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Second Generation Ethanol Production Using Subcritical Water Hydrolysis on Sugarcane Bagasse and Straw

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Second Generation Ethanol Production Using Subcritical Water Hydrolysis on Sugarcane Bagasse and Straw

A Major Qualifying Project Report

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Abstract

Subcritical water is potentially an efficient, environmentally-friendly alternate to acid and enzymatic hydrolysis of lignocellulosic biomass. Here, water was run over sugarcane bagasse and straw at 200 °C, 15 MPa, and 20 mL/min with the resulting hydrolyzate being collected, analyzed, and fermented. Analysis of the hydrolyzate included determining the TRS and inhibitor concentrations via UV-Vis spectroscopy and HPLC. During fermentation, yeast yields were measured as a proxy for ethanol production using UV-Vis spectroscopy. Finally, a compositional analysis of the feedstock and reactor residue was carried out using TGA and FTIR. We found that subcritical water has the potential to compete with acid and enzymatic hydrolysis, being both efficient and more environmentally-friendly.

Figure 1: Process flow diagram of experimentation and analysis. This diagram highlights the similarity between the experiments and analyses applied to both feedstocks under investigation. The green boxes represent major steps of the experimental processing, whereas orange represents materials to be analyzed.
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Chapter 1: Introduction

As more of the world’s population develops, energy consumption will rise in unison. This will place an intense strain on the world’s energy supplies and will eventually act as a major constraint on economic progress. Furthermore, as most of the world’s energy comes from fossil fuels, rising energy consumption poses deep concerns for the environment, as the increases in greenhouse gases produced could cause irreversible damage to the Earth’s biosphere.

To resolve these issues, alternative sources of energy are being developed to reduce global dependency on fossil fuels. The use of ethanol as a replacement or supplement for gasoline is one alternative which has become commercialized. Most of the world’s fuel ethanol is produced by the United States and Brazil via fermentation of corn and sugarcane respectively. Of course, diverting land resources once allocated to food production to fuel instead raises new socioeconomic issues. As more land is used to make fuel, food prices increase economically burdening a society. Higher food prices also begin to make it unattractive for ethanol production to increase slowing its adoption [1] [2].

One way to alleviate this issue is to improve ethanol yields per acre by attempting to ferment what would traditionally be considered agricultural waste products. Agricultural food waste represents an underutilized energy resource that is ready for exploitation. In particular, sugarcane bagasse and straw are bulk materials which have proven suitable for ethanol fermentation [3].

During fermentation, simple sugars are fed to yeast (such as *S. cerevisiae*) which then produce ethanol as a metabolic byproduct. Because yeast require simple sugars, feedstocks must be pretreated before fermentation. For typical feedstocks such as sugarcane and corn this process is usually mechanical. However, the sugars in bagasse and straw are not as easily liberated as they are bound up in a lignocellulosic complex. The lignocellulosic complex is the structural component of a plant and is composed of hemicellulose, cellulose, and lignin. Each of these compounds is unable to be digested by yeast, and is too structurally stable to be broken down via mechanical means. In order to make sugarcane straw and bagasse suitable for fermentation, chemical pretreatment processes are used [4].

The feedstock is typically subjected to either an acidic or enzymatic pretreatment. The goal is to hydrolyze the bonds in hemicellulose and cellulose to form simple sugars which can be fermented. Both have issues regarding their use. Acidic pretreatments are quick but result in dangerous waste which must be neutralized and disposed. Furthermore, use of strong acids in large quantities provides challenges in equipment design due to its highly corrosive nature. Enzymatic pretreatments are more environmentally friendly but operate on slow time scales. Furthermore, the enzymes required to breakdown straw and bagasse are typically expensive, hindering economic viability [5].
An alternative under study is subcritical water hydrolysis. It is a novel option which may offer solutions to both issues where it is more environmentally friendly than acidic treatment and faster than enzymatic treatment. Subcritical water is water with temperature above boiling, but is kept in the liquid state by increased pressures. In this state, water takes on certain properties which allow it to hydrolyze lignocellulosic biomass. The resulting solution can then be used as feed for the fermentation process for ethanol generation [4] [6].

