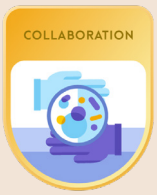
# Engineering an Enzymatic Process for Paper Waste Management





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Paper towels, which are more difficult to recycle than other paper products due to their short fibers, are dumped in landfills, contaminating the environment. However, paper, a cellulosic waste which makes up 40% of municipal solid waste in the United States, is a potential energy source. This study aims to engineer an alternative solution to paper towel disposal, in the form of an enzymatic process using a commercially available cellulase enzyme (CTec2 from Novozymes), to divert paper towels and produce energy by means of a microbial fuel cell or ethanol production. Specifically, this study determines the optimal environment for microbial digestion and attempts to recreate these parameters in an out-of-lab setting. In this study, we test eighteen paper towel digestion scenarios in triplicate, with varying buffer types, temperatures, solution ratios, agitation frequencies, pretreatment methods, duration of incubation, and sample sizes. We record the concentration of sugar from hydrolyzing cellulose (brix), pH, and appearance at each interval. We determine success of each scenario through increase in brix and visual degradation.

**Keywords:** Cellulase (CTec2), paper saccharification, paper towel, microbial fuel cell, ethanol fermentation, enzymatic digestion, waste management

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Watch a video introduction by the authors at:
https://bit.ly/2N3dRqB

## Background

The United States has just 5% of the world's total population and generates around 30% of the world's trash. About 55% of this waste will go directly into a landfill, 40% of which is paper waste (Artula Institute, 2013). Paper towels and tissue grade paper in particular are difficult to recover from the waste stream because their fibers are too short. While paper towels can be taken out of the waste stream and repurposed, all present methods are costly and energy intensive (Smithers 2011). Therefore, used paper towels end up in landfills where they contribute to the formation of toxic sludge and leaching.

Lignocellulose, which composes paper products, is the most abundant renewable biomass on earth, with a worldwide annual production of 1010 MT. It is composed mainly of cellulose, hemicellulose, and lignin. Cellulose can be hydrolyzed (broken apart with water) by cellulase enzymes, enabling the release of sugars (glucose, xylose, arabinose) used to fuel bacteria in power or ethanol production. Cellulose can be difficult to hydrolyze due to its strong crystalline structure resulting from the dense packing of cellulose chains, requiring pretreatment (Harmsen et al. 2010). Pretreatment methods include heat, acid, and alkali treatments (Kumar et al. 2009). Lignin, once removed from lignocellulose, has many commercial applications as a binder, dispersant, emulsifier, or sequestrant (Purkiss).

Cellulases such as Novozymes' CTec2 (Denmark) have been engineered as cost-efficient enzymes to enable the mass production of biofuel from lignocellulosic biomass (Launch of Cellic CTec2 2010). While there are some types of cellulosic mass that are currently being hydrolyzed into ethanol, such as corn, many other potential energy sources that make up the waste stream are being overlooked, including paper towels. Harnessing energy from these overlooked biomasses would save costs in two systems: waste disposal and power production.

Aside from ethanol production, energy can be harvested by the biological consumption of sugars in a microbial fuel cell. This form of power production is considered energy efficient and environmentally friendly when compared to conventional methods of acquiring electricity, as it produces no harmful waste and is renewable (Chaturvedi and Verma 2016).

This study aims to engineer an alternative solution to paper towel disposal, in the form of an enzymatic digestion process using CTec2 (Novozymes, Denmark), to divert paper towels from the waste stream. This process converts cellulose into a sugar solution which is then used to collect energy by means of a microbial fuel cell. Additionally, this study shows that cellulosic waste can be a valuable resource for green energy.

## Systems Level

This study is an engineering project in which we are making use of biological enzymes to address a municipal waste issue. Our system, summarized in Figure 1, uses a commercial cellulase (CTec2) in an inexpensive, *in vitro* bioreactor to degrade paper towels and release sugars for energy collection. The CTec2 enzyme is able to directly convert cellulose into simple sugars like glucose, xylose, and arabinose, leaving behind the remaining lignin. This biological degradation process is the key component of the system, and central to our engineered solution. The process we design requires us to fine tune our process parameters and enables the digestion of paper towel waste in an out-of-lab setting for validation.

