cassava peels). The pretreatment of the cassava peels was carried out by chemical hydrolysis followed by the process of distillation of water and ethanol mixture. It was discovered that *Pichia caribbica* have higher ethanol yield 14.8% in (100g) of substrate of cassava peels than *Candida tropicalis* which have ethanol yield 10.2% in the same (100g) used for both fermentation. The proximate and elemental analysis of the cassava peel revealed high tendency of ethanol production based on the values obtained. Hence this study indicates that cassava peels can be a good feedstock for sustainable bioethanol production.

Key words: Bioethanol, Proximate, Cassava peels, Genera, substrate

Introduction

In recent years, the annual energy consumption from petroleum sources has increased many fold resulting not only in continuous depletion of limited fossil fuel stocks but also a cause of concern for the saver, better and greener environment (Lynd & Wang, 2003). Further, the high prices of fossils have led to energy crises in both developing and developed countries that are oil dependent. Reserve of fossil fuel is going to be depleted fast which may lead to the increase in fuel price and simultaneously unfolding energy crises. Naylor *et al.*, (2007), stated that biofuels will remain critical energy development target in many parts of the world, if petroleum price exceed $55 – 60 per barrel. Therefore, there is a considerable emphasis on the development of biofuel production technologies from plant source and bioethanol production from plant biomass. Bioethanol is an alcohol produced by fermentation of plant biomass, containing starch or sugars. And its production depends upon feed stock availability, variability and sustainability (Liimatainen *et al.*, 2004).

Bioethanol is produce by fermentation of sugar by microorganisms, as opposed to synthetically produce ethanol from petrochemical sources. It is produce by the

process of distillation of fermented sugars, which can be utilized in a liquid fuel in internal combustion engines either neat or in blends with petroleum (Graeme & walker, 2010). It is a cleaner transportation fuel than non-renewable gasoline, Biomass-based ethanol or bioethanol is well entrenched in policy as a potential substitute for petroleum based gasoline. One of the primary benefit of switching to this fuel is that biomass can be renewed and can potentially provide a sustainable fuel supply over a long term period (Farrel *et al.,* 2006). As an alternative source of energy, bioehtanol can meet the fuel demand as well as can save the environment from pollution problem. There are two type of ethanol: denatured ethanol and Hydrous ethanol, (Maclean & Lave, 2004). Denatured ethanol is the one that is used in blending premium motor spirit to E10, E20, but unfit for human consumption.When the bioethanol replaces aromatic and sulfurs containing compounds used in gasoline, it may also reduce nitrogen oxide emissions to improve air quality, which can reduce urban smog, (Thatoi *et al*., 2014). The high oxygen content in bioethanol could reduce the generation of known hazardous volatile organic compounds and carbon monoxide in vehicle exhaust. It is usually mixed with 2 – 5 low – cost hydrocarbon that make it unfit for human consumption.On the other hand,hydrous ethanol is the natural ethanol used as industrial raw material in the production of alcoholic beverages, cosmetics perfumes, drinks, medicaments and other industrial uses (Thatoi *et al*., 2014).

Justification

Large tons of cassava peels wastes are discarded annually in Nigeria when cassava is processed to various food products. These wastes end up in open dumps or draining system threatening both surface and ground water quality, it is therefore necessary to convert these wastes into useful products since the local material of cassava peels waste which yield high quality of starch for the production of ethanol through fermentation, rather than allowing them to becoming aesthetic pollution to the environment. At present, there is no commercial production of alcohol from cassava in Nigeria, Huge amount of money are been spent to import ethanol for medical, pharmaceutical, research and industries (Williams, 2013). The aim of this research is to produce ethanol from cassava peels using *Candida tropicalis* and *Pichia caribbica.* Candida tropicalis is a potentially useful organism for the commercial production of ethanol as it is capable of fermenting starch at a low rate, again *C. tropicalis* does not require the saccharification step (Jamai *et al*., 2007).

Methodology

Collection of Sample (Cassava Peels) for the Fermentation Process

4000g of fresh cassava peels were collected into clean polythene bag from cassava market waste dump site at Alkaleri local government area of Bauchi State. The waste was sun dried for three days after washing with distilled water in order for the soil and dirt to be removed and ready for the pretreatment.

Microorganism used for the Fermentation

Maize and kola nut were randomly bought from local markets within Gwallameji Metropolis, Bauchi State Nigeria. The samples were transported to the laboratory. All samples were washed using sterile water and grinded to powder form using mortar and pistil.These particles were then sieved to obtain average particle sizes of 300 μm in diameter.

Preparation of Media (Malt extract agar)

Malt extract agar was used as the medium for the isolation. The medium was prepared according to the manufacturer's instruction and sterilized at 121^{o}C for 15 minutes. The prepared medium was dispensed aseptically into sterile petridishes and left on the bench to solidify at room temperature.

