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Article

Effect of Time on the Enzymatic Saccharification and Fermentation of *Alstonia boonei* for Ethanol Production

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Abstract: Pre-treatment is an essential step in the enzymatic hydrolysis of biomass and subsequent production of bio-ethyl alcohol. The study investigated bio-ethyl alcohol production by means of two pretreatment processes of *Alstonia boonei* wood sawdust namely, alkali treatment and acid hydrolysis, before enzymatic hydrolysis and subsequently fermentation using *Saccharomyces cerevisiae and Zymomonas mobilis* isolated from freshly tapped palm wine. In one *Alstonia boonei* wood sawdust was treated with 1.5%(w/v) aqueous NaOH at two different temperatures of 100^{o}C for 2h and 28^{o}C for 18 h followed by acid hydrolysis 0.75%(v/v) H_2SO_4 at 100^{o}C for 2 h. In the second method of the pretreatment, the wood sawdust was first subjected to acid hydrolysis and thereafter alkali treatments at the same conditions. After the pretreatment, the residues obtained were subjected to enzymatic hydrolysis using accellerase 1500(commercial cellulase). *Saccharomyces cerevisiae* was identified using their morphological characteristics and by determining their pattern of fermentation and assimilation of glucose, sucrose, maltose and lactose. On the other hand, *Zymomonas mobilis* was identified using API ^{TM} test kit. Analysis of reducing sugar in the residues was performed using Dinitrosalicylic acid (DNS) method. The results revealed that

maximum yield of glucose (48.2g/L) after enzymatic hydrolysis was obtained with pretreatment of 0.75% H_2SO_4 at 100^0C for 2h followed by alkali pretreatment (1.5% NaOH) at 100^0C for 2h which produced 20.4g/L reducing sugars. The optimum condition for *S. cerevisiae* were pH 4.5, temperature of 35ºC and fermentation time of 72 h. Analysis of ethyl alcohol content was carried out using gas chromatography. The maximum ethyl alcohol yield of 3.24 g/L and efficiency of 62% was obtained for *S. cerevisiae* while yield of 3.82g/L and efficiency of 74.2% was obtained for *Z. mobilis*.

Keywords: *Alstonia boonei*, palm wine, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, fermentation, saw dust.

1. Introduction

Bio-energy is fast becoming one of the most dynamic and rapidly changing sectors of the global economy. The associated accelerated growth in the production, supply, conversion and use of bio-energy especially in the liquid bio-ethyl alcohol presents a new reality that is attracting interest from key stakeholders in developed and developing countries.

Energy, particularly one from fossil fuels has played very important roles in our lives. Consider the case of a baby Michael born in August 2003, a gasoline powered automobile rushed the mother to the maternity ward, a coal tar power plant light the hospital in which he was born and a central heating system burning natural gas warms the room in which he took his first breath. Had any of these sources of energy failed, little Michael's life would have been lost. This simple illustration underscores the essential role of energy in our lives.

Fossil fuel is the traditional source of fuel in Nigeria but it is being continuously depleted. The current known oil reserve as well as the reserves yet to be discovered were estimated and concluded that the world crude oil production will begin to decline in 2015.[1]

There have also been concerns about the pollution and various health risks associated with the use of petroleum as fuel. In view of these, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of the limited fossil fuel stock but also for the safe and better environment.[2]

The interest in biomass as the alternative source of energy is gaining momentum more and more over the last century and ethyl alcohol produced from renewable energy source is the most promising future bio-fuel.[3]

To be a viable substitute for fossil fuel, alternative fuel should not only have superior environmental benefits over the fossil fuel it displaces, be economically competitive with it, or be producible in sufficient quantities to make a meaningful impact on energy demands, but it should also provide a net energy gain over the energy sources used to produce it.[4] Bio-ethyl alcohol meets most of these criteria but the quantity of ethyl alcohol produced annually has not overtaken petroleum.

Crops such as maize have traditionally been utilized as the substrate for the production of ethyl alcohol. It has however been reported that the cost of raw material accounted for 70% of the total cost of producing the ethyl alcohol.[5] Research into alternative substrates such as agricultural waste, municipal waste, molasses etc is on-going to find low cost as well as efficient substrate for ethyl alcohol production.[5]

The problem however is the technology for conversion of the lignocellulosic part of these materials to bio-ethyl alcohol. The choice of the best technology for the conversion of lignocelluloses to bio-ethyl alcohol should be decided on the basis of overall economics(lowest cost), environmental safety(pollutants), and energy(higher efficiencies).

Many investigations have been performed on the appropriate technology for the conversion of lignocellulosic materials to ethyl alcohol as well as substrate with little or no lignin such as molasses.[2]

Lignocellulosic material consists of mainly three different types of polymers, namely cellulose, hemicelluloses and lignin, which are associated with each other.[2]

Alstonia boonei wood sawdust is the most attractive low cost feedstock for production of fuel alcohol. This is because of its abundance, low lignin content and renewability, but it has to be pretreated.