The first stage of the system is pretreatment, in which heat and acid are used to weaken the bonds in the lignocellulose. The input into this stage is lignocellulosic waste along with acid and energy in the form of heat.

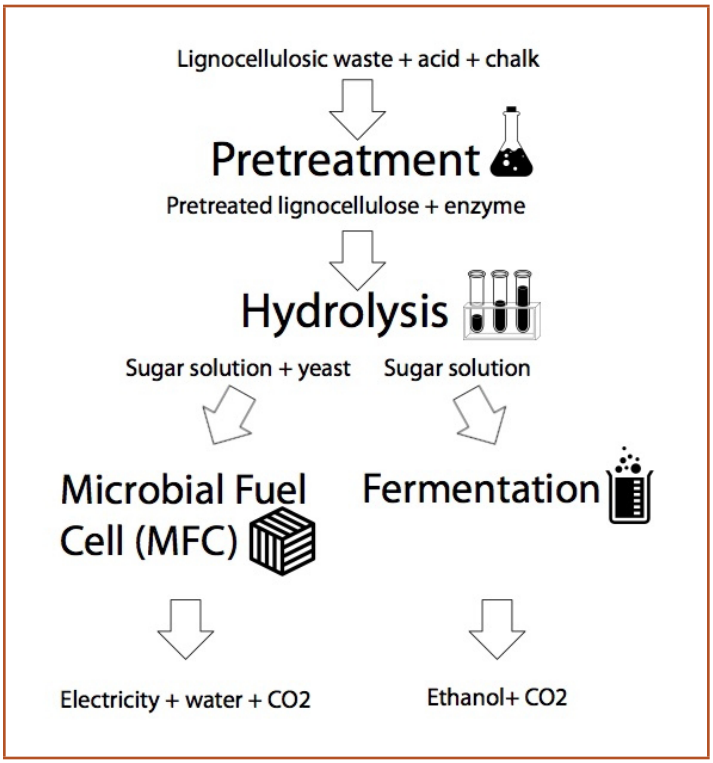


***Figure 1.*** *Complete digestion process diagram, from pretreatment to energy production, as well as the inputs and outputs for each respective step.*

Acid is able to break the bonds between the cellulose and lignin, and the heat accelerates this degradation process and shortens the overall pretreatment time. This step is critical for hydrolysis to occur because it reduces the overall recalcitrance (resistance to degradation) of the substrate. The output from this process is a weakened form of lignocellulosic waste in a solution at neutral pH.

The core stage in the system is the cellulose saccharification process, the hydrolysis of the polysaccharides to simpler sugar compounds. Upon neutralization of the pretreated substrate, the enzyme (CTec2), a buffer, and kinetic energy in the forms of heat and agitation are added. The heat and buffer are necessary to create the optimal operation environment for degradation by the cellulase. Periodic agitation accelerates the process by additionally breaking down the substrate, and increasing its surface area in contact with the enzyme. The enzyme, in the presence of water, is able to break the glycosidic

bonds (hydrolysis) that make up the complex polysaccharide sugar, cellulose, releasing simple sugars such as glucose, arabinose, and xylose. Therefore, the output of this step is a simple sugar solution, which also contains enzyme and lignin.

Once the sugar solution has been saccharified and filtered, it can be used in multiple ways to produce energy. One of the two methods we use in this study is the commercially available microbial fuel cell (MFC), shown in Figure 2. The input of a sugar solution into the MFC, as well as yeast and non-toxic chemicals, enables the movement of electrons from one chamber to another. The natural movement of electrons resulting from the consumption of sugar by microbes can be tapped and its energy harvested and stored in a battery. The non-toxic chemicals increase the efficiency of this movement and carbon fiber brush electrodes optimize the collection of electrons in the system via their large surface areas (Gunawardena et al., 2008). This is an emerging energy collection method that results in an environmentally low impact and consistent electrical current.

