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Laboratory Analytical Procedure (LAP)

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February 2015

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1. Introduction

1.1. This procedure describes the enzymatic saccharification of native or pretreated lignocellulosic biomass to glucose, cellobiose, and xylose in order to determine the comparative digestibility/conversion extent of the feedstock or the efficacy of various enzyme samples.

1.2. The older filter paper unit assay is no longer considered a meaningful option for determining cellulase activity. It only converts 3.6% of the substrate and the substrate is not a real-world feedstock.

2. Scope

2.1. This procedure is appropriate for lignocellulosic biomass and model cellulose (e.g., Avicel). If the biomass has some starch content, the dry weight percent cellulose calculated from total glucan must be corrected by subtracting the starch contribution to total dry weight percent glucose.

2.2. All analyses should be performed in accordance with an appropriate laboratory specific Quality Assurance Plan (QAP).

3. Terminology

3.1. *Pretreated biomass*: biomass that has been chemically and/or thermally altered, changing its physical structure and/or chemical composition.

3.2. *Lignocellulase enzyme cocktails*: an enzyme preparation exhibiting synergistic cellulolytic, hemicellulolytic, and other lignocellulose-degrading activities. Different enzyme activities are present at varying ratios in different enzyme preparations.

4. Significance and Use

4.1. The extent of digestibility is used in conjunction with other assays to determine the appropriate enzyme loading for the saccharification of biomass.

4.2. This procedure may also be used with a “standard” cellulase preparation to measure the efficacy of a given pretreatment based on a preset/standard enzyme loading.

4.3. This procedure is designed for biomass solids loadings of 1.0% (w/v) or 10 mg of dry biomass/mL of reaction slurry. Enzyme activity generally decreases with increasing solids content.

5. Interferences

5.1. Test specimens not suitable for analysis by this procedure include unwashed acid- and/or alkaline-pretreated biomass samples. Free acid or alkali may change the solution pH to values outside the range of enzymatic activity and the hydrolysate may contain inhibitory degradation products. Glucose, xylose, and other sugars present in unwashed pretreated biomass may influence the final result.

5.2. Air-drying of biomass samples prior to saccharification will quite likely have a negative impact on conversion due to hornification of the cellulose component and is therefore **not** recommended.

5.3. This procedure is developed for the use of fungal secreted enzymes with temperature optima near 50°C and pH optima near 5.0; other enzyme cocktails may have different temperature and pH optima, which should be determined prior to using this assay.

5.4. Enzymatic hydrolysis of biomass is an exceedingly complex biochemical process, with wide variability present in the substrate and the enzyme complex. Great care must be taken when making comparisons between assays.

5.5. In general, cellulases are strongly inhibited by cellobiose; therefore, if a sufficient amount of β-D-glucosidase activity is not present in the enzyme cocktail then supplementing the reaction with β-D-glucosidase may be necessary to achieve conversion levels representative of the reactivity of the substrate.

5.6. Glucose can inhibit β-glucosidase, so high solids loadings may lead to overall enzyme inhibition as cellobiose levels build due to inhibited β-glucosidase activity.

5.7. Other conditions, such as excessive temperature swings in the incubator, rotational distance and speed differences in shaking/rotating, reactor vial configuration (aspect ratio or geometric conformation), feedstock particle size, and evaporative losses from reactor vessels, can also greatly impact the results.

5.8. *Trichoderma reesei* (*Hypocrea jecorina*) produces a cellulase cocktail that is roughly 50% cellobiohydrolase I (Cel7A). Cel7A is a difficult protein to quantitate by chemical colorimetric methods as it reacts poorly to most commercial kits. Other GH7 cellobiohydrolases may be similar and care should be taken when quantifying enzyme cocktails. Bicinchoninic acid (BCA) seems to give the best response. Dye-based assays (Lowry's, Bradford, and others) are poor at detecting Cel7A.

5.9. The hydrolysis process releases sugars, which can be consumed by microbes present in the hydrolysis mixture. While antibiotics and/or sodium azide can be used to help control this, sterile materials should be used when possible to minimize potential contamination. Sterile vials, buffers, enzyme solutions,

reagents, water, pipet tips, etc., are strongly encouraged. The main source of contamination is the biomass itself, as it is generally not sterile, especially if it has been washed/neutralized and stored for any length of time.