Pretreatment disrupts the naturally resistant carbohydrate lignin shield that limits the accessibility of enzymes or chemicals to the cellulose and hemicelluloses. The main goal of pretreatment is to increase enzyme accessibility and improve the digestibility of cellulose.[6] Each pretreatment has a specific effect on the cellulose, hemicelluloses and lignin fraction and thus, different pretreatment methods and conditions should be chosen according to the hydrolysis.[6]

Acid hydrolysis removes the hemicellulosic portion and some fraction of lignin. The remainder of the lignin remains intact to the cellulosic substrate.[7] During enzymatic hydrolysis of lignocellulosic biomass cellulose components glucosidase and endoglucanase have more binding affinity towards lignin than to the carbohydrates, resulting in a lower efficiency of saccharification. Hence, to achieve maximum hydrolysis of cellulosics, which is a prerequisite for ethyl alcohol fermentation, an appropriate delignification treatment of biomass is required.[8]

Alkali treatment disrupts the cell wall through dissolving hemicelluloses, lignin, silica and hydrolyzing uronic and acetic acid esters. Alkali swells cellulose, decreasing ester linked substituent's of the hemicelluloses and other cell wall components.[9]

Several species of bacteria and fungi are able to produce cellulase and hemicellulases which are used for enzymatic hydrolysis of cellulose to glucose. Fungi like Trichoderma reesei or trichoderma viride have been the most broadly studied and best characterized and the best vehicles for cellulose production.[10]

A full complement production of cellulase stability under the enzymatic hydrolysis conditions and resistance of the enzyme to chemical inhibitors are the advantages of the cellulase produced by Trichodemia.[11,12]

Fuel ethyl alcohol produced from plant biomass hydrolyzates by Saccharomyces cerevisiae and Zymomonas mobilis(a Gram negative bacterium) grows and ferments glucose very fast, its preference for low pH prevents contamination and grows in high glucose and ethyl alcohol concentration are of great economic and environmental significance.[13]

2. Materials and Methods

2.1. Sample Collection

Alstonia boonei wood was collected at Ikot Ekpene timber market Akwa Ibom State. The wood sample was identified at the Department of Botany University of Nigeria, Nsukka. The wood was air-dried and cut into 1-3 cm bits in a laboratory pulverizer followed by sieving to make the wood sawdust free and then oven-dried at 60 ± 0.5ºC for 18h.

Fresh palm wine samples were obtained from 20 palm wine tappers in four localities(Etim Ekpo, Essien Udim, Abak and Ikot Ekpene) in Akwa Ibom State. The freshly tapped palm wine samples were collected in pre-sterilized 500 ml capacity bottles. The wine samples were transported to the laboratory in a cooler equipped with ice-blocks to slow down fermentation. Analysis was carried out in triplicate.

2.2. Media Preparation

2.2.1. Inoculum media for organism

Medium used for the inoculum preparation for ethyl alcohol fermentation was YEPD-agar plates containing 10 g/L yeast extract, 20 g/L peptone, 20g/L dextrose, pH 5.00±0.5 for 24 h at 28±0.5ºC with agitation at 150 rpm following the method of Pasha, et al.[14].

2.2.2. Fermentation media

The hydrolyzate was taken along with the supplementation of 1.5 g/L yeast extract, 1 g/L (NH4)2SO4, 0.5 g/L K2HP04, 1g/L peptone, 0.5g/L MgSO4.7H20 and 0.5g/L MnS04. pH was adjusted to 5.0.

2.2.3. Malt yeast peptone glucose media (MYPG)

500 mL of MYPG was prepared by dissolving 1.5g of yeast extract, 2.5 g peptone, 1.5g malt extract, and 10 g of glucose in 500 ml conical flask containing about 150 mL distilled water. Exactly 10g of agar (melted) was added and made up to 500 ml mark and heated at 121ºC for 15 min. The pH of each medium was adjusted to the appropriate pH values using 1M of NaOH and 1M of HCL.

2.3. Pre-treatment of Wood Sawdust

The pre-treatment of Alstonia boonei wood sawdust was done by two methods namely;

1). Alkaline treatment followed by Acid hydrolysis

2). Acid hydrolysis followed by Alkaline treatment

2.3.1. Alkaline treatment followed by acid hydrolysis

The wood sawdust was treated initially with 1.5%(w/v) NaOH at room temperature(28 ± 2 ºC) for 18h and at 100 0C for 2 h separately, followed by 0.75%(v/v) sulfuric acid hydrolysis.

The alkali pretreatment was carried out by taking two sets of 25 g dried wood sawdust at 10 % substrate level(1:10 ratio) in 500 ml flasks separately, the flasks were treated with 3.75g NaOH. One flask was placed at room temperature(28 ± 2 ºC) for 18 h and the other sample in the flask at 100oC for 2 h. The residual substrates were neutralized and washed with water and oven dried at 50oC for further acid hydrolysis. The alkali treated substrate was subjected to acid hydrolysis at 10% substrate level with 0.75%(v/v) H_2SO_4 at 100ºC for 2h.

2.3.2. Acid hydrolysis followed by alkaline treatment

50g of dried wood sawdust was treated with 500ml of 0.75 %(v/v) H_2SO_4 in boiling water bath maintained at 100ºC for 2h. After acid hydrolysis, the residual substrates were washed, neutralized, dried and differentiated into two equal parts then subjected to alkali treatment with 1.5%(w/v) NaOH at room temperature (28 ± 2ºC) for 18h and at 100ºC for 2h separately. The contents were squeezed using cheese cloth and the biomass was repeatedly washed with tap water until the pH became neutral.