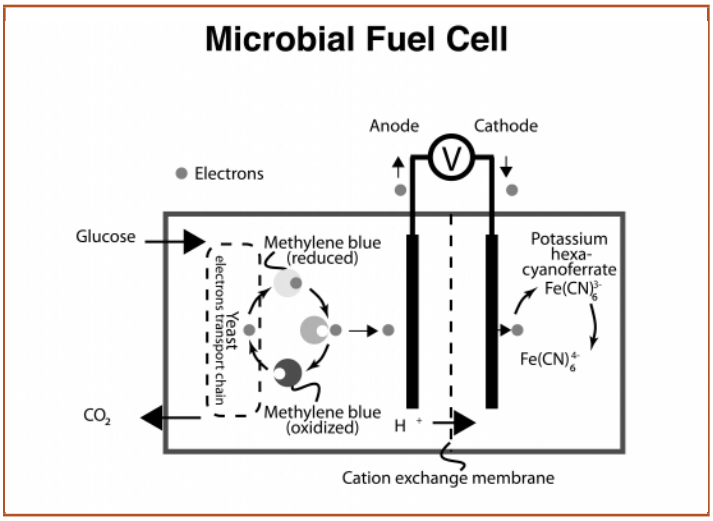


***Figure 2.*** *The microbial fuel cell system, including electron movement.*

Another method of energy collection is to ferment the saccharified sugar solution into ethanol. This process is performed anaerobically, as the lack of oxygen requires the yeast to resort to ethanol production. Without oxygen, cells need an alternative method of creating adenosine triphosphate (ATP) in order to live. The anaerobic consumption of 1 mol of glucose results in 2 moles of CO2, 2 ATP molecules, and 2 moles of ethanol. The ethanol acts as an efficient electron acceptor for the electrons carried by the nicotinamide adenine dinucleotide (NAD) molecules that facilitate the reaction, as seen in Figure 3. After fermentation, the solution must be distilled to increase the alcohol concentration. While ethanol production is a more efficient form of energy collection than the microbial fuel cell, its creation and use results in the release of more carbon dioxide. It is more efficient because energy can be harvested in larger batches through the fermentation process as compared to the MFC. However, this method is more easily scalable and has many existing applications for combustion. This is a viable method of converting cellulose-derived sugars into a cleaner burnable energy source with few byproducts.

## Device Level

During pretreatment and hydrolysis we use an incubator to accelerate the substrate degradation. In the

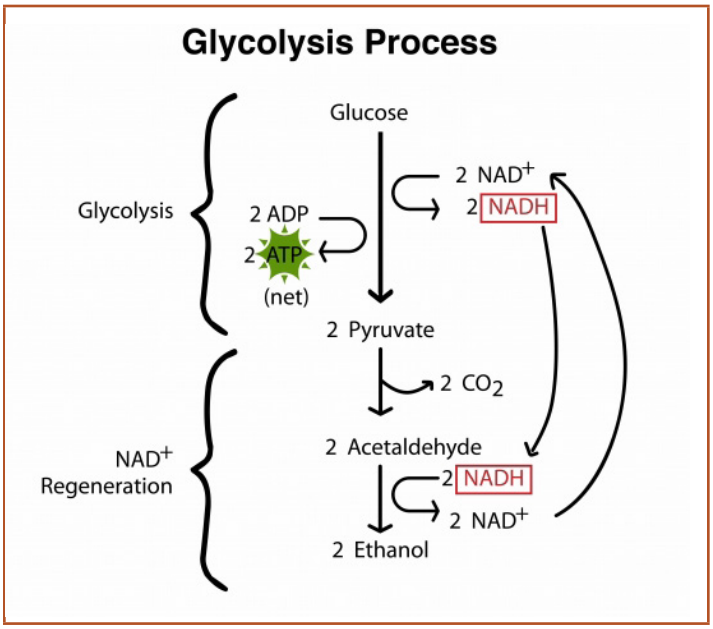


***Figure 3.*** *The biochemical pathways of ethanol fermentation process.*

in-lab studies, we use a small electric incubator to reach 70-90ºC for acid pretreatment and 50ºC for hydrolysis. However, during the out-of-lab experiments, we use a thermostatically controlled space heater inside of a larger insulated box, and we measure temperature using an electronic thermometer. Additionally, the out-of-lab samples are significantly larger, needing to be contained in a watertight bucket as opposed to the in-lab test tubes.

