EVALUATION OF BIOETHANOL PRODUCTION POTENTIAL FROM *EUCALYPTUS TERETICORNIS* BIO WASTE USING *Bacillus subtilis, Escherichia coli* and *Sacharomyces cerevisae.*

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Abstract

The use of bark and leaves of Eucalyptus tereticornis (forestry waste with no appreciable value to industries or competitive use as food) as alternative and cost effective feedstock for bioethanol production was examined. Eucalyptus tereticornis biomass (bark and leaves) were pretreated with microwave irradiation (250V, 50Hz) prior to fermentation with Bacillus subtilis and E.coli isolated from soil, and Saccharomyces cerevisae purchased from market. The highest reducing sugar yield of 43.5 % and 40 % were obtained from bark and leaves of Eucalyptus tereticornis pretreated with microwave irradiation as compared to untreated biomass. Bioethanol production concentration of 0.52% was higher in microwave irradiated bark of E .tereticornis during 21 days of fermentation while the least concentration of 0.13% was obtained from combination of bark and leaves of E .tereticornis at 21 days when B. subtilis and E. coli were used in synergy. The study concludes that synergy between B. subtilis and E. coli may be a better combination for bioethanol production from E. tereticornis Bio waste.

Key Words: *Microwave, reducing sugar, bioethanol, Eucalyptus tereticornis, fermentation*

1.0 INTRODUCTION

Energy, which is the ability to work plays significant roles in our life. Most energy demand is met by fossil fuels (oil, coal and natural gas). Petroleum products are the main transportation fuel and coal is mostly used for producing electricity whereas natural gas is increasingly used for heating (Environmental Literacy Council, 2008). As a result of improved standard of living, proliferation of world's population and rapid industrialization in most countries, the use of fossil fuels has increased significantly in recent years (Environmental Literacy Council, 2008). This has led to the belief that the current use of fossil fuels will not only deplete the world´s oil reserves but also have serious impact on the environment, leading to increased health risks and global climate change (Panwer *et al.,* 2010). These, among other factors have made the world to start moving from petroleum-based to a bio-based global economy (Gomez *et al.,* 2008). For example, biological wastes (biomass) such as bark and leaves of *Eucalyptus tereticornis* which are usually seen as low-valued materials are now being transformed to usable forms for the production of eco-friendly and sustainable fuels (Gomez *et al.,* 2008) . This is because most biological wastes contain high level of cellulose, hemicelluloses, lignin, starch, proteins, and lipids, which provide good options for the biotechnological production of liquid biofuels without interfering with the ever-increasing

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need for world's food supply (Leandro and Andriana 2009).

However, the major challenge in the utilization of these abundant raw materials is freeing the reducing sugar content of the biomass due to their complex structures. As the result of this, pretreatment is necessary for effective utilization of these lignocellulose materials to obtain high degree of fermentable sugars. Therefore, the objective of this research is to assess the possibility of using microwave hydrolysis as a pretreatment method in bioethanol production from the complex organic waste.

2.0 MATERIAL AND METHODS

2.1 Collection and Processing of Samples

One kilogram (1kg) each of bark and leaves of *E. tereticornis* were collected separately in clean polyethene bags from Afaka Forest Reserve, Kaduna State and transported immediately to Centre for Energy and Environment Nigerian Defence Academy, Kaduna. Samples collected were washed several times with distilled water to remove adhering dirt and later chopped into small pieces using a sharp knife. Chopped samples were oven dried at 150 °C for 6 hours, pulverized to powder using mortar and pestle, and stored in capped plastic containers (Galbe and Zacchi, 2007).

2.2 Isolation of Bacteria from Soil

2.2.1 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 method described by Musliu and Salawudeen, (2012). The soil samples were collected at 5 different locations within Afaka Forest Reserve, Kaduna. The soil samples were collected from upper layer where most microbial activities take place and thus where most of the bacteria population is suspected to be concentrated. Soil samples (5g), at each location, was collected using a clean and dry plastic sample tubes using a sterile spatula. The soil samples collected were mixed together, and 1 g of the mixed sample was dissolved in 10 ml of sterile water to make suspension of 1: 10 (10^{-1}). Serial dilution of the 10^{-1} suspension was used to prepare dilutions of 10^{-2} , 10^{-3}, 10^{-4} and 10^{-5}.

2.2.2 Preparation of media

Preparation of media was carried out in accordance with procedure as described by Musliu and Salawudeen, (2012). The nutrient agar (28 g) was weighed and transferred to a beaker containing distilled water (1000 ml). The mixture was stirred vigorously and dissolved using hot plate after which it was autoclave for 15 minutes at 121 °C. This was allowed to cool after which it was dispensed into petridishes and allowed to solidify.

