Sodium Bicarbonate Pretreatment, Enzymatic Saccharification and Fermentation of Mesquite (Prosopis chilensis) for Bioethanol Production

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Abstract—This study aimed to assess the use of mesquite tree pretreated as biomass for the growth of Saccharomyces cerevisiae and bioethanol production using anaerobic fermentation condition, in a pH-controlled; 500 ml shake flask cultivations with a working volume of 350 ml under anaerobic condition at pH 6.5, 35 °C. The effects of Sodium bicarbonate doses (25, 50, 75, and 100) on the pretreatment of mesquite for 10, 20, 40, and 60 min at 100 °C were evaluated. The yields of glucose as well as total sugars increased with increasing of Sodium bicarbonate concentration. No furfural and hydroxymethy furfural were detected in any of the Sodium bicarbonate-pretreated mesquite tree hydrolyzates, pH 5.0 and Temperature 35 °C are optimal for saccharification of Sodium bicarbonate-pretreated mesquite tree by the commercial enzyme Saccharomyces cerevisiae. The concentration of ethanol was 8.5 g/l with a yield of 0.40 g/g of available sugars in 30 h by Separate hydrolysis and fermentation (SHF). According to these results, the mesquite tree could be known as an alternative substrate to be used for biotechnological purposes, mainly for ethanol production.

Index terms: Ethanol, Mesquite tree, Saccharomyces cerevisiae.

I. INTRODUCTION

Currently, the world’s energy demand is focused on the use of fossil fuels, which are inevitably depleting [1, 2]. Fossil fuels have the big disadvantage that; they are depleting source of energy and some of them have high sulfur content, nitrogen and metal and its burning results in extensive amount of SO₂ and NOx emissions to the atmosphere, which is causing global warming and resultant changes in our climate. Additionally, CO₂ is released which is considered to have undesirable climatic consequences [3]. Energy consumption is growing at rising rates at the same time, leading to increased interest of renewable alternatives to fossil-based fuels. Bioethanol are promising CO₂ neutral types of biofuels since they are derived from renewable sources [4, 5]; bioethanol, at the expense of fossil fuels, contribute in a meaningful way to reducing greenhouse gases (GHG) emissions; This is because the biomass feedstock’s employed fix carbon dioxide photosynthetically during their growth and this leads to significant reductions in CO₂-equivalent GHG emissions compared to oil and gas combustion [6]. The primary biomass feedstock’s for the current ethanol industry have been corn grain glucose (In the United States) and sugar cane sucrose (in Brazil) [7]; but any country with a significant agronomic-based economy can use current technology for fuel ethanol production [8-10]. Mesquite trees are leguminous plants trees that are widespread in arid and semi-arid zones of the world [11]. Mesquite trees, a complex lignocellulosic material with high lignin (11-28%) and extractives content (3-15%), contain (40-45%) cellulose and (25-30%) hemicelluloses [12]; and have a great potential to serve as a low-cost feedstock for production of bioethanol. The total area covered by Mesquite in Sudan was estimate at 1,551,000 hectares, while in Eastern Sudan alone (Gash, Tokar, Red Sea and Hafla) in year 2005; Mesquite coverage was estimate to be about 1.4 million hectares [13]. Utilization of mesquite trees for bioethanol production greatly decreases economical and environmental cost of mesquite control. Considering the high amount of carbohydrates present in mesquite leaves [14] and their high production in different countries, they could arise as a feasible alternative source for bioethanol production;
The objective of this work was to develop methods for cost effective pretreatment and enzymatic saccharification of mesquite tree cellulose and hemicellulose into fermentable sugars, and fermentation of the hydrolyzate to ethanol. In previous studies, dilute acid pretreatment, enzymatic saccharification and fermentation of mesquite to ethanol was evaluated, [15]. Mesquite can be enzymatically saccharified to sugars with a maximum yield of 70% after dilute acid pretreatment under optimized conditions. More than 50% of the cellulose in the dilute acid-pretreated mesquite remained unhydrolyzed by enzymes. Moreover, inhibitors were also produced during dilute acid pretreatment which needed to be removed by over liming before fermentation.