Subcritical water hydrolysis is not a very controlled process, however, and several byproducts form such as various organic acids and phenols. Some of these compounds are toxic to yeast, inhibiting fermentation [7]. The question then arises on whether the sugar concentrations from the hydrolyzate are great enough in order to overcome the inhibition effects of the toxic products.
Chapter 2: Background

2.1 Bioethanol in Brazil and the United States

Bioethanol (or ethanol) is ethyl alcohol used as fuel for passenger vehicles and is most widely used in Brazil and United States [8]. The generation of bioethanol is the same as regular alcohol. A feedstock is harvested, pretreated, and then is fermented using yeast (S. cerevisiae). In both Brazil and the United States, legal mandates exist requiring ethanol to be blended into gasoline. These fuels are produced primarily from sugarcane and corn (in Brazil and The United States respectively) raising questions on the validity of using food sources as fuel [9]. Lately, there have been efforts made to shift the source of ethanol from food crops to less, high-value materials such as lignocellulosic biomass [5].

2.2 Sugarcane Bagasse and Straw as Feedstocks

2.2.1 Collecting Feedstock

Figure 2: Structure of sugarcane plant. [10]

The bagasse and straw are remnant products of the sugarcane milling, which slices the length of the plant and crushes the plant matter to extract the sugar-containing juice. The sugarcane plant is grown densely in fields (due to the small arable area required, Figure 2), and harvested through increasingly mechanical means, though a large portion of production is manual labor [10]. Sugarcane bagasse is the fibers within the stalk of the plant that contain the sugar juice principally desired and ultimately processed. The straw is the green and dead plant matter associated with the sugarcane plant, such as leaves and dried stalk [11].
2.2.2 Feedstock Production and Availability

In the 2018-2019 crop year, the nation of Brazil cultivated 610 million metric tons (MMT) of sugarcane [14] – nearly 90% of which is from the state of São Paulo alone. For 2018, Brazil produced 36 MMT of sugar, making up about 20% of the world’s sugar production [15]. Similarly, 30 billion liters of fuel ethanol were produced, making Brazil the second largest producer at 25% of the world’s production [15]. Some of this fuel was fermented from the sugar itself, but there is a sizable portion from hydrolysis of bagasse and straw [15]. Due to legal statues (Brazilian Federal Law 2661/98 and State of São Paulo Law 11241/02), the burning of these agricultural waste products is illegal, and alternate routes to manage this material must be found [16] [10]. This statute cannot go unanswered, considering 170 MMT of bagasse and 100 MMT of straw produced in Brazil in 2018/2019 need to be managed alone [15] [14].

2.3 Lignocellulosic Biomass

Lignocellulosic biomass (biomass) is the material which composes the bulk of plant material. It is composed of hemicellulose, cellulose and lignin, all of which are tightly bound to one another chemically to form the main scaffolding of the plant [9]. Despite containing the bulk of the sugars in a plant, biomass is typically considered a waste product, either burned off for energy or left on the ground for soil treatment because of the difficulty in breaking down the bulk material into usable monomers and oligomers for further chemical processing [9] [5]. Despite this, it has recently been looked into and been used as an alternate feedstock for the production of bioethanol.

2.3.1 Hemicellulose

Hemicellulose is a relatively amorphous heteropolysaccharide composed of a wide variety of monosaccharides such as xylose (a pentose), glucose (a hexose) and D-glucuronic acid (a uronic acid). These sugars form highly branched polymer chains which are easily hydrolyzed. Each hemicellulose chain is only about 100-200 monomers [17] [18] [19].

2.3.2 Cellulose

Cellulose is the main component of most plant material and forms the bulk of the structure. The structure of cellulose is that of a linear polymer composed of glucose subunits. Individual cellulose
chains are attracted to one another via hydrogen bonding and van der Waal forces and naturally pack themselves together to form crystalline microfibrils. This crystalline nature of cellulose makes it more difficult than hemicellulose to hydrolyze and process. A single cellulose chain can be composed of anything from 500 to upwards of 25,000 glucose monomers [17] [18] [19].