When agitating the small in-lab samples, we are able to shake the test tubes by hand. However, the larger out-of-lab samples require agitation using a hand crank attached to the bucket stand inside the incubator, as shown in Figure 4. We do not change the intervals in which we agitate between in-lab and out-of-lab trials.

As demonstrated in Figure 5, engineering and optimizing the out-of-lab incubator is difficult due to its many variables and design constraints. When building the incuba-

tor, the most important aspects of the system are how well it can agitate the digestion solution and maintain a consistent temperature, how stable it is, and how easy it is for people to use. In addition to these goals, we also focus on making it as environmentally friendly by making the outside dark as to facilitate passive solar heating and replacing the space heater with a solar powered heating depending on location.

The MFC from a Carolina Kit consists of a fuel cell that has two small chambers with enough space for flat carbon fiber electrodes, solutions, and a semipermeable membrane. Solutions include sugar from saccharification, a mediator dye, and an electron acceptor. The sheet electrodes are functional, but the increased surface area of brush electrodes results in increased voltage. The kit MFC is too small to accommodate the brush electrodes, so we build a new, larger MFC with a high surface area to volume ratio that fits the carbon fiber brushes. We laser cut acrylic chambers, include a larger semipermeable membrane, and use aquarium sealant to make the new MFC watertight.

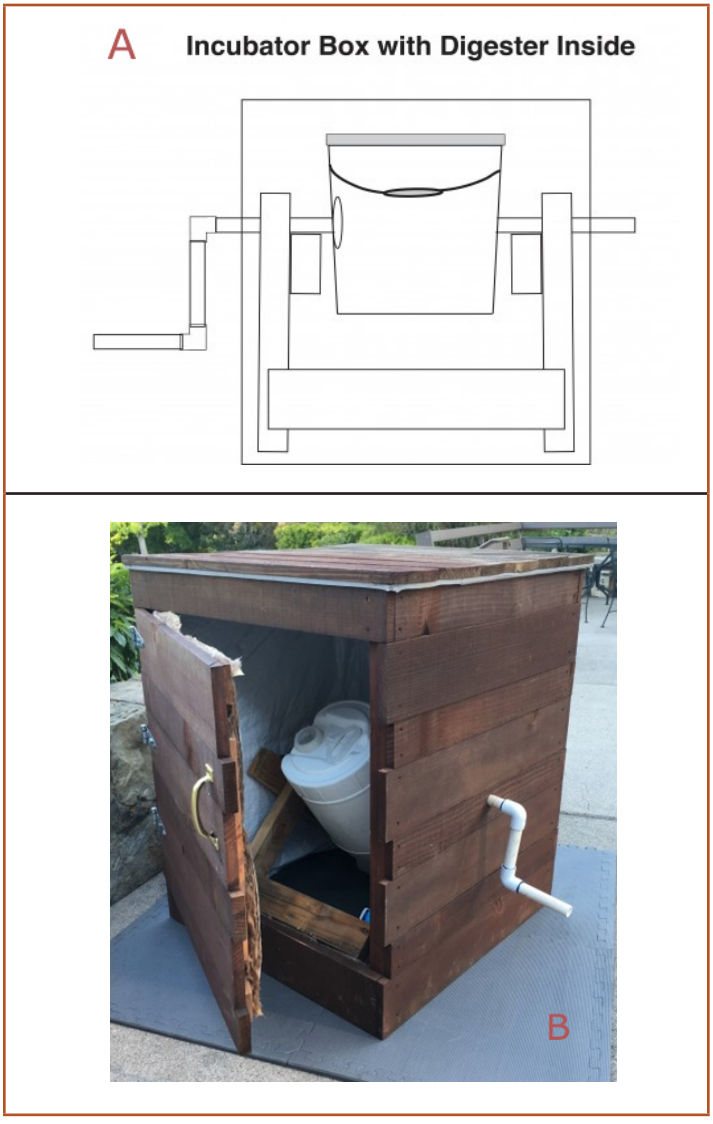


***Figure 4.*** *(A) Internal design of the out-of-lab incubator prototype. (B) External design of the out-of-lab incubator prototype.*