The filtrate was used to estimate the amount of reducing sugars by Dinitrosalicylic Acid (DNSA) method. The residual substrates were dried at 50ºC constant weight and later subjected to enzyme hydrolysis.

2.4. Enzymatic Hydrolysis

The 4 residual substrates obtained after pretreatment were subjected to enzymatic hydrolysis in separate flasks, using Accelerase 1500 (commercial cellulase). The loading of enzyme was 261 U/g of substrate taken for hydrolysis in the ratio of 1:20 (0.5ml enzyme + 19.5ml acetate buffer pH 5) (w/v)

(substrate: enzyme), the temperature was maintain at 50ºC, pH 5.0 and incubated for 48h. The samples were collected at regular intervals of 0h, 18h and 24h for glucose yield.

2.5. Optimization

2.5.1. Testing for ethyl Alcohol in fermented sample

10ml of fermented sample was taken into test tube, a pinch of potassium dichromate and 2 drops of H_2SO_4 was added. The colour of the sample in the test tube changed from brown to green which indicated the presence of ethyl alcohol.

Optimization of Fermentation Period (Time)

Fermentation time was carried out using *S. cerevisiae* at different time periods, 24, 48, 72 and 96h under optimum conditions of pH 5 and temperature 30^0C and the fermented solution was analyzed using brix value for every 24h intervals

2.6. Isolation of Microorganism from Palm Wine

2.6.1. Isolation of bacteria

1ml aliquot of each palm wine sample was taken aseptically into test tubes. From 10-fold serial dilution in peptone, 0.1ml portion was surface-spread onto MYPG agar according to Cheesbrough(2003)[15]. The inoculated plates were incubated aseptically at 30^0C for 72h. 14 colonies were randomly selected from plates with distinct colonies, recultivated in MYPG agar at 30^0C for 24 h and further purified on MYPG agar.

2.6.2. Isolation of yeasts

1ml aliquot of each palm wine sample was taken aseptically into test tubes. These samples were serially diluted 10-fold in sterilized distilled water. 1ml of the serially diluted sediment was inoculated by streaking on plates of standard media, media were supplemented with chloramphenicol 0.05mg/L and incubated at 28ºC for 24h

2.7. Identification of Isolates

2.7.1. Identification of bacteria isolate

Purified isolates from fresh plates of Malt, Yeast, Peptone and Glucose (MYPG) medium were subjected to the following tests for the characterization of the isolates: Gram stain, catalase, motility, oxidase, urease, indole, and carbohydrate fermentation using API™ test kit.

2.7.2. The principles of API 20 test for bacterial identification

The API 20 strip consisted of 20 micro-tubes containing dehydrated substrates. The conventional

test was inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produced colour changes that are spontaneous and revealed when reagents was added. The assimilation test was inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate.

The reaction was read according to reading table and the identification was obtained by referring to the analytical profile index or using the identification software.

2.7.3. Carbohydrate (Sugar) fermentation

The ability of the bacterium to ferment various carbohydrates using glucose, fructose, sucrose, maltose, lactose and arabinose were determined by growing the isolate in liquid standard medium containing 1%(w/v) of the particular carbohydrate according to Obire(2005).[16] Durham-tubes were inserted into the culture tubes for gas collection. Incubation was at 30^0C for 24h and uninoculated broths were used as control.

2.7.4. Identification of yeast isolates

Colonies suspected to be yeast were isolated from the spread plates and purified by streaking on freshly prepared media and incubated for 24h at 30^0C. Isolates from such fresh plates were subjected to the following tests for the characterization; morphology, surface characteristics, presence of pseudohyphae, ascospore formation and vegetative reproduction. Fermentative tests included sugar such as glucose, lactose, sucrose and maltose.

2.7.5. Evaluation of isolates for ethyl alcohol fermentation

To evaluate ethyl alcohol fermentation by different strains of *Klebsiella pneumoniae, Enterobacter aerogenes, Saccharomyces cerevisiae and Z. mobilis,* 100ml of rich medium (RM) containing 5g/L of glucose was places into 100ml of Erlenmeyer flask. After inoculation with 5%(v/v) seed culture, the cultures were incubated at 30ºC without agitation for 24h. The yield of ethyl alcohol was used to assess the fermentation performance. The ethyl alcohol yield was determined by gas chromatography.

2.8. Fermentation Process

The enzymatic hydrolyzate with the highest sugar concentration was used for ethyl aclcohol fermentation with *saccharomyces cerevisiae and Z. mobilis* isolated from palm wine. Fermentation was carried out in 500ml Erlenmeyer flasks. The fermentation bubble trap consisted of rubber stopper(with hole) through which a tube was inserted. A cotton plug was inserted in the tube and the tube was connected to silicone tubing. The other end was submerged in a test tube containing water.

They were cooled to a temperature between 27-30^{0}C after liquefaction and saccharification and the pH adjusted with HCl. They were then inoculated with 10ml pre-culture *Saccharomyces cerevisiae and Zymomonas mobilis* in separate set ups. Fermentation was performed in an incubator with intermittent shaking at optimized conditions. The fermentation process was monitored by measuring the sugar content and ethyl alcohol content.

