Bioethanol Production from *Ziziphus abyssinica* Agroresidues: a Comparison of Effect of Different Pretreatment Approaches on Amount of Glucose

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Abstract: ***The aim of this research was to optimize bioethanol production from Ziziphus abyssinica agroresidues (inner stone). Proximate composition of the agroresidues was determined. Seven pretreatment approaches comprising of single-step (Aspergillus niger, sodium hydroxide, sulphuric acid) and multi-step (sodium hydroxide + A. niger, sulphuric acid + A. niger, sulphuric acid + sodium hydroxide, sulphuric acid + sodium hydroxide + A. niger) were carried out. Fermentation was carried out for three (3) days using Saccharomyces cerevisiae. Proximate analysis of these agroresidues revealed that it contain high amount of crude fibre (35.59 %) and non-fibrous carbohydrates (36.60 %) making it potential feedstock for bioethanol production. Pretreatment with dilute sulphuric acid produced the highest amount of glucose (286.80 mg/g biomass) while the unpretreated biomass yielded the lowest amount of glucose (10.87 mg/g biomass). Fermentation under optimized condition yielded bioethanol of concentration 4.5133 % (v/v).***

Keywords: Pretreatment**,** Bioethanol, *Ziziphus abyssinica*, Agroresidues, Glucose

1. Introduction

Overdependence of the transport sector on fossil fuel has resulted into rapid depletion of fossil reserves and environmental pollution [1]. These reasons have triggered researchers to investigate into potential replacement for fossil fuels such as renewable energy which includes water, wind, geothermal heat, sun, and plant biomass [2]. The use of plant biomass for biofuel production is receiving increasing attention because it does not produce a net addition of carbondioxide to the atmosphere. As such, does not contribute to global warming [3]. Efforts are geared towards bioethanol production because ethanol can effectively replace gasoline in the transport sector [4]. Ethical concerns about the use of food a raw material for ethanol production have encouraged research efforts to be more focused on the potential of inedible feedstock alternatives such as lignocellulosics [5].

Lignocellulosic materials constitute a substantial renewable substrate for bioethanol production that do not compete with food production and animal feed. These cellulosic materials also contribute to environmental sustainability. Additionally, lignocellulosic biomass can be supplied on a large scale basis from different low-cost raw materials such as municipal and industrial wastes, wood and agricultural residues [6]. Agricultural wastes and forest products (soft and hard woods) are the major lignocellulosic materials found in abundance worldwide [7]. Lignocellulosic materials consists of three (3) types of polymers; cellulose, hemicellulose and lignin [8]. Lignocellulosic materials contain cellulose and hemicellulose that are bound together by lignin. Cellulose and hemicellulose are both polymers built up by long chain of sugar monomers, which after pretreatment and hydrolysis can be converted into ethanol by microbial fermentation [9] [10].

Ziziphus abyssinica is a species of shrub in the *Rhamnaceae* family [11]. It is endemic to the dryer parts of Africa. The fruits are almost spherical, 2-3cm in diameter, shiny red or reddish brown when mature, smooth, and contain 1 or 2 light brown glossy seeds inside the inner stone [12][13]. The fruit of *Ziziphus abyssinica* tree (catch thorn) contains an edible outer part and an inedible inner stone which contains the seeds [14]. This inner inedible part is normally thrown away after the edible outer part of the fruit has been consumed. Therefore, the inner stone is a waste and a contributing factor to environmental pollution. Using the stone for bioethanol, will help in providing an alternative source of fuel and reducing pollution to the environment. One of the major challenges of bioethanol production from agricultural wastes is development of efficient pretreatment methods for total delignification of lignocellulosics [15]. Proper pretreatment of lignocellulosic biomass can increase concentrations of fermentable sugars, consequently, improving ethanol yield [16]. The aim of this research was to optimize bioethanol production from *Ziziphus abyssinica* agroresidues.

2. Materials and Methods

2.1 Sample Preparation

The inedible part (inner stone) of the locally consumed *Ziziphus abyssinica* fruit was thoroughly washed with distilled water and sun dried. A mortar and pestle was used to manually grind the biomass (*Z. abyssinica* agroresidues) into a powdered form. The powder was then sieved with a 0.3 mm mesh diameter test sieve. The dried, powdered, and sieved sample was stored in a cool, dry place for later use.

