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DELIGNIFICATION PRETREATMENT OF *ACANTHOSPERMUM HISPIDUM* BIOMASS FOR BIOETHANOL PRODUCTION

***[1]Ahmad M. A., [1,2]Tambuwal A. D., [1]Sokoto A. M., [1]Sanda A.**

[1]Department of Pure and Applied Chemistry, Usmamu Danfodiyo University
[2]Sokoto Energy Research Center, Usmanu Danfodiyo University

*Corresponding authors' email: awwalsharp@gmail.com Phone: +2347030168232

ABSTRACT

Bioethanol from lignocellulosic biomass is environmentally friendly and a renewable source of energy. This study used delignification as a pretreatment process to obtain the optimum conditions for reducing sugar production. The selected delignification parameters, including temperature (^{o}c), time(hours) sodium chlorite concentration (g/dm^3) and Volume of acetic acid (cm^3). The Optimum conditions for delignification were: 70^{o}c, 6 hr at -0.1 g/ dm^3 sodium chlorite concentration and acid-3 cm^3 of acetic. The R^2 value obtained from the correlation between reducing sugar concentration and time was R^2 =0.796 The maximum bioethanol concentration was at 66h and the bioethanol productivity was 0.0304 g/L/h. The result obtain pointed that, the *Acanthospermum hispidum* can be used for both economic and environmental footprint studies and real world application.

Keywords: Bioethanol, *Acanthospermum hispidum*, Delignification, Pretreatment, Reducing sugar

INTRODUCTION

Ethanol is now the most important renewable fuel in terms of volume and market value. Nowadays it is produced from sugar and starch-based materials such as sugarcane and corn. Bioethanol is the largest produced liquid biofuel in the world. As a transportation fuel, it can be either used in blended form along with gasoline (gasohol) or as pure ethanol. Ethanol fermentation is one of the most mature and well-established bioprocesses (Swana *et al.*, 2011). With global production crossing 100 billion liters in 2016 (Renewable Fuels Association 2016). Bioethanol is expected to remain the most prominent and cost-effective biofuel for the foreseeable decades, with prices approaching that of gasoline(Eisentraut *et al*. 2011).

Bioethanol is produced via microbial degradation of carbohydrate-rich substrates by yeast, bacteria and fungi. *Saccharomyces cerevisiae* is the most commonly used yeast in small-scale as well as industrial bioethanol production. *Saccharomyces cerevisiae* produces ethanol as its major fermentation product. The robust yeast can operate in a wide pH range and can tolerate high levels of ethanol and other inhibitory compounds when compared to other fermentative microbes (Almeida *et al*., 2007; Lin *et al*., 2012; Prasertwasu *et al*., 2014; Tesfaw and Assefa 2014).

First-generation bioethanol production utilizes edible crops such as sugarcane, corn, wheat, rice and sorghum as a feedstock. The Majority of the ethanol plants across the world are first-generation. Lignocellulosic materials and residues from agriculture and forests are used to produce second-generation bioethanol, while third generation biofuels use algal feedstocks (Jambo et al. 2016). US and Brazil together account for more than 85% of the world's ethanol producers, however, they employ largely different processes. While Brazil uses a year-round supply of sugarcane feedstock, North American ethanol industries produce ethanol from corn grain. In comparison, the process of corn grain to ethanol in the us achieves only one-sixth of the energy efficiency (energy invested to energy returned) of sugarcane to ethanol .The potential increase in prices of corn (Renewable Energy Association 2016; World Energy Council 2016), a food crop, which along with wheat and rice contributes two-third of the world's calorific intake, undermines the need for developing alternate energy crops for ethanol production.

Many substrate are used for bioethanol production globally. Recently, research on the production of ethanol from lignocelluloses biomass has been accelerating for both energy and economic reasons.

Bioethanol from *Acanthospermum hispidum* can be used as transport fuel and in the production of hand sanitizer. The weedy biomass (*Acanthospermum hispidum*) is grown in the wild, in a semi-arid region of Sokoto State ,Nigeria . During the COVID-19 pandemic, there was a high demand for hand sanitizers and one key chemical used in production of hand sanitizer is bioethanol.

