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Potential of bioethanol production using millet husk

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The use of guinea corn husk and millet husk (agricultural waste with no appreciable value to industries or competitive use as food) as alternative and cost-effective feedstock for the production of bioethanol was examined. The methods used, included: enzyme hydrolysis, and simultaneous saccharification and fermentation with Aspergillus niger and Zymomonas mobilis isolated from soil and palm wine, respectively. The present study revealed that fermentation using Saccharomyces cerevisiae has the highest percentage concentration of bioethanol (2.5 mg/l) than Zymomonas mobilis with (1.4 mg/l) at pH 6, 7 and 8. The study indicated that millet husk is a suitable agricultural waste for bioethanol production process.

Key words: Bioethanol, Millet husk, Enzyme hydrolysis, Simultaneous saccharification.

INTRODUCTION

Bioethanol is a renewable energy source produced mainly by the sugar fermentation process; although it can also be synthesized by chemical processes such as reacting ethylene with steam (Anuj et al., 2007). Ethanol fuel blends are widely sold in the United States of America. The most common blend is 10% ethanol and 90% petrol (E10). Vehicle engines require no modification to run on E10 and vehicle warranties are not affected. Only flexible fuel vehicles can run on up to 85% ethanol and 15% petrol blends (E85) (Tanaka, 2006). Bioethanol is a renewable energy source produced mainly by the sugar fermentation process; although it can also be synthesized by chemical processes such as reacting ethylene with steam (Anuj et al., 2007). Ethanol fuel blends are widely sold in the United States of America. The most common blend is 10% ethanol and 90% petrol (E10). Vehicle engines require no modification to run on E10 and vehicle warranties are not affected. Only flexible fuel vehicles can run on up to 85% ethanol and 15% petrol blends (E85) (Tanaka, 2006).

The natural energy resources such as fossil fuel petroleum and coal are being utilized at a rapid rate and these resources have been estimated to last only a few years. Therefore, alternative energy sources such as ethanol, methane and hydrogen are being considered. Some biological processes have rendered possible routes for producing ethanol and methane in large quantities. Worldwide interest in the utilization of bioethanol as an energy source has stimulated studies on the cost and efficiency of industrial processes for ethanol production (Tanaka, 2006).

Ethanol production processes only use energy from renewable sources and there is no net CO₂ emission to the atmosphere, thus making ethanol an environmentally beneficial energy source. In addition, ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the greenhouse gas effect. This reduction of greenhouse gas emission is the main advantage of utilizing biomass conversion into ethanol (Anuj et al., 2007).

Traditionally, ethanol has been produced in batch fermentation with fungal strains such as Aspergillus niger, Mucor mucedo and Saccharomyces cerevisiae, which cannot tolerate high concentrations of ethanol. Therefore, improvement programmes are required in order to obtain alcohol-tolerant strains for fermentation (Gunasekaran and Chandra, 2007).

Human activities generate large amounts of waste such as crop residues, solid waste from mines and municipal waste. They may become a nuisance and sources of
pollution. It is therefore important to handle them judiciously to avoid health problems, since these wastes may harbour pathogenic microorganisms (Ledward et al., 2003). Agricultural wastes, including wood, herbaceous plants, crops and forest residues, as well as animal wastes are potentially huge source of energy. In Nigeria, large quantities of these wastes are generated annually and are vastly underutilized. The practice is usually to burn them or leave them to decompose. However, studies have shown that these residues could be converted into liquid fuel such as biogas and bioethanol, or combusted to produce electricity and heat (Soltes, 2000).

Several agricultural wastes have been tested for their bioethanol-producing potential. In the present study, the utilization of some agricultural residues (millet husk) for the production of bioethanol will be evaluated. Therefore, to complete and efficient conversion of both 5- and 6-carbon atom sugars, co-culture fermentation between both hexose and pentose fermenter should be carried out, even though few experiments were succeeded (Fu and Peiris 2008; Fu et al., 2009).