2.2.3 Sample Inoculation

Sample inoculation was carried out in accordance with the method described by Musliu and Salawudeen, (2012). Plates containing nutrient agar were inoculated by streaking with 10^{-5} dilution and incubated at room temperature for 24 hours, after which the plates were examined for bacteria colonies.

2.2.4 Fermentation Test

To determine the fermentation ability of bacteria isolates, each of the bacteria isolated from soil were screened by Carbohydrate Fermentation test using Triple Sugar Iron (TSI)

as described by Manga and Oyeleke, (2008). The culture medium was prepared as agar slope and the test organisms inoculated by stabbing the medium with the aid of a sterilized straight wire loop. Inoculation was carried out by streaking the surface of the slope and the medium incubated at 37ºC for 24 hours. Gas production was determined by cracking of the medium, formation of H_2S was determined by the blacking at the slant butt junction while glucose fermentation was determined by yellowing of the butt. The fermentation of lactose or sucrose or both was determined by the yellowing of both the butt and the slant, motility was determined by observing the line of inoculation where sharp defined line of inoculation indicates positive motility.

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 techniques describe by Chitra *et al.,* (2014); Musliu and Salawudeen, (2012) respectively.

2.3 Pretreatment of *Eucalyptus tereticornis* wood waste

2.3.1 Microwave irradiation

The bark (10 g) and leaves (10 g) of *E. tereticornis* were weighed separately into glass beakers and microwaved (model no-QMWO-25L) for 3 minutes at 250 V, 50 Hz (Nivedita *et al.,* 2013). Distilled water (100 ml) was added to each sample and autoclaved at 121 ºC for 15 minutes. The samples were filtered with Whatman filter paper (No. 1) in a conical flask and the hydrolysate collected.

2.4 Hydrolysate detoxification

The hydrolysate from each of bark and leaves samples were separately heated to 60 ºC and basified by adding gradually solid NaOH (0.5 g) 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 whatman No.1 filter paper to remove insoluble residues. The filtrates which contained the fermentable sugars were stored in capped plastic containers for determination of reducing sugar (Martinez *et al.,* 2000).

2.5 Determination of reducing sugar

The reducing sugar content of the hydrolysates was assayed by adding 3 ml of 3,5-dinitrosalicylic acid (DNS) to 3 ml of each sample. The mixture was heated in hot water bath for 10 minutes until red-brown color was observed and potassium sodium tartarate (40 %, 1 ml) solution was 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 making reference to a standard curve of known glucose concentration (Miller, 1959).

2.6 Fermentation of the Hydrolysate

Fermentation of the hydrolysed samples was carried out in accordance with the methods described by Brooks(2008). Fifty milliliters (50 ml) of bark, leaves and combination of bark and leaves of *Eucalyptus tereticornis* hydrolysates were separately dispensed into four 100 ml capacity conical flasks. Each conical flask was replicated three times. The flasks were then covered with cotton wool, wrapped in aluminium foil, and autoclaved at 120ºC for 15

minutes. The tubes were allowed to cool at room temperature and aseptically inoculated with the fermentative organisms (6.00×10^2cfu/ml for each organism) as follows:

A: Saccharomyces cerevisiae (GYMA CARPENTRAS TEWEX 431184, France)

B: Bacillus subtilis

C: E. coli

D: Bacillus subtilis + E. coli

E: Saccharomyces cerevisiae + E. coli

F: Saccharomyces cerevisiae + Bacillus subtilis

All the flasks were incubated anaerobically at 25 ^{o}C. One flask was removed every seven days for a period of twenty one days. The fermented broth was distillated at 78^{o}C and the distillate was collected for the determination of bioethanol concentration in the fermented medium

2.7 Determination of the Concentration of Bioethanol in the Distillate

Bioethanol concentration determination was carried out using UV-VIS quantitative analysis using chromium VI reagent according to the method described by Oyeleke and Jibrin, (2009). Standard ethanol (1 ml) was diluted with distilled water (99 ml) to give a concentration of 1 %. Then each of 0, 2, 4, 6 and 8 ml of the 1 % ethanol was diluted to 10 ml with distilled water to produce 0, 0.2, 0.4, 0.6 and 0.8 % of the ethanol. To each of the varying ethanol concentrations, 2 ml of chromium reagent was added and allowed to stand for an hour for color development. The absorbance of each concentration was measured at 588 nm using UV-VIS spectrophotometer (Model no: 6405, Denway) and the readings were used to develop a standard ethanol curve. Then 4 ml of each bioethanol samples were put in test tubes 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.