## Parts Level

We fine tune specifications in this system from the Device Level in order to optimize processes including the pretreatment, hydrolysis, and energy collection. For pretreatment, these parameters include pretreatment type, temperature, duration, and neutralization. Both 0.83 M acetic acid (for 2.5 h) and 0.1 M sulfuric acid (for 30 min.) are tested as pretreatment at temperatures between 70-90ºC, with sulfuric acid being the more effective of the two. Digestion solutions are subsequently neutralized with calcium carbonate in order to prevent denaturation of the cellulase used in the next step.

We optimize hydrolysis by testing varied agitation rates, durations of incubation, pH levels and buffer types, enzyme concentrations, and concentrations of lignocellulosic mass. We take 50ºC to be the optimal incubation temperature for CTec2, according to its manufacturer (Novozymes). During the 5-12 hour incubation, we cover samples to prevent evaporation and periodically agitate them by hand every 2.5-1.5 h. Higher frequencies of agitation and increased incubation length aid in accelerating hydrolysis. We find optimal digestion environment for paper towel waste to be at an approximate pH of 5.4, contrary to Lan 2013's research (Lan et al. 2013). This environment is best maintained using a sodium acetate buffer (pH 5.5 as compared to pH 6.0 sodium acetate and Hydrion buffers). After testing digestion with enzyme concentrations between 0% and 30% (~390 U/mL), we find levels as low as 0.25% (~3.25 U/mL) to be effective. Sugar yield is positively correlated with increased concentrations of lignocellulosic mass, with tested levels as high as 1.7% sugar release by mass.

The environmental factors we optimize in the microbial fuel cell system include presence of mediator fluid (methylene blue dye), electrode, semipermeable membrane, and microbe preparation. We prepare the yeast for the microbial fuel cell by activating it in either a pure glucose solution, apple juice, or the sugar solution resulting from paper towel saccharification, all methods being effective. We then pipette the yeast suspension into one side of the double chambered microbial fuel cell along with 5 mL of 10 mM methylene blue solution. We add 10 mL of 0.02 M potassium hexacyanoferrate (III) solution to the other chamber of the fuel cell. Without the presence of this mediator fluid, electrical output is significantly lower. A semipermeable membrane, either from a MFC kit or one intended for a hydrogen fuel cell, separates the two chambers (the one from the MFC kit yields higher voltages). We insert an electrode, either a carbon fiber brush or a carbon fiber sheet, into each chamber. As a result of its greater surface area, the carbon fiber brush electrodes yield higher voltages.