2.9. Chemical Analysis

2.9.1. Estimation of reducing sugars

The amount of reducing sugars present in the wood sawdust delignified filtrate acid and enzyme hydrolyzates was estimated by dinitrosalicylic acid (DNSA) method.[17] DNSA and Rochelle salt were prepared as described in appendix B. about 0.5ml was drawn from every treatment into test tubes. The volume was made up to 3ml using distilled water. 3ml of DNSA reagent of was added to each sample, and mixed well. The reagent black containing 3ml of distilled water and 3ml of DNS reagent was also prepared. All tubes (samples, standards and blank) were kept on boiling water bath for 5 min. After 1ml of 40% Rochelle slat solution was added when the reaction mixture was still warm and was allowed to cool. The absorbance in terms of optical density of the standards and samples were read at 540 nm using Systronics-UV Spectrophotometer – 117. The standard glucose was also prepared similarly with concentration ranging from mg to mg/ml.

2.9.2. Estimation of ethyl alcohol concentration

Ethyl alcohol concentration was determined using a Perkin Elmer, Autosystem XL, Gas Chromatograph (USA) equipped with a flame ionization detector (FID), coupled to a Yokogawa 3021 pen recorder. A Chromopak K 80/100 CRS column was used. The flow rate of the carrier gas, N_2 was 42 ml/min. H_2 and air were the fuel used. The oven temperature, injector temperature and detector temperature were 130^{0}C, 200^{0}C and 200 ^{0}C respectively**.** The injected volume was 1µl and the run time was 8.5 minutes. Identification and quantification were based on direct comparison of the gas chromatogram response to ethyl alcohol standards.

2.9.3. Calculation of results from gas chromatography

The concentration of the analyte in the extract can be calculated as follows;

$$\text{Concentration (mg/L)} = A \times \frac{B}{C} \qquad \text{---------- (1)}$$

where A = Date generated by equipment

B = Total volume of extract in millimeters

C = Weight of sample extracted in grams

The equipment does automatic calculation after feeding in all the required information. The results are reported in mg/kg.

The expected ethyl alcohol amount was calculated after fermentation stoichiometry, assuming that 1.0 g of total sugars produced 0.511 g of ethyl alcohol. The ethyl alcohol yield was calculated as the actual ethyl alcohol produced and expressed as g ethyl alcohol per g sugar utilized(g/g). The volumetric ethyl alcohol productivity as actual yield g/L/h according to Onsoy, *et al.*[18]

The efficiency of reducing sugar conversion into ethyl alcohol by the microorganisms(%) expresses the amount of produced ethyl alcohol relative to the theoretical quantity expected based on the sugar content of the sawdust and it was calculated accordingly with the following equation;

$$\text{Efficiency (\%)} = \frac{ethyl\ \ alcohol\ \ produced\ \ (g/l)}{TRS_i - TRS_f} X\ \ 100 \quad \text{---- (2)}$$

Where, TRS_i is the initial sugar content and TRS_f is the final sugar content.

3. Results and Discussions

3.1. Percentages of Delignification and Saccharification in Alstonia boonei Wood Sawdust Pretreatment

Pretreatment was carried out to break down the crystalline structure of the lignocelluloses of the wood sawdust isolating the cellulose away from the lignin in the cell walls for hydrolysis. An efficient delignifier should remove maximum of lignin and minimum of sugar (not more than 5%) according to taharzadeh and karimi (2007).[19] In the present work 79% and 82% of lignin loss was achieved in treatment of *Alstonia boonei* wood sawdust with 1.5% NaOH at 100^0C for 2h and 28^0C for 18h respectively while the sugar loss were 0.7% and 0.82%. The results are similar to the percentage reported where about 60 -70% of wood sawdust lignin was removed when treated with 1.5% NaOH at 100^0C for 1h. Dilute acid pretreatment and enzymatic saccharification were evaluated for conversion of *Alstonia boonei* wood sawdust cellulose and hemicellulose to monomeric sugars, thus reducing sugars in the acid hydrolysis after two different alkali pretreatments in method I were 11.2g/L and 12.3g/L.(Table 1).

Acid hydrolysis is also based on the cleavage of either bonds by acids between hemicelluloses and lignin complexes. In method II, when the acid hydrolysis has done with 0.75% H_2SO_4 at 100^0C for 2 h, 20.4g/L reducing sugars were produced. Similar conditions were used in which 0.75% of H_2SO_4 at 120 – 180^0C has solubilized hard wood sawdust hemicelluloses to fermentable sugars. 82% and 86% of lignin was removed in the two subsequent alkali treatments and nearly 1% sugars were lost (Table 2).