Isolation and Identification of Microorganisms

One gram of each sample was soaked in distilled water in 250 ml conical flask for 72 hours at 28 ± 2°C. Serial dilution of the steep of each was carried out up to 10^{-5}An aliquot of 0.1ml of each dilution was placed on MEA plates (5 g/100ml using spread plate technique. The inoculated plates were incubated for 48 hours at 28 ± 2°C. Chloramphenicol at the rate of 30 μg/ml was added as an antimicrobial agent to inhibit all bacteria growth.

Subculture Technique

Isolates were subculture on MEA to check for purity and incubated at 28 ± 2°C for 48 hours. Purified cultures were routinely maintained on MEA slants and kept at 4°C in refridgerator. The strains were stained using methylene blue and viewed under a high power microscope (100× magnification). Colour, texture and other features were observed on the colonies. Biochemical tests of the selected yeast isolates were carried out by the means of fermentation of different carbon sources using the modified method of Olanbiwoninu and Odunfa (2012). The identities of the isolates were confirmed by comparing the characteristics with those of known taxa using the schemes of Rhode and Hartmann, (1980) & Ellis *et al.*, (2007).

Medium for Maintaining Yeast Culture

The same procedure and media type for isolation was used in maintaining the isolated yeast cells however, instead of using petridishes; malt extract agar slant was used. These were stored in refrigerator at 6°C.

Viability test of yeast strains

Test for Presence of Glycogen in Yeast Strains

Suspension of yeast cell was fixed on a microscopic slide and cover with lugol's iodine, under the microscope. This was done to determine the state of nourishment of the yeast cells. Yeast cells containing reserve polysaccharide can be seen stains dark brown, while the yeast cell without glycogen stains yellow. The two yeast strains was stained dark brown. If less than two third ones stain brown, this indicate insufficient nourishment (*Cheesbrough* 2000).

Test for the Presence of Lipid in the Yeast Stain

Suspension of yeast cells was fixed on a microscopic slide and cover with sudan III Solution, these were covered with cover slip and observed under the microscope. Yeast cells containing lipids are stain red while the yeast without lipid are colourless which was not observed. Also two third of the yeast cells should be stained red if less than two third of the yeast cells stain red indicates insufficient nourishment.

Sugar Assimilation and Fermentation Test

The basal medium used for sugar fermentation test was 9ml of peptone water dispensed into a test tube containing inverted Durham Tube and sterilized at 121ºC for 15 minutes. 10% of each of the test sugar (Glucose, Lactose, Maltose, Fructose and Galactose) was separately sterilized at 115^{o}C for 10 minutes. After which they were allowed to cool. 1ml of each test sugar was added into the medium. Two drops of bromothymol blue indicator was added. Each sugar was then inoculated and incubated at 37^{o}C for 24 hours. Change in colour indicated acid production while gas production indicated by displacement of the Durham tubes to produce bobble**.**

Pre-treatment of cassava peels

Pretreatment basically refers to the mechanical and physical actions to clean and shape the biomass and destroy the cells structure to make it more accessible for further chemical or biological treatment (Alvira *et al.,* 2010). In physical pre-treatment, chemical milling of the desired substrate was carried out for grounding the substrate, the mechanical milling was used to grind the dried tubers to a certain particle size that can pass through a mesh screen and then the flour was collected and used or further processing i.e. hydrolysis of the substrate (Sun & Cheng, 2002).

Chemical Hydrolysis of Cassava Peels

Pre-treated cassava peels was weighed 100g into 1L conical flasks and make up to mark with distilled water, corked and sterilized at 121^{o}C for 15 minutes, where sterile distilled water was added to the flask to make a final volume of 1 litre and was hydrolyzed with 100 ml of concentrated acid, 70 – 77% (w/w), at a low temperature around 50^{o}C (Sun & Cheng, 2002). After conversion to simpler monomers (glucose), it was fermented to produce ethanol with the help of ethanol-producing microorganisms (Lin & Tanaka, 2006).

Propagation of the Yeast Strain

Peptone water media was prepared according to the manufacturer instruction, where about 100mls of each was dispensed into two separate steriled bottle and they were inoculated with the microorganism (*C. tropicalis* and *P. caribbica*) separately and incubated at 37^{o}C for 48 hours before introducing-it into the fermentation medium.

Fermentation Technique

The hydrolysate from the above was divided into two and transferred into another set of conical flask and labeled correctly, it was properly covered and

autoclaved at 121^{o}Cfor 15minutes and allowed to cool. The flask was inoculated with *Candida tropicalis and Pichia caribbica* separately to carry out fermentation for five (5) days. The pH of the hydrolysate containing *Candida tropicalis and Pichia caribbica* was adjusted to 4.5 and fermentation carried out at 25^{o}C (Ramanand, 2008). The flask was shaken at interval to produce a homogenous solution. After fermentation, the ethanol and water from the sugar containing liquid mixture was separated into their component using distillation techniques.