This research serves as a platform to give basic information on the development of bioethanol and to create further research on areas of data deficiency. The indication of areas lacking sufficient data and potential areas for additional study would direct academic researchers to select priority areas and undertake further study. The aim of this study is to potentially produce bioethanol from millet husk. These will be achieved through the following objectives;

1. To produce bioethanol from millet husk residues through fermentation using A. niger and Z. mobilis
2. To determine the quantity of bioethanol produced from millet husk
3/ To determine the percentage of ethanol concentration.

MATERIALS AND METHODS

Millet husk were collected from waste dumping sites in Sokoto metropolis. It was packed in a polythene bag and transported and to microbiology laboratory in Usman Danfodiyo University, Sokoto for analysis. The samples were dried and ground to a powder form using a Waring blender (Binatone).

Media Preparation

Nutrient Agar

The nutrient agar medium was prepared according to manufacturer’s instruction that is 28g of nutrient agar was suspended in 1000ml of distilled water, which was then mixed thoroughly and heated on hot plate to dissolve completely. The preparation was autoclaved at 121°C for 15 minutes, and then allowed to cool at 45°C which was dispensed into sterile petridishes for isolation of bacteria. (Cheesbrough, 2006).

Potato Dextrose Agar

The media was prepared according to the manufacturers instruction that is 39gram of PDA was suspended in 1000mls of distilled water and heated to dissolve completely and autoclaved at 121°C for 15 minutes. Also 1gram of antibiotic (streptomycin) was added to the mixture to inhibit the growth of bacteria for isolation of fungi (Cheesbrough, 2000).

Isolation of and Characterization of Microorganisms

Isolation of Aspergillus niger

The soil sample collected was serially diluted; a sample suspension was prepared by adding 1.0g of sample to 10ml of distilled water and mixed well for 10minutes. The suspension was diluted serially 10⁻¹, 10⁻² and 10⁻³. One ml (from the dilution factor) was measured using a syringe and inoculated into a potato dextrose agar (PDA), a glass spreader used to spread and inoculated at 28°C in an incubating room for 5 days for colony observation (Jasuja et al., 2013).

Isolation of Zymomonas mobilis

The soil sample was serially diluted from 10¹ up to the fifth 10⁵. From the fifth dilution factor, 0.1ml was taken and inoculated into nutrient agar medium by using spread plate method. The plates were incubated in an incubator at 37°C for 24 hours. Colonies suspected to be Zymomonas mobilis was characterized on the basis of morphological and biochemical characteristics. The isolates were purified by streaking on a freshly prepared media and incubated at 37°Cfor 24 hours. The ability of Zymomonas mobilis to ferment various carbohydrates using glucose, fructose, sucrose, maltose and lactose was determined by growing the isolates in liquid standard medium (glucose broth) 1% (w/v) of the particular carbohydrates (Jasuja et al., 2013; Cheesbrough, 2003)

Biochemical Characterization

Biochemical tests that were performed on the bacterial isolates included: Gram staining, catalase test, oxidase test, urease test, motility test, carbohydrate fermentation
test, indole test and coagulase test. *Z. mobilis* was identified by comparing the characteristics of the isolates with those of known taxa using Bergey’s Manual of Determinative Bacteriology (Holt *et al.*, 1994; Obire, 2005).

**Gram Staining**

A thin smear of bacterial isolates was prepared on a glass slide, air dried and then fixed by passing over a burner flame at least three times. The smear was covered with crystal violet stain for 1 minute and then washed, it was then covered with Lugols’ iodine and washed after 1 minute, and it was then decolorized with acetone and washed immediately for few seconds. The smear was covered with safranin and washed after 1 minute. The back of each of the slides were wiped out with cotton wool and allowed to air dried. The dried smear was then examined microscopically with oil immersion and viewed with a microscope using x100 objective lens (Cheesbrough, 2000).

**Catalase Test**

The test is used to differentiate those bacteria that could utilize hydrogen peroxide into oxygen and water. A small portion of colony was picked using a sterile glass wire loop and transferred onto a drop of hydrogen peroxide on the glass slide. The immediate bubbling was observed and indicated catalase positive while negative test was indicated by absence of bubbles (Oyeleke and Manga, 2008).