2.3.3 Lignin

Lignin is an aromatic polymer composed of phenols found in the cell walls of plants providing structural support, impermeability, and microbial resistance. It is the most irregular component of lignocellulosic biomass as it no repeating subunits and linked together with ether bonds. Lignin is the most difficult compound to hydrolyze and being composed of phenols its derivate are not even usable by microbes for fermentation [17] [18] [19].

2.4 Pretreatment

The process of breaking down lignocellulosic biomass into its constituent monomers and oligomers is called pretreatment. In the industry, the typical methods of pretreatment are acid or enzymatic hydrolysis.

2.4.1 Acid Hydrolysis

Acid hydrolysis is the most straightforward method of breaking down biomass into simple sugars. The biomass is broken down quickly by an acid (or alkaline) catalyst and yields a high sugar concentration. However, the catalysts become used-up after the process requiring that it be neutralized, generating large amounts of environmentally hazardous waste. Furthermore, the acid catalysts cause corrosion on the reaction vessels and result in degradation [7]. These degraded products, along with the residual acid catalyst, act as inhibitors in the subsequent fermentation process [9].

2.4.2 Enzymatic Hydrolysis

Enzymatic hydrolysis is the preferred method of breaking down biomass. Unlike acid, hydrolysis enzymes do not leave behind hazardous waste which needs to be removed. Furthermore, enzymes are inert with regard to simple sugars preventing the degradation of the simple sugar products. Enzymatic catalysts, however, are expensive to produce and are also difficult to recover making them a costly investment for an ethanol plant [7]. They also have great difficulty in breaking down the bonds between hemicellulose, cellulose, and lignin before being able to directly form sugars. This necessitates another pretreatment step to separate the different components of biomass before enzymatic treatment [9].

2.4.3 Subcritical Water

A novel method under study for the hydrolysis of lignocellulosic biomass is subcritical water hydrolysis (SCW hydrolysis). Subcritical water is liquid water held at temperatures between 100 °C - 374 °C and at pressures higher than its saturation pressure. Under these conditions water molecules split apart more readily leading to an increase in the number of hydronium and hydroxide ions by an order of three. Additionally, the high amounts of energy in the system lead
to a natural increase in the diffusivity of water as well as a decrease in the density. Furthermore, as a reaction medium and catalyst, subcritical water is relatively easy to produce, leaves behind no toxic waste requiring special treatment, and is less corrosive compared to other varieties of pretreatment [7]. However, SCW hydrolysis is not a very controlled reaction and several byproducts are created during the process. These byproducts such as furfural and hydroxymethylfurfural (HMF) are known to act as inhibitors to the fermentation process.

2.5 Fermentation

Fermentation is the biological process by which simple sugars, such as glucose, are converted into ethanol.

\[
C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2
\]

In most industrial scale applications, *S. cerevisiae* (yeast) is the primary organism responsible for fermentation. Glucose bonds are broken down inside the yeast cells and the energy is then used to form ATP and NADH for use in various cell processes later. The result of the metabolic cycle is ethanol and carbon dioxide being expelled as waste products. In particular, ethanol acts as a natural inhibitor to yeast growth. Thus, as sugar concentrations fall and ethanol concentrations rise, yeast growth slows and eventually turns negative. If left alone, the fermentation process will eventually result in the complete death of the yeast colony.
Chapter 3: Materials and Methods

3.1. Raw Materials and Chemicals

In this investigation, the feedstock material was sugarcane bagasse for subcritical water hydrolysis pretreatment. Before hydrolysis, the feedstock was crushed using a knife mill (Marconi, model MA 340, Piracicaba, SP, Brazil) and separated via sieve agitator (Bertel, model N.1868, Caieiras, SP, Brazil) based on particle size. Particles between 297 micrometers and 710 micrometers in diameter were chosen for hydrolysis because this range contained the most amount of ground feedstock for use, in addition to providing an adequate balance of heat and mass transfer limitations as well as physical particle handling. Appendix A illustrates this process. This refined feedstock range was of optimal size and surface area based on similar experiments done by Torres-Mayanga et al. to characterize sample size in the same subcritical hydrolysis system [20]. After hydrolysis, the liquid portion of the product, hydrolyzate, was collected for analysis and use in the fermentation section of the experiment.

