



Enzymatic Hydrolysis and Bioethanol Production from Neem Tree Leaves (*Azadirachta Indica*)

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Abstract

Bio-ethanol was produced from Neems tree leaves (*Azadirachta indica*) through enzymatic hydrolysis using *Bacillus firmus* as fermenting agent. Dried powdered of Neem tree leaves (*Azadirachta indica*) was hydrolyzed using 1.0cm³, 1.5cm³ and 2.0cm³ of *Bacillus* suspension. After that the hydrolyzed samples were fermented using 3cm³ suspension of cultured bacteria (*Bacillus firmus*). After that, the fermented broths formed were distilled to obtained bioethanol. Acidified K₂Cr₂O₇ was used to determine the concentration of the bioethanol produced. In which the absorbance of the bioethanol obtained using UV-Visible Spectrophotometer was extrapolated with the series concentration prepared with standard glucose solution. The FTIR Spectroscopy analysis of bio-ethanol produced confirms the presence of alcohol functional group present in the sample. The percentage yield of the bioethanol from the leaves using 1.0cm³, 1.5cm³ and 2.0cm³ of *Bacillus* suspension were 1.42%, 1.85%, and 1.72% yield respectively. The result showed that the Neem tree leaves that are from lignocelluloses materials can produce some appreciable percentage of bio-ethanol. Among the samples 1.5cm³ of *Bacillus* suspension has shown the highest yield of 1.85%.

Keywords: *Azadirachta indica*; *Bacillus firmus*; Bio-ethano; Enzymatic Hydrolysis; Ligno-cellulosic Biomass.

1.0 INTRODUCTION

The increase in greenhouse emissions from industries and transportation has been linked to changes in the climate. This concern, coupled with limited fossil fuel reserves, has initiated a justifiable global refocus on research and development of other energy sources (İçöz, *et al.*, 2009). It was firmly established that bioethanol can be produced from various sources, like starch crops, sugar crops, fruit juices etc. Most of today's bioethanol production is derived from edible sources. However, perusal of literatures at our disposal exposed a dearth of research for the production of bioethanol from non-edible raw materials. Balat *et al.* (2008) suggested that the

quest for non-edible raw material is a useful and sustainable option that should be pursued, will all vigor as a good alternative to fossils fuel. Since crops can grow renewably in almost all climates around the world with considerable lower emission of poisonous gasses to our environment (Brooks, 2008; Rabahet *et al.*, 2011). Utilization of non-edible plants for bioethanol production is one of the best options for the fact that the fuel production will not pose food security issues or threats. Bioethanol has become one of the most promising biofuels today and is considered as the only feasible and sustainable alternative to fossil fuel all over the world. With advanced energy

saving production technology, bioethanol can considerably reduce the climate distorting greenhouse gas emissions from transport and other industries (Junchen, 2012). The use of edible plants for biofuels production has recently been of great concern because of the fact that, they compete with food materials. As the demand for these plants for food has increased tremendously in recent years, it is difficult to justify the use of these edible plants for fuel used purposes such as bioethanol production. Moreover, these edible plants could be more expensive to use as fuel. Hence, the contribution of plants such as neem leaves that is not consumed as a food will be significant as a non-edible plant source raw material for bioethanol production. Bio-ethanol is eco-friendly and such an alternative fuel that can be used in unmodified petrol engines with current fueling infrastructure and it is easily applicable in present day combustion engine as mixtures with gasoline (Hansen *et al.*, 2005). Combustion of ethanol results in low emission volatile organic compounds, carbon monoxide and nitrogen oxides. The emission and toxicity of ethanol are lower than those of fossil fuels (petrol and diesel) (Wyman and Hinman, 1990). Production of ethanol from ligno-cellulosic materials has received extensive interest due to their availability, abundance and relatively low cost (Pessoa *et al.*, 1997). Biofuels production has potential advantage in improving rural and agricultural economies, benefiting agricultural and industrial sectors by using by-products such as wood wastes, oil, waste products and municipal solid wastes, as well as a decrease in dependence on oil imports and therefore providing some energy security (Demirbas, 2008). To prevent fuel food crisis, lignocelluloses biomass, particularly agricultural residues is converted to useful products such as bioethanol (Clausen, 1989; Brown, 1999; Demirbas 2007).