2.2 Pretreatment

2.2.1 Sodium hydroxide pretreatment

Sodium hydroxide (NaOH) of concentration 7% (w/v) was used to treat *Z. abyssinica* agroresidues at a biomass loading of 10 % (w/v). Treatment temperature was maintained constant at 30 ^{o}c for a period of 48 hours. After pretreatment, the sample was filtered and the solid residues were washed with de-ionized water, until the pH of the flow through reached the neutral range. The solid residue was then dried at

105 °c for 2 days and weighed. The solid residue was stored in a zip lock sample bag for further treatment.

2.2.2 Sulphuric acid pretreatment

Sulfuric acid (H_2SO_4) of concentration 60 % was used to treat the *Z. abyssinica* agroresidues for 4 hours in order to solubilize the polymeric cellulose and hemicellulose at 30 °c. Deionized water was then added to reduce the concentration to10 % (v/v) acid concentration, and the temperature was raised to 100 ° c for 1 hour to accomplish the hydrolysis process. The resulting hydrolysate was then filtered and neutralized with $Ca(OH)_2$ and stored at 4 ° c for further analysis.

2.2.3 Concentration and detoxification of acid hydrolysate

The hydrolysate obtained from concentrated sulfuric acid hydrolysis was first concentrated by evaporation at 100 °c on a heating mantle to a threefold concentration. The toxic compounds were then removed by using charcoal powder. Five grams per liter of charcoal powder was mixed in hydrolysate and stirred at room temperature for 2 hours.

2.2.4 Saccharification

Aspergillus niger of concentration 1.96×10^6 cells/5 ml was used for saccharification of the *Z. abyssinica* agroresidues. The *A. niger* was inoculated into the biomass in a conical flask, distilled water was added and the flask was incubated for 48 hours at room temperature.

2.2.5 Multistep pretreatment

Multi-step pretreatment of the sample was carried out in the following manner: alkaline hydrolysis followed by saccharification (NaOH + *A. niger*), aid hydrolysis followed by saccharification (H_2SO_4 + *A. niger*), alkaline hydrolysis followed by acid hydrolysis (NaOH + H_2SO_4), and alkaline hydrolysis followed by acid hydrolysis followed by saccharification (NaOH + H_2SO_4 + *A. niger*).

2.2.6 Fermentation of pretreated sample

treated sample that gave the highest yield of glucose was fermented using 1.09×10^6 yeast cell / 10 ml brewer's yeast (*Saccharomyces cerevisiae*). Nutrient supplements were added to the pretreated agroresidues to enhance the fermentation process. These supplements included: 0.1 g KH_2PO_4, 0.5 g $CaCl_2$, 0.05 g Mg SO_4, 0.1 g Na_2SO_4 and 0.1 g $(NH_4)_2$ SO_4. One hundred milliliters of the sample was transferred into 250 ml conical flask and 100 ml of distilled water was added. The pH was adjusted to between 4.5 -5.5. The inoculum was then added. Fermentation was allowed to proceed for 3 days at 30 °c.

2.2.7 Analytical methods

The recommended methods of the association of official analytical chemists were used for the determination of moisture [17], ash [18], crude lipid, crude fiber, and carbohydrate [19]. Amount of glucose in the sample was determined by dinitrosalicylic acid method as described by Miller [20]. Ethanol concentration was determined based on the density of the alcohol distillate at 20 °C and expressed as % v/v [21].

3. Results and discussion

3.1 Proximate analysis

Proximate analysis of the biomass show that non-fibrous carbohydrate had the highest percentage composition yielding a mean value of 36.60 % while crude fibre (fibrous carbohydrate) had a mean value of 35.59 % (Table 1), second only to non-fibrous carbohydrate. These results reveal that carbohydrate (fibrous and non fibrous) accounts for 72.19 % of the total biomass, other components (moisture, crude protein, crude lipid, and ash) accounting for 27.81 %. This results show that *Z. abyssinica* residues is a good source for both fermentable sugars (non-fibrous carbohydrates) and lignocellulose (fibrous carbohydrates). Hence, *Z. abyssinica* agroresidues is a potential feedstock for bioethanol production.