The next phase of the experiment was the fermentation of the hydrolyzate into fuel ethanol, which called for yeast (S. cerevisiae) to be mixed with samples of bagasse hydrolyzate. Ideally, this material is a combination of 5- and 6-carbon sugars, such as hemicellulose and cellulose. These sugars are desirable to have in the hydrolyzate due to their ability to be converted to ethanol via fermentation. Some samples of the bagasse hydrolyzate were also subjected to the Cellic® CTec2 enzyme in order for the traditional enzymatic hydrolysis to produce any sugars that the subcritical hydrolysis may not have accomplished. This was due to lower sugar concentrations in the initial bagasse hydrolyzate, as shown via the Somogyi-Nelson method for determining total reducing sugars (TRS), than in comparable subcritical hydrolysis performed by Lachos-Perez et al. on orange peels [4].

3.2. Analysis of Feed Stock and Char Residue

Analysis of the composition of the feedstock and char residue was performed using Fourier Transform Infrared Spectroscopy (FTIR) and Thermogravimetric Analysis (TGA). In both cases an attempt was made to classify the composition of the material based on the broad categories of hemicellulose, cellulose, and lignin. FTIR and TGA were both used to qualitatively describe the material, while only TGA was used to quantify composition.

For TGA, ~10 milligrams of material were placed into the TG 209 F1 Libra under an inert nitrogen atmosphere. The temperature was then brought from 25 °C to 800 °C at a rate of 10 °C per minute. One analysis was performed for both feedstocks, while three were performed for both bagasse and straw residues. Each analysis of the char residue was from a separate hydrolysis trial.

A similar sampling regimen occurred for FTIR. One analysis was performed on the feedstocks, while three analyses were performed on the bagasse and straw residues using Bruker
Vertex 70FT-IR spectrometer with a La-DTGS detector. Each analysis consisted of 1024 scans observing the bands from 600 to 4400 cm\(^{-1}\).

### 3.3. Subcritical Water Hydrolysis Experimental Procedure

Sugarcane bagasse was hydrolyzed using subcritical water in five trials to produce a total of 1.25 liter of hydrolyzate. In this experiment, an apparatus designed by Lachos-Perez et al. (Figure 4) was used to hydrolyze the feedstock [4]. Appendix B depicts the apparatus in laboratory. Water was heated to 200 °C before being pumped into an autoclave reactor filled with 5 grams of feedstock at a rate of ~15-20 mL/min. The effluent from the reactor flowed out into a condenser before being collected. The product of each trial was 250 mL ± 20 mL of hydrolyzate. An aliquot of 10 mL from each sample was taken for further chemical analysis, while the remainder of the solution was reserved for use in fermentation. Both the aliquot and bulk product were frozen to prevent any degradation or side reactions from occurring. At the conclusion of all five trials and their analyses, the bulk samples were unfrozen and mixed together. Another aliquot of 10 mL was taken from this 1.25 L mixed solution and analyzed, while the remainder was reserved for fermentation.

![Figure 4: PFD of subcritical hydrolysis of sugarcane bagasse. V-101 – Deionized water container for feed flow; P-101A – Pump; E-101 – Electric heat exchanger; TC – Thermocouple for reactor entrance feed temperature; V-102 – 297 micrometer, pretreated bagasse; R-101 – stainless steel, pressurized reactor; E-102 – Condenser with water as coolant; V-103 – Subcritical hydrolyzate collection beaker.](image)

### 3.4. Analysis of Hydrolyzate

#### 3.4.1. Somogyi-Nelson Method for TRS

The total reducing sugars (TRS) of the hydrolyzate were analyzed using the Somogyi-Nelson method. The hydrolyzate is further hydrolyzed using acid to break down all complex sugars into simple glucose monomers. The solution is then colored and its absorbance measured at 540 nm using a spectrometer. A dilution factor was applied so that the spectral reading could accurately be determined. The concentration was calculated using a known glucose standard calibration curve.
and expressed in terms of equivalent sugar concentration since exact sugar identities were not known.