Lignocelluloses biomass consists mainly of mainly lignin, cellulose and hemicelluloses materials that are present in a different percentage according to the plant type and its parts. It was

reported that, the plant leaves contain 15-20% cellulose, 80-85% of hemicelluloses and 0% of lignin (Junchen *et al.*, 2012). Presently, more researches are focused on non-edible biomass due to their availability and low cost in procurement. Bioethanol is a volatile and flammable liquid produced through microbial fermentation process, which has a molecular formula of C_2H_5OH (Greame, 2012).

Neem tree (*Azadirachta indica*), is a tree in the mahogany family Meliaceae. It is one of two species in the genus *Azadirachta*. It is a native to India, Pakistan and Bangladesh which is widely growing in tropical and semi-tropical regions. Neem tree is the official tree of the Sindh Province and is very common in all its cities. Neem tree also grows in islands of the southern part of Iran (USDA, 2014). It grows widely in northern Nigerian region, its fruits and seeds are the source of Neem oil. The neem tree leaves were reported to have contained 60% of H_2O , 23% carbohydrates, 7% proteins and more than 3% minerals and 1% fat (Heinrich, 2005). The Figure 1 below shows Neem tree leaves.

The major process for conversion of lignocelluloses to bioethanol requires the following process; delignification to liberate the cellulose and hemicelluloses, depolymerisation of carbohydrate polymers to produce free sugars (which is known as hydrolysis of cellulose to simple sugar) and finally, the fermentation of mixed simple sugar to ethanol (Chandel *et al.*, 2012). The Pretreatment of lignocelluloses biomass in bioethanol production is done not only to enable break the β -1,4-glycosidic linkages linkage of the polysaccharide but also contribute in generating high yield of bioethanol (Zhao, 2012; Uduak *et al.*, 2008).

This research aimed to assess the potential of the Neem tree leaves (*A. Indica*) for bioethanol production using cultured bacteria (*Bacillus firmus*).



Figure 1. Neem tree leaves sourced from Usmanu Danfodiyo University main campus.

2.0 MATERIAL AND METHODS

2.1 Sample Collection and Sample Preparation

Three different types of Neem tree leaves were collected in polythene bags and were taken to the laboratory for analysis at Usmanu Danfodiyo University permanent site. The Neem leaves were air dried and grounded to powder and sieved with mesh to obtained fine particles.

2.2 Preparation of Nutrient Agar (NA)

To prepare the nutrient agar 28g of nutrient agar was diluted using 1000cm³ of distilled water and heated with constant stirring until the agar was dissolved completely. The agar was autoclaved for about 15minutes at 121°C.

2.3 Sand Samples Collection for Isolation of Bacterium

Five (5) different decayed sand samples were obtained from five different places, each under the dead decayed trees within Sokoto Metropolis, three decayed sand samples were obtained inside Usmanu Danfodiyo University main campus and the rest of the two, were obtained in Kantindaji area, Sokoto.

2.4 Isolation of Bacillus Species From the Decayed Sands

One (1g) of each decayed sand samples were dissolved in 9cm³ of distilled water. The serial dilutions were carried out 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ respectively. Two lowest dilution factors were carried out 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ and inoculated in different sterilized petridishes containing nutrient agar medium by spreading

method using bend glass rod and incubated at 37°C for 24h.

After 24h, different colony were observed and sub cultured into another sterilized Petri-dishes by streaking method to obtained pure culture and then incubated for 24h. The pure culture was used for Microscopy and biochemical characterization.

2.5 Characterizing of Bacterial Isolates

Bacterial isolates were characterized based on colonial characteristics, gram staining and morphological characteristics. Biochemical test were also carried out to identify the generic level of each isolate according to the Cheesebrough (2003). The following biochemicals Characterization were done to the isolates which are Gram Staining, Urease Test, Indole Test, Citrate Test, Catalase.

2.6 Starch Hydrolysis

After incubation, iodine solution was added to each growth and blue black colour was observed. Development of a blue-black colour indicated the presence of starch (Oyelekeet *et al.*, 2008).

