Enzymatic Hydrolysis of Agave Salmiana Cellulose

Leonardo Chávez-Guerrero\textsuperscript{1*}, Julio Silva-Mendoza\textsuperscript{2}, María E. Cantú-Cárdenas\textsuperscript{2} and Selene Sepúlveda-Guzmán\textsuperscript{1}

\textsuperscript{1} Electrical and Mechanical Department, CIIDIT, University of Nuevo Leon, Mexico.
\textsuperscript{2} Chemistry Department, University of Nuevo Leon, Mexico.

Abstract. Cellulose is the most abundant biopolymer on earth, since its crystallinity is a key factor to the enzymatic efficiency to produce glucose, in the present work cellulose morphology was studied. One sample is mostly amorphous and the other semicrystalline in order to evaluate influence of structure on the glucose production by enzymatic hydrolysis. Cellulase from \textit{Aspergillus niger} at 28 °C was used to produce glucose at 4 different times. Also, present sugars before hydrolysis and the impact of low lignin/hemicellulose amount on agave were analyzed. Best results were found after 60 min under enzymatic hydrolysis, showing a production of 3.5 mg/ml and 1.5 mg/ml of glucose for agave cellulose (AC) and microcrystalline cellulose (CMC) respectively. Raman spectra show a crystallinity of 29.5% and 48.3% in the case of the AC and CMC respectively, showing a direct correlation between crystallinity and glucose production.

Keywords: bagasse, reducing sugar, renewable energy, Raman crystallinity.

1. Introduction

The introduction of alternative sources of energy to substitute fossil fuels are of high importance, thus in an attempt to reduce the CO\textsubscript{x} and SO\textsubscript{x} in the atmosphere, which have important effects on global warming and acid rain [1]. Another reason is that fossil fuels are limited, and then it is necessary to develop options of renewable energy while the use of fossil fuels is still economically viable. Energy crops can be used to produce biomass and ethanol, which have a very important participation in the supply of clean energy [2]. Sugarcane and corn are crops commonly used to produce bioethanol, but they are in the human food chain, and require an important amount of cultivable land and water [3]. Bioethanol is produced by microorganisms using the present free sugar in the process, where sugars are mainly produce by enzymatic hydrolysis of starch or cellullosic biomass (trees, agricultural byproducts and algae). Biomass harvest from semi-arid lands is very promising, leaving cultivable lands for food production. The xerophyte plants like \textit{Agave americana} or \textit{Agave tequilana}, growing in semi-arid lands, have been used as a source of food and fibers by ancient humans for the last seven thousand years [4]-[8]. More recently (XVIII century), energy and spirit beverages like Tequila and Mezcal have been obtained, with alcohols as main component. Agave juices contain several types of sugar such as, inulin, sucrose, glucose, galactose and fructose [9]. It is known that 8 to 15 kg of agave are needed to produce one liter of Tequila. The making of Tequila produces a byproduct or waste (bagasse) representing about 40% (w/w) of the total weight of agave used, also vinasse and CO\textsubscript{2} as byproducts [7], plus all the leaves disposed at the field during harvest. Recently, several applications have been developed to take benefit of the agave such as, fuel [10], papermaking [11], biofuels production [9], renewable source of calcium compounds such as CaCO\textsubscript{3} and Ca(OH)\textsubscript{2} [12], [13]. Using coniferous, eucalyptus and deciduous trees as source of cellulose, enzymatic hydrolysis efficiency has been studied. Also, commercial microcrystalline cellulose was used to obtain glucose, analyzing the influence of crystallinity and the relationship between amorphous and crystalline regions present inside the fibres [14], showing that