The study aimed to produce bio-ethanol from *Acanthospermum hispidum*. This investigation also carried out pretreatment on *Acanthospermum hispidum* biomass.

MATERIALS AND METHODS

Collection of substrate

Acanthospermumpidum was selected as a substrate in this work because it is inedible by humans and animal and available in the wild of Sokoto State, Nigeria . *Acanthospermum hispidum* was collected from Runjin Sambo area of Sokoto State , Nigeria . The weed can also be cultivated as a reducing sugar source for biofuel production on open land and possess some significant advantages over existing sources of bioethanol such as Sugar beet juice, Sweet sorghum juice, corn , Sugar cane and Oil palm frond juice. The weed grows with less water input(without irrigation) ; and does not compete with food crops and requires little quantity of fertilizer to grow.

Determination of Lignin

The sample (3 g)was weighed into a beaker. To the beaker containing the test specimens, H_2SO_4(72%,40.0cm^3) solution was added. The acid was added gradually in small increments while stirring and macerating the material with a glass rod. After the specimen was dispersed, the beaker was covered for 2 h at room temperature. The material was frequently stirred to form a mixture. Water (400 cm^3) was added to the flask and then transferred the mixture from the beaker into the flask. Sulphuric acid(3%) was added.The solution was boiled for

4h, constant volume was maintained by frequent addition of hot water. The insoluble material (lignin) was allowed to settle and then filtered. Then 3 g of insoluble residue (lignin) was weighed placed in an oven at 105 ± 3°C for 2h; a crucible containing the dried sample was cooled in a desiccator and weighed. To determine Acid-soluble lignin (ASL) the content of acid-soluble lignin in the first filtrate was subjected to spectrophotometry at 205 nm according to (TAPPI UM 250)

Acid-insoluble lignin (AIL)

AIL= $\frac{m}{M}$×1000 (mg/g) (1)

Where : m= the weight increase (ie. the residue after drying), in g

M = Oven-dry weight of sample (ie. as 100% dry matter) before acid hydrolysis/suspension, in g

Acid-soluble lignin (ASL):

ASL= $\frac{A.D.V}{a.b.m}$.1000 (mg/g). (2)

where

A = Absorption at 205 nm

D = Dilution factor

V = Volume of the filtrate, in litre (here: 0,029 L)

a = Extinction coefficient of lignin, in g/l cm (here: 110 g/l cm, according to TAPPI UM 250)

b = cuvette path length, in cm (here: 1 cm)

M = Weight of sample (as 100% dry matter) before acid hydrolysis/suspension, in g

Total lignin content = AIL + ASL (3)

Delignification of the *Acanthospermumpidum* biomass

Acanthospermumpidum (110g) was washed several times with hot distilled water (80^{O}C) to remove free non-structural sugar that may be present in the sample. The washed sample substrate was squeezed by hand to remove excess water and then dried at room temperature for three days in an oven. The dried sample(5.0g) was weighed into a flask(250cm^3) and water(160 cm^3) was added to form a slurry,then each sample (slurry) was heated at 70±2^{O}C on a hotplate, followed by adding sodium chlorite (0.1g/dm^3) and also acetic acid (5cm^3). The slurry was thoroughly mixed by shaking the flasks. Sodium chlorite and acetic acid were added to the reaction every 2 hour for up to 6 hour during deligninification. The slurry was filtered on a filter paper to separate the liquid from the residue and the residue was washed with distilled water and then dried for 3h at room temperature (TAPPI UM 250).The dried residue was weighed as acid insoluble lignin (AIL) TAPPI UM 250.

Design of Experiment

A central composite experimental design was used to optimize the temperature (X1), time (X2), Sodium chlorite concentration (X3) and acetic acid volume (X4) in reducing sugar yield from delignified weeds. Reducing sugar recovery was used as a dependent output variables. 21 experiments were performed for *Acanthospermumpidum*. A quadratic model (Box *et al*., 1978) was used to evaluate the optimization of key parameters using the following equation (equation iv):

$Y = \beta_0 + \sum \beta_{1\text{-}44} X_{1\text{-}44}$ (4)

Where, Y = predicted response; X1, X2, X3 and X4 = parameters, $\beta_0$is the intercept and β is to be found. The response variable (Y) was fitted using a predictive polynomial quadratic equation in order to correlate the response variable to the independent variables (Lay, 2000). The Y values were regressed with respect to Temperature , Time , Sodium chlorite concentration and Acetic acid concentration . Design expert software version 6.0.6 (Stat-Ease. Inc., MN, USA) was used for regression analysis of the experimental data obtained. The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing it. The quality of the fit of quadratic model was expressed by the coefficient of determination(R square).