Table 1: Percentage of delignification and saccharification in alkali followed by acid pretreatment

Treatment	Delignification (%)	Sugar loss (%)	Sugar (g/L)	Saccharification (%)
1.5% NaOH at 28^0C for 18h	79± 0.03	0.70± 0.26	0.87± 0.04	-
0.75% acid hydrolyzate	-	-	11.2± 0.03	10.7± 0.52
1.5% NaOH at 100^0C for 2h	82 ± 0.40	0.82 ±0.06	0.90± 0.09	-
0.75% acid hydrolyzate	-	-	12.2± 0.04	10.4± 0.06

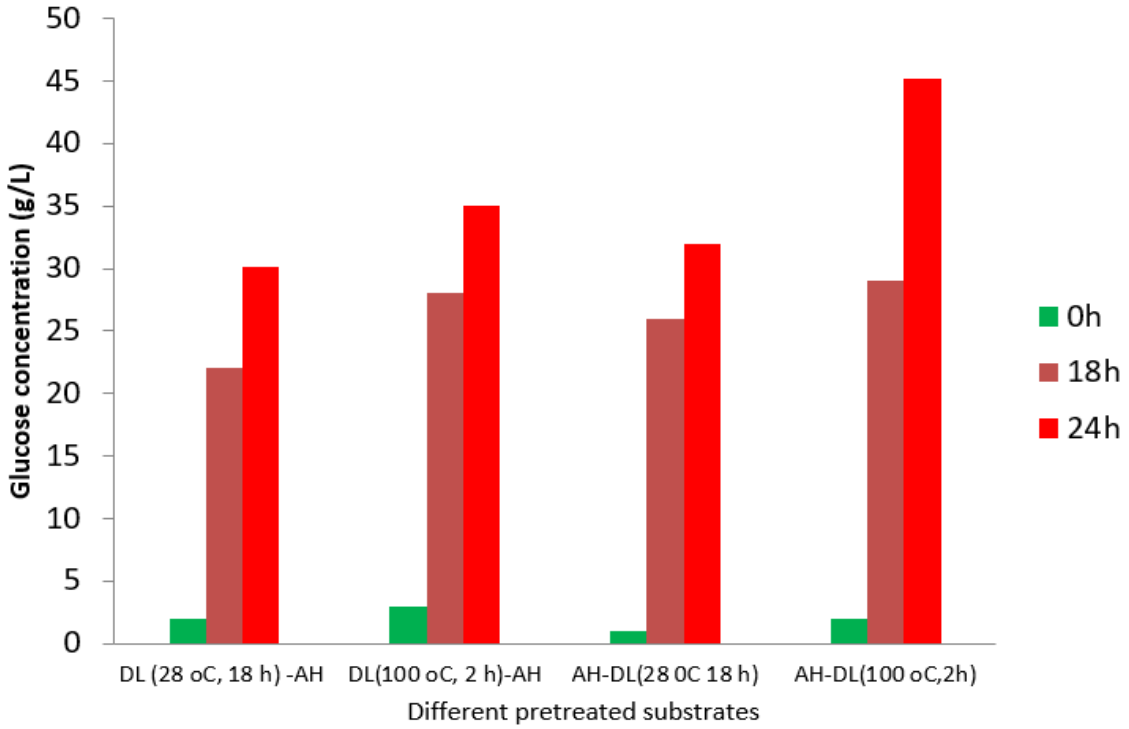
Table 2: Percentage of delignification and saccharification in acid followed by alkali pretreatment

Treatment		Delignification (%)	Sugar loss (%)	Sugar (g/L)	Saccharification (%)
Acid Treatment	0.75% acid hydrolyzate	-	-	20.4 ±0.32	20.8 ± 0.25
Alkaline Treatment	1.5% NaOH at 28^0C for 18h	86.2 ± 0.4	1.5 ± 0.03	2.03 ±0.06	-
	1.5% NaOH at 100^0c for 2h	82.0 ± 0.02	0.88 ± 0.02	1.46 ±0.03	-

3.2. Enzymatic Hydrolysis

The glucose yields in each interval of enzymatic hydrolysis (0h, 18h and 24h) were shown (Figure 3). The neutralized substrates residue from pretreatments were oven dried at 45^0C for 18 h and further subjected to enzyme hydrolysis with Accellerase 1500(commercial cellulose) and was incubated at 50^0C for 48h. An increase in temperature from 30^0C to 50^0C improved the hydrolysis of cellulose in the enzymatic treatment, this could be explained by an increase of enzymatic activities which have an optimal temperature close to 50^0C. Hydrolysis using enzyme increased the release of glucose by 48.2g/L at 24h. It was found that about 92% of cellulose in the pretreatment *Alstonia boonei* wood sawdust residue which initially treated with H_2SO_4 0.75%(v/v) at 100^0C for 2h and then alkali at 100^0C for 2 h in method II were converted to glucose by cellulose enzymes. Similar result was found in the work carried, where 95 % of cellulose in the pretreated bagasse pulp residue was converted to glucose by cellulose enzyme, according to Assam,(2003).[20] Hence, in the present study the substrate was

evaluated for maximum production of ethly alcohol by adopting acid and alkali pretreatment methods followed by fermentation. The saccharification in the other pretreatment methods such as, wood sawdust first pretreated with dilute H_2SO_4 followed by NaOH at 28^0C for 18h was 32g/L, the substrate treated with 1.5% NaOH at 100^0C for 2h followed by dilute H_2SO_4 treatment was 30g/L.



