International Journal of Sustainable and Green Energy
2015; 4(2): 40-46
Published online March 28, 2015 (http://www.sciencepublishinggroup.com/j/ijrse)
doi: 10.11648/j.ijrse.20150402.13

Bioethanol Production from *Eucalyptus camaldulensis* Wood Waste Using *Bacillus subtilis* and *Escherichia coli* Isolated from Soil in Afaka Forest Reserve, Kaduna State Nigeria

Usman Yahaya[1, *], Umar Yahaya Abdullahi[2], Denwe Samuel Dangmwan[2], Muhammad Muktar Namadi[3]

[1]Forestry Research Institute of Nigeria, Trial Afforestation Research Station, Kaduna, Nigeria
[2]Department of Biological Sciences, Nigerian Defence Academy, Kaduna, Nigeria
[3]Department of Chemistry, Nigerian Defence Academy, Kaduna, Nigeria

Email address:

usmanyahayaks@yahoo.com (U. Yahaya), umaryahaya09@gamil.com (Y. A. Umar), dangmwansamuel@gmail.com (S. D. Denwe), ammimuktar@yahoo.com (M. M. Namadi).

To cite this article:

Usman Yahaya, Umar Yahaya Abdullahi, Denwe Samuel Dangmwan, Muhammad Muktar Namadi. Bioethanol Production from *Eucalyptus camaldulensis* Wood Waste Using *Bacillus subtilis* and *Escherichia coli* Isolated from Soil in Afaka Forest Reserve, Kaduna State Nigeria. *International Journal of Sustainable and Green Energy.* Vol. 4, No. 2, 2015, pp. 40-46. doi: 10.11648/j.ijrse.20150402.13

Abstract: The economic and ecological problems associated with fossil fuel have raised interest in biofuel research in recent times in different parts of the world. The use of *Eucalyptus* forest waste biomass with no appreciable value to industries or for food as alternative and cost effective feedstock for bioethanol production was evaluated in this study. *E. camaldulensis* biomass (bark and leaves) were pretreated separately with acid (2M H_2SO_4) and Microwave irradiation (250V, 50Hz) prior to fermentation with *Bacillus subtilis* and *Escherichia coli* isolated from surrounding soil. Higher yield of reducing sugar were obtained from bark (43 %) and leaves (38.5 %) pretreated by microwave irradiation as compared with acid treated plant biomass. Similarly, Bioethanol volume and concentration of 34.89 g/l and 0.51 % respectively were higher in Microwave irradiated bark of *E. camaldulensis* at 21 days of fermentation when *E. coli* and *B. subtilis* were used in synergy The least bioethanol volume yield of 18.79 g/l and concentration of 0.12 % when bark and leaves of *E. camaldulensis* were combined was obtained on day 7 of fermentation using *E. coli.* The study concludes that the amount of dried wastes generated (37.8 kg) from one average stand of *Eucalyptus* tree could yield significant volume (131,884.2 g/l) of bioethanol when *B. subtilis* and *E. coli* are used in synergy.

Keywords: *Eucalyptus*, Biomass, Bioethanol, Fermentation

1. Introduction

The world is facing the crisis of global warming and environmental degradation which mainly has been associated with excessive use of fossil fuels. Alternative sources of energy are being explored the world over in order to reduce oil dependence and increase energy production [1]. Among the various sources been explored, biofuels offer one of the best alternative options as they have much lower life cycle Green House Gas (GHG) emissions compared to fossil fuels [2]. Biofuels which could be solid, liquid or gaseous fuel derived from biological materials can be used to generate energy [3]. Energy produced through these processes could help to reduce world's dependence on oil and therefore cut CO_2 emission, thus mitigating global warming. In addition, bi-products of biofuel production can provide new income and employment opportunities in rural areas [4].

