Photographic Assay Methods for Quantifying Enzymatic Breakdown of Cellulose

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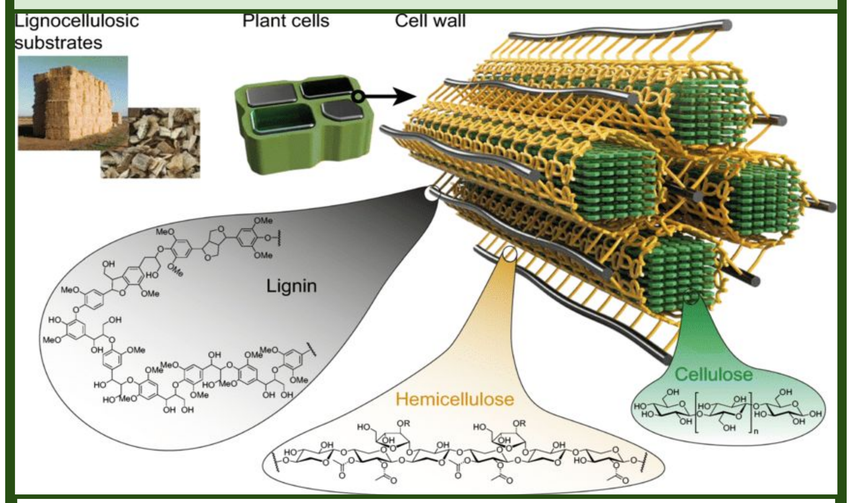
Appalachian STEM Academy at Oak Ridge 2022

Introduction

Biofuels are of interest as a renewable solution to increasing carbon emissions. However, current corn based ethanol biofuels are not economically competitive with petroleum based fuels, primarily due the inefficiency of separating plant biomass into its component parts. Research is being done to develop methods to overcome recalcitrance of plant biomass to cellulase through pretreatment methods and development of mutant strains. One crop of interest is the native perennial switchgrass *Panicum virgatum* which has potentially higher yields of biofuels, while maintaining currently utilized agricultural equipment and reducing soil erosion compared to annual crops. Butanol, aromatic compounds, and advanced materials can be produced in addition to traditional enzyme/yeast conversion of cellulose into ethanol. As new methods are developed, tools for analyzing the efficiency of these conversions are needed. Current methods of analysis include using plate readers to quantify colorimetric assays, but these methods are costly and are problematic when working with solid, opaque substrates like plant biomass. Our goal is to develop a method which uses photographs captured from webcams and smartphones that can quantify the amount of digestion of plant biomass by enzymes.

Background

Plant cell walls are made of a matrix of cross-linked lignocellulose, which is in turn comprised of three polymers: **Cellulose**, **Hemicellulose**, and **Lignin**. Each component has multiple applications, but the complex must be broken down and separated before they can be utilized.



Examples of the three plant cell wall polymer structures: **Cellulose** (right), **Hemicellulose** (center) and **Lignin** (left). Note regular crystalline structure of **Cellulose** versus random amorphous structure of **Hemicellulose** and **Lignin**. **Hemicellulose** and **Lignin** can block the enzyme cellulase from breaking **Cellulose** down to fermentable sugars. Graphic taken from Reference 1.

Cellulose, a polysaccharide, is produced by plants, fungi, and some algae, and makes up the greatest percentage biomass of the lignocellulose (38%-50%) with currently the broadest array of applications. **Cellulose** is a linear chain of glucose units and readily forms hydrogen bonds. These chains form bundles with high tensile strength and give rigidity to the structure of the cell wall. **Lignin** fills in the gaps in between cellulose in the cell walls and acts as a glue in the matrix. **Lignin** bonds covalently with **Hemicellulose**, crosslinking the units within and providing additional strength to the structure. It is used to produce solvents, biofuels, and the sweetener, xylitol. While **Lignin** is being studied for applications such as a bonding agent in plywood, resins, and adhesives, it presents a challenge to breaking down lignocellulose into its component parts through enzymatic digestion. **Lignin**'s resistance to digestion is a challenge that must be overcome to make the production of biofuels cost effective.