5.10. Microbial contamination is also a concern for samples awaiting high-performance liquid chromatography (HPLC) analysis. Samples should be frozen if there will be a significant delay in having them analyzed. Be sure to mix well after thawing; re-filtering may be necessary if precipitates have formed. A refrigerated auto-sampler is highly recommended, especially if sample sets requiring more than a few hours are analyzed.

6. Apparatus and Materials

- pH meter
- A shaking, rotating, or static incubator with temperature control ± 1°C
- Analytical balance, accurate to 1.0 or 0.1 mg
- BCA protein quantitation kit (Thermo Fisher Scientific) or equivalent
- HPLC system with refractive-index detection containing Aminex HPX-87H column (Bio-Rad Laboratories, Inc.)
    - Other sugar analysis columns may be used, however the details of their operation are left to the operator
    - Other sugar analysis methods (gas chromatography [GC], enzyme-linked, etc.) may be used, however accuracy and precision are dependent on the methodology employed
- 2.0 mL glass HPLC vials with watertight caps capable of withstanding digestion temperatures
- 20, 200, and 1,000 µL pipettors with tips.

7. Reagents

- 5% sodium azide (50 mg/ml in distilled water)
- (Alternative antibiotic) tetracycline (10 mg/mL in 70% ethanol)
- (Alternative antibiotic) cycloheximide (10 mg/mL in distilled water)
- Sodium citrate buffer (1.0M, pH 5.0)
- (Alternate buffer) sodium acetate buffer (1.0M, pH 5.0)
- Lignocellulase enzyme(s)
- (If necessary) β-glucosidase enzyme of known activity, pNPGU/mL
- (If necessary) hemicellulase enzyme of known protein concentration (mg/ml).

8. Environment, Safety, and Health Considerations and Hazards

8.1. Cycloheximide, tetracycline, and sodium azide are hazardous and must be handled with appropriate care.

8.2. Follow all applicable safe chemical handling procedures.

9. Sampling, Test Specimens, and Test Units

None.

10. Procedure

10.1. Perform biomass chemical compositional analysis laboratory analytical procedures (LAP) to determine the amount of glucan and hemicellulose in the biomass.

10.2. Perform LAP *Determination of Total Solids in Biomass and Total Solids in Liquid Process Samples* for all biomass samples to be digested. Note: all lignocellulosic materials that have undergone some aqueous pretreatment must never be oven- or air-dried prior to enzyme digestibility, since irreversible pore collapse (hornification) can occur in the microstructure of the biomass leading to decreased enzymatic accessibility to the substrate.

10.3. Determine protein content of the enzyme solution(s) being used. Commercial enzymes typically contain high levels of hygroscopic stabilizing agents such as glycerol, sorbitol, glucose, and other low molecular weight compounds. These compounds can interfere with protein assays and also affect digestion rates, especially in high-solids digestions, and so should be removed prior to determining protein content. The sample used to determine total protein content is sacrificed during the analysis with the resulting number used to back-calculate the original solution concentration. The preferred protocol for protein content determination is as follows:

10.3.1. For highly concentrated commercial enzymes, the stock solution should be diluted to a range of 0.5–5 mg/mL in 50 mM sodium citrate buffer pH 5.0 to reduce the viscosity and allow accurate pipetting of the enzyme solution. Diluted commercial enzyme preparations should be used immediately, as the enzymes lose activity over time.

10.3.2. Protein concentration is determined using the Pierce BCA protein assay according to the manufacturer's instructions. It is well known that different protein assays (Bradford, BCA, Lowry, A280, etc.) will yield different results. Our experience is that the BCA assay is the most consistent and accurate on fungal cellulases. If the enzyme preparation is a commercial or other highly concentrated sample, additional dilutions may be needed to get within the linear range of the assay.

10.4. If the removal of soluble sugars, pretreatment chemicals, and degradation products is required prior to enzymatic hydrolysis, wash the biomass three times with 30 mM sodium citrate pH 5.0 containing 0.002% sodium azide to inhibit microbial growth and then resuspend the solids in a 2.0% slurry (20 mg/ml) in the same buffer.

10.5. Pipette or weigh out a biomass slurry sample containing 0.014 g of total biomass on a dry weight basis and quantitatively transfer to a 2.0 mL glass HPLC vial.