Finally, the factors that affect the ethanol fermentation

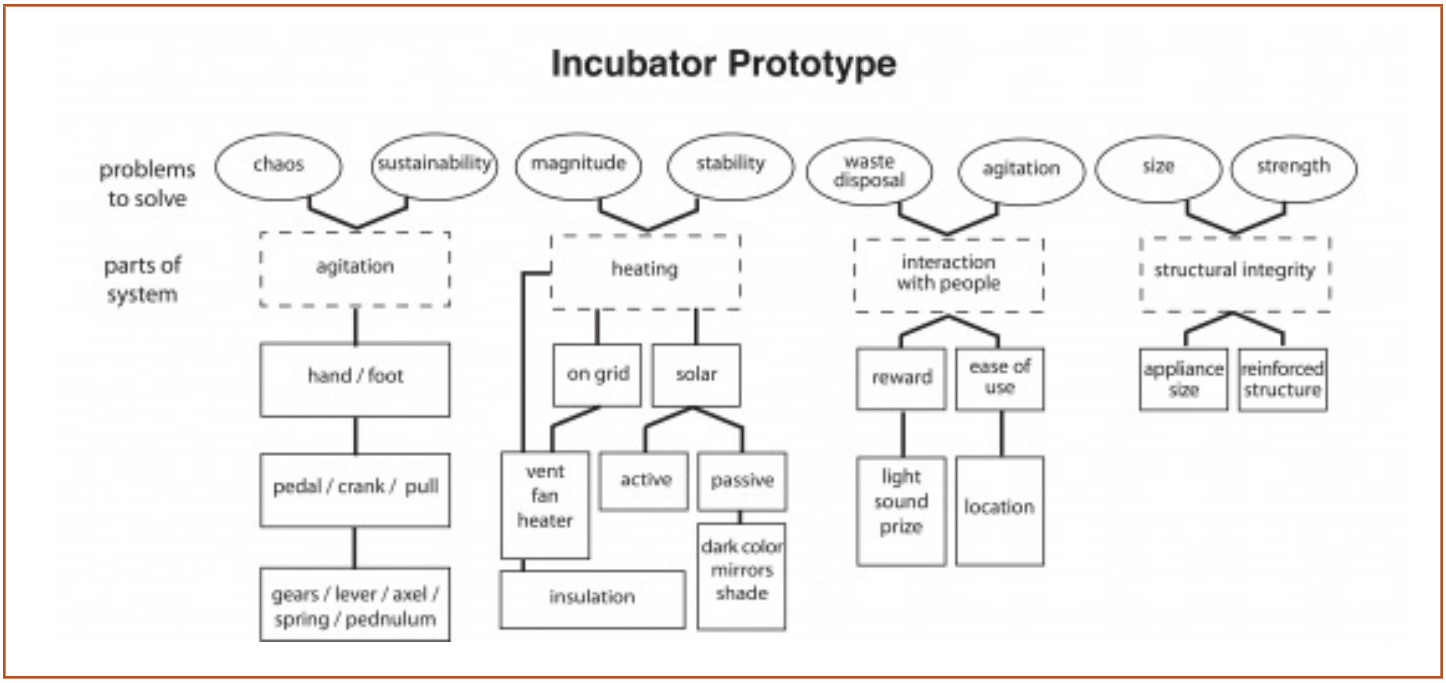


***Figure 5.*** *Variables to be considered, implemented and tested when designing the incubator prototype.*

system are both the concentration of sugar and duration of fermentation. We test samples for ethanol using high-performance liquid chromatography (HPLC). Although pretreatment and hydrolysis are optimized for increased sugar concentration, the ideal conditions for ethanol production are not experimentally determined yet.

## Safety

The potentially hazardous substances used in this study include the Ctec2 cellulase enzyme and sulfuric acid. Substances will be handled with caution at all times. Risks include eye and respiratory tract irritation, chemical burn, and harmful ingestion. Gloves, goggles, lab coats, and closed toed shoes are requirements when working in the lab to decrease risk of exposure. Additionally, a fume hood is used, along with the assistance of an advisor, when diluting acid in order to prevent exposure to sulfuric acid. The disposal procedure for the sulfuric acid is neutralization using calcium carbonate.

## Discussion

The aims of this study are to address waste management, explore a source for alternative energy, achieve high precision and replicability, and recreate an effective digestion environment in an out of lab setting. The objectives of both pretreatment and hydrolysis are to achieve visual degradation and high yields of sugar, measured in triplicate using a refractometer (in Brix). Ideally, the lignocellulose that composes paper towel waste can be broken down entirely, via pretreatment and hydrolysis, until only lignin remains. Once separated from cellulose, lignin is also a viable economic resource that has many applications, including as a binder or sequestrant.

We consider the standard deviation of brix readings for each trial and use unpaired T-tests to inform the significance of our conclusions. Through comparing trials with incrementally increasing concentrations of biomass, we find that greater amounts of substrate are positively correlated with sugar yield. As shown in Figure 6, the most effective concentrations of enzyme are lower than 1% (~13 U/mL) and as low as 0.25% (~3.25 U/mL); samples with 0.25% enzyme demonstrating a significantly greater change in brix than those with 1% ($P < 0.001$). Due to enzyme cost, lower concentrations are also more efficient.

