FUDMA Journal of Sciences (FJS)
ISSN online: 2616-1370
ISSN print: 2645 - 2944
Vol. 6 No. 2, April, 2022, pp 262 - 267
DOI: https://doi.org/10.33003/fjs-2022-0602-1753

THE POTENTIAL OF *Azadirachta indica* (NEEM TREE) LEAVES FOR BIOETHANOL PRODUCTION USING *Zymomanas mobilis* AS FERMENTING ORGANISMS

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ABSTRACT

Bioethanol is a renewable energy produced from lignocellulosic biomass mainly by the fermentation process. It is used as an alternative to fossil fuel which doesn't contribute to global warming. This research work was carried out to determining the potential of Neem tree leaves for bioethanol production. The process used in this study include pretreatment, hydrolysis fermentation and distillation. Hot water and Acid methods were used for the pretreatment. The hydrolysis of the substrate was carried out using Aspergillus niger for five days. Benedict test method was used to determine the reducing sugar concentration of the hydrolysate. The highest yield of 1500-2000 (mg/dl) was obtained equally for both the pre-treatment method. After hydrolysis, fermentation process was carried out for seven days using *Zymomonas mobilis* to ferment the hydrolysate. The fermented broth was separate using fractional distillation. Acid potassium dichromate was used to determine the concentration of the bioethanol produced. The results obtained shows that liquid hot water pretreatment produced the highest yield of 0.294±0.882 (mg/L) whereas alkaline method produced 0.282±.882 (mg/L) yield. Gas chromatography and mass spectroscopy analysis was also carried out to determine the presence of volatile compounds in the bioethanol produced. The highest volatile compound was Ethanol with 33.12% where Butyl Glycol was found to be least volatile compound with 6.22%. This result shows that Neem tree leaves is a good biomass for bioethanol production and pretreatment using Hot water method produced more bioethanol than Alkaline method of pretreatment.

Keywords: Neem tree leaves, Bioethanol, Pretreatment, Hydrolysis, Fermentation, Distillation, *Zymomonas mobilis*

INTRODUCTION

Biofuel has been a source of energy that human beings have used since ancient times. Increasing the use of bio-fuels for energy generation purposes is of particular interest nowadays because they allow mitigation of greenhouse gases, provide means of energy independence and may even offer new employment possibilities. Biofuels are being investigated as potential substitutes for current high pollutant fuels obtained from conventional sources (Blackwell, 2008). The quest for alternative energies has provided many ways to produce electricity, such as wind farms, hydropower, or solar cells. (Anuj *et al.*, 2007).

Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. It is a renewable energy source produced mainly by the fermentation process but can be synthesized by chemical processes such as reacting ethylene with steam (Anuj *et al*., 2007). Bioethanol produced from different biomass materials. It is renewable and it has lower energy density that gasoline and availability of feedstock for bioethanol can vary considerably from season to season. The production of ethanol from lignocellulosic biomass has received considerable attention because of the potential of producing large quantities of ethanol for use as a transportation fuel. Hemicellulose and cellulosic components of lignocellulosic biomass are hydrolyzed to their component sugars for subsequent conversion to ethanol by a fermentative process. Hemicellulose and cellulose are usually hydrolyzed with a chemical process (acid) or biological (enzyme) attack. The economic success of ethanol production will depend on efficient conversion of cellulose and hemicellulose to their monomeric sugars and the efficiency of fermenting those sugars to ethanol, while also reducing capital and operating costs.

Neem, *Azadirachta indica*, is a member of the mahogany family Meliaceae comprising fifty genera and five hundred species. The neem tree was thought to have originated from Assam in India and Burma where it is most widely used (Oparaeke, 2004). *A.* indica belongs to a large family of wood species, height ranged from 15.4 to 46.0 m. The buttress measures from 0.1m to1.14m in height; while the bark relatively thin (Al-hassan and Usman, 2012). For centuries, the people of India cleaned their teeth with neem twigs, topically applied neem-leaf juice to treat skin disorders, took neem tea as tonic, and placed neem leaves in their beds, books, grain bins, cupboards, and closets to keep away troublesome bugs. Extract from the tree had been reported to relieve pains, fever, infections and other complaints (Ajeet and Abdul 2014).