Table 1. Independent Variables and their Coded levels used for Delignification.

Independent Variable	Code	Lower level	Upper level
Temperature (^{o}c)	X_1	70	90
Time(hours)	X_2	2	6
Sodium chlorite(gram/ dm^3)	X_3	0.05	0.1
Acetic acid(cm^3)	X_4	0.50	3

Determination of Reducing Sugar from Delignified *Acanthospermumpidum*

This was carried out according to the method described by Humphrey and Caristas (2007), Gupta *et al.*,2009 and Oyeleke and Jibrin (2009) .Each of the deligninfied samples (5g) was weighed into conical flasks(250 cm^3), and Sulphuric acid(5.0 % , 150 cm^3) was added into the conical flasks. The flask was covered with cotton wool, wrapped in aluminium foil, heated in a water bath for 2 hours at a temperature 50 ^{O}C . The flask was allowed to cool, and filtered through No1 Whatman filter paper(No1).The pH of the hydrolysate was adjusted to 4.5 with sodium hydroxide (0.4 M).

The reducing sugars was estimated using dinitrosalicylic (DNS) reagent. DNS(10g) was dissolved in 2M NaOH (200 cm^3) with shaking and heated for 15min. Sodium potassium tartrate tetrahydrate(40) was dissolved in distill water(100 cm^3). The two solutions were mixed and made up to 1L with distilled .The reducing sugar content of the hydrolysate was assayed by adding DNS reagent (3 cm^3) into the sample (3 cm^3).The mixture was heated in boiling water for 10 minutes to develop the red brown colour, then, cooled to room temperature under running water tap. The absorbance of the sample was measured at 540nm using UV-VIS spectrophotometer. The reducing sugar content was subsequently determined by referring to a standard curve of known glucose concentration (Ahmad *et al*., 2015).

Reviving the yeast

The yeast used in this study was purchased in a bakery shop in Sokoto, Sokoto State. Prior fermentation , the yeast was revived in the mycology laboratory of Usmanu Danfodiyo University , Sokoto (UDUS). The reviving was done using warm water (36^{o}C) using method reported by Rabah, et al (2011).The yeast dextrose agar was prepared according to the method described by Ahmad et al (2015) and incubated at room temperature for 24hrs, thereafter a single colony of the yeast was inoculated into the yeast dextrose agar broth and incubated at room temperature overnight in an incubator.

Fermentation Process

The fermentation process was carried out in 100cm^3 conical flasks (Batch). Each flask contains the hydrolysate of the weed. The hydrolysate was sterilized at a temperature of 125 ◦C in the autoclave for 35 min. The sterilized solutions was inoculated with activated yeast(g). Then, the flasks were monitored every 6h . Finally, the hydrolysate was fermented at room temperature. The Samples were analyzed every 6h. Aliquots were withdrawn for sugar, ethanol and biomass analysis every 6h and the fermentation was for 90h

Plate 1:Experimental setup for fermentation

Quantification of bio-ethanol

Bio ethanol from the samples was estimated by potassium dichromate and sulphuric acid method. Bio-ethanol (1 cm^3) was taken and made up the volume to 5 cm^3 with distilled water then followed by 3cm^3 of $K_2Cr_2O_7$ solution(dichromate reagent). .The intensity of the color was read at 595 nm in UV/VIS spectrophotometer. This intensity of the color was measured in triplicate. Bioethanol produced was extimated by extrapolation with standard ethanol curve.