**Oxidase Test**

One percent (1%) solution of aqueous tetramethyl-p-phenylenediamine hydrochloride. (The regent used) this test is particularly useful for differentiating pseudomonas from certain other enteric or gram negative bacteria. On a nutrient agar plate containing 24 hours culture streaking from a nutrient agar slant, a few drops the reagents was placed on the line of streaking of each culture. Oxidase positive colonies developed a pink colour which progressively became purple within 30 seconds, while oxidase negative colonies did not produce this purple colouration (Cheesbrough, 2003).

**Urease Test**

A little portion of the organism was picked using a sterile wire loop and inoculated into prepared urease medium. It was then incubated at 37°C for 24 hours. A change in the colour of the medium from yellow to pink indicated urease positive test (Oyeleke and Manga, 2008).

**Motility Test**

A 24 hours old colony was inoculated in nutrient broth medium and incubated at 37°C for 24 hours. A drop from overnight cultured broth was picked and placed on clean grease free slides and covered with cover slip, then viewed under X40 lens. Motile bacteria were seen moving randomly (Cheesbrough, 2003).

**Sugar Fermentation Test**

A small portion of each isolate was picked using sterile inoculating needle and stabbed into triple sugar agar medium and incubated at 37°C for 24 hours. Fermentation of any sugar (Glucose, lactose or sucrose) was observed by colour change of the medium from red to yellow. TSI glucose fermenters were indicated by the butt becoming becoming yellow, lactose and sucrose fermentation was indicated the slope becoming yellow. However fermentation of lactose and sucrose but not glucose was indicated with red butt and yellow slope (Cheesebrough, 2006).

**Bioethanol Production**

Methods used for production of bioethanol include hydrolysis, fermentation and fractional distillation.

**Enzymatic Hydrolysis**

Here, ten grams (10g) of pre-treated millet husk sample was poured in twenty different 250ml conical flask containing 100ml of distilled water, the pH was adjusted in triplicates, 3 samples was adjusted to pH 6, 7, 8 using potassium hydroxide (KOH) and salicylic acid, and two samples was left at their normal pH, where each of them was serve independently as control for a distinct fermentation. The samples was sterilized at 121°C for 15 minutes, using autoclave, then the conical flasks was allowed to cool and then inoculate with *Aspergillus niger*, covered with cotton wool and wrapped with aluminium foil and incubated at 37°C for five days, (Humprey and Caritas, 2007).

**Fermentation**

The fermentation was carried out along with saccharification (simultaneous saccharification and fermentation (SSF), as described by Kroumov *et al.* (2006); Oghgren *et al.* (2006). The flasks containing the hydrolyzed samples were covered with cotton wool, wrapped in aluminium foil, autoclaved for 15 min at 121°C, and allowed to cool at room temperature. *Z. mobilis* and a spore suspensions of *A. niger* and were aseptically
inoculated into each flask and incubated at 30°C. Two flasks of each sample (millet husk) were removed after every 24 hours, up to 7 days.

Fractional Distillation

The fermented broth was dispensed into round-bottom flasks fixed to a distillation column enclosed in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. A heating mantle with the temperature adjusted to 78°C was used to heat the round-bottomed flask containing the fermented broth.

Determination of Quantity of Bioethanol Produced

The distillate collected over a slow heat at 78°C was measured using a measuring cylinder, and expressed as the quantity of ethanol produced in g/l by multiplying the volume of distillate collected at 78°C by the density of ethanol (0.8033 g/ml), g/l is equivalent to the yield of 100 g of dried substrate (Humphrey and Caritas, 2007).

Determination of Percentage Ethanol Concentration

A standard ethanol density curve was prepared by taking series of percentage (v/v) ethanol solutions, which were prepared in volumetric flasks, and the weight was measured. The density for each of the prepared ethanol solutions was calculated and a standard curve of density against percentage ethanol was plotted. The percentage ethanol concentration of ethanol produced was obtained by comparing its density with the standard ethanol density curve (Oyeleke and Jibrin, 2009).

RESULTS

The result obtained in this study revealed bioethanol production using Zymomonas mobilis and Aspergillus niger. Isolation and identification of Zymomonas mobilis from soil was presented in table 1. The organism was identified base on cultural and morphological characterization; a motile gram negative rod bacteria, catalase and glucose positive, urease, oxidase, and lactose negative.