2.6.1 Triple Sugar Iron Agar Test

The sugar and protein were attacked oxidatively to release ammonia. Through this medium the production of H₂S can be detected by the presence of a black colour in the media along the stabbed line. Motility was detected by the presence of growth along the area been stabbed by the straight wire loop. Gas production was detected by the presence of gas bubbles or crack on the agar in the test tube or complete disruption of the medium. Colonies from the sub-cultured plate was picked

with a sterile straight wire loop and stabbed on the butt, streak on the surface of the slope. This was incubated at 37°C for 24h (Cheesebrough, 2002).

2.6.2 Preparation of *Bacillus firmus* Suspension

The media (Nutrient Agar) was poured into the petridishes and then allowed it to be completely solidified. Sterile wire loop was used to pick a colony from the bottle and strike into petridishes and then incubated for 24h for organism to grow. Ten (10cm³) of distilled water was pipetted into five clean test tubes. The test tubes were covered with cotton wool wrapped in an aluminum foil, then autoclaved for 15 minute at 121°C and then allowed to cool. The sterile wire loop was used to pick cultured bacteria from the petridishes and inoculate into five clean test tubes, shake and the turbidity was observed.

2.6.3 Determination of Inoculum size

The test tubes containing the inoculum suspension was shaken and the bottles of McFarland suspension also shake. The turbid observed in the test tube was compared with the turbid observed in the McFarland control standard bottle. Where 0.5 was selected, 0.5 McFarland standards are comparable to a bacterial suspension of 10⁸cfu/cm³.

2.6.4 Enzymatic Hydrolysis

Powdered leaves (30g) of neem samples were taken into three 500cm³ conical flasks and 300cm³ of distilled water was added. The flasks were plugged with cotton wool and wrapped in aluminum foil and sterilized at 121°C for 15

minutes, the containers were allowed to cool. Each flask was inoculated with 1.0cm³ suspension of *Bacillus firmus*. The same procedure was repeated using 1.5cm³ and 2.0cm³ of *Bacillus* suspension. The flasks were incubated at 37°C for 5 days. After the period of 5 days the content of each flask was filtered and pH was then adjusted to 4.5 (Zhao *et al.*, 2012). The important of pretreatment is shown in the Figure 2, also the equation 2.2 defines the pretreatment process.

2.6.5 Determination of Reducing Sugar

The glucose standard was prepared by dissolving 1g of glucose to 100cm³ of distilled water, 10cm³ of the portion was pipetted and transfer into 100cm³ volumetric flask and made to the mark using distilled water, series of standard were then prepared by pipetting 0, 0.3, 0.6, 0.9 and 1.2cm³ from second standard glucose into five clean test tubes. The volume was then diluted by adding 3, 2.7, 2.4, 2.1 and 1.8cm³of distilled water to each test tube. To each test tube 3cm³ of DNS reagent was added. The content of each test tube was placed in boiling water bath for 10min to develop red brown colour. Then 1cm³ of 40% Potassium sodium titrate solution was added to stabilize the colour. The mixture was cooled at room temperature and the absorbance was measured at 540nm with a UV-visible spectrophotometer. Determination of sugar was done by adding 3cm³ of DNS solution to 3cm³ of each filtrate sample. The mixture was heated in a boiling water bath for 10 minutes to develop (Akpina *et al.*, 2013; Ohgren, 2006).