Distillation of the Mixture

The fermented liquid was transferred into round bottom flask and place on the heating mantle fixed to the distillation column enclose in running tap water, another flask was fixed to the other end of the distillation column to collect the distillate at 78^{o}C (standard temperature for ethanol production).

Determination of Ethanol Yield

The method of Oyeleke and Jibrin (2009) as used to determine the percentage of ethanol yield, where the mass of ethanol over the mass of substrate used in the fermentation in other to determine the percentage yield by the two respective microorganism (*C. tropicalis* and *P. caribbica*) that was used for the process of fermentation, using the following formula:

$$\text{Ethanol (\%)} = \frac{A}{Z} \times 100$$

Where, A = Total Mass of Ethanol

Z = Total Mass of Substrate (Cassava peels).

Proximate Analysis of the Cassava Peels

Total Ash, Crude Fat and Crude Fibre

Total ash Crude Fat and Crude Fibre content was determined using the method of AOAC (2000).

Moisture Content

The crucible was heated at 100^{o}C in an oven for 60 minutes and cooled in desiccator and weighed. About 2 g of the sample was weighed into the cleaned crucible (W_1) and dried in an oven at temperature of 100^{o}C for eight hours and reweighed (W_2) the weight was expressed according to AOAC (2000).

$$\text{Moisture content (\%)} = \frac{W_1 - W_2}{\text{Weight of sample}} \times 100$$

Where, W_1 = Initial weight of sample + Crucible before heating

W_2 = Final weight sample + Crucible after heating

Crude Protein

Kjeldahl's method was used in order to estimate the percentage of protein present in cassava peels.

Carbohydrate

Determination of the carbohydrate contents in the cassava peels is the total sum of all the values of the proximate analysis which includes: ash content, crude protein, crude fat, moisture content and crude fibre subtracted from 100%.

Determination of the Nitrogen, Phosphorus and Potassium

Determination of Phosphorus and potassium in Cassava peels were achieved using the method of (Ademoroti, 1996).

Determination of Nitrogen in Cassava peels

The nitrogen content of the cassava peels was determined using Kjeldahl method The sample cassava peels (2g) was weighed into a 250cm^3 beaker, 25.00 cm^3 of concentrated nitric acid was added for pre-oxidation, covered with a sizeable watch glass and swirled to mix well. The mixture was set on a hot plate regulated to 200°C and heated for 30 minutes. The beaker was set down from the hot-plate and cooled. After all the brown fumes initially produced have ceased, 5.00 cm^3 of concentrated sulphoric acid and 5.00 cm^3 of concentrated perchloric acid were added, replaced on the hot plate, covered with watch glass and the digestion was continued for 90 minutes. Thick white fumes initially set but subsided as the digestion progressed and finally leaving colorless residue behind (Ademoroti, 1996).

Results

The two yeast strains were isolated, purified and further identified from different food crops produced in Nigeria eg, Maize and sorghum. Different test were applied including morphological, physiological which facilitate the identification of the yeast.

These test allowed for baseline information for the studies and the determination of the systematic status of the yeast. Distinct yeast was isolated from each of the food crops used with each belonging to separate genus which includes *Candida* and *Pichia*. Only the isolates of *Candida* showed dark bluish colony while *Pichia* showed a whitish creamy. Isolate of *Candida tropicalis* showed pseudohyphae formation. Both organisms, isolated show elliptical round spores belonging to *Deutromycotina* and *Ascomycotina*.

The physiological and biochemical tests of the yeasts carried out on Table 1 showed that both isolates ferment respective sugar for their growth, sugar assimilation and fermentation by the isolates including glucose, lactose and maltose but was unable to ferment galactose in *P.carribica* and fructose in *C.tropicalis*. Both isolates are able produce acid by changing the colour of the indicator in the tubes. The result of physiological and biochemical tests are presented in the Table 1.

The Result of the proximate analysis of the peels before the fermentation process is repesented in the table 2 below. The average value of ash, fat content, moisture content, fibre crude protein and carbohydrate are 0.56$\pm$ 0.0%, 3.33$\pm$0.8%, 8.3$\pm$5.6%, 16.6$\pm$5.6%, 0.79$\pm$0.03% and 70.4% respectively. This result was higher than other parameters suspected to be present in the cassava peels sample before the fermentation by its respective microorganism (*C. tropicalis* and *P. caribbica*). The result of mineral composition of the cassava peels is represented in Table 3. The average value of phosphorus (mg/l) is higher with 155.1$\pm$5.0% while average value of potassium (mg/l) is 0.37$\pm$0.0% while nitrogen having the lowest average value 0.13$\pm$0.03%.