\* Corresponding author. Tel.: + (81) 13404000, ext. 1531
\textit{E-mail address:} guerreroleo@hotmail.com
pre-treated eucalyptus gives the best results with 10.5 mmol of glucose per liter, indicating the effects of the hemicellulose and lignin present in the wood. In another research, agave leaves were used to produce biofuels [9], showing the low recalcitrance of this plant and the presence of different sugars even before hydrolysis. There are important quantities of arid and semi-arid lands in Mexico, South America, Africa and Australia, where every year these amounts are increasing because of the desertification or due the agricultural lands abandoned by farmers. These lands are able to produce fuels, fibers, food, chemicals and other products, and then plants that grow in those environments (xerophytes) can be used as cellulose suppliers to produce renewable energy. Agave leaves are cover by a transparent cuticle which is the external waxy layer made of cutin, then a thick layer of chlorophyll which gives the green colour to the agave. This structure was “designed” in order to protect the cellulose (68.4 wt%) and the matrix (water, sugar and other components) inside the leaves, that is why the agave matrix does not have an important amount of lignin (only 4.9 wt%), instead leaves have about 20 mg/l of sugars (glucose, fructose and inulin), which make them easier to use as a source of cellulose to produce glucose. It has been reported that ethanol yield using agave is up to 3273 gallons/(hectare year), compared with 1273 gallons/(hectare year) for poplar and 1403 gallons/(hectare year) for grass, which makes agave a great option as a source of biomass to produce ethanol [9].

The purpose of this research is to explore a fraction of the agave leaves as a source of cellulose, which is highly amorphous, improving the conversion of cellulose into sugar by enzymatic hydrolysis. Raman characterization helps to understand the influence of the cellulose crystallinity on the glucose production.

2. Materials and Methods

2.1. Cellulose Source

Cellulose microcrystalline was obtained from sigma Aldrich and used as received, without further purification.

2.2. Agave Cellulose

Agave leaves were cut from the plant and the skin was removed. Then samples of 10 cm$^3$ were cut from the leaves and freeze dried for 24 hrs. Then the fibre is separated from the matrix by mechanical pulping.

2.3. Cellulase

Cellulase from Aspergillus niger was obtained from Sigma-Aldrich.

2.4. DNS Solution

Solution 1.- 2g NaOH was dissolved in 50 ml distilled water, later 2g of 3,5 dinitrosalicylic acid was added and mixed.

Solution 2.- 40 g sodium potassium tartrate was dissolved in 100 ml distilled water.

Both solutions were mixed and diluted to a final volume of 200 ml using distilled water.

2.5. Glucose Calibration Curve

A glucose stock solution was prepared at 1% (w/v). Standards at different concentrations (0.1 - 5 mg/ml) were prepared from the stock.

200 µl of each standard were mixed with 200 µl DNS solution, the mix was boiled for 5 minutes. 5 ml of cold distilled water was added in order to stop the reaction. Absorbance was obtained at 540 nm using a spectrophotometer Spectronic 20 Genesys. A mix of distilled water and DNS solution was used as blank.

2.6. Enzymatic Hydrolysis Assay

Cellulose dispersion was prepared in 1 wt% in distilled water. In test tubes 950 µl of buffer with 0.1 M solution of citrate-phosphate at pH 6 were added to 50 µl of cellulase at 10 mg/ml (8 U/ml), then 1 ml cellulose dispersion was added and mixed. Tubes were incubated at 28°C at times of 0, 15, 30, 45 and 60 minutes. After incubation, 3 ml of DNS solution were added and immediately the test tubes were placed on ice in order to end the enzymatic reaction, then the test tubes were boiled for 5 minutes. 1/10 dilutions were
made before reading the absorbance at 540 nm. Tests were carried out by triplicate. Commercial microcrystalline cellulose (CMC) was used as reference in order to compare amount of glucose released.

2.7. Raman Spectroscopy

Raman spectroscopy measurements were recorded at room temperature using a Thermo Scientific DXR Raman microscope using a 532 nm laser excitation. The spectral data were scanned for the acquisition of up to 30 accumulations and 30 s laser exposure time.

2.8. Scanning Electron Microscope (SEM) and Confocal Microscope (LSM)

The morphology of the samples were determined using a HITACHI UHR FE-SEM SU8020 with acceleration voltage of 10 kV with spot size of 3 and working distance of 5 mm. Samples were glued with an adhesive carbon tape to an aluminum holder. A laser scanning microscope (LSM) was used to obtain height profiles with a ZEISS LSM 700, in order to study the samples topography.