DL = Alkaline Treatment; AH = Acid hydrolysis

Figure 1: Enzymatic hydrolysis of pretreated *Alstonia boonei* wood sawdust at 0h, 18h and 24h

3.3. Isolation and identification of bacteria

Microscopic examination of palm wine samples showed that palm wine serves as a good medium for the growth of numerous microorganisms which included Gram-positive and Gram-negative bacteria mostly in chains and clusters(rods and cocci). According to the different morphology of the colony and fermentation of different carbohydrates, seven strains of bacteria were isolated and three were identified from four localities Essien Udim(EU), Etim Ekpo(EE), Abak(A), Ikot Ekpene(IK) in Akwa Ibom State. The codes represent the locality from which the isolate was obtained. From the Gram staining examination, three isolates(EU1, IK4, and A3) were Gram-negative and bacillus. Four isolates(IK2, EE1, and EE3 and EE4) were Gram-positive. Isolates EE1, EE2 and IK2 were bacilli, EE4 was cocci. The ability of the bacterial isolates to ferment various carbohydrates aerobically and anaerobically is presented in Table 3.

All the bacterial isolates were able to ferment glucose and fructose with only three isolates(EE1,

A3 and IK4) which produced gas from the fermentation both aerobically and anaerobically. Isolates A3 and IK4 were able to ferment all the four carbohydrate both aerobically and anaerobically with gas production. Isolate EU1 was able to ferment glucose, fructose and sucrose but not lactose both aerobically and anaerobically with gas production. The results therefore indicated that all the three isolates (EU1, A3 and IK4) were facultative anaerobe. AP1^{TM} analysis revealed that the isolate EU1 was urease, oxidase and indole negative while IK4 was urease, oxidase and indole negative and catalase positive.

Confirmatory test with AP1^{TM} 20 revealed isolate EU1 to be Zymomonas mobilis, IK4 to be Klebsiella pneumoniae, A3 to be Enterobacter cloacae. The isolation of *Klebsiella and Zymomonas* sp from the fresh palm wine agrees with the work done by Okafor, (1975) and Obire,(2005).[21,22]

Table 3: Fermentation of different sugars by bacteria isolates under aerobic and anaerobic conditions

	Isolates													
	EU1		**IK4**		**IK2**		**A3**		**EE1**		**EE3**		**EE4**	
Sugar	Aerobic	Anaerobic	Aerobic	Aerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
Glucose	+ Gas	+ Gas	+ Gas	+ Gas	+ No Gas	-	+ Gas	+ G Gas	+ No Gas	-	+ No Gas	-	+ NNo Gas	-
Fructose	+ Gas	+ Gas	+ Gas	+ Gas	-	-	+ Gas	+ GGas	+ No Gas	-	+ No Gas	-	+ NNo Gas	-
Lactose	-	-	+ Gas	+ Gas	-	-	+ Gas	+ GGas	+ No Gas	-	+ No Gas	-	+ NNo Gas	-
Sucrose	+ Gas	+ Gas	+ Gas	+ Gas	-	-	+ Gas	+ GGas	+ No Gas	-	+ No Gas	-	+ NNo Gas	-

No gas = no gas was produced during fermentation
Gas = gas was produced during fermentation
+ = substrate was fermented indicated by colour change
- = substrate was not fermented
EU = Essien Udim
IK = Ikot Ekpene
A = Abak
EE = Etim Ekpo

Enterobacter cloacae and klebsiella pneumonia were found to be facultative anaerobic producing gas from glucose, fructose, lactose and sucrose. Z. mobilis was found to be facultative anaerobic since it fermented glucose, fructose and sucrose both aerobically and anaerobically, heterofermentative, producing gas from glucose, fructose and sucrose. These observations were similar to those reported by Swings (1977) and Obire,(2005)[22]

3.4. Isolation and Identification Yeast

A total of seven yeast isolates were identified. Based on the colony and cell morphology including the growth of isolates in liquid medium as well as the assimilation and fermentation of carbohydrates, four different types of yeasts were recognized in the palm wine samples. Nearly, all the yeast isolates fermented glucose, fructose and sucrose, but not lactose. Results presented in Table 4 indicated that all the isolates were aerobes.

Table 4: Characteristics of Isolates

Isolate	Colour of colony	Shape	Gram-stain
EU1	Cream/white	Rod	-
IK4	Cream/white	Rod	-
IK2	Cream/white	Rod	+
A3	Cream/white	Rod	-
EE1	Cream/white	Rod	+
EE3	Cream/white	Rod	+
EE4	Cream/white	Circular	+

Table 5: Fermentation of different sugar by yeast isolates under aerobic condition

Isolate	Glucose	Fructose	Lactose	Sucrose
A1	+ gas	+ gas	-	+ gas
A2	+ gas	+ gas	-	+ gas
EU2	+ gas	+ gas	-	+ gas
EU3	+ gas	+ gas	-	+ gas
IK3	+ gas	+ gas	-	+ gas
EU4	+ gas	+ gas	-	+ gas
EE2	+ gas	+ gas	-	+ gas

Gas = gas was produced during fermentation
\+ = substrate was fermented indicated by colour change
\- = substrate was not fermented
EU = Essien Udim
IK = Ikot Ekpene
A = Abak
EE = Etim Ekpo

They were tentatively identified as *Saccharomyces cerevisiae* based on the AP1 database. Five(A1, A2, EU4, EE2 and IK3) out of the 7 isolates identified were *S. cerevisiae*, EU3 was identified as *Kloechera apiculata*, and EU2 could not be identified. The results indicated that *S. cerevisiae* is the dominant microorganism at the four locations sampling was done.