3.0 RESULTS

3.1 Reducing sugar yield

The reducing sugar yields from the hydrolysates of bark and leaves of *E. tereticornis* pretreated with microwave irradiation and untreated biomass are presented in Figure 1. Microwave irradiation produced the highest (40 – 43.5 %) yield of reducing sugar when compared with untreated (07% - 08%). The difference in reducing sugar yield between microwave treated and untreated biomass is statistically significant ($p<0.05$).

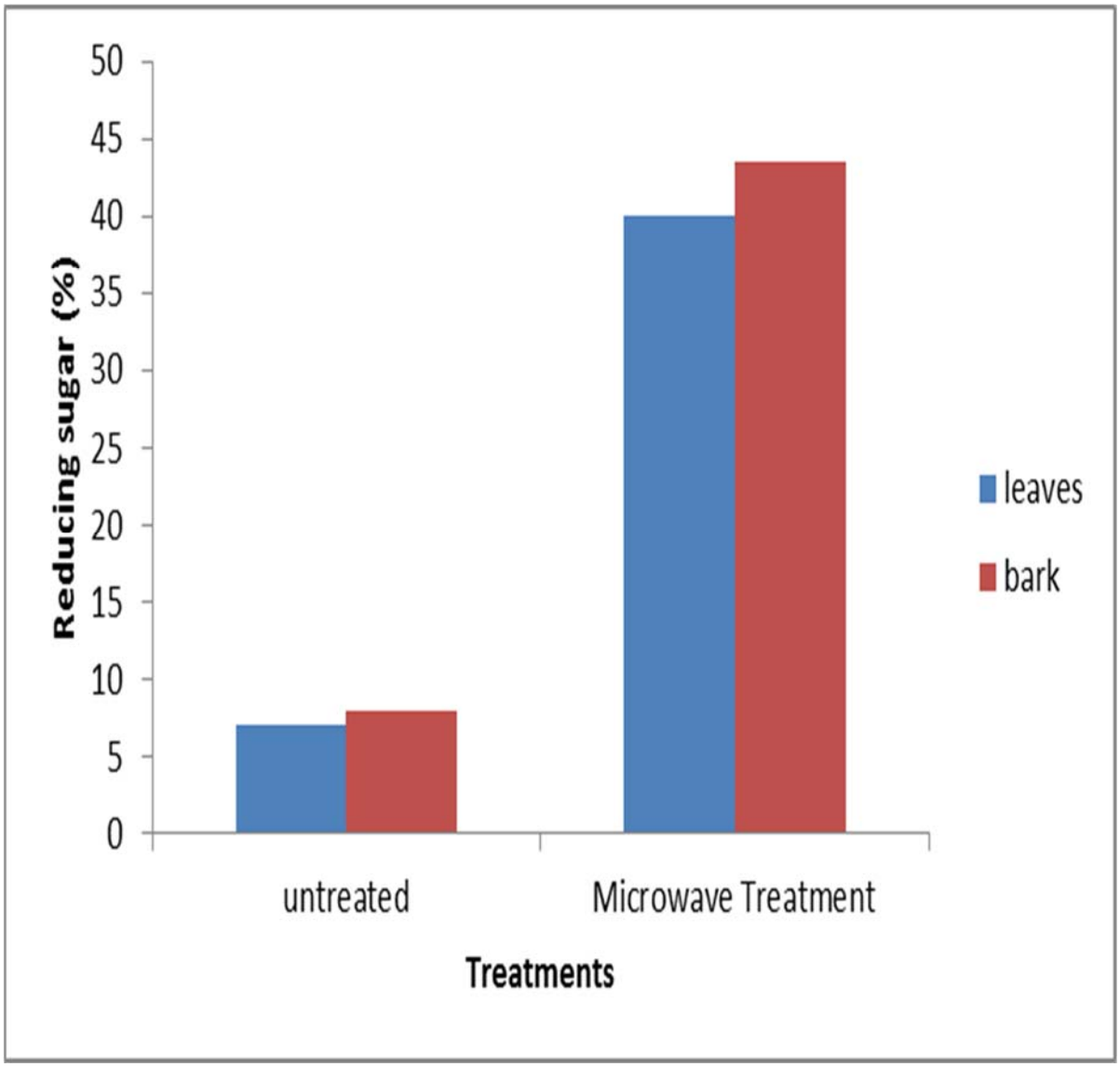

Figure 1: Percentage yield of reducing sugar from *E. tereticornis* treated and untreated wastes using microwave irradiation.