Bioethanol which is one of the biofuel derived from plant biomass is an ethyl alcohol, grain alcohol, CH_3–CH_2–OH or ETOH. It is a liquid biofuel which is produced from several different biomass feedstocks and conversion technologies. Bioethanol has been reported as an attractive alternative fuel because of its renewable bio-based resource and its oxygenation which provides the potential to reduce particulate emissions in compression–ignition engines [5]. It is one of the promising future energy alternatives that could

contribute to the reduction of negative environmental impacts generated by the use of fossil fuels [6]. Bioethanol has been produced from a variety of raw materials containing fermentable sugars.

Eucalyptus species is one of the commercially important fast-growing trees in Nigeria. It provides raw material for papermaking and is widely used in the construction industries, although large amounts of wood residue, such as bark, leaves, cork residue, cross-cut ends, edgings, grinding dust and saw have not been efficiently utilized [7].

Considering the high cellulose content, fast growth of Eucalyptus trees and the fact that waste generated during wood processing has no human and animal food values, the plant could serve to provide the much needed feedstock for bioethanol production in Nigeria.

2. Methodology

2.1. Study Area

Afaka Forest Reserve occupies about 7,093.1366 hectares of land (Fig. 1). It lies on latitudes 10^0 33'N and 10^0 42'N; Longitudes 7^0 13'E and 7^0 24'E. The Forest Reserve provides a mixture of both natural and man-made vegetation characteristic of guinea savannah vegetation. Some of the indigenous and exotic plants in the forest reserve include *Pakia biglobolsa*, *Ceiba petandra*, *Azadirachta indica*, *Mangifera indica*, *Eucalyptus spp*, *Tectona grandis*, *Pinus caribae*, *Gmelina arborea* among others

The forest reserve is the main source of electricity pole for most part of the state in addition to providing wood for the construction industry.