Recently, we have shown that alkaline peroxide pretreatment under optimized conditions released about 90% of the sugars present in mesquite [16]. However, alkaline peroxide pretreatment of mesquite under the conditions used is not cost effective. Sodium bicarbonate offers certain advantages such as: inexpensive (0.09 $/kg), safe to handle and can be recovered easily. In the present study, the efficiency of Sodium bicarbonate as pretreatment option, enzymatic saccharification and fermentation of Sodium bicarbonate-pretreated mesquite were examined. The production of ethanol from any lignocellulosic biomass generally involves four process steps—feedstock pretreatment, enzymatic saccharification, fermentation, and ethanol recovery [17]. In order to reduce the cost of ethanol production from lignocellulosic biomass, integration of these process steps is essential. Moreover, during enzymatic saccharification, the cellulases and hemicellulases are severely inhibited by their own products (sugars) [18]. In order to relieve the product inhibition, simultaneous saccharification and fermentation (SSF) of the pretreated hydrolyzate is preferred where the fermentative microorganism would convert the sugars into ethanol as soon as they are formed. However, the optimal conditions (mainly pH and temperature) for enzymatic saccharification and fermentation are different [19]. In this study mesquite tree was assessed as biomass for the growth of *Saccharomyces cerevisiae* and also for production of ethanol by anaerobic fermentation. Moreover, an analysis of main effects was carried out in order to identify the best conditions for the ethanol production regarding the alkaline pretreatment (Sodium Bicarbonate), condition of fermentation and the time of fermentation of mesquite for bioethanol production.

II. MATERIAL AND METHODS

*Raw material collection and preparation*

Mesquite tree (branches) were collected from the vicinity of houses in Alshagara area; Sudan. Leaves removed, cleaned, dried at 45°C for 48 hours in Oven, then raw materials (branches) were ground with a hammer mill to a particle size between 1 mm and 5 mm and dried overnight in oven at 50 °C, and kept in polyethylene pages for subsequent analysis, The ground material had a dry matter (DM) content of 44.4%.

**Pretreatment Method**

Production of bioethanol from mesquite tree is done by pretreating the mesquite and then hydrolyzing it and finally fermenting it. The pretreatment is necessary because the cellulose, whose glucose building blocks are used in the fermentation, is well shielded by a matrix built up by the hemicellulose and lignin.

The ground material (200 g of mesquite powder) was pretreated by addition of 600 ml of sodium bicarbonate (NaHCO$_3$) solution (7.5%/w/v). The mixture was stirred manually during the addition of sodium bicarbonate and heated (100°C) for 30 min; then mechanical stirring was used to mix the contents of the reactor. The reactor was heated to the desired temperature with a water circulation thermostat (C6CS, Lauda). A homogeneous mixture formed, and kept for 3hr at room temperature, and then diluted with 625 ml of distilled water; Samples from the liquid phase were taken for analysis. Afterwards, the remaining solids were separated from the solution using a pressure filter. Samples were taken from the filtered solutions to see if any monosaccharide losses occurred during the filtering.

**Simultaneous Saccharification and Fermentation (SSF)**

After the pretreatment the glucose molecules are still imprisoned in long chains of cellulose and hemicellulose and therefore not readily available for fermentation. This is why hydrolysis is necessary. The enzymatic saccharification of the sodium bicarbonate-pretreated mesquite tree was performed by shaking gently (100 rpm) at 45 °C after adjusting the pH to 5.0 with HCl and adding a commercial *Saccharomyces cerevisiae* enzyme preparation at dose of 0.05ml/g of mesquite tree for 72 h. Samples (1 ml) were withdrawn and kept at -20 °C until analyzed.
**Experimental Procedure**

Separate hydrolysis and fermentation (SHF) was performed in anaerobic shake flask cultivations. The fermentability of ethanol production and capability of the yeasts were investigated. The yeasts were supplied gently (by the Microorganisms Collection, Department of Antibiotics, International University of Africa, Khartoum–Sudan) capable of natural xylose utilization.

The fermentation was performed in a pH-controlled 500 ml shake flask cultivations with a working volume of 350 ml under anaerobic condition at pH 6.5, 35 °C [20]. The liquid portion of the pretreated mesquite hydrolyzate after separating it from the solids by filtration over glass fiber filter (1.0–1.5 mm pore size, 75mm diameter) was used as substrate. The medium was prepared by dissolving 10 g tryptone and 5 g yeast extract in 1 l hydrolyzate and autoclaving at 100 °C for 15 min. The pH was controlled at 6.5 using 4M KOH. Samples were withdrawn periodically to determine cell density, ethanol, organic acids and residual sugars and stored at 20 °C prior to analysis.

During the fermentation experiments, the amount of ethanol produced was determined by measuring the weight loss of the fermentation flasks. The weight loss is caused by CO₂ release, and the mole amount of released CO₂ equals the mole amount of ethanol produced. The weight loss was measured daily. In addition, the ethanol production was analysed with HPLC using an Aminex HPX-87H column at 55 °C, with 20 μL injection volume, with 2.5 mmol/l H₂SO₄ as an eluent, and with a flow rate of 0.3 ml/min. HPLC samples were centrifuged before the analyses. Cell growth during the fermentation experiments was followed optically by spectrophotometer (UV-1201 UV-VIS, Shimadzu) at 600 nm. The quantitative analysis of carbohydrates (rhamnose, arabinose, galactose, glucose, mannose and xylose) was carried out with high performance anion exchange chromatography (Dionex ICS-3000, Dionex) with a pulse amperometric detector (HPAECPAD) using a CarboPac PA-1 (Dionex) column at 30 °C. Purified water was used as eluent [20].