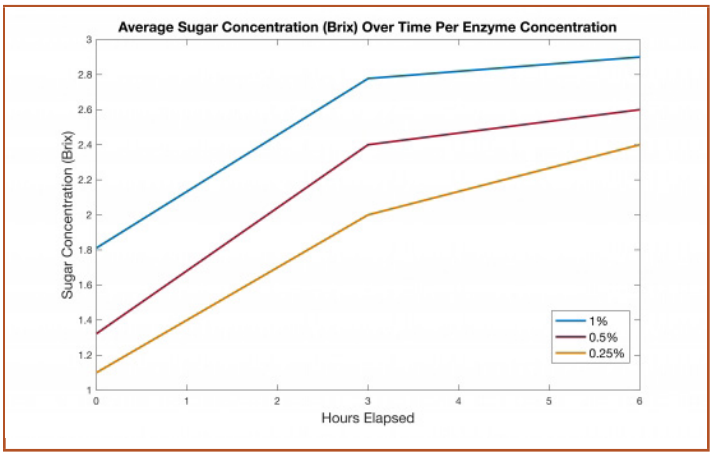


***Figure 6.*** *Average sugar concentration over time per enzyme concentration. Lower concentrations of enzyme, as low as 0.25%, are more efficient.*

We find a reduction in sugar release when the reaction pH is uncontrolled, while the use of a buffer stabilizes pH and enhances sugar production. Our results, in Figure

7, show that the use of a sodium acetate buffer (pH 6.0) significantly enhances sugar production compared to the Hydrion buffer (pH 6.0) ($P < 0.01$). Note that in Figure 7, the three trials start with different sugar concentrations due to differing brix of the buffers.

According to Figure 8, a comparison between average change in brix and pH suggests that the ideal pH is between 5 and 5.8, likely around 5.4 or 5.5. This pH differs from those suggested by T. Q. Lan, et. al, which suggested a pH 5.5-6.2. Including estimated brix changes during pretreatment, the brix of samples pretreated with sulfuric acid increased significantly greater than that of the control (untreated samples) ($P < 0.001$).

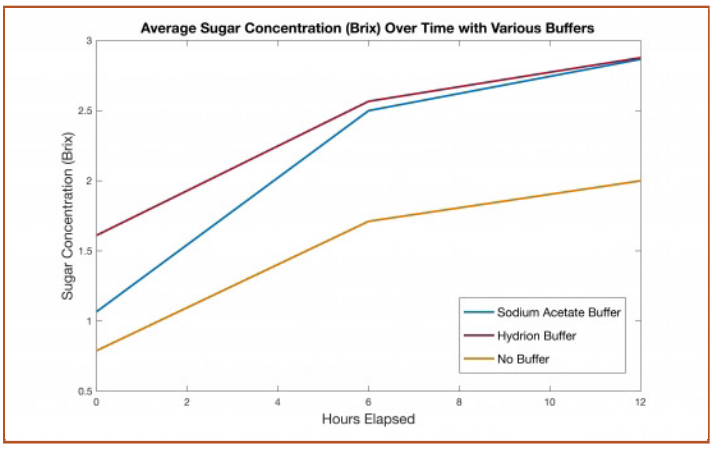


***Figure 7.*** *Average sugar concentration over time with various buffers. Sodium acetate buffer is required for pH stability and is most effective buffer tested.*

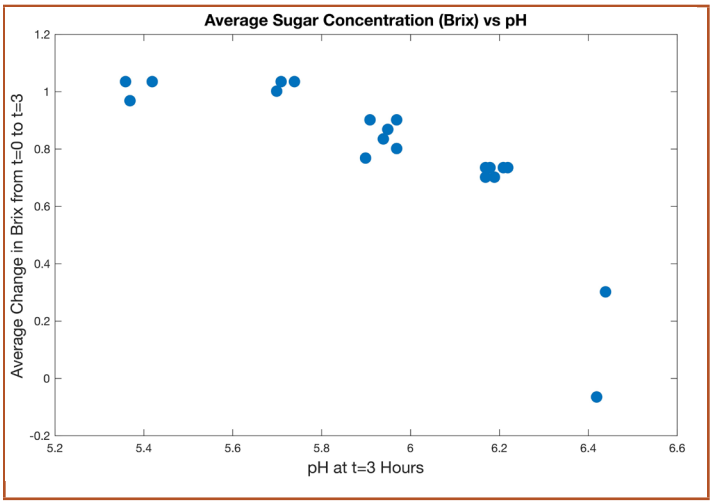


***Figure 8.*** *Average sugar concentration over time with various pH. A pH of 5.4-5.5 is the ideal environment for enzymatic digestion.*