10.6. To each vial add 42 µL 1.0 M, pH 5.0 sodium citrate buffer (or alternative buffer). (Note: Sodium citrate is inhibitory to fermentative organisms. Sodium acetate is inhibitory to acetyl xylan esterase. If the biomass slurry does not contain antimicrobial agents, add 5.6 µl of a 5.0% sodium azide solution to each vial. Alternatively, a combination of tetracycline/cycloheximide may be added. If required, add 5.6 µL (56 µg) tetracycline and 4.2 µL (42 µg) cycloheximide to prevent the growth of organisms during the digestion. Also note: tetracycline and cycloheximide both pose reproductive hazards; do not combine sodium azide with tetracycline/cycloheximide).

10.7. Calculate the amount of enzyme solution required to provide 20 mg protein per g of glucan in the biomass according to the following equation:

$$enzyme\ volume = \frac{1.0\ mL}{X\ mg\ protein}\ x \frac{20.0\ mg\ protein}{1.0\ g\ glucan}\ x\ \ g\ glucan$$

where:

X = mg protein in 1.0 mL of enzyme sample.

The original enzyme sample may need to be diluted (or concentrated) in order to obtain a suitable concentration to maintain a 1.4 mL total volume. Do not add enzyme yet.

10.8. Add distilled water to bring the total volume in each vial to 1.4 mL minus the calculated volume of enzyme determined above. All solutions, including the biomass slurry, are assumed to have a specific gravity of 1.000 g/mL. Thus, if 0.5 g of biomass slurry is added to the vial, it is assumed to occupy 0.5 mL and a total of 0.9 mL of additional liquid is to be added.

10.9. Prepare substrate blank(s) containing buffer, water, antimicrobials, and the identical amount of substrate in 1.4 mL total volume (no enzyme).

10.10. Prepare enzyme blank(s) with buffer, water, antimicrobials, and enzyme (no substrate). Some commercial enzymes are stabilized with glucose and this must be accounted for in the final conversion yield. If multiple enzyme volumes are used for different reactions, separate enzyme blanks should be prepared for each level.

10.11. After all other reaction components are loaded into the vials, initiate the reaction by adding the lignocellulase enzyme preparation to equal 20 mg of protein per g of glucan as determined above. (Hemicellulases and β-glucosidase may be added at the same time). Close the vials tightly and place them in an HPLC vial rack suitable for the shaking incubator or fixed speed rotator that has been placed in the incubator. Set the temperature to 50°C and incubate with shaking or rotation sufficient to keep solids in constant suspension for a period of 72 to 168 hours or until additional release of soluble sugars from the sample(s) becomes negligible when measured by HPLC, as described in the next step.

10.12. If reaction rate is to be determined, withdraw a 0.1 µL aliquot at each predetermined time interval after the vial contents have been well mixed. Use a 1000 µL pipet-tip (wide bore or with the tip cut off) to provide an orifice large enough to allow solids, as well as liquid, to be sampled while constantly suspending the contents of the vial. Pay attention to pipetting accurately because 100 µL is at the lower end of the accuracy range of a 1,000 µL pipet. Make sure biomass solids do not clog the tip; if this happens, widen the orifice by cutting the tip with scissors. Removal of a consistent solid:liquid ratio is critical. Avoid over-sampling. A rule of thumb is to not remove more than 50% of the starting volume over all samples taken. Dilute samples 10-fold with double-distilled water (ddH_2O) into 2.0 mL vials, then seal the vials and immerse in a boiling-water bath for 10 minutes to inactivate the enzymes and terminate the reaction. Filter the diluted and terminated digestion aliquots through 0.2 µm nominal-pore-size nylon syringe filters to remove residual substrate and other insolubles prior to sugar analysis. Process substrate blank and enzyme blank vials in the same manner as the reaction vials.

10.13. Released cellobiose, glucose, and xylose in the diluted samples can be determined by HPLC analysis on an Aminex HPX-87H column operated at 55°C with 0.01 N sulfuric acid as the mobile phase at 0.6 mL/min in an Agilent 1100 HPLC system with refractive-index detection. Other sugars, such as arabinose, galactose, mannose, and others may be determined using other HPLC methods and columns (such as Shodex SP-0810).

11. Calculations

11.1. To calculate the percent conversion, first correct the sugar concentrations determined in 10.13 above by subtracting the sums of the sugar concentrations, if any, from the diluted aliquots of the substrate and enzyme blanks. Then correct for the 10-fold aliquot-dilution to obtain the concentrations of the sugars in the digestion vials.