**Analysis**

The HPLC machines fitted with an Aminex HPX-87P column (Bio-Rad, Hercules, CA, USA) were used for analyses of the liquid samples. The machine was calibrated to be able to detect the following compounds: Cellobiose, xylose, galactose, arabinose, mannose, lactic acid, glycerol, hydroxymethylfurfural (HMF), acetic acid and furfural. From monosaccharides, only glucose and xylose can be analysed with HPX-87H column because other monosaccharides elute under xylose peak.

### Results and Discussion

**Raw Material**

Table 1 shows the composition of the raw and pretreated materials. These values are susceptible to variation caused by genetic and environmental variability of the raw material. The composition of the pretreated Mesquite varies with pretreatment method and conditions. The amount of Glucan and Lignin showed no significant difference (p > 0.05) as the amount of mesquite ground was 30 g/100 ml or more. Xylan, Galactan, Mannan, and Arabinan shows reduction pattern, glucose and mannose shows increasing due to hydrolysis of cellulose. It suggests a possible saturation of these compounds (resulting in glucose and mannose found in pretreated materials due to hydrolysis of cellulose in the raw materials).

**Table 1.** The raw material and pretreated mesquite dry material compositions presented in weight percentage

<table>
<thead>
<tr>
<th>Element</th>
<th>Raw Material (wt, %)</th>
<th>Pretreated mesquite (wt, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>40.5</td>
<td>48.5</td>
</tr>
<tr>
<td>Xylan</td>
<td>5.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Galactan</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Mannan</td>
<td>10.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Arabinan</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Lignin</td>
<td>28.5</td>
<td>33.6</td>
</tr>
<tr>
<td>Liquid Fraction (g/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>30.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
<td>22.5</td>
</tr>
</tbody>
</table>
**Effects of Sodium bicarbonate doses on the pretreatment of mesquite**

Initially, the effects of Sodium bicarbonate doses (25, 50, 75, 100 mg/g mesquite tree) on the pretreatment of mesquite for 10, 20, 40, and 60 min at 100 °C were evaluated. The resultant yield of glucose and total sugars in terms of mg/g mesquite tree after enzymatic saccharification using the commercial *Saccharomyces cerevisiae* enzyme preparations at a dose level of 0.05 ml at 45 °C and pH 5.0 for 72h is shown in Figure 1 and 2, respectively. The yields of glucose as well as total sugars increased with increasing of Sodium bicarbonate concentration for pretreatment. There was not much difference between 40 min and 1h pretreatments. The yield of total sugars was increased from 80±1 to 110±1mg (27% increase) with the increase of Sodium bicarbonate dose from 75 mg to 100 mg/g of mesquite tree for 1h pretreatment (Figure 1). Using the same Sodium bicarbonate dose (100mg/g of mesquite tree), the yield of glucose increased from 70±1 to 80±1mg by increasing the pretreatment time from 10 min to 1 h (Figure 2).

The maximum yield of total sugars (110±1mg/g mesquite tree; glucose, 80±1 mg; xylose, 20±0 mg; arabinose, 40±0 mg) was achieved at 100 mg Sodium bicarbonate per gram mesquite tree and 1h pretreatment time. Thus, it was decided to use 100 mg Sodium bicarbonate per gram mesquite tree and pretreatment time of 1h for subsequent studies. No furfural or HMF (detectable limit, 1 mg/ml) was detected in any of the Sodium bicarbonate -pretreated mesquite tree hydrolyzates. No galactose (detectable limit, 25 mg/ml by HPLC) was detected in any of the Sodium bicarbonate pretreated hydrolyzates even though acid pretreatment released 16 mg galactose/g of mesquite tree [21].
Thus Sodium bicarbonate pretreatment aided in 2.4-fold increase in the saccharification of mesquite tree by enzymes over the control (without Sodium bicarbonate).

**Effect of pH and temperature on enzymatic saccharification of Sodium bicarbonate-pretreated mesquite tree**

The effects of pH (2.5–7.5) and temperature (25–70 °C) on the enzymatic hydrolysis of Sodium bicarbonate-pretreated mesquite tree using the commercial enzyme *Saccharomyces cerevisiae* at dose level of 0.05ml/g substrate were investigated. Figures 3 and 4 show the release of glucose, xylose, and total sugars at 72h for various pH and temperatures, respectively. The commercial enzyme worked well over a pH range of 4.0–5.5 with an optimum pH of 5.0 for the release of all sugars (Figure 3). The relative sugar yields at pH 6.0 and 6.5 were 80% and 60% of the maximum level observed at pH 5.0, and the commercial enzyme worked better at the lower pH side of optimum pH 5.0 than at the higher pH side. With regard to temperature, the commercial enzyme worked optimally at 35 °C (Figure 4). The yields of total sugars at 40, 45 and 50 °C were 110%, 100% and 80% of that at 35 °C, respectively. Thus, pH 5.0 and 35 °C are optimal for saccharification of Sodium bicarbonate-pretreated mesquite tree by the commercial enzyme *Saccharomyces cerevisiae*.