Table 1: Proximate composition of *Ziziphus abyssinica* agroresidues

Parameter	Amount (%)
Moisture	6.17 ± 0.0676
Crude protein	16.33 ± 0.0926
Crude fiber	35.59 ± 0.3706
Crude lipid	2.94 ± 0.0406
Ash	2.37 ± 0.0050
Carbohydrate	36.60 ± 0.0140

Values are means of three (3) replications ± S.D

3.2 Effect of pretreatment on amount of glucose

Analysis of the biomass for the presence of glucose revealed that pretreatment of the biomass with sulphuric acid (H_2SO_4) produced mean glucose value of 286.80 mg/g of biomass while multi-step pretreatment with sulphuric acid, sodium hydroxide, and *A. niger* (H_2SO_4 + NaOH + *A. niger*) had a mean glucose value of 285.79 mg/g of biomass (Table 2). Both of these pretreatment approaches were the most effective since they yielded the highest mean glucose value compared to other pretreatment methods used. However, considering chemical cost, and the fact these two pretreatment approaches produced statistically similar mean glucose value ($p > 0.05$) as shown in Table 2, H_2SO_4 pretreatment can be said to be more cost-effective than the multi-step pretreatment approach. It is pertinent to mention that the unpretreated biomass had the lowest mean glucose value (10.87 mg/g biomass) compared with the pretreated biomass (Table 2).

Table 2: Amount of glucose obtained from analysis of unpretreated, single-step and multi-step pretreated biomass.

Treatment	*Amount of glucose (mg/g of biomass)*
Unpretreated biomass (control)	$10.87^{h} \pm 0.811$
A. niger	$15.10^{g} \pm 1.000$
NaOH	$19.55^{f} \pm 0.829$
NaOH + *A. niger*	$27.68^{e} \pm 1.300$
H_2SO_4 + *A. niger*	$207.51^{d} \pm 1.809$
NaOH + H_2SO_4	$236.02^{c} \pm 2.301$
NaOH + H_2SO_4 + *A. niger*	$285.79^{a,b} \pm 3.518$
H_2SO_4	$286.80^{a} \pm 0.695$

Values are means of three (3) replications ± S.D, means with different letters are significantly different at 5% probability level with 'a' being the highest value and 'h' being the lowest