3.4.2. High Performance Liquid Chromatography (HPLC)

Furfural and Hydroxymethylfurfural (HMF) concentrations in the hydrolyzate are analyzed using the EXTRACT-US system (FAPESP 2013/04304–4 – Patent pending). The primary function of the EXTRACT-US system in the context of this experiment was as an HPLC. Concentrations of Furfural and HMF were measured by matching retention times of compounds in the hydrolyzate solution to known standards. The device is depicted in Appendix C.

3.6. Fermentation Procedure

The straw and bagasse hydrolyzates were divided into 90 mL aliquots between six Erlenmeyer flasks, making a total of twelve flasks. The two sample sets were placed into an autoclave and subjected to temperatures of 121 °C and pressures of 0.11 MPa for 15 minutes to disinfect them. Once cooled, three straw and three bagasse flasks had cellulase enzyme (Cellec CTec2) mixed in and were agitated for 24 hours at 50 °C to break down any remaining complex sugars into glucose monomers. Afterwards, 10 mL of yeast inoculant (YPD) was given to seven flasks from each set. The inoculum medium (20 g-glucose/L) was also used as a control for both bagasse and straw. Before being placed inside a shaker operating at 150 rpm and 30 °C for 96 hours, two mL samples were then taken from each of the now 13 flasks (see Table 1) and analyzed for yeast and sugar concentration. After placement in the shaker, samples were then taken at the third, sixth, twelfth, and twenty-fourth hour of fermentation. Following this, samples were only taken and analyzed every twenty-four hours. The yeast petri dish, inoculum, and fermentation flasks are depicted in Appendix D.

**Table 1: Fermentation Summary.** This table summarizes the division of samples for fermentation.

<table>
<thead>
<tr>
<th>SOLUTION TYPE</th>
<th>Enzyme</th>
<th>Yeast</th>
<th>Straw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Yeast</td>
<td>-</td>
<td>Yeast</td>
<td>Straw</td>
</tr>
<tr>
<td>Enzyme Yeast</td>
<td>-</td>
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<td>Yeast</td>
<td>Straw</td>
</tr>
<tr>
<td>Enzyme Yeast</td>
<td>-</td>
<td>Yeast</td>
<td>Bagasse</td>
</tr>
<tr>
<td>Enzyme Yeast</td>
<td>-</td>
<td>Yeast</td>
<td>Bagasse</td>
</tr>
<tr>
<td>Enzyme Yeast</td>
<td>-</td>
<td>Yeast</td>
<td>Bagasse</td>
</tr>
</tbody>
</table>

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3.7. Analysis of Fermentation Products

A measure of the completion of the conversion of sugars to products such as ethanol by the yeast is through extracting aliquots at periodic times. From each sealed Erlenmeyer flask, 2 mL of the solution was withdrawn via syringe. The samples were centrifuged at 10,000 RPM at 25 °C for 10 minutes to separate dead yeast cells that fell out of the solution. An absorbance test was then conducted on each flask’s sample, with dilution factors considered so as not to exceed the range of the spectrometer.
Chapter 4: Results and Discussion

4.1. Composition of Feedstocks and Char Residue

4.1.1 Thermogravimetric Analysis

Thermograms of the feedstocks and char residue were converted into derive thermograms (DTGs) for analysis (Figure 5). The char residue DTGs were averaged together into two curves: straw and bagasse. The resulting peaks were then identified as either belonging to hemicellulose, cellulose, or lignin using literature results of pure compounds. The peaks were then fitted using Gaussian curves and integrated to obtain relative mass compositions (Figure 6). The residue designation in Figure 6 represents the compounds too heavy for gasification after thermogravimetric analysis, such as ash content and carbon content locked in the form of aromatic rings.