In Nigeria, neem is locally known as "dogonyaro", "darbejiya" in Hausa, "igi-oba" in Yoruba and "ogwu-akom" in Igbo. The neem tree is common in the north-east, north-west and north central geopolitical zones of the country where it is used as shelter-belts to control desertification (Anuj *et al.*, 2007). The tree remain leafy except during extreme drought, when the leaves fall. Neem trees are found generally in every state of the country growing wild. Despite the abundance of this tree, its economic value has not been fully exploited (Al-hassan, 2003).

This research work was aimed to determine the potential of neem tree leaves for bioethanol production. The methods used include pretreatment, hydrolysis (enzymatic), fermentation and finally distillation.

MATERIALS AND METHODS

Sample Collection

The Neem tree leaves samples were aseptically collected in new clean polythene bag from U~~n~~iversity of Maiduguri Botanical Garden. The leaves were then transported to the Microbiology laboratory, University of Maiduguri for the research analysis.

Sample Preparation

The samples were air dried under room temperature for about 2-3 days, grinded using pestle and mortar. The grinded sample was labelled and stored in sealed polythene bag. The samples were then collected and weighed each, before taken to laboratory for the analysis.

Isolation and Identification of Microorganism for the research work

Isolation and Identification of Zymomonas mobilis

Z. mobilis was isolated from *Hibiscus sabdariffa* drink using standard solid medium (5 g/l yeast extract, 20 g/l glucose, 20 g/l agar; pH 6.8) as described by Rabah *et al.,* (2014). The medium was supplemented with actidione (cycloheximide) to inhibit yeast growth. One ml (1 ml) of the juice was serially diluted in sterile distilled deionized water and aliquots of the dilutions was aseptically plated onto the medium using the pour plate technique. The agar plates were incubated at 37°C in an anaerobic jar for 24 - 48 h. After incubation, the bacterial colonies that grew on the agar medium was counted using a colony counter and expressed as colony forming units (cfu)/ml of sample. Colonies differing in size, shape and color was selected from different agar plates and was sub-culture on standard solid medium by the streak plate technique. The agar plates were incubated at 37°C in an anaerobic jar for 24 h. The subsequent pure cultures were maintained on agar slant for further characterization and identification. Biochemical tests were also carried out on bacterial isolates. (Oyeleke and Manga 2012). *Z. mobiles* was identified by comparing the characteristics of the isolates with those of known taxa using Bergey's Manual of Determinative Bacteriology (Elijah 2010).

Isolation and Identification of Aspergillus niger

Macro culture method and micro methods were used to identify the organisms according to the method describe by (Musa *et al.,* 2019). The soil sample was collected from botanical garden, University of Maiduguri transferred to a sterilized sample container. The soil sample was collected from the upper layer where most microbial activities take place and thus where most of the bacteria population is suspected to be concentrated (Oyeleke and Manga 2012). The soil sample was serially diluted; a sample suspension was prepared by adding 1.0g of sample to 10ml of distilled water and mixed well for 10 minutes. The suspension was diluted serially 10^{-1}, 10^{-2} and 10^{-3}. 1ml (from the third dilution factor) was measured using a syringe and inoculated into a Potato Dextrose Agar (PDA) plate and incubated at 37^{o}C for five days. The isolate was identified by comparing their characteristics with those of known taxa (Oyeleke and Manga, 2012). The initial white color of the colonies that later turns black at the top with pale yellow color at the bottom confirm the organism to be *Aspergillus niger*. Microscopically the isolates were stained with lacto-phenol cotton blue and was observed under a compound microscope for the conidia, conidiophores and arrangement of spores (Singh *et al.*, 2015).

Sample Pretreatment

All of the samples were subjected to Acid and Hot water pretreatment.

Dilute Acid Pretreatment

Dilute acid pre-treatment was carried out according to the method describe by (Rabah *et al.,* 2014). In this method, 0.5ml % of sulfuric acid was added into different conical flasks of size 250 ml containing each of the samples. The flasks were cover with cotton wool, wrap in aluminum foil, heated in a water bath at 60 ^{o}C for 1 hour and then autoclaved at 121 ^{o}C for 15 minutes. The flasks were allowed to cool and the pH was adjusted to 7 using NaOH solution (
Zhang and Feng 2010).

Liquid Hot water Pretreatment

Liquid Hot water Pre-treatment method was also carried out on substrate according to the method describe by (Farouq *et al.,* 2019). In this method, 10g of the grounded neem leaves was put in to conical flasks of size 250 ml in triplicate and diluted each with 100 ml of hot sterile distilled water. The conical flasks were then covered with aluminum foil paper and capped with rubber plugs for 24 hrs. The treated samples were filter with sieve to collect the soluble potion while the insoluble biomass was made to hydrolyze in the next step which is hydrolysis (Farouq *et al.,* 2019).