11.2. The resulting glucose, cellobiose, and xylose concentrations (mg/mL) calculated for each digestion mixture should be converted to anhydro-glucose, anhydro-cellobiose, and anhydro-xylose concentrations, respectively, by subtracting out the proportional weight added to each molecule by the water of hydrolysis (i.e., multiplying by 0.95 for cellobiose, by 0.90 for glucose, and by 0.88 for xylose),

which will convert the concentrations (mg/mL) of sugars found in the digestion liquid to the "concentrations" of material removed from the polysaccharides (*H* in equation below).

To calculate percent conversion, the sum of the concentrations of anhydro-glucose and anhydro-cellobiose or anhydro-xylose is equivalent to the weight-concentration of the glucan and xylan chains hydrolysed to produce the soluble sugars, and is then divided by the initial weight-concentration of glucan or xylan in the digestion mixture and multiplied by 100% to yield activity results as percent conversion of glucan or xylan.

$$\%\ conversion = \frac{\frac{mg\ sugar}{mL}\ x\ 1.4\ mL\ x\ H}{\frac{g\ polysaccharide}{g\ biomass}\ x\ 0.014\ g\ biomass}\ x\ 100$$

where:

H = the hydrolysis factor specific for each polysaccharide/sugar combination

mg/mL sugar is from HPLC analysis

g polysaccharide/g biomass is from the compositional analysis.

11.3. To report or calculate the root mean square deviation (RMSdeviation) or the standard deviation (stdev) of the samples, use the following calculations.

First find the root mean square (RMS), of the sample using:

$$RMS = x_m = mean = \sqrt{\left(\frac{\Sigma_1^n x}{n}\right)^2}$$

Then find the root mean square deviation, or standard deviation, using:

$$RMSdeviation = \sigma = stdev = \sqrt{\frac{\Sigma_1^n (x_i - x_m)^2}{n}}$$

where:

x_m = the root mean square of all x values in the set

n = number of samples in set

x_i = a measured value from the set.

12. Report Format

12.1. Report the percent glucan or xylan digested in the sample to two decimal places on a dry weight basis. Cite the basis used in the report.

12.2. For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

13. Precision and Bias

13.1. The precision of this protocol has not been defined because it is dependent upon pipetting errors, cellulase source, and substrate composition. Not only will different preparations of cellulase hydrolyze identical substrates to different extents, but different preparations of pretreated biomass also exhibit different degrees of homogeneity.

14. Quality Control

14.1. *Reported significant figures or decimal places:* Typically results are reported as percentages, calculated to two decimal places, along with the standard deviation and RPD. The assay conditions, specifically digestion time, must be defined when reporting the results.

14.2. *Replicates:* It is recommended the samples be run in triplicate to verify reproducibility.

14.3. *Blank:* Enzyme and substrate blanks are run to correct for glucose contributions other than that produced by cellulose hydrolysis.

14.4. *Relative percent difference criteria:* Not defined; dependent on the substrate being tested. Different preparations of pretreated biomass will exhibit different amounts of homogeneity, which will influence the extent to which they are hydrolyzed.

14.5. *Method verification standard:* Solka-Floc 200 NF is digested alongside the biomass samples. Hydrolysis is expected to be in the range of 94.00%–96.00%.

14.6. *Calibration verification standard:* None.

14.7. *Sample size:* Dependent on percent dry weight cellulose composition. Typically between 0.010 g and 1.00 g of sample will be required.

14.8. *Sample storage:* Pretreated biomass samples should be stored moist, or frozen not longer than one month. Hydrolysis samples for sugar analysis should be stored cold if analysis is short term, frozen if analysis will be delayed.

14.9. *Standard storage:* None.

14.10. *Standard preparation:* None.

14.11. *Definition of a batch:* Any number of samples that is analyzed and recorded together. The maximum size of a batch will be limited by equipment constraints.

14.12. *Control charts:* Percent hydrolysis of Solka-Floc 200 NF will be charted; use of different preparations of cellulase enzyme and total hydrolysis time will be noted.

15. Appendices

None

16. References

16.1. Selig, M.; Weiss, N.; Ji, Y. (2008). *Enzymatic Saccharification of Lignocellulosic Biomass*: *Laboratory Analytical Procedure (LAP).* NREL/TP-510-42629. Golden, CO: National Renewable Energy Laboratory.

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