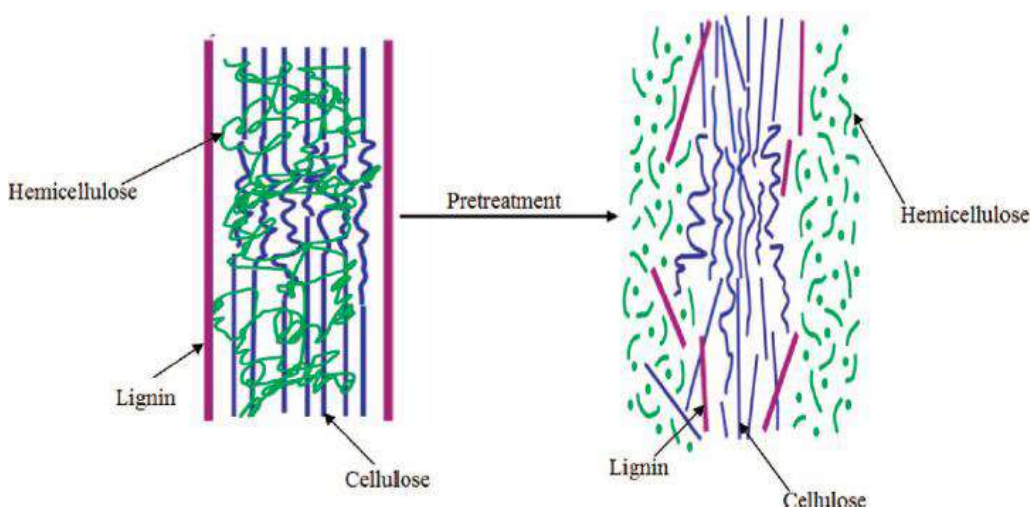


Figure 2. Representative section of lignocelluloses Biomass. Source: Manta *et al.* (2013).

2.7 Fermentation process

In this analysis the conical flask containing the hydrolyzed filtrate samples were covered with cotton wool, wrapped in aluminum foil and sterilized at 121°C. After cooling of the flasks at room temperature the pH of each flask was adjusted to 4.5 with NaOH. The samples were inoculated with 3cm³ suspension of culture

bacteria (*Bacillus firmus*). Then, it was incubated aerobically at 37°C for five days. After that, the broth obtained was distilled to obtain a pure ethanol (Oyeleke and Jibrin, 2009). The fermentation process is shown using equation 2.3 below.



2.8 Qualitative and Quantitative Test for Ethanol

2 drops of acidified 0.1M K₂Cr₂O₇ was added to the 2cm³ of distillate produced and heated for 30 minutes on a water bath. The content of the test tube changed to green colour indicating the presence of ethanol. Equation of the reaction is represented below (Raymon, 2003). However, the quantity of ethanol produced was determine using UV-visible quantitative analysis of alcohols using potassium dichromate VI an Oxidizing reagent in whereby the ethanol will be oxidized to ethanoic acid. This was carried out using UV-VIS quantitative analysis. 1cm³ of absolute ethanol (98% v/v) was dilute to 100cm³ using distilled water to give a concentration of 1% (ethanol stock solution) through which Series of standard were prepared. The content of each test tube was then heated in a boiling water bath for five (5) minutes, for developed full colour development. The absorbance of each concentration was measured at 585nm using UV-VIS Spectrophotometer and the

reading was used to develop standard ethanol curve. Consequently, Five (5cm³) of each of the sample were taken in the test tubes, and then 2cm³ of Dichromate reagent was added to each. The content of each test tube was then heated in a boiling water bath for five (5) minutes, for the reaction to complete and developed colour. The absorbance of each concentration was measured at 585nm, using U-V Visible spectrophotometer (Miller, 1959).

2.9 FTIR Spectroscopy

The sample of bioethanol produced was taken for The FTIR analysis which was carried out at Sokoto Energy Research Centre using MB 3000 Model spectrometer (Shimadzu) Japan that was equipped with pike technologies diffused reflectance accessory at 30 scans and resolution 2cm⁻¹. A small amount of the pyrolysis oil was mixed with 0.25-0.50 teaspoon of the potassium bromide (KBr) then it was placed in the die and kept for 2-3 minutes after that it was taken into the sample holder

3.0 RESULTS AND DISCUSSION

3.1 Results

Table 1. Result of Biochemical test of the isolate.

Parameters	Results
Morphology	Small colony white
Cell Gr react	Gram +ve rods in pairs
Spores Strain	+
Citrate	+
Starch Hydrolysis	-
Urease	+
Glucose	+

Indole

-

Species

*B. firmus***Key** + = positive, - = Negative**Table 2:** Reducing Sugar concentrations bio-ethanol produce and percentage yield.