This show that pretreatment had a significant effect ($p < 0.05$) on the amount of glucose. Reasons being that, pretreatment results into solubilization and separation of one or more of the three main components of the lignocellulosic biomass (lignin, hemicellulose and cellulose) thereby, making the remaining solid biomass more accessible to further chemical or biological treatment [3]. Furthermore, pretreatment causes changes in chemical composition, including microscopic, macroscopic and submicroscopic changes in size and structure of lignocellulosic biomass with the resultant effect of increasing yields of fermentable sugars such as glucose [22]. Of the two classes of pretreatment approaches (biological and chemical) investigated, single-step pretreatment of the biomass with *A. niger* produced the least mean glucose value (15.10 mg/g of biomass) as shown in Table 2. This is because it is a biological pretreatment approach. Hence, it is mild relative to the chemical pretreatment approaches (NaOH, H_2SO_4, NaOH + H_2SO_4) investigated, which are harsh. The finding that single-step biological pretreatment is mild is further corroborated by results obtained when the approach was modified to multi-step pretreatment by carrying out NaOH + *A. niger*, and H_2SO_4 + *A. niger* which produced significant increase ($p < 0.05$) in mean glucose to 27.68 mg/g (83.31 % increase) and 207.51 mg/g (1,274.24 % increase) of biomass respectively (Table 2). Investigations on selective breakdown of lignin using cellulase-less mutant to prevent loss of cellulose showed that hydrolysis rate was low [23]. However, biological pretreatment is cost effective and safe [24][23]. Zhang *et al*. [25] reported the effectiveness of pretreatment of Bamboo culms with white rot fungi. *Phlebia* sp. MG-60 has also been successfully used for the pretreatment of sugarcane bagasse [26]. Singh *et al*. [27], showed that biological pretreatment of sugarcane trash resulted in delignification value of 92 %. We thought that H_2SO_4 + *A. niger* would produce higher glucose yield than single-step sulphuric acid pretreatment, however, results show otherwise which could be due the inhibitory effects of the degradation products of carbohydrates (such as formic acid, acetic acid, levulinic acid, furfural, 5-hydroxymethyl furfural, vanillin, syringlaldehyde, and 4-hydroxybenzaldehyde) on hydrolytic enzymes (cellulases, xylanases, hemicellulases) produced by *A. niger*. Vanillic acid, syringlaldehyde, and formic acid, cause significant inhibition of xylanases whereas cellulases are found to be significantly inhibited by formic acid [28][29]. Results show that NaOH pretreatment produced less glucose (19.55 mg/g biomass) than H_2SO_4 pretreatment (Table 2). This observation could be due to variation in mechanism of action of these chemicals, while NaOH acts mainly on lignin (delignification) and to a less extent hemicellulose, H_2SO_4 attacks mainly hemicellulose and to a less extent cellulose. More so, H_2SO_4 can significantly hydrolyze cellulose and hemicellulose. Hence can be used as the actual method of hydrolysis of lignocellulose to fermentable sugars [30]. Thus, NaOH pretreatment can be said to be somewhat mild relative to H_2SO_4 pretreatment. Results also show that NaOH + H_2SO_4 pretreatment yielded less glucose compared to H_2SO_4 pretreatment (Table 2). This observation could be due to interplay of various parameters which affect pretreatment of lignocellulosic biomass. NaOH pretreatment could have increased the crystallinity of cellulose microfibrils and consequently, decreased the amenability of the resulting biomass to sulphuric acid (H_2SO_4) hydrolysis. This also explains the reason for having mean glucose for NaOH + H_2SO_4 + *A. niger* pretreatment not significantly higher ($p > 0.05$) than single-step H_2SO_4 pretreatment. Kim and Holtzapple [31] reported that delignification of Corn stover with calcium hydroxide increased its crystallinity form 43 % to 60 %. Fan *et al*. [32] observed a positive correlation between crystallinity of cellulose and its enzymatic hydrolysis. NaOH + H_2SO_4 pretreatment involved partial drying of the resulting biomass after the first step (NaOH pretreatment) this could have adversely affected the capillary structure of cellulosic fibers resulting into decrease in accessible surface area. The accessible surface area of lignocellulose and its interaction with the hydrolyzing agent (chemical or enzyme) significantly affects effectiveness of hydrolysis [24][32]. Fan *et al*. [32] also reported that drying of fibers can cause irreversible collapse and shrinking of capillary consequently, reducing the accessible surface area. This Sequential pretreatment approach (NaOH + H_2SO_4) involved sequential wetting, partial drying and wetting again of the biomass which can magnify the effect of wetting on the crystallinity of cellulose microfibrils. Fan *et al*. [32] reported that wetting increases crystallinity of cellulose due to recrystallization of highly amorphous cellulose.

3.3 Fermentation under optimized condition

The optimized pretreatment approach (sulphuric acid pretreatment) was employed prior to fermentation with *Saccharomyces cerevisiae*. Bioethanol concentration of 4.5133 % (v/v) was obtained after fermentation. This shows the potential of *Ziziphus abyssinica* agroresidues as a feedstock for bioethanol production. Akin-Osanaiye *et al*. [33] obtained similar results when *Carica papaya* agro-waste was fermented using *S. cerevisiae*.

4. Conclusion

This research has demonstrated an approach for ethanol production from *Z. abyssinica* agroresidues. Investigations on the effect of pretreatment approach on the amount of glucose released from the lignocellulosic biomass showed that sulphuric acid hydrolysis had the most significant effect. Thus is recommended for pretreatment of *Z. abyssinica* agro residues, prior to fermentation. The use of *Z. abyssinica* agro residues for bioethanol production will not only provide another alternative feedstock but will also help in reducing the negative environmental impacts this agricultural waste.

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6. Future Studies

Future studies would focus on the use of a more robust and efficient fermenter such as *Geobacillus* as a means of investigating the feasibility of commercializing bioethanol production from *Z. abyssinica.*

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