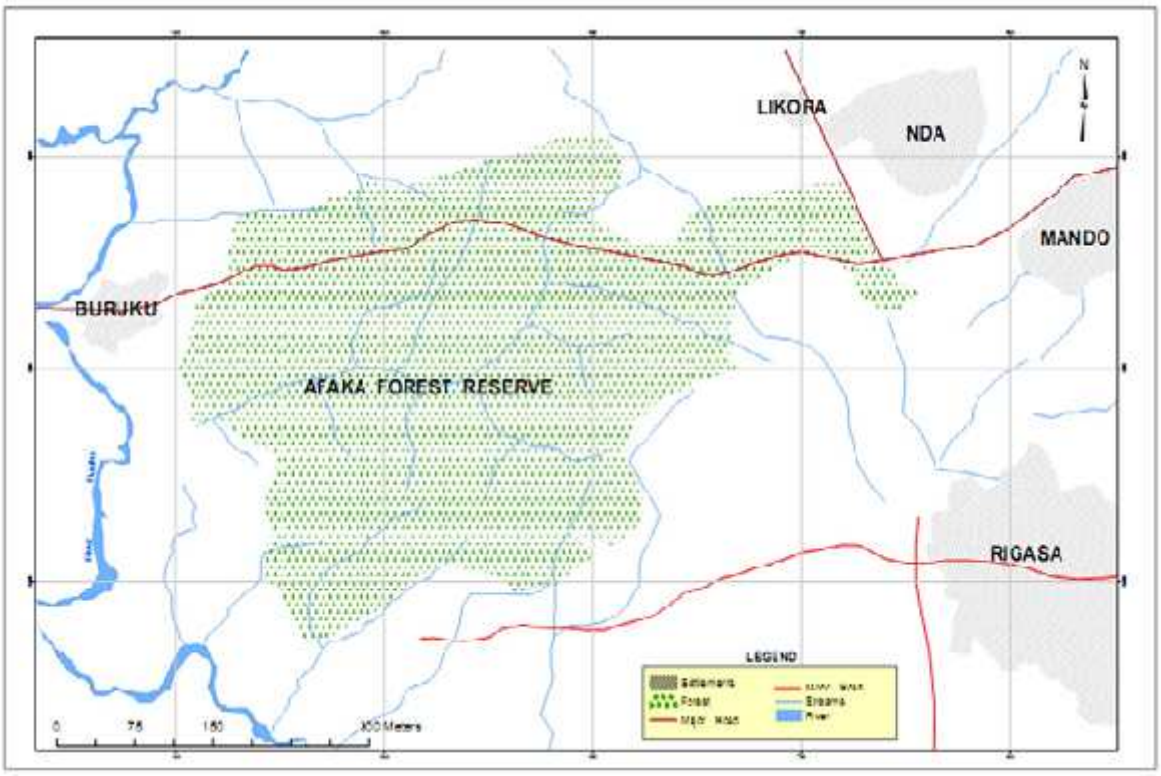

Figure 1. *Map of Afaka Forest Reserve and Immediate communities.*

Source: Department of Geographgy, NDA Kaduna.

2.2. Sample Collection and Processing

One kilogram (Kg) each of Bark and leaves of *E. camaldulensis* were collected separately in clean polyethene bags from Afaka Forest Reserve, Kaduna State and transported immediately to the Centre for Energy and Environment, Nigerian Defense Academy - Kaduna. Samples collected were washed several times to remove adhering dirt and later chopped into small pieces using a sharp knife. Chopped samples were oven dried in an oven at 150 ^{0}C for 6 hours, pulverized to powder using mortar and pestle, and stored in capped wide mouthed plastic containers until needed [8].

2.3. Isolation and Identification of Fermenting Bacteria from Soil

2.3.1. Collection and Preparation of Soil Sample for Serial Dilution

Collection and preparation of soil sample for serial dilution was carried out in accordance with the standard method described by [9]. Briefly, five (5) grams of upper soil layer were collected at 5 different locations within Afaka Forest Reserve using clean, dry plastic sample tubes with the aid of a sterile spatula. Soil samples collected were mixed together to form composite soil from which 1g was suspended in 10ml of sterile water in a ratio of 1:10 (10^{-1}). Further dilution of

10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were prepared from the stock (10^{-1}) preparation.

2.3.2. Preparation of Media

Preparation of nutrient agar media was carried out in accordance with the standard procedure described by [9]. Twenty eight (28) grams of nutrient agar was added to 1000ml of distilled water in a beaker, stirred vigorously and dissolved by heating on a hot plate. This was later sterilized by autoclaving for 15 minutes at 121 ^{0}C and allowed to cool before dispensing into petri-dishes. The preparations were allowed to solidify at room temperature.

2.3.3. Inoculation of Media

Media inoculation was done by streaking different dilutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) on solidified nutrient agar in petri-dishes and incubated at 37 ^{0}C for 24 hours (9). Each petri-dish was later observed for appearance of colonies.

2.3.4. Fermentation Test

Each bacteria isolated from soil was screened for fermentation ability by Carbohydrate Fermentation Test using Triple Sugar Iron agar (TSI) prepared as agar slope [10]. Test organisms were inoculated by stabbing and streaking the medium with the aid of a sterilized straight wire loop and then incubated at 37 ^{0}C for 24 hours. Gas production was determined by cracking of the medium while H_2S formation was determined by blackening at the slant butt junction. Determination of glucose fermentation was achieved by yellowing of the butt. Fermentation of lactose or sucrose or both was determined by yellowing of both the butt and the slant, while motility was determined by observing the line of inoculation. Sharp defined line of inoculation indicates positive motility.

2.4. Morphological and Biochemical Characterization of Bacteria Isolates from Soil Samples

Colonies of bacteria with fermenting ability were characterized and identified based on morphological and biochemical characteristics using standard techniques described by [11] and [10] respectively.

