Enzymatic Hydrolysis of Cellulose to Glucose

NSF ATE Project 1601636
Chemical and BioEnergy for Sustainability

Introduction:

Cellulases are one type industrial enzymes with significant economic importance in industries spanning forest products (pulp and paper), dish and clothing detergents, textile finishing, personal care products, animal feed, leather making, beer brewing, wine/cheese/liquor making, baking, sweetener production, bioethanol, biogas and biodiesel synthesis[1]. Enzymes are also used extensively pharmaceutical therapy, some examples include insulin, interferons and blood clotting agents. The enzymes used in our lab experiment were purchased from Novozymes, a Danish company with a large U.S. office and research lab in eastern North Carolina. Novozymes is the largest producer of industrial enzymes worldwide.

To produce lignocellulosic biofuels and/or bioproducts using the *sugar platform*, cellulose and hemicellulose derived from biomass pretreatment muse be hydrolyzed to glucose and other fermentable monosaccharides[2,3]. This *enzymatic saccharification* technique relies on specially designed enzymes called cellulases that cleave cellulose into cellobiose (a disaccharide of β-glucose). Subsequently, β glucosidases cleave cellobiose into glucose. In biorefineries, enzymatic "cocktails" are often used, and in addition to cellulase and β glucosidase, the cocktails contain hemicellulases to hydrolyze hemicellulose into monomeric sugars (i.e. glucose, xylose, mannose). The monosaccharides are then fermented into biofuels (such as ethanol) or bioproducts.

Once the saccharification reaction is complete, you must determine the glucose yield from the original mass of biomass (i.e. willow oak). Glucose yields are important to understand the efficacy of your pretreatment reaction (i.e. soda pulping). Raw, untreated, biomass is often used as a control. In this lab we will use GC-MS to determine the yield of glucose as its pentaacetate derivative. HPLC in combination with Mass Spectrometry, Refractive Index or Evaporative Light Scattering is also commonly used for glucose yield determination because it does not require derivatization.

Prelab Questions:

1. Define the *sugar platform* and give an example of how this method is used to produce a commercially sold chemical.

2. What is meant by an enzymatic "cocktail" and how are they used in industrial applications?

3. Notice the ***concentration*** of enzyme used, and optimum ***pH*** and ***temperature*** for the hydrolysis of biomass. Why are these factors important for industrial production of biofuels such as ethanol?

4. How and why is glucose derivatized to the pentaacetate ester prior to analysis using GC-MS? Draw a reasonable reaction mechanism for the reaction.

Learning Objectives - The following experiments will:

- Analyze hexose sugars, e.g. glucose and mannose, using GC-MS by derivitization to the pentaacetate esters
- Analyze pentose sugars, e.g. xylose and arabinose using GC-MS by derivitization to the tetraacetate esters
- Hydrolyze cellulose pulp generated from the soda pulping process to monosaccharides

Caution – pyridine and acetic anhydride are toxic reagents and have associated stenches. It is best to work with these reagents in the chemical fume hood.

Pentose and Hexose Standard Curve Procedure:

1. Using an analytical balance, prepare calibration curves for glucose and xylose by weighing 10.0 mg, 20.0 mg and 30.0 mg of each sugar into 6 separate vials.
2. Add 2.00 mL of pyridine to each vial
3. Add 1.00 mL of acetic anhydride to each vial
4. Heat with stirring for 2 hr at 60^{o}C (you can then let the reaction stand overnight at room temperature)

5. Dilute 15 μL of each into 1 mL of acetonitrile into a GC vial and inject 1 μL on the GC-MS.

6. Make calibration curves for glucose and xylose data from the respective GC-MS chromatograms.

7. GC-MS method:

Enzymatic Saccharification of Biomass Pulp Procedure:

1. Weigh 500 mg of biomass pulp from the soda lab experiment, 500 mg of Avicel (microcrystalline cellulose control) and 500 mg of Raw Biomass (untreated control)

2. Prepare 100 mL of 0.05 M citrate buffer at pH 4.8 by combining 0.05 M citric acid with 0.05 M sodium citrate. Use the pH meter to determine pH of final buffer solution.

3. Add 100 mg of sodium azide (or another appropriate antibiotic) to 100 mL the citrate buffer.

4. Into a 15mL (blue, screwcap) Falcon Tube, add biomass samples + 10 mL of citrate buffer. It would be preferred to do this into a 20mL scintillation vial that could be Speed-Vacd dry.

5. Group 1: Add 10 μL of Novozymes Cellulase Enzyme (CTec) to each sample.
   Group 2: Add 20 μL of Novozymes Cellulase Enzyme (CTec) to each sample.
   Group 3: Add 30 μL of Novozymes Cellulase Enzyme (CTec) to each sample.

   *Note: for complete sugar profiles, Hemicellulase (HTec) enzymes should be added in 25-50% of the concentration of CTec enzymes.

6. Collect 2.0 mL samples from each experiment at 24, 48 and 72 hr. Filter through 0.2 micron syringe filter into separate round bottom flasks and dry extensively on the rotary evaporator.

7. Dilute each sample into 2.0 mL of pyridine and transfer to a 4 mL screw cap scintillation vial. Add 1.0 mL of acetic anhydride to each sample and heat with stirring for 2 hr at 60^{o}C (you can then let the reaction stand overnight at room temperature).

8. Dilute 100 μL of each sample into 900 μL of acetonitrile and inject onto GC-MS.

9. Quantify and compare sugar yields from each experiment using standard curve.

Post Lab - State your conclusions:

What was the overall percent yield of glucose yield from the original biomass sample?

What was the glucose yield from the pretreated biomass sample?

How did the glucose yield from pretreated biomass compare to that of raw biomass or Avicel?

Why do we use Avicel as a control?

What could have been done to improve this experiment?

References:

1. *Enzymes at Work*: 4th Edition of Novozymes Industrial Enzyme Catalogue. **2013**, pp. 26-35.
2. Wilson, D. Cellulases and Biofuels. *Curr. Op. Biotech*. **2009**, 20, 295-299.
3. *From the Sugar Platform to biofuels and biochemicals*. Final report for the European Commission Directorate – General Energy. **2015**