Microbial Hydrolysis

Microbial hydrolysis was carried out according to the method describe by (Singh *et al.,* 2015).
In this method, the treated watermelon peels were inoculated with 0.5 ml suspension of the pure culture of *Aspergillus niger*. Hydrolysis was carried out at room temperature for five days. Samples were taken daily for reducing sugar determination. The samples were then filtered using What man filter paper No. 1 and the filtrates were used for fermentation (Sun and Cheng, 2011).

Determination of the Reducing Sugar

Benedict test method was used to determine the reducing content of the hydrolysates. One (1) ml each of the hydrolyzed sample was diluted to 1ml of Benedict reagent in the test tube, after which the samples was boiled in water bath at 70^{o}c for 1 hour. The samples were removed from the water bath and the color observed from the samples was compared with the Benedict table of reading sugar for results (Rabba *et al.,* 2014).

pH Adjustment

The pH of the samples was adjusted to 5.5 before the addition of the fermenting organism otherwise the bacteria may be denatured in hyper acidic or basic state. A highly concentrated NaOH and HCL solution were prepare to adjust the pH and was regularly check using a digital pH meter (Rabba *et al.,* 2014).

Sterilization

All the hydrolysate samples were sterilized by autoclaving at 121^{o}C for 15 minutes prior to fermentation (Rabba *et al.,* 2014).

Fermentation of the Hydrolysates

The fermentation of the hydrolyzed samples was carried out in accordance with the methods described by Musa *et al.* (2019). The hydrolysates from each conical flasks were aseptically inoculated with 1ml suspension (6.0×102cfu/ml) of *Zymomonas mobilis* using McFarland standard. Fermentation was carried out anaerobically at room temperature for 5 days (Farouq *et al.,* 2019).

Fractional Distillation

Fractional distillation apparatus was set up in the laboratory. The fermented broth was dispensed into round-bottom flasks fixed to a distillation column that was enclose in running tap water. A conical flask was fixed to the other end of the distillation column to collect the distillate. The temperature of the heating mantle was adjusted to 78oC which was used to heat the round bottomed flask containing the fermented broth, since ethanol can only be distilled at 78oC. Further, extraction was carried out using Soxhlet apparatus after which the ethanol produced was pour into conical flask and then label (Nata'ala *et al*., 2018).

Determination of Percentage Concentration of the Bioethanol Produced

Percentage concentration was carried out using acid potassium dichromate reagent according to the methods of (Oyeleke and Jibrin, 2009). About (1ml) of standard ethanol was diluted with 99 ml of distilled water to give a concentration of 1 %. Then 0, 2, 4, 6, and 8 ml each of the 1% ethanol was diluted to 10 ml of distilled water to produced 0, 0.2, 0.4, 0.6, and 0.8 ml of the ethanol. To each of the varying ethanol concentrations, 1 mls of acid potassium dichromate was added and allowed to stand for an hour for color development. The absorbance of each concentration was measured at 580 nm using UV-VIS spectrophotometer and the reading was used to develop standard ethanol curve (Oyeleke and Jibrin, 2012).

RESULTS

Table 1 and 2 shows the colonial and morphological characterization of *Zymomonas mobilis* isolated from *Hibiscus sabdariffa* (Sobo drink). The organism shows a brilliant white to cream color, plump with rounded ends.

Table 1: Colonial and Morphological Characteristics of the isolated Microorganisms from *Hibiscus sabdariffa*

Isolate	Results
Colony shape	Brilliant white to cream color
Cell shape	Plump with rounded ends
Gram's reaction	-
Endospore formation	-
Motility test	+

Table 2: Biochemical Characterization of the isolated Organism from Hibiscus sabdariffa

Isolate	Test Results
Coagulase	-
Glucose	+
Sucrose	+
Lactose	–
Urease	–
Oxidase	–
Methyl red	–
Endospore	–
Suspected organism	*Zymomonas mobilis*

Table 3 shows the morphological characterization of Aspergillus niger isolated from soil. The organism shows a black mycelium on the agar medium, it had septate hyphae, long and smooth conidiospores, and long unbranched porangiospores with a large and round head.

Table 3: Morphological Characterization of *Aspergillus niger* isolated from soil.