Table 2 showed the macroscopic and microscopic characteristics of the isolated specie of Aspergillus niger. It was found to be haploid filamentous fungi with septate hyphae; produced colonies that are composed of yellow felt, covered by dark asexually produced fungal spores.

The concentration of the reducing sugar produced from the treated milk husk revealed that Saccharomyces cerevisiae has the highest reducing sugar yield of 0.126mg/L presented in table 3, when compared to the to the value of reducing sugar obtained from Zymomonas mobilis presented in table 4 with lowest reducing sugar (0.106mg/L).

The quantity of bioethanol produced in quantity of bioethanol produced that was presented in table 3, showed that the quantity of bioethanol produced from Saccharomyces cerevisiae of 180ml/L has the highest yield as compared to the quantity of bioethanol yield by Zymomonas mobilis which has 175ml/L presented in table 4.

The densities of bioethanol produced from millet husk revealed that samples with low densities tend to produce more bioethanol and the density should be less than the density of water which is one (1). The results further revealed that bioethanol produce through fermentation with Saccharomyces cerevisiae has the lowest density of 8.0 g/ml, presented in table 4, whereas bioethanol produce through fermentation with Zymomonas mobilis has the highest density of 9.11 g/ml, presented in table 3.

The viscosities of bioethanol produced from millet husk presented in table 3 and 4 revealed that viscosity of bioethanol produce through fermentation with Zymomonas mobilis yields the highest viscosity of (9.11 g/ml) which is greater than that of Saccharomyces cerevisiae (5.8g/ml).

The concentration of bioethanol produced from millet husk presented in table 3 and 4 revealed that concentration of bioethanol produce through fermentation with Zymomonas mobilis yield the lowest concentration of (1.4mg/l) which is greater than that of Saccharomyces cerevisiae (2.5 mg/l).

DISCUSSION

The result showed that Aspergillus niger have a black mycelium on the gar medium, it had septate hyphae, long and smooth conidio spores, and long unbranched sporangiospores with a large and around head. The results obtained in this present studies on morphological characteristics of Aspergillus niger is similar to the findings of Oyeleke and Jibril (2009).

Zymomonas mobilis was found to be gram negative short rod, catalase-positive, oxidase and urease negative, motile and hetero- fermentative, producing gas from glucose, fructose and sucrose. This finding is in conformity with that of Obire (2005), who reported the isolation of Zymomonas mobilis from fresh wine saps. Its therefore reveals that bioethanol can be produced any time Zymomonas mobilis was used. This is in agreement with the findings of Rabahet et al., (2011) who reported the microbial pretreatment of millet husk and ground nut shell for bioethanol production using Zymomonas mobilis.
Table 1: Biochemical Characterization of *Zymomonas mobilis*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Gram rxn</th>
<th>Motility</th>
<th>Catalase</th>
<th>Glucose</th>
<th>Urease</th>
<th>Oxidase</th>
<th>Lactose</th>
<th>Organism identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-ve rod</td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td><em>Zymomonas mobilis</em></td>
</tr>
</tbody>
</table>

Table 2: Macroscopic and Macroscopic Characteristics of *Aspergillus niger* Isolated from Millet husk

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Macroscopic Characteristics</th>
<th>Microscopic Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus</em></td>
<td>Pin like or black powdery myceliated, spreading and zonated colonies</td>
<td>non branched conidiophores with bulb end carries conida like sunrays</td>
</tr>
<tr>
<td><em>niger</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Result for Bioethanol Produced from *Zymomonas mobilis*

<table>
<thead>
<tr>
<th>S/NO</th>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reducing sugar (mg/ml)</td>
<td>0.106</td>
</tr>
<tr>
<td>2</td>
<td>Quantity (ml/l)</td>
<td>480</td>
</tr>
<tr>
<td>3</td>
<td>Viscocity (g/ml)</td>
<td>4.9</td>
</tr>
<tr>
<td>4</td>
<td>Density</td>
<td>9.11</td>
</tr>
<tr>
<td>5</td>
<td>Concentration (mg/l)</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 4: Result for Bioethanol Produced from *Saccharomyces cerevisiae*

<table>
<thead>
<tr>
<th>S/NO</th>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reducing sugar (mg/ml)</td>
<td>0.126</td>
</tr>
<tr>
<td>2</td>
<td>Quantity (ml/l)</td>
<td>486</td>
</tr>
<tr>
<td>3</td>
<td>Viscocity (g/ml)</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>Density</td>
<td>8.0</td>
</tr>
<tr>
<td>5</td>
<td>Concentration (mg/l)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

(Negative rod, motility, catalase, glucose, fructose and sucrose positive, maltose, arabinose, urease, oxidase, lactose negative) and other ruminant microorganisms.