2.5. Pretreatment of E. camaldulensis Wood Waste

2.5.1. Microwave Irradiation

Ten grams each of dried bark and leaves of *E. camaldulensis* were taken in separate glass beakers and microwaved (model no-QMWO-25L) for 3 minutes at 250V, 50Hz [12]. To the content in each of the beakers, 100 ml of distilled water was added and autoclaved at 121 ^{0}C for 15 minutes. The mixtures were then filtered through No1 Whatman filter paper into a conical flask and the hydrolysate collected for further analysis.

2.5.2. Acid (2M H_2SO_4) Pretreatment

Ten grams each of dried bark and leaves of *E. camaldulensis* were soaked separately in 100 ml of 2M H_2SO_4 in a beaker. The mixtures were allowed to stand for 4 hours and later autoclaved at 121 ^{0}C for 15 min. The mixtures in each beaker were then filtered into a conical flask through a Whatman No.1 filter paper. Hydrolysates collected were subjected to further analysis.

2.6. Hydrolysate Detoxification

The hydrolysate from bark and leaves of *E. camaldulensis* collected from both microwave irradiated and acid treated biomass were separately heated to 60 ^{0}C and basified by adding at intervals 0.5g solid NaOH until a pH of 5.5 was achieved. To the solution, 1g of $Ca(OH)_2$ was added to detoxify harmful materials present in the hydrolysate and then filtered through a Whatman No.1 filter paper to remove insoluble residues. The filtrates containing fermentable sugars were then stored in capped plastic containers for determination of reducing sugar [13].

2.7. Determination of Reducing Sugar

The reducing sugar content of the hydrolysates was assayed by adding 3ml 0f 3, 5 - dinitrosalicylic acid (DNS) to 3 ml of each hydrolysate sample. The mixture was heated in hot water bath for 10 minutes until red-brown color was observed. To the mixture, 1 ml of 40 % potassium sodium tartrate solution was then added to stabilize the color and the mixture cooled to room temperature under running tap. Absorbance of each sample was measured at 491 nm using UV-VIS spectrophotometer. The reducing sugar content was subsequently determined by reference to a standard curve of known glucose concentration [14].

2.8. Fermentation of the Hydrolysate

Fifty (50) milliliters of bark, leaves and combination of bark and leaves of *E. camaldulensis* hydrolysates were separately dispensed into three 100 ml capacity conical flasks and each flask replicated three times. The flasks were then covered with cotton wool, wrapped in aluminium foil, and autoclaved at 120 ^{0}C for 15 minutes. The flasks were allowed to cool at room temperature and aseptically inoculated with the fermentative organisms (6.00×10^2cfu/ml) isolated from soil as follows:

a) *Bacillus subtilis*
b) *E.coli*
c) *Bacillus subtilis* + *E.coli*

All flasks were incubated anaerobically at 30 ^{0}C and each examine at seven days interval for 3 weeks. The fermented broth was distillated at 78 ^{0}C and the distillate collected for determination of bioethanol concentration in the fermented medium.

2.9. Determination of Concentration of Bioethanol

The concentration of bioethanol in distillates was carried out by the method described by [15] using UV-VIS quantitative analysis of alcohols. This involves taking 1 ml of standard ethanol and diluting with 100 ml of distilled water to produce 1% stock solution. To obtain 0%, 0.2%, 0.4%, 0.6% and 0.8% of the stock ethanol solution, 0 ml, 2ml, 4ml, 6ml and 8ml of the stock solution was diluted in 10ml of

distilled water. To each of the varying ethanol concentrations, 2 ml of chromium reagent was added and allowed to stand for an hour and the absorbance of each concentration measured at 588 nm using UV-VIS spectrophotometer. Readings obtained were used to develop standard ethanol curve. To determine the concentration of bioethanol produced, 4 ml of each bioethanol sample was transferred into a test tube and treated with 2 ml of the chromium reagent. The mixture was allowed to stand for an hour and the absorbance measured at 588 nm using the UV-VIS spectrophotometer.