Samples	Reducing Sugar (g/kg)	Bioethanol Produced (g/kg)	% Yield
1.0cm ³ <i>B.firmus</i>	1.42± 0.05 ^{bc}	14.22 ± 2.02	1.42
1.5cm ³ <i>B.firmus</i>	2.85± 0.14 ^a	18.46 ± 1.10	1.85
2.0cm ³ <i>B.firmus</i>	1.97± 0.20 ^b	17.16 ± 5.53	1.7

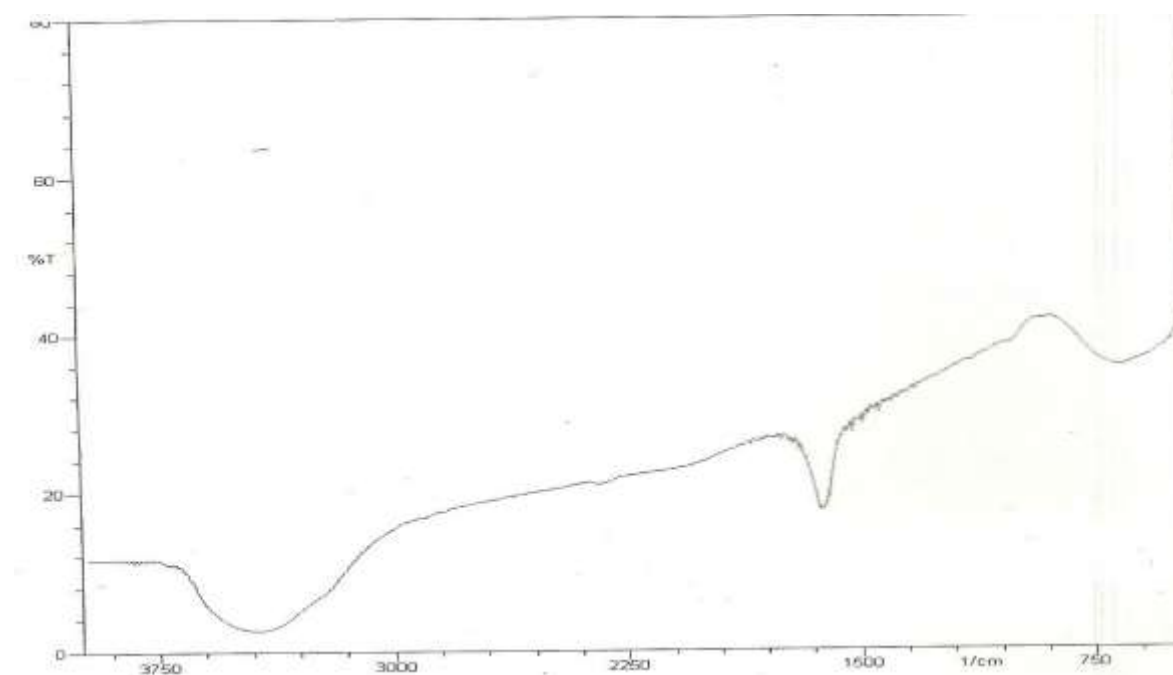


Figure 3. FTIR Spectrum of the Bioethanol Produce.

3.2 DISCUSSION

3.2.1 Reducing Sugar

Neem leaves hydrolyzed with 1.5cm³ inoculum suspension released the highest yield of reducing sugar (2.85g/kg), followed by 2.0cm³ (1.97g/kg) and 1.0cm³ (1.42g/kg). No significant ($P > 0.05$) variation was observed using different concentration of *B. firmus*. The results were closed to result obtained by Rabahet *al.* (2011), using cellulose enzymes as hydrolyzing agent on millet husk, this is because some enzyme find it more difficult to break down the lignin when compared to acid during hydrolysis (Kim and

Holtzapple, 2006 ; Dashtbanet *al.*, 2009 ; Akpanet *al.*, 2005).

3.2.2 Bioethanol Produced

The results of bioethanol as shown in the Table 2 indicate a linear relationship between the volume of ethanol produced and the reducing sugar. The amount of ethanol increase with increase in the mass of reducing sugar, beyond this level the ethanol became toxic to the organism (*B. firmus*) and kills them before the total sugar present is fermented (Graham and John, 2000)

The highest yield of ethanol reported for enzymatic hydrolysis and *B. firmus* fermentation was in 1.5cm³ inoculum suspension, which produced (18.46g/kg), this result is in agreement with what is reported by Oyeleke and Jibrin (2009), where he obtained 25.30cm³bioethanol generated from guinea corn husk. Nimbkaret *al.*(2009), also reported 12.45% as bioethanol from unspecialized juice of sweet sorghum. The findings of their research coincide with this study by giving maximum yield at 1.5cm³ inoculum levels. The highest yield obtained also agrees with that reported by Rajendran and Saravana (2013) were 4% yield of bioethanol obtained from Agave leaves using *S.cerevisiae*. Anbuselvi and Balamurugan (2013), also reported 5.89% yield bioethanol obtained from cassava leaves using *S.cerevisiae* which is closed to the higher yield obtained in this study.