Materials and Methods

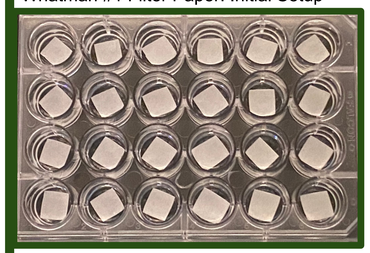
Step 1: Set up Cellulase Digestions of Cellulose from Two Sources: Whatman #1 Filter Paper and Wild Type Switchgrass

For Whatman #1 Filter paper: Filter paper was cut into 1 cm squares. Each square was weighed, recorded, placed in each of 24 wells in a 24-well plate. Plate was photographed to show the starting dimensions of the paper.

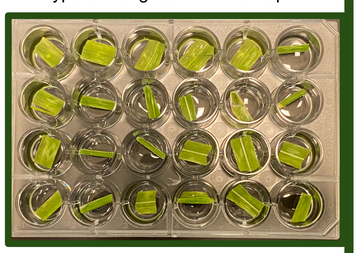
For Wild Type Switchgrass: A second well plate was prepared using wild type switchgrass. Switchgrass was cut from sections with a relatively uniform width of 1 cm. Sections were cut into 1 cm lengths. Each piece of switchgrass was weighed, recorded, and placed into each of 24 wells in a 24 well plate. Plate was photographed to show the starting dimensions of the switchgrass.

Both preparations were given the following treatment: 1 mL of Sodium Acetate buffer was added to each well in Columns 1 & 2. 1 mL of Cellulase (1/100 dilution in Sodium Acetate buffer) was added to each well in Columns 3&4. 1 mL of Cellulase (1/50 dilution in Sodium Acetate buffer) was added to each well in Columns 5&6. Samples were placed on a rotating platform and agitated at 100 rpms at 24.5° Celsius ambient.

Whatman #1 Filter Paper: Initial Setup



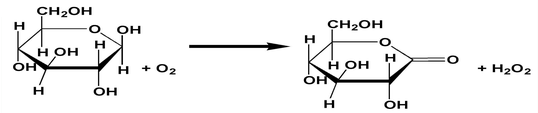
Wild type Switchgrass: Initial Setup



Step 2: Create a Standardized Assay with Known Quantities of Glucose

A 24-well plate was set up with known concentrations of glucose in sodium acetate buffer as follows:

Column 1	1 mL Sodium Acetate buffer (control)
Column 2	1 mL 0.25 mM glucose
Column 3	1 mL 0.5 mM glucose
Column 4	1 mL 1.0 mM glucose
Column 5	1 mL 1.5 mM glucose
Column 6	1 mL 2.0 mM glucose



Glucose (left) in the presence of the enzyme glucose oxidase and oxygen is converted to gluconic acid (right) and hydrogen peroxide. This hydrogen peroxide further reacts with ferrous ammonium sulfate yielding a yellow ferric solution with a 340 nm absorbance peak.

Each well was treated with 0.5 mL of 10 mM Ferrous Ammonium Sulfate in Sodium Acetate. Ferrous Ammonium Sulfate turns yellow when oxidized by Hydrogen Peroxide and is therefore useful as an indicator. Each well was then treated with 0.5 mL 4 mg/mL Glucose Oxidase extracted from *Aspergillus niger*, an enzyme that hydrolyzes glucose into gluconic acid and hydrogen peroxide. The reaction was allowed to proceed for 30 minutes. The well plate was then examined visually and the results were checked using a Cary UV Spectrophotometer at a wavelength of 340 nm. Additionally, the assay was repeated at a 1:1 dilution of Sodium Acetate buffer.