2.10. Quantification of Ethanol

To determine the quantity of ethanol produced, distillate from hydrolysate of bark, leaves and a combination of bark and leaves fermented with *B. subtilis*, *E. coli* and combination of the two bacteria were collected over a slow heat at 78 ^{0}C. The quantity of ethanol produced in g/l was then obtained by multiplying the volume of distillate collected at 78°C by the density of ethanol (0.8033 g/ml) [16].

2.11. Statistical Analysis

Data obtained were statistically analyzed by one-way analysis of variance. Comparison of means were done by the New Duncan's multiple range test (P = 0.05).

3. Result

The amount of waste generated from one average fell stand of *Eucalyptus* plant in Afaka forest Reserve is presented in Fig. 2. Fresh Bark, leaves and unused branches of felled *Eucalyptus* plant produced 38.0 kg, 7.0 kg and 26.0 kg respectively while the dried bark, leaves and unused branches produced 32.0 kg, 5.8 kg and 20.8 kg respectively.

The reducing sugar yields from the hydrolysates of bark, leaves and, bark and leaves of *E. camaldulensis* pretreated with acid (2M H_2SO_4), microwave irradiation and untreated biomass are presented in figure 3. Microwave irradiation produce the highest (38.5% - 43%) yield of reducing sugar compared with untreated (07% - 08%) and acid (2M H_2SO_4) (35.5% - 38%) pretreated biomass. Although there was no significant difference in reducing sugar yields of acid (2M H_2SO_4) treated and microwave irradiated biomass ($P>0.05$), the difference in reducing sugar yield between treated and untreated biomass is statistically significant ($P<0.05$).

Table 1 shows the quantity in volume (g/l) of ethanol produced over a 3 weeks period from microwave treated *E. camaldulensis* wood waste biomass using bacteria isolated from soil sample. Although the performance of individual fermenting bacteria is lower than when both are used in synergy, the volume of ethanol produced by these organisms irrespective of plant biomass used increased steadily over the 3 wks period.

However, highest volume (31.22 ± 0.54g/l – 34.89 ± 0.07g/l) of ethanol production was achieved when microwave irradiated bark was fermented by *B. subtilis* + *E. coli* than leaves (25.95 ± 0.04g/l – 33.95 ± 0.02g/l) or bark and leaves (26.18 ± 0.50g/l – 29.05 ± 0.02g/l) using the same fermenting organisms.

Similarly, there was an increase in percentage concentration of bioethanol produced from day 7 to day 21 irrespective of plant part or fermenting organism used. However, microwave irradiated bark biomass when fermented by *B. subtilis* + *E. coli* had higher percentage concentration of bioethanol produced at day 7 (0.42 ± 0.02), day 14 (0.47 ± 0.03) and day 21 (0.51 ± 0.02) than either of the plant parts fermented by individual bacteria (Table 2).

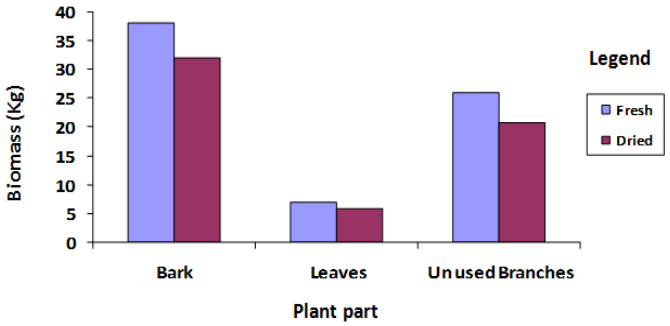

Figure 2. *Biomass by weight generated from an average fell stand of E. camalendulensis in Afaka Forest Reserve, Kaduna*