![Figure 5: Comparison of Derivative Thermograms](Image)

**Figure 5: Comparison of Derivative Thermograms.** Here the DTGs of the untreated and treated straw and bagasse are presented offset together. The solid curves are the untreated feedstocks, while the dashed curves are post-treatment. The regions where the peaks of hemicellulose, cellulose, and lignin have been demarcated. In both bagasse and straw, it can be seen that the hemicellulose curve almost entirely disappears, while the cellulose peak rises after hydrolysis.

As can be seen in Figures 5 and 6, the feed straw and bagasse are primarily composed of hemicellulose and cellulose, with very little lignin content. Of the two components, cellulose is
the majority quantity. After subcritical water hydrolysis has been applied, however, the data shows that almost no hemicellulose can be found in the residues of either straw or bagasse.

**Figure 6: Compositional Comparison between Feedstocks and Residue.** This bar graph shows the percent composition of the feedstock pre and post-subcritical hydrolysis. Again, it can be seen that the hemicellulose has been almost completely hydrolyzed and the cellulose now takes up a much larger percentage of the composition.
4.1.2 Fourier Transform Infrared Spectroscopy

Figure 7: Compositional Comparison of Feed and Residue using FTIR. The feed and residue spectra of bagasse (left) and straw (right) are presented here along with the spectra of pure cellulose for comparison. Both Bagasse and Straw have very similar structures and compositions both with each other and cellulose. Furthermore, in both cases after subcritical water hydrolysis, the residue bagasse and straw almost perfectly mirrors that of pure cellulose.

FTIR spectroscopy was used to qualitatively confirm the results of the thermogravimetric analysis. Each spectra was normalized by using the height of the 1029 cm\(^{-1}\) band as the basis. In a similar manner to the thermograms, the normalized values of the straw and bagasse residues were averaged together and compared to the feedstock (Figure 7). Using literature values (Appendix E) and direct comparison between spectra of pure compounds, bands were identified as either belonging to hemicellulose, cellulose, or lignin.

The information found by FTIR corroborates qualitatively what was found by TGA. The band peaks from 1029 to 1200 cm\(^{-1}\) become much rougher and more pronounced post hydrolysis indicating an increase in the relative composition of cellulose. The collapse of the 1242 cm\(^{-1}\) peak corresponds to the total removal of hemicellulose from the bagasse and straw matrix. The relatively unchanged peak at 1512 cm\(^{-1}\) indicates that the lignin content of the bagasse and straw remains relatively untouched.

4.2. Composition of the Hydrolysate

4.2.1 Determination of Quality Hydrolyzate Concentration

Each trial of hydrolysis produced about 250 mL of usable hydrolyzate. Figure 8 below indicates how usable hydrolyzate was determined; all 12 batches of hydrolysis for both feedstocks are similarly plotted in Appendix F. Some trials were inaccurate due to leaks in the pressurized reaction vessel, which was indicated by a visible vapor effluent and hissing sound. These
necessitated repeated trials so that a total of 1.25 L hydrolyzate could be collected for each feedstock.

Figure 8: Bagasse hydrolysis experiment 1. Here, T1 is reactor exit temperature, T2 is reactor entrance temperature, and T3 is heat exchanger temperature. The reaction time for hydrolysis is divided into the static region where the pump and heat exchanger bring the water to the correct temperature and pressure; the dynamic region where the water is introduced to the feedstock in the reactor and low-concentration liquid is produced; and the hydrolyzate region is where there is a noticeable change in fluid color density and frothiness occurs. This hydrolyzate region is the amount collected to contribute to each round’s ~250 mL of hydrolyzate.

4.2.2 Total Reducing Sugars Determination via the Somogyi-Nelson Method

Figure 9: Sugar concentration of bagasse hydrolyzate. Here, batch # 2 was discounted due to deviation in executing the experimental procedure during hydrolysis. Mixture batch # 7 never included batch # 2.

