



Ethanol Project

***Chemical Analysis and Testing Task
Laboratory Analytical
Procedure***

LAP-009

Enzymatic Saccharification of Lignocellulosic

Procedure Title: Biomass	
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Enzymatic Saccharification of Lignocellulosic Biomass

Laboratory Analytical Procedure #009

1. Introduction

1.1 This procedure describes the enzymatic saccharification of cellulose from native or pretreated lignocellulosic biomass to glucose in order to determine the maximum extent of digestibility possible (a saturating level of a commercially available or in house produced cellulase preparation and hydrolysis times up to one week are used).

2. Scope

2.1 This procedure is appropriate for lignocellulosic biomass. If the biomass is suspected to have some starch content, dry weight percent cellulose calculated from total glucan (LAP-002) must be corrected to subtract the starch contribution to total dry weight percent glucose.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Grohmann, K., Torget, R., and Himmel, M. (1986), Biotech. Bioeng. Symp. No. 17,

135-151.

- 3.2 Ghose, T.K. (1987), Pure & Appl. Chem., **59**, 257-268.
- 3.3 Stockton, B.C., Mitchell, D.J., Grohmann, K., and Himmel, M.E. (1991), Biotech. Let., **13**, 57-62.
- 3.4 Adney, B. and Baker, J. (1993), Ethanol Project Laboratory Analytical Procedures, LAP-006, National Renewable Energy Laboratory, Golden, CO, 80401.
- 3.5 Ehrman, C. I. (1996), Ethnaol Project Laboratory Analytical Procedures, LAP-016, National Renewable Energy Laboratory, Golden, CO, 80401.

4. Terminology

- 4.1 Pretreated biomass - Biomass that has been subjected to milling, chemical treatment with water or steam, strong or dilute acid or alkali, or other physical or chemical methods to render the cellulose content of the material more accessible to enzymatic action.
- 4.2 Cellulase enzyme - an enzyme preparation exhibiting all three synergistic cellulolytic activities: endo-1,4- β -D-glucanase, exo-1,4- β -glucosidase, or β -D-glucosidase activities, which are present to different extents in different cellulase preparations.

5. Significance and Use

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

6. Interferences

- 6.1 Test specimens not suitable for analysis by this procedure include acid- and alkaline-pretreated biomass samples that have not been washed. Unwashed pretreated biomass samples containing free acid or alkali may change solution pH to values outside the range of enzymatic activity.

7. Apparatus

- 7.1 VWR model 1540 incubator set at $50^{\circ} \pm 1^{\circ}\text{C}$.

- 7.2 Cole-Parmer model 7637-20 "Roto-Torque" Fixed Speed Rotator.
- 7.3 A 24-slot large-holed test tube rack that can be attached to the "Roto-Torque" Rotator.
- 7.4 Eppendorf model 5414 microcentrifuge.
- 7.5 pH meter.
- 7.6 Analytical balance, sensitive to 0.0001 g.
- 7.7 Yellow Springs Instrument, Inc., Model 27 Glucose Analyzer or Model 2700 Select Biochemistry Analyzer.
- 7.8 Drying oven adjusted to $105^{\circ}\text{C} \pm 2^{\circ}\text{C}$.
- 7.9 A 200 μL and a 1000 μL Eppendorf Pipetman pipet with tips.

8. Reagents and Materials

- 8.1 Tetracycline (10 mg/mL in 70% ethanol).
- 8.2 Cycloheximide (10 mg/mL in distilled water).
- 8.3 Sodium citrate buffer (0.1M, pH 4.80).
- 8.4 Cellulase enzyme of known activity, FPU/mL.
- 8.5 \square -glucosidase enzyme of known activity, pNPGU/mL.
- 8.6 Solka Floc 200 NF, FCC (microcrystalline cellulose) from Brown Company with ash, moisture, and xylan contents determined (see Ethanol Project Laboratory Analytical Procedures, LAP-001, -002, and -005).
- 8.7 Eppendorf Safe-Lock 1.5-mL microcentrifuge tubes.
- 8.8 20-mL glass scintillation vials equipped with plastic-lined caps.

9. ES&H Considerations and Hazards

- 9.1 Cycloheximide and tetracycline are hazardous and must be handled with appropriate care.

9.2 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

10. Procedure

10.1 Total solids must be determined for all cellulose containing samples to be digested (LAP-001).

Note: all lignocellulosic materials which have undergone some aqueous pretreatment must never undergo any drying whatsoever prior to enzyme digestibility, since irreversible pore collapse can occur in the micro-structure of the biomass leading to decreased enzymatic release of glucose from the cellulose. Additionally, all frozen lignocellulosic materials which are to be subjected to digestibility tests can not have been frozen for more than one month prior to analysis, since, depending on the environment, sublimation could have occurred, leading to possible irreversible collapse of micropores in the biomass.