Saccharomyces cerevisiae had creamish colony with spherical shaped. The organism was glucose, fructose, sucrose and lactose fermentor with gas production (Table 4). The results agree with

Chilaka *et al*,(2010)[23] who reported the isolation of *Saccharomyces cerevisiae* and other yeast from palm wine. Elijah,(2010)[24] also reported isolation of Saccharomyces cerevisiae from palm wine.

3.5. Evaluation of Isolates for Ethyl Alcohol Production

Enterobacter cloacae, Klebsiella pneumonia, Zymomonas mobilis, Saccharomyces cerevisiae and kloechera apiculata were selected and their performance in ethyl alcohol production was assessed. The result indicated that *Z. mobilis* produced the highest ethyl alcohol concentration of 1.4v/v, followed by *S. cerevisiae* with ethyl alcohol concentration 0.834v/v. The rest of the isolates; *Enterobacter cloacae, Klebsiella pneumonia and kloechera apiculata* produced ethyl alcohol concentraton below 0.5v/v.

The difference in ethyl alcohol concentration produced by the different isolates might be due to the different pathways employed by the isolates in conversion of glucose to ethyl alcohol and the major product of such pathway. In the case of *Z. mobilis and S. cerevisiae* the major products from fermentation were ethyl alcohol and carbon dioxide. In *enterobacter cloacae and Klebsiella pneumonia* the main fermentation products are acetoin,2,3 butanediol, ethyl alcohol, lactic acid, formic acid and acetic acid[25].

The proportion of these compounds varies with oxygen supply and the pH. [26] In the absence of air, growth was slow in this organism and most of the carbon was converted into ethyl alcohol formate, butanediol acetoin and acetate[27]. These observations might account for the low ethyl alcohol concentration in these organisms.

Table 6 Evaluation of yeast and bacterial strains for ethyl alcohol

Microorganism	Actual ethyl alcohol produced v/v	Ethyl alcohol yield g/g	Volumetric ethyl alcohol productivity g/l/h	Conversion efficiency %
Zymomonas mobilis	1.40	0.28	0.0058	54.90
Klebsiella pneumonia	0.29	0.057	0.0012	11.17
Enterobacter cloacae	0.32	0.064	0.0013	12.54
Saccharomyces cerevisiae	0.83	0.166	0.035	32.62
Kloechera apiculata,	0.28	0.056	0.0012	10.98