3.2 Concentration (%) of bioethanol from combination of bark and leaves of *E. tereticornis* using either *S. cerevisiae, B. subtilis, E. coli*, or combinations therefrom.

Table 1 shows the concentration of bioethanol produced over a three weeks period from microwave treated bark and leaves of *E. tereticornis* using treated with *B. subtilis, E. coli* and *S. cerevisiae.* Although the performance of individual fermenting organisms is lower than when both are used in synergy, the concentration and volume of bioethanol produced by these organisms irrespective of plant biomass used increased steadily over the three weeks period. However, *S. cerevisiae* and bacteria isolated from soil in synergy performance better at 7 days of fermentation after which there was a gradual decrease in concentration and volume over the three weeks.

Highest concentration (0.41 %) of bioethanol production was achieved when microwave

irradiated bark and leaves of *E. tereticornis* were fermented by *S. cerevisiae* and *B. subtilis* in synergy at 7 days than other fermenting organisms. *S. cerevisiae* and *E. coli* produced the lowest concentration of 0.13% at 7 days of fermentation.

Table 1: Concentration of bioethanol (%) from bark and leaves of *E. tereticornis* using single and combined fermenting organisms.

FERMENTING ORGANISMS	FERMENTATION DAYS		
	7	14	21
S. cerevisiae	$0.22^{b} \pm 0.00$	$0.35^{d} \pm 0.02$	$0.40^{c} \pm 0.01$
B. subtilis	$0.13^{a} \pm 0.02$	$0.17^{a} \pm 0.00$	$0.25^{b} \pm 0.01$
E. coli	$0.13^{a} \pm 0.01$	$0.16^{a} \pm 0.01$	$0.23^{b} \pm 0.03$
B.subtilis and E.coli (in synergy)	$0.24^{b} \pm 0.00$	$0.31^{c} \pm 0.01$	$0.39^{c} \pm 0.02$
S.cerevisiae and B. subtilis (in synergy)	$0.41^{c} \pm 0.03$	$0.23^{b} \pm 0.03$	$0.22^{ab} \pm 0.02$
S. cerevisiae and E. coli (in synergy)	$0.38^{c} \pm 0.02$	$0.22^{b} \pm 0.01$	$0.19^{a} \pm 0.02$

Note: a,b,c,d means within a column with different superscripts are significantly different ($P<0.05$). Values are means ± standard deviation of three replicates.

3.3 Concentration (%) of bioethanol from leaves of *E. tereticornis* using either *S. cerevisiae, B. subtilis, E. coli,* or combinations therefrom.

Table 2 shows the concentration of bioethanol produced over a three weeks period from microwave treated leaves of *E. tereticornis* using treated with *B. subtilis, E. coli* and *S. cerevisiae*. Highest concentration (0.48 %) of bioethanol production was achieved when microwave irradiated leaves of *E. tereticornis* were fermented by *E. coli* and *B. subtilis* in synergy at 21 days than other fermenting organisms. *E. coli* produced the lowest concentration of 0.15% at 7 days of fermentation.

Table 2: Concentration (%) of bioethanol from leaves of *E. tereticornis* using single and combined fermenting organisms.

FERMENTING ORGANISMS	FERMENTATION DAYS		
	7	14	21
S. cerevisiae	$0.23^{b} \pm 0.02$	$0.39^{c} \pm 0.00$	$0.45^{c} \pm 0.02$
B. subtilis	$0.16^{a} \pm 0.01$	$0.26^{a} \pm 0.02$	$0.34^{b} \pm 0.02$
E. coli	$0.15^{a} \pm 0.01$	$0.24^{a} \pm 0.02$	$0.32^{b} \pm 0.03$
B.subtilis and E.coli (in synergy)	$0.27^{b} \pm 0.04$	$0.41^{c} \pm 0.02$	$0.48^{e} \pm 0.01$
S.cerevisiae and B. subtilis (in synergy)	$0.42^{d} \pm 0.02$	$0.39^{c} \pm 0.02$	$0.38^{c} \pm 0.01$
S. cerevisiae and E. coli (in synergy)	$0.37^{c} \pm 0.03$	$0.31^{b} \pm 0.01$	$0.19^{a} \pm 0.02$

Note: a,b,c,d,e means within a column with different superscripts are significantly different ($P<0.05$). Values are means ± standard deviation of three replicates.

3.4 Concentration (%) of bioethanol from bark of *E. tereticornis* using either *S. cerevisiae, B. subtilis, E. coli,* or combinations therefrom.