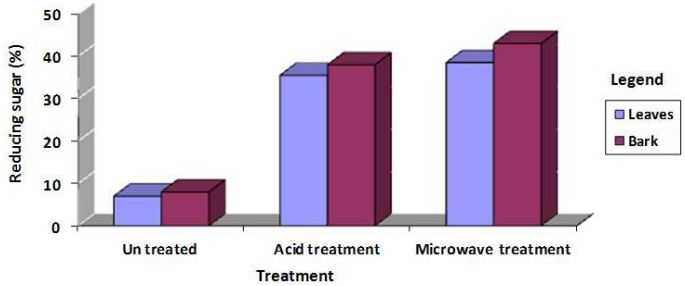

Figure 3. *Percentage yield of reducing sugar from E. camaldulensis waste using different treatment methods.*

Table 1. *The volume of bioethanol (g/l) produced from E. camaldulensis biomass using bacteria isolated from soil at Afaka Forest Reserve Kaduna, Nigeria.*

		Fermentation days		
Plant part	**Fermenting Organisms**	**7**	**14**	**21**
	B. subtilis	$19.28^{a} \pm 0.02$	$21.13^{a} \pm 0.01$	$25.18^{a} \pm 0.05$
Bark and leaves	*E. coli*	$18.79^{a} \pm 1.10$	$20.09^{b} \pm 0.04$	$22.88^{b} \pm 0.51$
	B.subtilis+ E.coli	$26.18^{b} \pm 0.50$	$27.70^{c} \pm 0.06$	$29.05^{c} \pm 0.02$
	B. subtilis	$21.09^{a} \pm 0.04$	$25.28^{a} \pm 0.07$	$27.82^{a} \pm 0.01$
Leaves	*E. coli*	$19.80^{b} \pm 0.06$	$25.72^{a} \pm 0.07$	$27.28^{a} \pm 0.09$
	B.subtilis+ E.coli	$25.95^{c} \pm 0.04$	$30.58^{b} \pm 0.01$	$33.93^{b} \pm 0.02$
	B. subtilis	$25.84^{b} \pm 0.06$	$27.50^{c} \pm 1.04$	$28.93^{b} \pm 0.05$
Bark	*E. coli*	$25.26^{b} \pm 0.02$	$26.25^{bc} \pm 0.08$	$27.55^{b} \pm 0.09$
	B.subtilis+ E.coli	$31.22^{e} \pm 0.54$	$31.77^{d} \pm 0.41$	$34.89^{c} \pm 0.07$

a,b,c, means within a column in each plant part with different superscripts are significantly different ($P<0.05$). Values are means ± standard deviation of three replicates

Table 2. *Percentage concentration of bioethanol produced from E. camaldulensis wood waste biomass at Afaka Forest Reserve Kaduna, Nigeria.*

		Fermentation days		
Plant part	**Fermenting Organisms**	**7**	**14**	**21**
	B. subtilis	$0.13^{a} \pm 0.01$	$0.16^{a} \pm 0.01$	$0.25^{a} \pm 0.02$
Bark and leaves	*E. coli*	$0.12^{a} \pm 0.03$	$0.15^{a} \pm 0.02$	$0.22^{b} \pm 0.02$
	B.subtilis+ E.coli	$0.23^{b} \pm 0.02$	$0.30^{b} \pm 0.02$	$0.39^{c} \pm 0.01$
	B. subtilis	$0.16^{a} \pm 0.01$	$0.25^{a} \pm 0.02$	$0.33^{a} \pm 0.02$
Leaves	*E. coli*	$0.14^{a} \pm 0.02$	$0.23^{a} \pm 0.02$	$0.31^{a} \pm 0.02$
	B.subtilis+ E.coli	$0.27^{b} \pm 0.01$	$0.42^{b} \pm 0.03$	$0.47^{b} \pm 0.01$
	B. subtilis	$0.25^{a} \pm 0.03$	$0.34^{a} \pm 0.02$	$0.38^{a} \pm 0.01$
Bark	*E. coli*	$0.22^{a} \pm 0.03$	$0.32^{a} \pm 0.02$	$0.36^{a} \pm 0.03$
	B.subtilis+ E.coli	$0.42^{b} \pm 0.02$	$0.47^{b} \pm 0.03$	$0.51^{b} \pm 0.02$

a,b,c, means within a column in each plant part with different superscripts are significantly different ($P<0.05$). Values are means ± standard deviation of three replicates