RESULTS AND DISCUSSION

Lignin Content of the Substrate

Lignocellulose, the principal component of the plant cell walls, is mainly composed of cellulose (40–60% of the total dry weight), hemicellulose (20–40%), and lignin (10–25%)(Mahmoudi *et al* .,2010). Lignins are phenolic compounds that are formed by polymerization of three types of monomers (p-coumaryl, coniferyl, and synapyl alcohols)(Wyman *et al.*2005).Research *revealed that* high lignin content are unfavorable for bioethanol production because of the recalcitrance of lignin. (Demirbas,2004).The results in Table 1 indicate that *Acanthospermum hispidum* has high lignin contents (Acid-insoluble lignin + Acid-soluble lignin) .Indeed, a couple of studies showed that lignin content varies from plant to plant. This may be due to some factors like plant species, plant parts, growth conditions, etc. (Ding and Himmel, 2006; Zhang and Lynd, 2004).

Table 1: Lignin Content of the Substrate.

	Acanthospermum hispidum
AIL (mg/g)	126.30±13.61
ASL (mg/g)	5.51±0.64
Total lignin content = **AIL** + **ASL**	131.81

Means that do not share a letter are significantly different.
± standard deviation

Reducing sugar concentration (%) of Delignified *Acanthospermum hispidum*

Table 2 shows the reducing sugar recovery from the delignified *Acanthospermum hispidum.* The Result in Table 2 reveled that *Acanthospermum hispidum* has a maximum reducing sugar yield of 33.22 % at 70^{o}c, 6 hr at 0.1 g/ dm^3 sodium chlorite concentration and acid-3 cm^3 of acetic.

Table 2: Production of Reducing Sugar from Delignified *Acanthospermum hispidum*

Run	X_1(oC)	X_2 (hours)	X_3 (g/dm^3)	X_4 (cm3)	Reducing sugar yield(%)
1	70.00	2.00	0.05	0.50	4.56
2	80.00	4.00	0.075	1.75	28.84
3	80.00	4.00	0.075	0.50	17.84
4	80.00	4.00	0.1	1.75	17.68
5	90.00	2.00	0.1	3.00	18.94
6	80.00	4.00	0.075	1.75	18.24
7	90.00	4.00	0.075	1.75	32.02
8	90.00	6.00	0.1	0.50	6.28
9	70.00	2.00	0.1	0.50	14.24
10	70.00	4.00	0.075	1.75	18.22
11	**70.00**	**6.00**	**0.1**	**3.00**	**38.22**
12	80.00	4.00	0.05	1.75	18.02
13	80.00	4.00	0.075	3.00	16.22
14	80.00	6.00	0.075	1.75	16.26
15	70.00	6.00	0.05	3.00	38
16	80.00	4.00	0.075	1.75	20.12
17	80.00	4.00	0.075	1.75	24.84
18	90.00	6.00	0.05	0.50	7.5
19	90.00	2.00	0.05	3.00	18.24
20	80.00	2.00	0.075	1.75	32.24
21	80.00	4.00	0.075	1.75	28.86

Table 3 showed that the p-value is less (0.05) , which implies that the model is significant. In this case X_2, X_1 X_2, X_1 X_4, X_2 X_4 are significant model terms. Using regression co-efficient ,the regression square(R^2) was calculated to be 0.9467. Statistical analysis revealed that the maximum reducing sugar yield of 38.22% was optimum at 70^{o}c, 6hr and 0.1g/ dm^3 of sodium chlorite concentration and acid-3cm^3 of acetic.

Table 3: Analysis of variance(ANOVA) for Reducing Sugar Yield from Delignified *Acanthospermum hispidum*

Sources	Sum of Squares	DF	Mean square	F-Value	P-Value Prob>F
Model	**4.22**	**14**	**0.30**	**6.58**	**0.01**
X_1	0.24	1	0.24	5.20	0.0627
X_2	**0.32**	**1**	**0.32**	**7.01**	**0.0381**
X_3	0.019	1	0.019	0.42	0.5421
X_4	3.280E-003	1	3.280E-003	0.072	0.07978
X_1^2	0.050	1	0.050	1.09	0.3377
X_2^2	0.023	1	0.023	0.50	0.5048
X_3^2	0.13	1	0.13	2..81	0.1445
X_4^2	0.18	1	0.18	3.92	0.0950
X_1 X_2	**0.48**	**1**	**0.48**	**10.47**	**0.0178**
X_1 X_3	0.032	1	0.032	0.71	0.4324
X_1 X_4	**0.60**	**1**	**0.60**	**13.21**	**0.0109**
X_2 X_3	0.042	1	0.042	0.92	0.3739
X_2 X_4	**0.62**	**1**	**0.62**	**13.53**	**0.0104**
X_3 X_4	0.019	1	0.019	0.41	0.5440
Residual	0.27	6	0.046		
Lack of Fit	0.035	2	0.017	0.29	0.7617
Pure error	0.24	4	0.060		
Cor Total	4.49	20			