Isolate	Colony Characterization	Cell Shape	Organism
1.	Black dotted surface as conidia	Filamentous with septate hyphae	*Aspergillus niger*

Table 4 and 5 shows the concentration of reducing sugar from liquid hot water and acid pre-treatment using Benedict test was carried out for two days. A reducing sugar of 1500-2000 mg/dl was the highest obtained from both pre-treatments.

Table 4: Concentration of Reducing Sugar from Liquid |hot Water and Acid Pre-treatment for Day 1

Sample	Color observed	Approximate glucose	Indication (mg/dl)
liquid hot water 1	Orange ppt	1500-2000 mg/dl	+++
liquid hot water 2	Orange ppt	1500-2000 mg/dl	+++
liquid hot water 3	Orange ppt	1500-2000 mg/dl	+++
Alkaline 1	Orange ppt	1500-2000 mg/dl	+++
Alkaline 2	Orange ppt	1500-2000 mg/dl	+++
Alkaline 3	Orange ppt	1500-2000 mg/dl	+++

Table 5: Concentration of Reducing Sugar from Liquid Hot Water and Acid Pre-treatment for Day 2

Sample	Color observed	Approximate glucose	Indication (mg/dl)
liquid hot water 1	Orange ppt	1500-2000 mg/dl	+++
liquid hot water 2	Orange ppt	1500-2000 mg/dl	+++
liquid hot water 3	Orange ppt	1500-2000 mg/dl	+++
Alkaline 1	Orange ppt	1500-2000 mg/dl	+++
Alkaline 2	Orange ppt	1500-2000 mg/dl	+++
Alkaline 3	Orange ppt	1500-2000 mg/dl	+++

Table 6 shows the Percentage Concentration of bioethanol produced using hot water and acid pre-treatment. The result shows that liquid hot water pre-treatment produced the highest bioethanol yield of 0.294 ± 0.882mg/l whereas acid pre-treatment has the lowest yield of 0.281 ± 0.882mg/l.

Table 6: Percentage Concentration of Bioethanol Produced from Liquid Hot Water and Acid Pre-treatment

Concentration of Bioethanol Produced (mg/l)		
liquid hot water	**Acid**	***P* Value**
0.294 ± 0.882	0.281 ± 0.882	1.011

The Values are Mean ± Std, T-Test of difference = 0.05, there is significant difference using t-test ($p>0.05$)

Table 7 shows the spectrum of the separated compound was compared with the database of the spectrum of known compound saved in the NISTO2 Reference Spectra Library. Data analysis and peak area measurement was carried out using Agilent software (NARICT, 2014). Ethanol was the highest volatile compounds with 33.12 % and the least volatile compound was found to be Ethyl acetate Glycol with 7.32 %.

Table 7: Volatile Profile of the Produced Bioethanol Using Acid and Liquid Hot Water Pre- treatment.

S/N	Retention time	Peak Area	Volatile Compounds	%
1	3.145	4433654	Ethanol	33.12
2	4.262	565334421	Butyl Glycol	8.14
3	6.544	55643327	Acetone	18.51
4	7.764	45609864	Ethyl acetate	7.32
5	9.237	3966113	Isobutanol	14.26
6	12.871	4312543	Isopropanol	9.43
7	13.345	4536543	Methanol	11.20

DISCUSSION

The research work was carried out to produced bioethanol from Neem tree leaves. Cellulose, hemicellulose and lignin are the major components of the substrate (Patel *et al.*, 2006). *Aspergillus niger* and *Zymononas mobilis* are the microorganisms used for this research. *Aspergillus niger* are mostly found in a conducive environment especially in the soil and plant debris, whereas *Zymononas mobilis* are found in fermented product such as palm wine, Burukutu or *Hibiscus drink*) (sobo drink). (Yasir and Hind 2018).

Tables 1 and 2 shows the morphological and biochemical characterization of *Zymomonas mobilis* isolated from *Hibiscus sabdarifa*. The organism shows a brilliant white to cream colored, plump with rounded ends when grow on nutrient agar. The organism also shows a positive reaction towards Catalase, Glucose and Sucrose test and shows negative reaction towards Gram, Coagulase, Lactose, Urease, Oxidase and Methyl red test. The organism is motile and has no spore. The ability of this microorganism to grow in this medium is because they derive their nutrient from it (Obianwa *et al.,* 2016). These 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, Oyeleke, Manga and Hassan (2014) that reported the isolation of *Zymomonas mobilis* from rotten sweet orange and stated that the organism can thrive in sweet mediums because they love sugar and receive available juices that they utilize as their growth factor. Table 3 shows the macroscopic identification of *Aspergillus niger* based on colony pigmentation and the structure of the conidial head. This corresponds with the report gave by (Lide *et al.,* 2012), that *Aspergillus niger* are carbon black with a dark globular conidial head. He stated that the organism has a potential to produce amylase which is important in hydrolyzing starch. Sing *et al.,* (2014) also stated that A. *niger* can be used in industries for the production of ethanol and some acids because of its high tolerant to acidity, thus, preventing bacterial contamination.