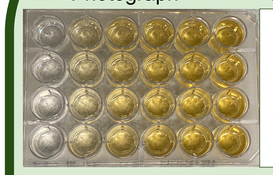
Step 3: Produce Assays to Quantify Cellulase Digestion of Whatman #1 Paper and Wild Type Switchgrass

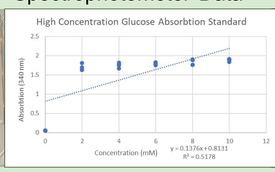
24 Well plates containing treatments of Whatman #1 filter paper and wild type switchgrass from Step 1 were removed from the rotating platform. Samples were visually examined and photographed. Aliquots of supernatant from each well were removed, centrifuged to remove solids, added to corresponding wells in a new plate, and treated with 0.5 mL of 10 mM Ferrous Ammonium Sulfate in Sodium Acetate. Then 0.5 mL of glucose oxidase was added to each well for both Whatman #1 and wild type switchgrass samples. The reaction was allowed to proceed for 30 minutes. The well plate was examined visually and photographed. Photographs were compared to glucose standard and checked for absorbance using a Cary UV spectrophotometer at a wavelength of 340 nm. Samples were also diluted with 1 part sodium acetate buffer to 1 part sample and checked for absorption.

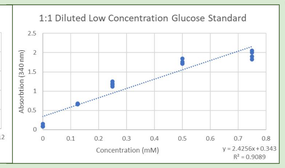
Results

Glucose Standard:

Photograph | Spectrophotometer Data | 1:1 Dilution

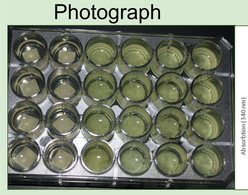


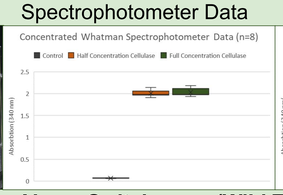


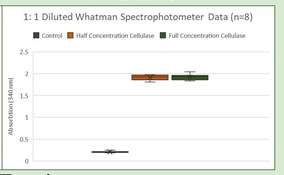


Whatman #1 Filter Paper:

Photograph | Spectrophotometer Data | 1:1 Dilution







Alamo Switchgrass (Wild Type):

Photograph | Spectrophotometer Data | Cary UV Spectrophotometer

The Standard Glucose Assay produced a color differential that was observable to the naked eye, as well as through photography. Likewise, assays of Whatman #1 filter paper and Alamo Switchgrass created observable color differentials relative to the concentration of cellulase used to digest the biomass. Spectrophotometry revealed that our Glucose Standard Samples were too concentrated to create a standardized absorption curve. Further analysis after 1:1 dilution of glucose standards show a linear relationship between concentration of glucose and absorption at 340 nm.

Conclusions

Our goal was to develop a method which uses webcam and smartphone photography to quantify enzyme digestion of plant biomass. We used the enzyme cellulase to breakdown cellulose from plant biomass into glucose. We then used glucose oxidase with ferrous ammonium sulfate as an indicator of the concentration of glucose present in the product. This method shows promising results and warrants further research to refine the protocols. We successfully created an observable color differential relative to the concentration of glucose present. We were not able to correlate the photographic results to absorptions using spectrophotometry. We believe that this is due to high concentration of color that is beyond the range of the spectrophotometer we used to analyze the samples. More research is needed to find a concentration of glucose standard that falls within the range of the spectrophotometer.

Acknowledgements

Thank you to Dr. Barbara R. Evans of Oak Ridge National Laboratory for mentoring this research project. The SFA Biofuels project is supported by the Genomic Science Program, Office of Biological and Environmental Research, U.S. Department of Energy. We appreciate the opportunity provided to us by Oak Ridge National Laboratory, Oak Ridge Associated Universities and the Appalachian Regional Commission.

References

1) Brethauer, Simone & Shahab, Robert & Studer, Michael. (2020). Impacts of biofilms on the conversion of cellulose. Applied Microbiology and Biotechnology. 104.10.1007/s00253-020-10595-y.