Effect of fermentation period on reducing Sugar concentration

Figure 1 show a relationship between reducing sugar concentration and fermentation period for *Acanthospermum hispidum.* A rapid decrease in reducing sugar from 3.5g/dm^3 to 1.0 g/dm^3 was observed after 35hr of fermentation. At 54h to 60h a stationary phase was observed, at this phase, the size of the yeast population remained constant, even though some cells continued to reproduce and others began to die. Since the R^2-value obtained from the graph was 0.796. it mean that the correlation between reducing sugar concentration against time was moderate.

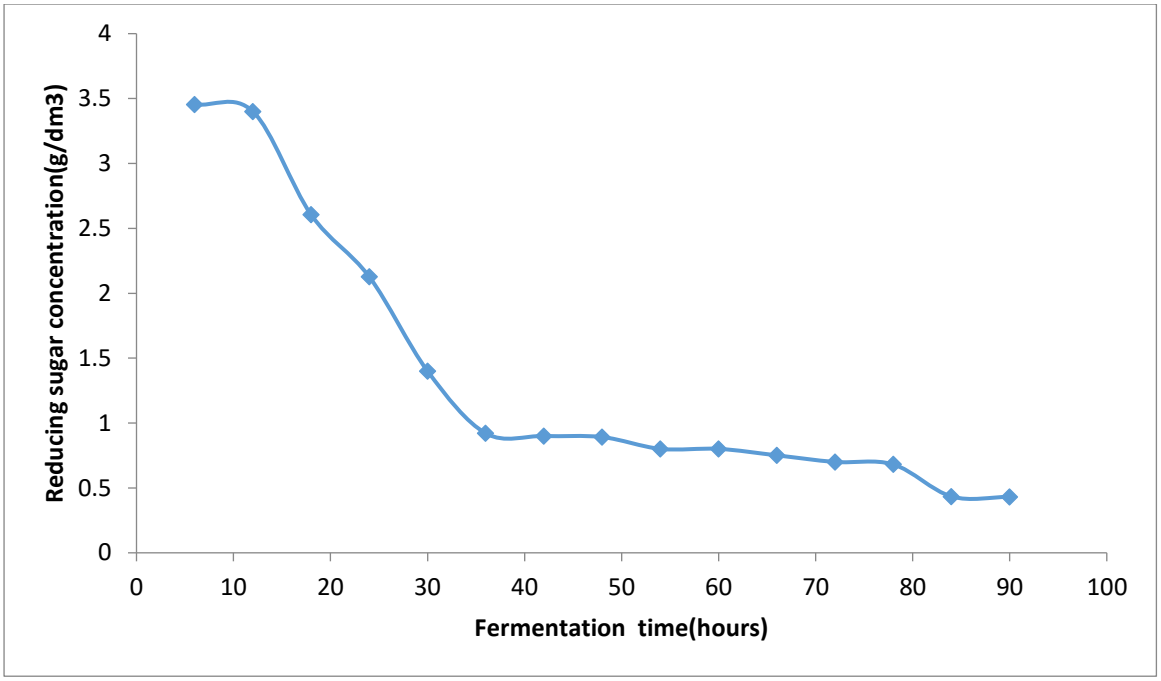


Figure 1. A graph showing the relationship between reducing sugar concentration against time for *Acanthospermum hispidum.*