Discussion of Result

In this study, two yeast species from two genera were isolated from *Cola acumunata* and *Zea mays* respectively; they include *C. tropicalis* and *P. Caribbica.* The yeast was found to be fermentative in the breakdown of hexose and pentose sugar.

Firstly, several researchers such as Oyeleke and Jibrin, (2009), Mohammed *et al.,* (2011) have reported the activities of some yeast strain in bioethanol production. The species of yeast like *Candida* have not been extensively reported as fermentative agent for industrial utilization such as the production of bioethanol nor in the production of other useful organic compounds.

Table 1: Biochemical test of the isolate showing fermentation by the two organisms

Parameters	***C. tropicalis***	***P. caribbica***
Glucose	+	+
Lactose	+	+
Galactose	+	–
Maltose	+	+
Fructose	–	+

+ = Ferment, – = Non-Ferment.

Table 2: Proximate analysis of cassava peels before fermentation

Parameters	**Values (%)**
Ash	0.56±0.0
Fat	3.33±0.8
Protein	0.79±0.03
Crude Fibre	16.6±5.6
Moisture	8.3±5.6
Carbohydrate	70.4±0.0

Table 3: Concentration of Nitrogen, potassium and phosphorus in cassava peels

Parameters (mg/l)	**Concentration (%)**
Nitrogen	0.13±0.03
Potassium	0.37±0.0
Phosphorus	155.1±5.0

The species of yeast like *Candida* have not been extensively reported as fermentative agent for industrial utilization such as the production of bioethanol nor in the production of other useful organic compounds. Elis *et al.,* (2007) reported *C. tropicalis* as the causal agent of *candidiasis* in man, they are opportunistic fungi which live in most human organs. However, recent reports by Senthilraja and Saravanakumar, (2011) have shown that species of *Candida* are not just pathogens but can be useful tools for bioethanol production, as they were able to used *C. tropicalis* and *C. albicans* isolated from marine environment to produce bioethanol. There had also been reports of their isolation from dairy products such as yoghurt and milk (Gadaga *et al.,* 2000). *P. caribbica* and *C. tropicalis* were isolated from *Zea mays* and *Cola acuminata*, respectively. This is probably the first report of isolation and characterization of yeast from this substrate that can be used in fermentation for the production of bioethanol in Nigeria. Most workers had reported the use of *S. serevisiae* for fermentation in the production of bioethanol Adesanya *et al.,* 2008, Oyeleke & Jibrin, 2009). This report therefore gives an array of prospective fermentation species of yeast from locally available substrates which can be of industrial benefits. The microorganisms were able to degrade the carbon sources because they contain the enzymes necessary for the conversion of sugars to other products.

The analysis of the nutrient composition in the substrate that was used for the process of fermentation was recorded as shown in Table 3. The fermentation medium increases in addition of microorganism in which by their activity may also increases the protein content, or the increase in growth and proliferation of the microbial complex in the form of single cell protein might contributed to increased in protein content after fermentation of the substrate, which can be either lesser or higher than 6.19±00% for the fermented cassava peels as reported by Shanavas *et. al.,* (2011) who stated that the increase in protein is associated to nitrogenous and non-nitrogenous remains locked up within the cell as a result of the cellulose and hemicellulose of the wall components, showing up after the activities of the microbes. Based on the metabolic activities of the microbes which was also recorded by (Lynd *et al.,* (2002)that the increase value of the protein content in the substrate (cassava peels) leading to the considerable reduction in some of the other parameters like the carbohydrate as a result of the treatment process performed on the substrate which include conversion of the starch by enzymes or chemicals into simple sugar (glucose) which serves as the carbon source for those microorganism that lead to their proliferation and subsequently converting the monomer of sugar into ethanol in an anaerobic condition.

The mineral analysis of the substrate before fermentation reveals that nitrogen concentration (0.13±0.03%) was the lowest because of the decrease in the value of protein of the substrate before the fermentation, which is meant to increase after fermentation with specific

microorganism as reported by (Shanavas *et al.,* (2011). While the potassium concentration (0.37±0.0%) which is higher than (0.20±0.01%) reported by Wantanee, (2004). The higher concentration was recorded, in the substrate before the fermentation for phosphorus was (155.1±5.0%). The result of ethanol yield in this research indicated that *Pichia caribbica* produced higher ethanol yield of 14.8% (14.8g/l) than *Candida tropicalis* 10.2% (10.2g/l) which was then used to compared with the previous work of Szambelan, Nowak, and Czarnecki (2004) by using *Kluyveromyces fragilis*, a yeast with commercial yeast and *Zymomonas mobilis* producing an ethanol concentration of (0.48g/l) for the mixed population and (0.46g/l) for the single population.

Conclusion

Conclusively, the results of the present research revealed that cassava peels have the potential of producing reasonable ethanol using these microorganisms (*C. tropicalis* and *P. caribbica*).

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