Table 3 shows the concentration of bioethanol produced over a three weeks period from microwave treated bark of *E. tereticornis* using treated with *B. subtilis*, *E. coli* and *S. cerevisiae*. Highest concentration (0.52%) of bioethanol production was achieved when microwave irradiated bark of *E. tereticornis* was fermented by *E. coli* and *B. subtilis* in synergy at 21 days than other fermenting organisms. *S. cerevisiae* produced the lowest concentration of 0.14% at 7 days of fermentation.

Table 3: Concentration of bioethanol (%) from bark of *E. tereticornis* using single and combined fermenting organisms.

FERMENTING ORGANISMS	FERMENTATION DAYS		
	7	14	21
S. cerevisiae	$0.14^{a} \pm 0.02$	$0.18^{a} \pm 0.01$	$0.20^{a} \pm 0.01$
B. subtilis	$0.25^{b} \pm 0.01$	$0.35^{c} \pm 0.02$	$0.39^{b} \pm 0.01$
E. coli	$0.23^{b} \pm 0.01$	$0.33^{c} \pm 0.02$	$0.37^{b} \pm 0.02$
B.subtilis and E.coli (in synergy)	$0.42^{e} \pm 0.03$	$0.48^{d} \pm 0.03$	$0.52^{c} \pm 0.02$
S.cerevisiae and B. subtilis (in synergy)	$0.38^{d} \pm 0.02$	$0.32^{bc} \pm 0.02$	$0.20^{a} \pm 0.03$
S. cerevisiae and E. coli (in synergy)	$0.31^{c} \pm 0.03$	$0.29^{b} \pm 0.01$	$0.20^{a} \pm 0.02$

Note: a,b,c,d,e means within a raw with different superscripts are significantly different ($P<0.05$). Values are means ± standard deviation of three replicates

4.0 DISCUSSION

Bioethanol is ecofriendly and proposed to overcome the problem of energy crisis. It can be produced from lignocellulose biomass rich in sugars with the action of different fermenting organisms. Lignocellulosic ethanol can be produced from various feedstocks such as lignocellulose biomass, starchy materials, sucrose containing feedstocks and microalgae (Mathiyazhagan *et al.,* 2011, Templer and Murphy, 2012). Microwave irradiation is one of the methods employed in pretreatment of lignocellulose biomass in which the cellulosic and hemicellulosic substances in the biomass had been broken down into simple sugars which the fermenting organisms can utilize than untreated biomass (Xiang *et al.,* 2013).

The amount of reducing sugar from microwave irradiation pretreatment of *E. tereticornis,* 40 to 43.5 %, is higher when compared to the findings of Lima *et al.* (2013) who reported that the bark of *Eucalyptus grandis* produces 39 %, and hybrid between *Eucalyptus grandis* and Europhylla produces 40% when treated with acid (1% HCl). The findings of Arumugan and Manikandan (2011) revealed that Banana fruit peels yielded 36.67% of reducing sugar while mango fruit peels yielded 31.29% when treated with dilute H_2SO_4.

The result obtained from the concentration of bioethanol shows progressive increase in the amount of bioethanol produced with prolongation of fermentation time from 7 to 21 days when the organisms are used separately in bark, leaves, and combination of bark and leaves of *E. tereticornis*. Also, the combination of *B. subtilis* and *E. coli* shows progressive

increase in the amount of bioethanol produced with time from 7 to 21 days. However, the combination of *S. cerevisiae* and *B. subtilis;* and *S.cerevisiae* and *E. coli* produced the highest yield at 7 days after which there was a gradual decrease in the amount of bioethanol produced with time to 14 and 21 days. The decrease could be due to the consumption of accumulated ethanol by the organism. It had been observed that when ethanol accumulates in a medium, the microbial population present adapts to simultaneously consume sugar and ethanol which enables the bacteria starts another phase of growth.

The concentration (%) of bioethanol produced from bark (0.42 %), leaves (0.27 %), combined bark and leaves (0.23 %) of *E. tereticornis* at 7 days in this study are higher than that of millethusk (0.11 %) reported by Rabah *et al.* (2011) but similar to the findings of Usman *et al.*,2015 for *E. camaldulensis.*

5.0 CONCLUSION

Bioethanol from lignocellulose biomass has a great potential as an environmentally sustainable alternative to fossil fuels. The opportunity to reduce dependence on fossil fuels, while reducing CO_2 is of strategic important today. Microwave irradiation could serve as a pretreatment option in freeing reducing sugar as it was revealed in the study that *E. tereticornis* biomass shows reasonable percentage of reducing sugar which is the key indicator for conversion to bioethanol. Similarly synergy between fermenting organismsmay serve as a good mixed culture for the production of bioethanol.

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