The total reducing sugar (TRS) concentrations were found for each of the batches conducted for the subcritical hydrolyses of the sugarcane bagasse and straw. The Somogyi-Nelson method was used for each of these trials, in addition to mixture solutions of the bagasse batches.
and straw batches. The resulting concentrations are shown in Figures 9 above and 10 below. The glucose curves used for standardization may be found in Appendix G.

![Sugar concentration of straw hydrolyzate](image)

**Figure 10: Sugar concentration of straw hydrolyzate.** Here, batch # 5 was discounted due to deviation in executing the experimental procedure during hydrolysis. Mixture batch # 7 never included batch # 5.

The TRS concentration for bagasse was 1.23 ± 0.04 g/L. This was the result for both the physical mixture of the five batches, as well as the arithmetic average of the TRS concentrations of those same batches. The TRS concentration for straw was 1.02 ± 0.14 g/L. Similarly, this was the results for both the physical mixture of the five batches, as well as the arithmetic average of the TRS concentrations of those same batches.

4.2.3 Inhibition of Hydroxymethylfurfural and Furfural via HPLC

High performance liquid chromatography (HPLC) was used on samples during the fermentation process in order to track the growth of inhibitors. Inhibitors found in hydrolysis of agricultural waste during the fermentation process are known to include hydroxymethylfurfural (HMF) and furfural (FF) [7]. Figure 11 shows the degradation of HMF and FF over time for sugarcane bagasse in both the regular subcritical water hydrolyzate and that in which enzymatic hydrolysis was applied. The same is true for Figure 12 about sugarcane straw.
Figure 11: Inhibitor growth during fermentation of bagasse hydrolyzate.

In Figure 11, while the HMF curves seem to not be following a similar path the error bars indicate that the data is essentially the same. This means that the data supports the claim that there is not significant difference between the concentration of HMF, and FF for that matter, in the hydrolyzate with and without the enzymatic hydrolysis applied.
Figure 12: Inhibitor growth during fermentation of straw hydrolyzate.

In Figure 12, there seems to be minimal difference between the hydrolyzate with and without enzymatic hydrolysis applied in terms of both HMF and FF. However, it should be noted that the trials for straw have not yet been run in triplicate, so further testing other trials will increase the confidence in this statement.
Figure 13: Comparison of TRS and inhibitor concentrations. This data is the average TRS concentration from the subcritical hydrolyzates without any enzymes. Similarly, the inhibitor concentrations are those which were present prior to any enzymes were added or the fermentation process began.

In actuality, the levels of HMF and FF being reported are reasonably low in comparison to the TRS data, as Figure 13 summarizes. For bagasse and straw, FF concentrations were about 0.52 and 0.32 g/L, and HMF concentrations were 0.046 and 0.043 g/L. Since the inhibitor data presented in Figure 13 is essential higher than any inhibitor concentration in the previous two Figures, then it may be said that HMF and FF inhibitors only decrease as fermentation occurs. That is, the levels reported in Figure 13 are the maximum, and even so are quite minuscule compared to the TRS concentration.

4.3 Yeast Yields

Yeast yields were used as a proxy for ethanol production in this experiment and were measured using UV-Vis spectroscopy. Figure 14 plots the absorbance of the samples and the control (YPD) versus time in hours. While the maximum growth of the yeast extract surpasses that of all the experimental samples, which is to be expected due to how much more sugar the control has, the initial rates of yeast growth are actually all very similar. This suggests that the concentrations of HMF and furfural present in the hydrolyzate solution currently do not act as major inhibitors to yeast growth. It may then be inferred that the major limiter on ethanol production using subcritical hydrolysis are the low sugar concentrations found in the hydrolyzate. Another point is that the growth curves of the pure hydrolyzate and enzymatically improved hydrolyzate track each other very closely indicating a low concentration of oligomers in the initial solutions.
Figure 14: Ethanol Generation Using Yeast Yields as a Proxy. The multiple trials behind each sample were averaged together for the purposes of this plot. The Yeast Extract acted as the control and, unsurprisingly, had the longest growth phase and the highest growth yield. In contrast, the experimental samples’ yeast growth all tracked closely to one another despite the addition of enzymes to some of the hydrolyzate samples. Furthermore, the initial growth rates for the first twelve hours matched that of the yeast extract.
Chapter 5: Conclusions and Recommendations