The highest reducing sugar yield of 0.126mg/L was obtained after the treated millet husk was hydrolyzed with *Aspergillus niger*. This might due to the hydrolyzing microorganisms use the sample as their source of carbon and at the same time produce enzymes that hydrolyze the millet husk into glucose during the process of feeding. The 0.126mg/L reducing sugar obtained is almost in agreement with the 0.120mg/L reducing sugar obtained by Thompson *et al*. (2008) and lower than 0.128mg/L reported by Brooks *et al*. (2008). The studied further showed that hydrolyzing the millet husk with *Aspergillus niger* produce more reducing sugar.

The highest concentration of 2.5% mg/l was obtained after the hydrolysates were fermented with *Saccharomyces cerevisiae*, which is greater than that of *Zymomonas mobilis* with 1.4% mg/l. The result revealed that higher production through *Saccharomyces cerevisiae* might due the enzymes that possess which can reduce a carbonyl group into a hydroxyl group in properly high yield reported by Shang Xueng (2011) which tends to facilitate the breaking down of the sugars into alcohol. All these might be responsible for high bioethanol produced from hydrolysates.

The fermentation of the hydrolysates through *Zymomonas mobilis* had a maximum yield of 0.076% bioethanol after 24 hours of fermentation. The highest bioethanol yield obtained was lower than 0.095% reported by Rabah *et al*. (2011). This may be associated to environmental factors and differences in methods of fermentation. In this study, it revealed that bioethanol can be produced from millet husk with maximum yield obtained using *Saccharomyces cerevisiae*. This is because *Saccharomyces cerevisiae* can ferment the glucose more efficiently to break down the sugar into minor metabolite such alcohol and CO2 to produce ethanol.

The highest density obtained from *Zymomonas mobilis* is 9.11 mg/ml which is greater than the highest density obtained from *Saccharomyces cerevisiae* which is 8.0
g/ml. The results of this study disagree with the findings obtained by Oyeleke and Jibrin (2009) which in his work got 0.8035 from millet husk substrate. This might be as a result of difference in the manure of substrate, there was no pretreatment in Oyeleke and Jibrin (2009)while there was pretreatment in this work, the hydrolysis in Oyelekeand Jibrin (2009), work was done using *Gloeophyllums epiairium* (0.8035g/ml) and *Pleurotusos tretus* (0.8023g/ml) from millet and guinea husk. While in this work *Aspergillus niger* was used in hydrolysis, and 5% H$_2$SO$_4$ was also used for pretreatment of the millet husk.

**CONCLUSION**

In this research bioethanol was produced from millet husk. Enzymatic hydrolysis with dilute acid was carried out. The result in this study revealed that fermentation using *Saccharomyces cerevisiae* has the highest percentage concentration of bioethanol (2.5 mg/l) than *Zymononas mobilis* with (1.4 mg/l). This is because *Saccharomyces cerevisiae* can ferment the glucose more efficiently to break down the sugar into minor metabolite such as alcohol and CO2. Also, the study indicated the potentiality of millet husks as cheap raw materials for the bioethanol production process.

**RECOMMENDATIONS**

This research would like to suggest the following recommendations.

i. Millet husk should be utilized for ethanol production.
ii. Works done with regard to bioethanol production should not remain on paper and they should be implemented.
iii. The government should involve in bioethanol production from wastes generated from home, restaurant, juice house, hotels, cafeterias and shops to encourage business owners.
iv. Further researches have to be done on hydrolysis of starch so as to get high amount of glucose concentration which gives high amount of ethanol.

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