3. Results and Discussion

3.1. Enzymatic Hydrolysis assay

Fig. 1 shows coloring changes in test tubes at the different times. With this test, the production of reducing sugars can be detected easily, where a solution with a darker coloration, like the one AC at 60 min depicted in Figure 1b, will present a higher production of reducing sugars, compared with the CMC under similar conditions, Figure 1a.

![Fig. 1: Enzymatic hydrolysis of a) microcrystalline cellulose and b) Agave cellulose.](image)

Fig. 2 shows the amount reducing sugars obtained at different times, indicating a higher liberation of glucose using agave cellulose compared to the commercial microcrystalline cellulose. Even if the initial sugars (2.5 mg/ml in AC) were not included in the chart, as seen in AC 2, the production of glucose by AC (3.5 mg/ml) was more than two times higher than the CMC (1.5 mg/ml), reaching a 46% and a 15.5% of conversion by AC and CMC respectively. The efficiency of cellulose breakdown was increased due to the cellulose morphology, obtained in a very simple way, without any chemical treatment.

3.2. Raman Analysis

Raman spectra for all samples are shown in Fig. 2, which depicts the effect of the cellulose type, allowing measurement of the cellulose crystallinity. The peak from 3280 cm\(^{-1}\) to 3400 cm\(^{-1}\), corresponds to the intramolecular OH stretching, while the peak at 2890 cm\(^{-1}\) is related to a CH and CH\(_2\) stretching. The region between 1800-700 cm\(^{-1}\) is the cellulose fingerprint region [15], where the band found around 1621 cm\(^{-1}\) corresponds to the lignin fraction [16], which is present only in AC, because in CMC the pretreatment eliminated those components, which are only removed under acid hydrolysis.
The peak at 896 cm$^{-1}$ is for β-glycosidic link in cellulose, which appears weaker for CMC due to the acid treatment of wood to obtain the cellulose. The relative band intensity at 1121 cm$^{-1}$ is due the glycosidic linkage, where changes are consequence of the hydrogen bonding pattern in the cellulose chains. The band at 1121 cm$^{-1}$ is shared by non-cellulosic carbohydrates and lignin, which is coupled with the glucopyranose rings at 1096 cm$^{-1}$, then the relative intensity between those bands is useful to determine delignification [17], where the delignification is higher for CMC, because the cellulosic contribution at 1096 cm$^{-1}$ is more important than the non-cellulosic depicted at 1121 cm$^{-1}$.

The stretching vibration of lignin is located around 1621 cm$^{-1}$, which is revealed by the Raman Spectra for AC but not for CMC. The intensity registered at the band located at $\approx$ 380 cm$^{-1}$ (inset in Figure 3) represents the order of crystallinity [18], [19]. The crystallinity (Cr Raman) was determined using the equation 1 and the data at Table 1, which gives a crystallinity of 48.31 for CMC and 29.54 for AC.

<table>
<thead>
<tr>
<th></th>
<th>Intensity*</th>
<th>Percentage</th>
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<tbody>
<tr>
<td></td>
<td>1096 cm$^{-1}$</td>
<td>380 cm$^{-1}$</td>
</tr>
<tr>
<td>CMC</td>
<td>300.06</td>
<td>102.79</td>
</tr>
<tr>
<td>AC</td>
<td>23.34</td>
<td>5.15</td>
</tr>
</tbody>
</table>

*Intensity expressed in arbitrary units
Bands found at 1490 cm$^{-1}$ and 1463 cm$^{-1}$ correspond with the calcium oxalate monohydrate [20], which is present in the raw material (AC).

### 3.3. Cellulose Morphology

Superficial area is another factor to take into account, which can be inferred by the images obtained by SEM in Figure 4b. The CMC depicted in Figure 4c shows part of a fiber and other small particles product of the acid treatment. Figure 6c indicates a profile with the MCM dimensions, having a very heterogeneous material, in this case it is clear that the available area in contact with the enzyme will be limited, and then a slow degradation will be produced, as seen previously in Figure 3. While in Figure 5d the AC morphology is totally different, which consist of a small fibers (3 µm) joining together by platelets, as seen in the profile of the Figure 4d. Here the non-cellulosic materials can be appreciated (top left), which have a different structure. Even if hemicellulose avoids contact between the cellulosic material and the enzyme [21], the results indicate that is not an important factor for this material obtained by this specific procedure, which has not been reported before.