Sugarcane straw and bagasse were hydrolyzed with subcritical water using a semi-continuous reactor. It was found through TGA and FTIR that the process hydrolyzed the majority of the hemicellulose in straw and bagasse, but left lignin and cellulose mostly untouched. The hemicellulose was converted into sugars, some of which degraded further into furfural and hydroxymethylfurfural (HMF). The majority of the sugars in solution were found to be simple monosaccharide solutions. Both straw and bagasse hydrolyzate had reduced sugar concentrations of ~1g/L and the majority of these sugars were monomers. For the inhibitors, the hydrolyzate was a furfural concentration of ~ 0.4 g/L and an HMF concentration of ~0.04 g/L. It can be inferred that furfural is the main side reaction product. Furthermore, under these operating conditions, it does not seem to form appreciable enough amounts to meaningfully prevent growth. It was found that the low concentration of sugars in the solution played a more important role in limiting yeast growth rather than the furfural or HMF concentrations. This was evidenced by the initial rates of yeast growth remaining similar to that of the control, but the maximums falling short of the extract. Possibilities for improving sugar yields are two-fold: first, moving to supercritical water hydrolysis to possibly liberating the sugars found in cellulose; and second, using feedstocks with a higher hemicellulose to cellulose ratio.
References


Appendices

Appendix A: Mechanical Processing of Feedstock

A.1: Source Feedstock from GranBio (Bagasse)

A.2: Knife Mill for Sizing Feedstock for Reactor
A.3: Sieve for Isolating Desired Feedstock Size

A.4: 297 Micron Feedstock (Bagasse)
Appendix B: Subcritical Hydrolysis Apparatus

B.1: Process Flow Diagram:

B.2: Laboratory Photograph:
B.3: Laboratory Photograph of Low Quality Subcritical Hydrolyzate (Dynamic)

B.4: Laboratory Photograph of High Quality Subcritical Hydrolyzate
Appendix C: HPLC for Inhibitors HMF and FF

C.1: Laboratory Photograph of Samples of Hydrolyzate-Yeast Mixture for Testing

C.2: Laboratory Photograph of HPLC Machine made for HMF and FF Detection
Appendix D: Fermentation Process

D.1: Laboratory Photographs of Yeast and Inoculum

D.2: Laboratory Photograph of Beaker Trials
## Appendix E: FTIR Analysis

### E.1 Band positions (cm⁻¹) and assignments of Fourier transform infrared (FTIR) spectroscopy of sugarcane straw and bagasse from subcritical water treatment.

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Appendix F: Temperature-Time Charts of Subcritical Hydrolysis

F.1: Bagasse Subcritical Hydrolysis Temperature-Time Charts
Bagasse Hydrolysis Experiment 3

Bagasse Hydrolysis Experiment 4
F.2: Straw Subcritical Hydrolysis Temperature-Time Charts

Straw Hydrolysis Experiment 1

Straw Hydrolysis Experiment 2
Appendix G: Somogyi-Nelson

G.1: Bagasse-Glucose Standard Curves and Corresponding TRS Plot

Absorbance vs. Concentration Curve

\[ y = 2.5507x + 0.006 \]
\[ R^2 = 0.9972 \]

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Sugar Concentration of Bagasse Hydrolyzate
G.2: Straw-Glucose Standard Curves and Corresponding TRS Plot

- **Absorbance vs. Concentration Curve**
  - Equation: $y = 2.5507x + 0.006$
  - $R^2 = 0.9972$

- **Sugar Concentration of Straw Hydrolyzate Test**
  - [Glucose] Standard
  - [Straw Sugars #1]
  - [Straw Sugars #2]
  - Linear ([Glucose] Standard)