3.2.3 FTIR Analysis

The highest yield of bioethanol obtained was subjected to FTIR Spectroscopy, all samples shows strong broad peak at 3450 - 2850cm⁻¹, therefore indicating -CH₂- and -CH₃ stretching vibrations and well resolved Peak around 3416cm⁻¹ can be assigned to alcoholic -OH vibrations. These values are in agreement with the values obtained According to Spectra (2014) free O-H stretching normally occurs at 3550 – 3200 cm⁻¹, while C-H stretch occurs at 3000 – 2840 cm⁻¹. Therefore, the production of ethanol was successful. In figure 3. Shows the spectrum obtained from the FTIR analysis.

5.0 REFERENCES

- Akpan, U.G., Kovo, A.S., Abdullahi, M. and Ijah, J.J. (2005). The production of ethanol from maize cobs and groundnut shells. *Assumption University Journal of Technology*. **9**(2): 106-110.
- Anbuselvi, S and Balamurugan, T. (2013) Study on ethanol production from cassava Leaves and Pulp using *S.cerevisiae*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*.**4**(2): 1755, 2013 ISSN: 0975
- Anuj, K.C.,Ohan, E.S., Rabinder, R., Lakshmi, M. N., Venkateswar, R and Pogaku, R. (2007). Economic and environmental impact of bioethanol production technologies,*an appraisal*, **2**: 14-32.
- Balat, M, Balat H. and Oz, C. (2008) Progress in bio-ethanol processing. *Progress in Energy and Combustion Science*, **34**:551-573.

The highest yield of ethanol reported from enzymatic *B. firmus* fermentation was in 1.5cm³ inoculum suspension, which produced (18.46g/kg), from Neem tree (*Azadirachta indica*) leaves sample.

Production of ethanol from ligno-cellulosic materials has received extensive interest due to their availability, abundance and relatively low-cost. Neem leaves (*Azadirachta indica*) is therefore an abundant and sustainable biomass and non-food material that could be exploited for bio-ethanol production especially in the northern part of the Nigeria. Neem leaves (*Azadirachta indica*) however could serve this purpose since from the study it is indicated that with proper pretreatment and appropriate method bio-ethanol could be obtained.

4.0 CONCLUSION

The population of human being is increasing on the average worldwide, hence the demand for energy source increases. It is apparent that current fuel bioethanol production from grain-based feedstock is not favorable as it may lead to food shortage to the teaming world populace. In order to avoid these foreseen worrisome, lignocelluloses biomass should be utilized in the production of bio-ethanol and biofuels in general. The research conducted using enzymatic hydrolysis and *B. firmus* fermentation showed that 1.5cm³ inoculum suspension, has the highest yield of ethanol in which (18.46g/kg) was produced from neem tree (*Azadirachta indica*) leaves sample.

- Brooks A.A. (2008), Ethanol Production Potential of Local Yeast Strains Isolated From Ripe Banana Peels. *African Journal of Biotechnology*, **7** (20): 3749-3752
- Chandel, A. K., Felipe A. F. A., Priscila V. A., Milessi, T. S., da Silvio S. S. and Maria G. (2012) Dilute Acid Hydrolysis of Agro-Residues for the Depolymerization of Hemicellulose: State-of-the-Art. *Springer-Verlag Berlin Heidelberg* DOI: 10.1007/978-3-642-31887-02, pp39-61
- Cheesbrough, M (2003). District Laboratory practice in tropical countries, part 2 low price edition. Cambridge University press, Cambridge, pp 28-90
- Demirbas, A. (2007) Bioethanol from Cellulosic materials: A Renewable Motor Fuel from Biomass. *Energy Sources***27**: pp327 – 337.