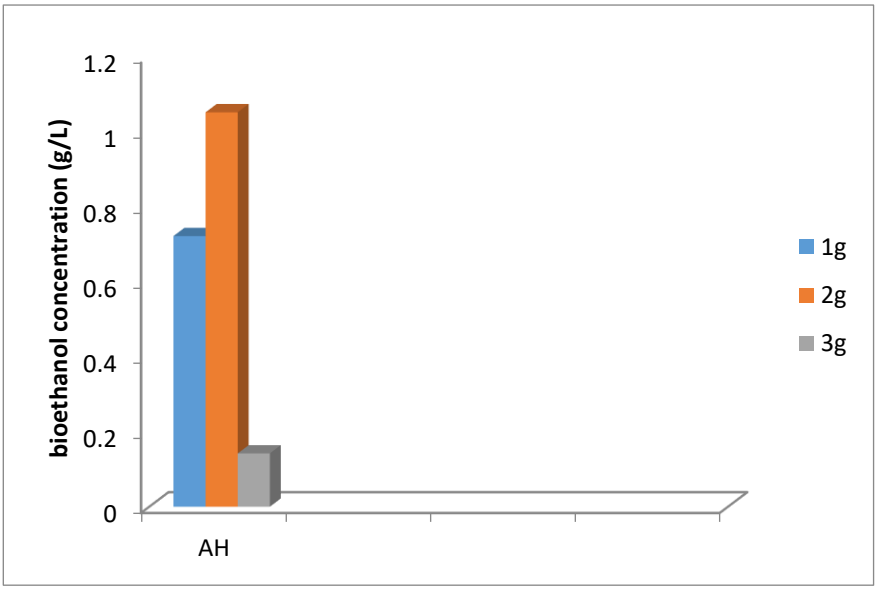
The change in reducing sugar concentration over time for *Acanthospermum hispidum* Substrate
is shown in Figure 1, From the graph , a short lag phase was observed. A lag phase of 6h was observed because the hydrolysate provided a suitable condition for yeast to thrive and multiply. From Figure1, as the reducing sugar concentration change with fermentation time, the concentration of bioethanol increases. The trend observed in Figure 1 is in agreement with the work of Pavlecic *et al.*, 2010 ; Ramos *et al.*,2013; Wu X,*et al*,2010 and Abdullah, *et al,2015*)

Bioethanol fermentation of *Acanthospermum hispidum* hydrolysate

Bioethanol yield depends on the rate at which the sugar is consumed or the rate at which the microorganism act on the sugar Therefore bioethanol yield can be define as the amount of bio ethanol produced per the sugar consumed during fermentation or the amount of sugar in which the organism acts during fermentation.
Bioethanol productivity is a key parameter to determine the cost- effectiveness of industrial production. Bioethanol productivity depend on the sugar content of the weedy biomass and the sugar content may vary as a result of geographical location, weather and other factor that affect weed growth. Sugar content is directly associated with bioethanol productivity (Ahmad *et al.*, 2015)
The significant fermentation parameters in ethanol production from *Acanthospermum hispidum* biomass are summarized in Table 3. The comparison with other feed stocks using the same *Saccharomyces cerevisiae* was made. The results of this study showed that *Acanthospermum hispidum* biomass has a potential as a non-food substrate for bioethanol production. The results is in agreement with that of (Wu *et al* 2010; Pavlecic *et al* 2010; Ramos *et al* 2013; Tussanee., *et al*.,2015).

Effect of yeast concentration on bioethanol yield from weedy biomass

To gain insight into the beneficial effect of the yeast, the yeast was activated. The reasons for the activation were to make the yeast more viable, and to replicative lifespan, which measures the number of times a yeast cell can produce offspring. Different concentration of activated yeast was prepared (1 g , 2 g and3 g) and was added to the same volume(100 cm^3) and concentration of hydrolyse. As it turns out, 2 g of activated yeast produced the highest concentration of bioethanol *Acanthospermum hispidum*(1.051%). It can be deduced that the lesser the concentration of yeast cells the more the bioethanol. What cause the anomaly is because that the total sugars /initial total reducing sugar have a lesser concentration of reducing sugar and less nutrient for the yeast to thrive.



CONCLUSION

The result obtained pointed that, the *Acanthospermum hispidum* can be used for both economic and environmental footprint studies and real world application. The Optimum conditions for delignification was at 70^{o}c, 6hr at 0.1g/ dm^{3} sodium chlorite concentration and acid-3cm^{3} of acetic . The R^2 value obtained from the correlation between reducing sugar concentration and time was R^2 =0.796.

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