10.2 Weigh out a biomass sample equal to the equivalent of 0.1 g of cellulose on a 105°C dry weight basis (the cellulose content of the sample is initially determined as glucose by LAP- 002, minus the contribution of any starch present, LAP-016) and add to a 20 mL glass scintillation vial. Also, weigh out 0.1 g of the Solka Floc MVS and add to another vial.

- 10.3 To each vial, add 5.0 mL 0.1 M, pH 4.8 sodium citrate buffer.
- 10.4 To each vial, add 40 μL (400 μg) tetracycline and 30 μL (300 μg) cycloheximide to prevent the growth of organisms during the digestion.
- 10.5 Calculate the amount of distilled water needed to bring the total volume in each vial to 10.00 mL after addition of the enzymes specified in the following step. Add the appropriate calculated volume of water to each vial. All solutions and the biomass are assumed to have a specific gravity of 1.000 g/mL. Thus, if 0.200 g of biomass is added to the vial, it is assumed to occupy 0.200 mL and 9.733 mL of liquid is to be added.
- 10.6 Bring the contents of each vial to 50°C by warming in the incubator set at 50° \pm 1°C. To each vial is added an appropriate volume of the cellulase enzyme preparation to equal approximately 60 FPU/g cellulose and the appropriate volume of β -glucosidase enzyme to equal 64 pNPGU/g cellulose.

Note: If the rate of enzymatic release of glucose is to be measured, all contents of the vial prior to the addition of the enzyme must be at 50°C. The enzymes are always added last since the reaction is initiated by the addition of enzyme.

- 10.7 Prepare a reaction blank for the substrate. The substrate blank contains buffer, water, and the identical amount of substrate in 10.00 mL volume.
- 10.8 Prepare enzyme blanks for cellulase and β -glucosidase with buffer, water, and the identical amount of the enzyme.
- 10.9 Close the vials tightly and place them in the "Roto-Torque" fixed speed rotator set at an approximate angle of 45°C that has been placed in the VWR incubator set at 50°C. Incubate with gentle rotation (68 RPM) for a period of 72 to 168 hours or until the release of soluble sugars from the sample(s) becomes negligible when measured by YSI, as described in the next step.
- 10.10 If the progress of the reaction is to be measured, a 0.3-0.5 mL aliquot is removed at each predetermined time interval after the vial contents have been well mixed by shaking. This is accomplished by using a 1.0-mL Eppendorf Pipetman pipet with the tip of the plastic 1.0-mL tip slightly cut off (to allow solids, as well as liquid, to be withdrawn into the orifice). The sample is expelled into a 1.5-mL microcentrifuge tube and centrifuged for 1.5 minutes. The supernatant is subjected to glucose analysis using the YSI glucose analyzer.

11. Calculations

- 11.1 To calculate the percent digestibility of the cellulose added to the scintillation vial, determine glucose concentration in the centrifuged supernatant by YSI. Subtract the glucose concentrations, if any, from the substrates and enzyme blanks.
- 11.2 Correct for hydration (multiply the glucose reading by 0.9 to correct for the water molecule added upon hydrolysis of the cellulose polymer) and multiply by 10 mL total volume of assay.

Example: If the glucose analyzer reading (corrected with blanks) is 9.9 mg/mL, then the amount of cellulose digested is:

$$0.0099 \text{ g/mL} \times 10 \text{ mL} \times 0.9 = 0.0891 \text{ g}$$

- 11.3 Calculate percent digestion:

$$\% \text{ digestion} = \frac{\text{grams cellulose digested}}{\text{grams cellulose added}} \times 100$$

12. Report

- 12.1 Report the percent cellulose digested in the sample, to two decimal places, on a 105°C 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 cellulase source and substrate composition. Not only will different preparations of cellulase hydrolyze identical substrates to different extents, but different preparations of pretreated biomass exhibit different amounts of homogeneity.

14. Quality Control

- 14.1 *Reported significant figures:* 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 duplicate 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 samples. Hydrolysis is expected to be in the range of 94.00 - 96.00%.
- 14.6 *Calibration verification standard:* Not applicable.
- 14.7 *Sample size:* Dependent upon percent dry weight cellulose composition. Typically between 0.10 and 1.00 grams of sample will be required.
- 14.8 *Sample storage:* Pretreated samples should be stored moist, or frozen not longer than one month.
- 14.9 *Standard storage:* Not applicable.
- 14.10 *Standard preparation:* Not applicable.
- 14.11 *Definition of a batch:* A batch is defined as the sample replicates and method verification standard hydrolyzed with an identical cellulase preparation and incubated during the same time.
- 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.