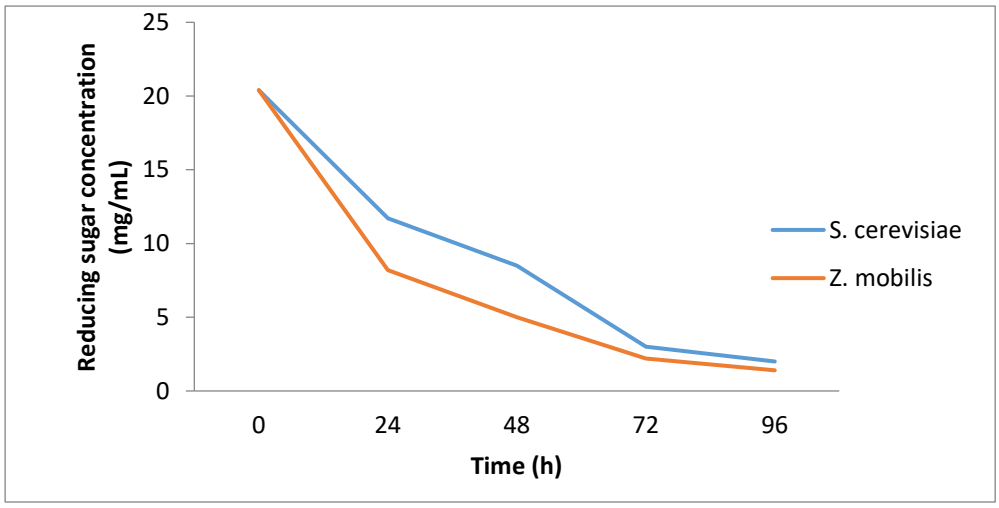


Figure 2: Sugar utilization by *Z. mobilis* and *S. cerevisiae*

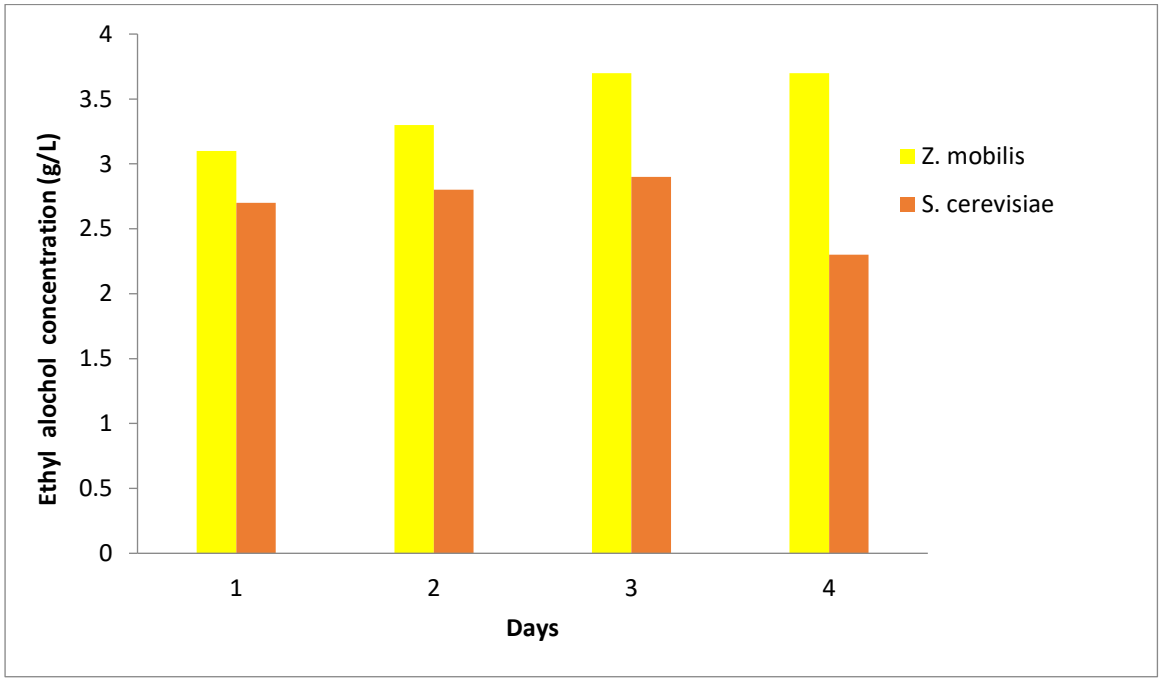


Figure 3: Ethyl alcohol produced from *Alstonia boonei* sawdust using S. cerevisiae and Z. mobilis separately.

3.7. Ethyl Alcohol Produced from Alstonia boonei Wood Sawdust using S. Cerevisiae and Z. mobilis Separately

Different microorganisms have been used in Bioethyl Alcohol production and each organism has exhibited different fermentation properties. In this work only one hydrolyzate which has had the maximum glucose after enzymatic hydrolysis (48.2g/L) was mixed with its acid hydrolyzate (20.4g/L) and inoculated with. *S. cerevisiae* and *Z. mobilis* separately. In both organisms a continuous increase in ethyl alcohol yield was accompanied with decrease in reducing sugar concentration during the whole

period of fermentation (Figure 2 and 3). The fermentation with *Z. mobilis* proceeded very rapidly and was essentially completed in 72h with maximum yield of 3.82g/L fermentation with *S. cerevisiae* was also successful and also required 72h to complete with a yield of 3.24g/L. *Z. mobilis* therefore showed quick rate of substrate utilization and could utilize glucose and other hydrolysate faster than S. *cerevisiae* and thus achieved higher fermentation efficiency than *S. cerevisiae* (Table 7). Bacteria are known to multiply faster than yeast thus Z. mobilis might reached the lag phase faster than *S. cerevisiae* and therefore utilized its substrate faster. The ethyl alcohol yield for *Z. mobilis* was higher than that of *S. cerevisiae* at all fermentation period.

Under the operative conditions, the efficiency of reducing sugar conversion to ethyl alcohol was found to be 62% in S. cerevisiae and 74.2% in Z. mobilis. The efficiency of conversion of sugar to ethyl alcohol by Z. mobilis was higher compare to S. cerevisiae. T-test analysis showed that at 95% confidence interval, there was significant difference between the amount of ethyl alcohol produced by Z. mobilis and S. cerevisiae on each day. From Table 7, it is clear that the overall performance of Z. mobilis was superior to S. cerevisiae making it attractive for large scale ethyl alcohol production.

This work therefore shows that under appropriate conditions and pretreatment methods *Alstonia boonei* wood sawdust can be used as alternative and cost effective feed stock for the production of bio-ethyl alcohol

Table 7: Comparison of ethyl alcohol production and efficiency between *S. cerevisiae* and *Z. mobilis*

Time (h)	Ethyl alcohol produced(g/l)		Microorganism efficiency%	
	Z. mobilis	S. cerevisiae	Z . mobilis	S. cerevisiae
24	3.18± 0.15	2.88 ±0.02	62.40	58.70
48	3.55±0.21	2.96±0.28	69.80	60.13
72	3.82±0.26	3.03±0.14	72.0	62.04
96	3.64±0.28	2.40±0.28	74.02	48.75

4. Conclusion

Pre-treatment of *Alstonia boonei* wood sawdust primarily done with 0.75% sulfuric acid at 100^0C for 2h then 1.5% NaOH at 100^0C for 2h was saccharification by enzymatic hydrolysis, when the mixture of both enzyme and acid hydrolyzates was fermented with *Saccharomyces cerevisiac* and *Zymomonas mobilis* isolated from palm wine it has produced high yield and better efficiency of ethyl alcohol. The study revealed that palm wine is a good medium for growth of ethanogenic microorganisms.

Thus, the production of ethyl alcohol from *Alstonia boonei* wood saw dust and other biomass is

possible locally in Nigeria, without depending on the edible ethyl alcohol sources. Even though it cannot yet replace saccharine and starchy materials, their consideration as possible substitute for grain based ethyl alcohol, stems from the fact that they reduce green gas emission calculation, and is a means of controlling environmental pollution, it can therefore be considered as good venture to undertake. Besides their collection and utilization, they can provide additional source of income to the farmers from existing acreage.

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