A good pretreatment technique determines the success of all bioethanol production; therefore, it is necessary to choose the best pretreatment technique available (Sabina *et al.* 2018). Three types of pretreatments were used to process the rice husks namely mechanical, alkaline and steam expulsion pretreatment. Sundry method is good in removing the moisture content of the rice husks, this is in agreement with the work of (Krishnan *et al.,* 2011) who observed that insufficient drying of the rice husks would encourage fungal growth and cause the husks to lose some of its sugars. Sodium hydroxide (NaOH) was used for alkaline pretreatment. This study reveals that treatment improves the carbohydrate content of samples with concomitant reduction in the cyanide levels allowing the microorganisms needed for hydrolysis and fermentation to attain optimum production of reducing sugars and final bioethanol yield. After pretreatment, hydrolysis was

done and a degree of colors were observed for three (3) days using Benedict's test to test for the percentage concentration of the reducing sugar. Hydrolysis was done using *Aspergillus niger (*Sirajo *et al.,* 2019).

Table 4 and 5 shows the concentration of reducing sugar from acid and hot water pretreatment of Neem tree leaves. The result was obtained using Benedict reagent. The scales of colors contained in the Benedict reagent starts with blue which shows no sugar, cream (> 500). Mg/dl, 500-1000 mg/dl, 1000-1500 mg/dl, orange 1500-2000 mg/dl and red show > 2000 mg/dl. The result after two days of hydrolysis show that the reducing sugar concentration varies with the methods of pretreatment and the duration of days used for the hydrolysis. The result shows Acids pretreatment produce highest amount of glucose with bricks red color which signifies the presence of the sugar > 2000 mg/dl while hot water pretreatment produced sugar of > 500- 1000 mg/dl indicated by a color green ppt. This result is similar to the finding of Fish *et al.,* (2009).

Table 6 shows that acids pretreatment produced the highest bioethanol yield of 0.294± 0.856 (mg/L) while hot water pretreatment produced bioethanol yield of 0.281 (mg/L). This is because there is variation and reduction of temperature that causes the show down in degradation of polysaccharides.

The fermentation of the hydrolysates with *Zymomonas mobilis* reveals that *Zymomonas mobilis* is able to produce bioethanol which may be because it possesses pyruvate decarboxylase and alcohol dehydrogenase as reported by Oyeleke *et al.,* (2012) as the key enzymes in ethanol production and they tends to facilitate continuation of fermentation at high concentration of ethanol, they also indicated the suitability of *Zymomonas mobilis* due to its higher sugar uptake and bioethanol yield, its lower biomass production and its higher ethanol tolerance, all these might be responsible for bioethanol produced from the neem tree hydrolysates (Sheelendra and Shilpa, 2014). This study revealed that bioethanol can be produced from rice husks using *Aspergillus niger* as hydrolyzing agent and *Zymomonas mobilis* for fermentation (William, 2011).

Table 7 **shows** the spectrum of the separated compound was compared with the database of the spectrum of known compound saved in the NISTO2 Reference Spectra Library. Data analysis and peak area measurement was carried out using Agilent software (NARICT, 2014). Ethanol was the highest volatile compounds with 33.12 % and the least volatile compound was found to be Ethyl acetate Glycol with 7.32 %.

CONCLUSION

In conclusion, this research work was carried out to determine the possibility of using neem tree leaves for bioethanol production. The methods used includes pretreatment (acid and hot water), hydrolysis (Enzymatic), fermentation (using *Z. mobilis*) and finally distillation. The result obtained reveals that neem tree leaves is a good biomass that has the potentials to produce bioethanol using *Aspergillus niger* and *Zymomonas mobilis* as hydrolyzing and fermenting organisms respectively. The results further revealed that acid pretreatment produced the highest bioethanol yield than hot water pretreatment.

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