- Dashtban, M., Schraft, H. and Qin, W. (2009). Fungal Biconversion of Lignocellulosic Residue; Opportunities and Perspectives. *International Journal of Biological Science*. **5**(6): 578-595
- Demirbas, A. (2008). Biofuels Sources, Biofuels Policy, Biofuels Economy and Global Biofuels Projections. *Energy Conversion and Management*, **49**:2106-2116.
- Graham, H. and John H (2000). Chemistry in context. Fifth edition. Thomas Nelson & Sons Ltd. Delter place 27 Bath road, Cheltenham GL53 7TH United Kingdom. 108
- Hasen, Alan C., Qia Zhang, Peter, W. L. Lyne (2005) Ethanol diesel fuel blends a review, *Bioresources Technology* **96**: 277-285
- Heinrich, M., Prance, G. and M. Nesbitt. (2005). "Plants as Medicines." The Cultural History of Plants. London: Routledge. pp228-239.
- İçöz, E, Tuğrul, K.M. and Saral, (2009). Research on ethanol production and use from sugar beet in Turkey. *Biomass and Bioenergy*, **33**:1-7.
- Kim, S. and Holtzapple, M T. (2006) Lime pretreatment and Enzymatic Hydrolysis of Corn Stover. *Bioresource Technology*, **96**: 1994-2006.
- Junchen, L., Muhammad, I. & Lin, F. (2012) Bioconversion of agricultural waste to ethanol: A potential source of energy. *Achives Des Sciences* **65** (12): 626-642
- Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analyst chemistry*. **31**: 436
- Mamta, A., Kauri, J. and Ramas, S. (2013), Bioethanol Production through water Hyacinth (*Eichornia Crassipes*) via optimization of the pretreatment conditions. *International Journal of Emerging Technology and Advance Engineering* **3** (3): 42-46
- Nimbkar, N., Ghanekar, A.R. and Joseph, R.D. (2009). Development of Improved Cultivars and Management Practices in Sweet Sorghum as a Source of Ethanol. National seminar held at marathwada Agriculture University, parbhana, February 2-3. 180-188
- Ohgren, K., Vehmaanpera, J., Siika-Alio, M. Galbe M. Viikari, L. and Zacchi, G. (2006) High temperature Enzymatic Pre-hydrolysis Prior to Simultaneous Saccharification and Fermentation of Steam Pretreated Corn Stover for Ethanol Production. *Enzyme and Microbial Technology*. **40**(4):607-613
- Oyeleke S.B and Jibrin N.M (2009), Production of Bio-ethanol from Guinea Corn Husk and Millet husk. *Africa Journal of Microbiology Research*, **3**(4):147-152.
- Oyeleke S.B and Manga S.B (2008), Essential laboratory Practical in Microbiology, 1st Edition. Tobest publishers, Minna Nigeria. Pp36-67.
- Pessoa, J.A., Mancilha, I.M, and Sato, S. (1997) Acid Hydrolysis of Hemicelluloses from Sugar Cane Baggase. *Brazil Journal of Chemical Engineering*. **14**: 590-663
- Rabah, A. B., Oyeleke, S. B., Manga, S. B. And Hassan, L. G. (2011) Utilization of Millet and Guinea Corn Husk for Bioethanol Production. *African Journal of Microbiology Research* **5**(31): 5721-5724.
- Rajendran, S. and Saravana, C.M. (2013) Bioethanol Production from Agave Leaves Using *Saccharomyces cerevisiae* and *Zymomonasmobilis*. *International Journal of Microbiological Research* **4** (1): 23-26,
- Raymond, C. (2003). General Physical Chemistry. 5th Edition MacGrawHill England. pp 245-247
- Spectra/IRintro, (2014). Spectra/IR. retrieved from <http://www.chem.ucla.edu> on 18/04/14.
- Uduak, G. A, Adamu, A. A and Udeme, J. J (2008) Production of Ethanol from Organic and Food Wastes. *Journal Practices and Technologies*. **13**:1-12.
- Wright, J.D. (2001) Ethanol from Lignocellulosics: An Overview, *Energy Progress* **8**: 71
- Wyman, C.E and Hinman, N .D. (1990). Ethanol: Fundamentals of ethanol production From renewable feedstocks and use as a transportation fuel. *Applied Biochemistry Biotechnology*, **24**(25): 735-754
- Zhao X, Zhang L & Liu D. (2012) Biomass recalcitrance. Part II: Fundamentals of different pre-treatments to increase the enzymatic digestibility of lignocellulose. *Biofuels, Bioprod. Bioref* DOI:10.1002/bbb.