4. Discussion

The basic structural framework of plants consists of cellulose, hemicellulose and lignin. The close and complex association between these three lignocellulosic materials causes physical and chemical barriers that have to be broken to release fermentable sugars for bioethanol production. Various pretreatment techniques have been developed for various biomass feedstocks [7]. The differences observed in the yield of reducing sugar in microwave and acid pretreatment in this study is indicative of the differences in efficiency of these techniques to release fermentable sugars from *E. camaldulensis* biomass. Yields of reducing sugar from lignocellulose biomass processed by different pretreatment methods including microwave irradiation, concentrated and dilute acids are well documented [7, 17, 18].

Microwave irradiation is known to enhance the digestibility of cellulosic biomass, increases surface area, decreases the degree of polymerization and crystallinity of cellulose, enhances hydrolysis of hemicelluloses and results in partial depolarization of lignin [7]. This is in contrast to acids which though are powerful agents for cellulosic hydrolysis results in the formation of degradation products and releases natural inhibitors which affect the yields of fermentable sugars [19].

The high volume (34.89 ± 0.07g/l) of bioethanol obtained from bark of *E. camaldulensis* in the present study using B. subtilis + E. coli in synergy at days 21 is higher than the volume of ethanol obtained for other plant biomass such as guinea corn husk (26.31g/l), sawdust (12.30g/l)[15]; Sweet potato peels (16.47g/l), rice husk (06.22g/l)[20]; and empty fruit branches of palm oil tree (10.32g/l)[21]. These differences in volume of bioethanol obtained from the different plant biomass could be associated with the major composition of the various feedstocks in addition to the fermenting organisms involved in the production process. *B. subtilis* and *E. coli* are excellent organisms that are resistant to salt/ toxic inhibitors in addition to being good anaerobic fermenters. According to [22], *B. subtilis* and *E. coli* are organisms that grow very fast and can utilize pentose (C_5) and hexoses (C_6) including glucose, xylose, mannose, cellobiose among other simple sugars. They are also reported to possess native hemicellulases. Although both *B. subtilis* and *E. coli* have excellent fermentation ability, *B. subtilis* is reported to produces larger number of polysaccharide degrading enzymes such as α amylase, pullulanase, endo β-1-4 mannase, levenase, pectate lyases, β-1-4-endogluconase, β-1,3-1,4-endogluconase, and endo-1,4- β- xylanases. The ability of *B. subtilis* to efficiently break down polysaccharides into soluble carbohydrates is reflected in the relatively high volume of bioethanol produce when these organisms are used individually in the present study.

5. Conclusion

This study revealed that *B. subtilis* and *E. coli* isolated from soil have a great potential in the fermentation of *E. camaldulensis* biomass into bioethanol. The amount (34.89 g/l) of bioethanol produced from 10g of *E. camaldulensis* biomass after 21 days of fermentation using *B. subtilis* and *E.coli* in synergy translates into about 131,884.2g/l of bioethanol derivable from waste generated from an average stand (37.80 kg) of *Eucalyptus spp*. Further studies would however be needed to enhance the performance of these organisms through genetic manipulation to achieve higher yields thus reducing over dependence on fossil fuel.

Acknowledgement

The Authors acknowledge the support received from staff and management of Forestry Research Institute of Nigeria and the Nigerian Defense Academy Kaduna. We are also grateful to the Department of Geography for providing the sketch map of the study area and to the Centre for Energy and Environment, NDA for allowing the use of their facilities.

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