![Fig. 4: Separated material of agave on its components of matrix and fibre (a). SEM image showing the microstructure of the matrix and the fibre (b). Height profile of CMC individual fibre and AC in (c) and (d) respectively. Scale bar of 10µm in (c) and (d).](image)

Profile of the CMC fiber indicates a length of 110 µm, a width of 33 µm and 14 µm height (c), while agave microfibers have 3 µm width and 1 µm high. Another important result is the lack of chemical pretreatment in the case of AC, which gives an important advantage compared with other lignocellulosic materials commonly used to produce glucose.

### 3.4. Experimental Conditions

Table 2 shows representative procedures and conditions where agave leaves show the lowest time and temperature to get a conversion of about 40%.

AC requires only one hour to get a 46% of hydrolysis, producing two times more glucose than commercial cellulose under same conditions. Wood as a raw material must be chopped in small pieces by
mechanical milling and have a chemical pre-treatment in order to obtain cellulose powder (Avicel), while other sources and by-products [22]-[24], must be pre-treated to take non-cellulosic materials and the colour given by lignin (rice straw, reed and wood) which may interfere with the cellulase. In the case of the agave matrix, chemical pre-treatment was not necessary because this material is whiter than other materials as seen in Figure 4a, mainly because to its low amount of lignin. When lyophilisation is used to dry the agave leaves, the material becomes brittle and is easy to separate the fibre/matrix system (Figure 4a), even by hand, avoiding the usual alkaline/hydrogen peroxide extraction and the consequent production wastewater, making the procedure described in this research a greener process.

Table 2: Comparative analysis for different pretreatments and hydrolysis percentage in a period of time.

<table>
<thead>
<tr>
<th>Cellulose source</th>
<th>Enzyme</th>
<th>Pretreatment</th>
<th>Conditions</th>
<th>Hydrolysis (%) /time (hrs)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eucalyptus Coniferous</td>
<td>Cellulase from <em>Aspergillus sp.</em></td>
<td>Mechanical milling and phosphoric acid.</td>
<td>45°C for 24 hrs.</td>
<td>93/24 ≈ 3.8 82/24 ≈ 3.4 85/24 ≈3.5</td>
<td>14</td>
</tr>
<tr>
<td>Coniferous Deciduous</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reed</td>
<td>Cellulase from <em>Trichoderma longibrachiatum</em></td>
<td>Milled and pretreated with liquid hot water (180° C)</td>
<td>50°C, pH 4.8 for 72 hrs</td>
<td>82/72 ≈ 1.1</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Cellulase from <em>Trichoderma longibrachiatum</em></td>
<td>Milled and pretreated with liquid hot water (180°)</td>
<td>50°C, pH 4.8 for 72 hrs</td>
<td>56/72 ≈ 0.8</td>
<td></td>
</tr>
<tr>
<td>Avicel PH101</td>
<td>Glucose-tolerant β-glucosidase from <em>T. thermosaccharolyticum</em></td>
<td>Cellulose was not pretreated</td>
<td>50°C, pH 5.2 for 48 hrs</td>
<td>58/48 ≈ 1.2</td>
<td>23</td>
</tr>
<tr>
<td>Rice straw</td>
<td>Crude cellulase from <em>T. harzianum</em> SNRS3</td>
<td>2% (w/v) NaOH</td>
<td>50°C for 96 hrs</td>
<td>60/96 ≈ 0.63</td>
<td>24</td>
</tr>
<tr>
<td>Agave leaves</td>
<td>Cellulase from <em>Aspergillus niger</em></td>
<td>Raw material was not pretreated after extraction</td>
<td>28°C, pH 6 for 1 hour.</td>
<td>46/1 ≈ 46</td>
<td>Present work</td>
</tr>
</tbody>
</table>

4. Acknowledgements
To PAICYT, FCQ and FIME-CIIDIT-UANL.

5. References