With sugar production optimized, two potential applications we see for harvesting energy from the sugar solutions are the microbial fuel cell (MFC) and ethanol fermentation. Voltage can be produced by using the sugar solutions from the digestion process in the MFC. We design and create a new MFC with an increased surface area to volume ratio to enable the use of carbon fiber brush electrodes. This new MFC produces double the watts per brix than the kit MFC. A 56k ohm load resistor in the circuit improves electrical output. Furthermore, the voltage produced by the microbial fuel cell is increased when a mediator dye and an electron acceptor fluid are used.

We show the possibility of large-scale ethanol production by successfully fermenting sugar solutions and finding positive readings of ethanol from HPLC tests. As shown in Figure 9, 7.35 ng/μL ethanol can be produced from a 5.22 brix solution, suggesting that ethanol can be produced from these solutions on a larger scale. Legal restrictions prevent the full distillation of ethanol, but the evidence of ethanol in the fermentations, as well as similar procedures in other studies and industries, allows for the extrapolation towards pure ethanol production.

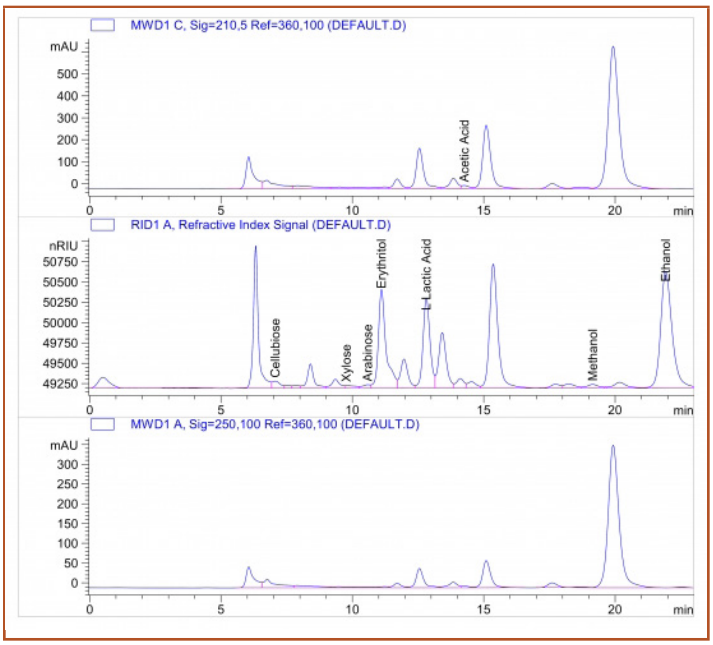


***Figure 9.*** *Absorbance (AU, top and bottom panels) and Refractive Index (RI, middle panel) measurements showing presence of ethanol following fermentation (middle panel).*

We successfully refine lab procedures over time in order to reduce error, resulting in a decrease in standard deviations over the course of the labs. These refinements include utilizing more precise equipment and increasing the number of repetitions and sample sizes.

Finally, we show the incubator prototype and bucket stand according to the diagrams and the box is stained

and insulated. The prototype is designed to be heated by the sun or by a thermostatically controlled heater, depending on location. We successfully recreate effective digestion environments and processes within the setting of the appliance prototype.

To make this digestion process economically and environmentally competitive with other paper towel disposal methods, such as composting or landfilling, efficiency must be further optimized by using lower concentrations of enzyme, and designing improvements for pretreatment and energy production. Future lab work will include testing contamination tolerance, finding maximum paper towel to liquid ratio, conducting multiple digestion cycles with the same solution, and testing lower concentrations of enzyme.

## Acknowledgements

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