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**3.4 Separate hydrolysis and fermentation (SHF) of Sodium bicarbonate-pretreated mesquite tree**

The time course of ethanol production by the *Saccharomyces cerevisiae* from Sodium bicarbonate-pretreated enzymatically hydrolyzed mesquite tree is shown in Figure 5. The concentration of ethanol from mesquite tree hydrolyzate by the bacterium was 8.5 g/l with a yield of 0.40 g/g of available sugars in 30 h by Separate hydrolysis and fermentation (SHF). Small amounts of acetic and succinic acids were produced by bacterium as by-products in addition to ethanol. The cell density (A660nm) reached a maximum of 5.6 in 30h after which it declined slightly to 5.0 in 36 h. There is little growth (A660nm, 0.33) of *Saccharomyces cerevisiae* in the control medium where water was substituted for the hydrolyzate. No detectable ethanol, succinic acid or acetic acid was found to be produced in the control medium by the bacterium. Regarding the mixed sugar utilization by the bacterium, glucose was utilized first (Figure 5). Xylose utilization only began after almost all the glucose was consumed. Similar patterns of mixed sugar utilization were also observed.
in the cases of fermentation of both dilute acid and
alkaline peroxy-pretreated enzymatically
saccharified mesquite tree hydrolyzates by the
Saccharomyces cerevisiae bacterium [22, 23].

![Image](image.png)

**Fig. 5:** Time course of ethanol production by the *Saccharomyces cerevisiae* from Sodium bicarbonate -pretreated enzymatically hydrolyzed mesquite tree hydrolyzate at pH 6.5 and 35 °C.

**Simultaneous saccharification and fermentation (SSF) of Sodium bicarbonate -pretreated mesquite tree**

For SSF, the concentration of ethanol was 10.5 g/l in 42h. There was no accumulation of glucose during the time period. However, there was slight accumulation of xylose during the initial phase of the SSF. These sugars disappeared slowly. Considering the time required for separate enzymatic hydrolysis (48 h) and fermentation (17 h), the total time of conversion of pretreated mesquite tree to ethanol by SHF was 65 h, whereas for the SSF the total time was 48h (Table 2). In this respect, SSF worked much better than SHF. Table 2 shows the results of both SHF and SSF pretreated mesquite tree hydrolyzate by *Saccharomyces cerevisiae*.

**Table 2.** Ethanol production from Sodium bicarbonate -pretreated mesquite tree hydrolyzate by *Saccharomyces cerevisiae*.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Separate hydrolysis and fermentation (SHF)</th>
<th>Simultaneous saccharification and fermentation (SSF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation time (h)</td>
<td>65</td>
<td>48</td>
</tr>
<tr>
<td>Total sugars (g/l)</td>
<td>16.5</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol (g/l)</td>
<td>9.1</td>
<td>12.3</td>
</tr>
<tr>
<td>Ethanol (g/g sugar)</td>
<td>0.43</td>
<td>-</td>
</tr>
</tbody>
</table>

**IV. CONCLUSIONS**

The feasibility of lignocellulosic bioethanol production using Sodium bicarbonate pretreatment with Enzymatic Saccharification and Fermentation was studied experimentally using authentic hydrolysates. Mesquite tree was used as raw materials. The fermentation was performed in pH-controlled 500 ml shake flask cultivations with a working volume of 350 ml under anaerobic condition at pH 6.5, 35 °C. Initially, the effects of Sodium bicarbonate doses (25, 50, 75, 100 mg/g mesquite tree) on the pretreatment of mesquite for 10, 20, 40, and 60 min at 100 °C were evaluated. The yields of glucose as well as total sugars increased with increasing of Sodium bicarbonate concentration for pretreatment. No furfural was detected in any of the Sodium bicarbonate-pretreated mesquite tree hydrolyzates. pH 5.0 and 35 °C
are optimal for saccharification of Sodium bicarbonate -pretreated mesquite tree by the commercial enzyme *Saccharomyces cerevisiae*. The concentration of ethanol from mesquite tree hydrolyzate was 8.5 g/l with a yield of 0.40 g/g of available sugars in 30 h by Separate hydrolysis and fermentation (SHF). No detectable ethanol, succinic acid or acetic acid was found to be produced in the control medium by the bacterium. The SHF worked better than the SSF method with respect to fermentation time. Attempts will be made to study the feasibility of using other inexpensive methods for pretreatment of mesquite tree.

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V. REFERENCES


