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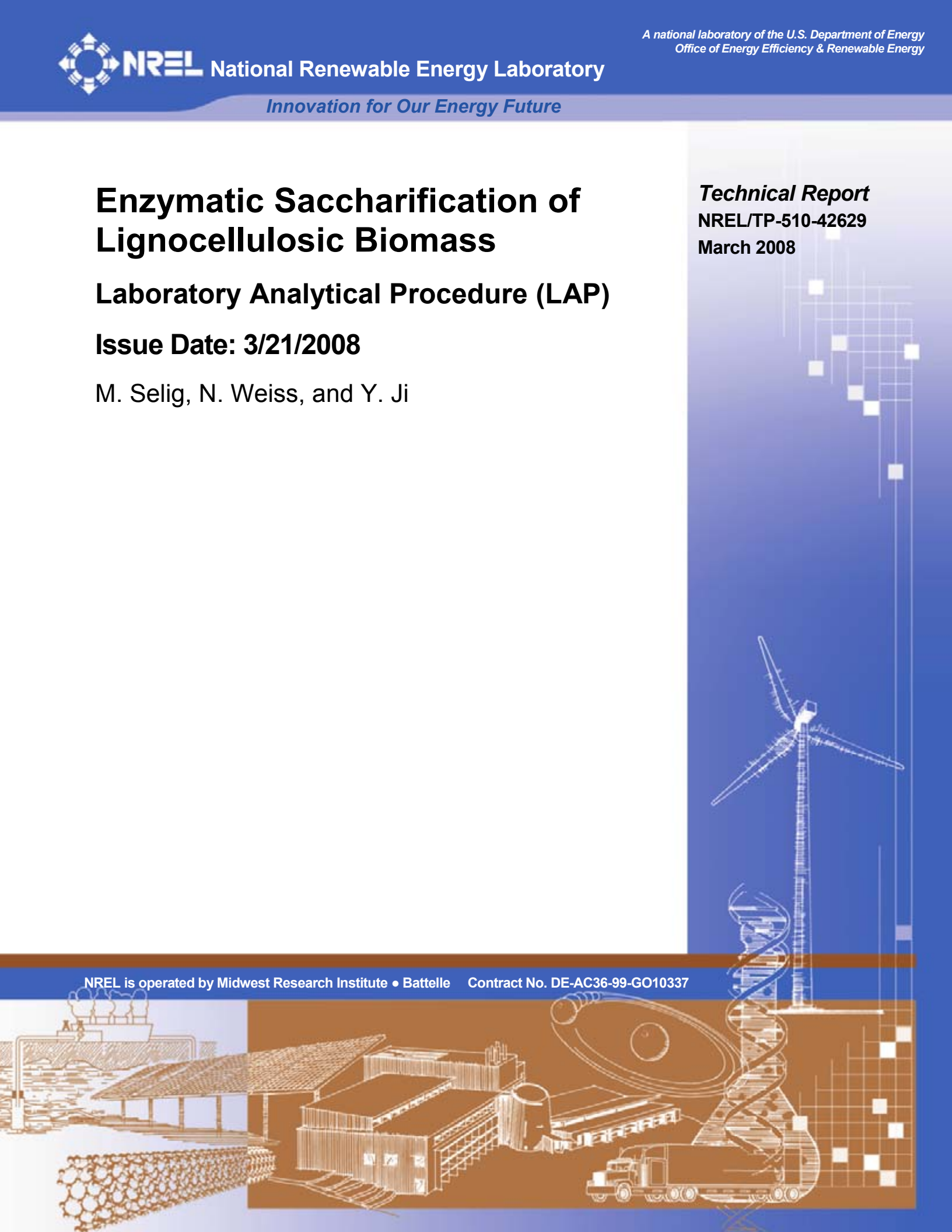
Enzymatic Saccharification of Lignocellulosic Biomass

Laboratory Analytical Procedure (LAP)

Issue Date: 3/21/2008

M. Selig, N. Weiss, and Y. Ji

Technical Report
NREL/TP-510-42629
March 2008



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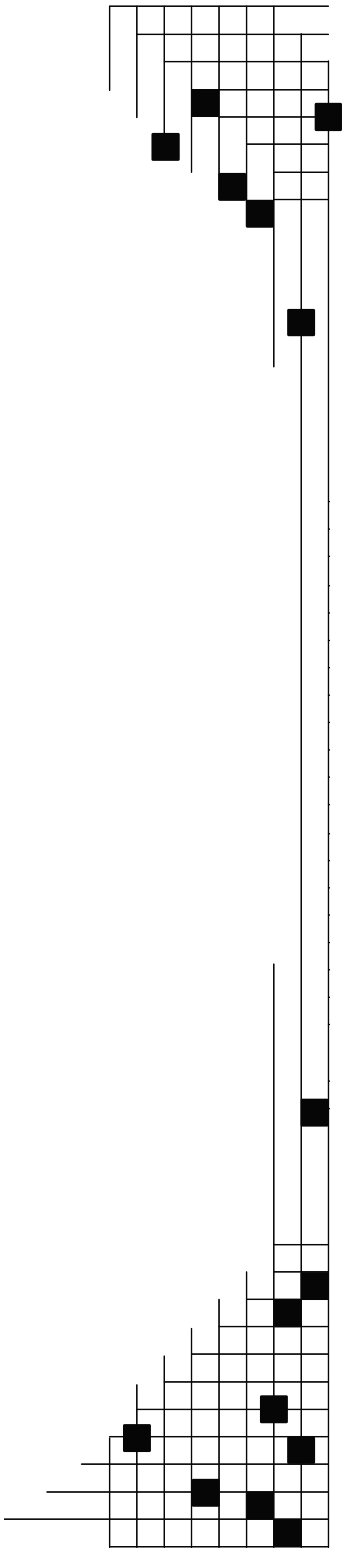

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Procedure Title: Enzymatic Saccharification of Lignocellulosic Biomass

Laboratory Analytical Procedure

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 must be corrected to subtract 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 or thermally altered, changing the structural composition

3.2 *Cellulase enzyme:* an enzyme preparation exhibiting all three synergistic cellulolytic activities: endo-1,4-β-D-glucanase, exo-1,4-β-glucosidase, and β-D-glucosidase activities, which are present to different extents in different cellulose preparations.

4. Significance and Use

4.1 The maximum 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 can also be used to measure the efficacy of a given pretreatment based on a maximum enzyme loading.

5. Interferences

5.1 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; and the unwashed glucose in the biomass may influence the final result.

5.2 Air drying of biomass samples prior to saccharification may have an impact on the maximal conversions achieved.

6. Apparatus and Materials

6.1 A suitable shaking or static incubator set at $50^{o} \pm 1^{o}C$

6.2 Any fixed speed rotator that can hold scintillation vials and operate in a static incubator.

6.3 Scintillation vial rack/tray

6.4 pH meter

6.5 Analytical balance, accurate to 1 mg or 0.1 mg
6.6 YSI analyzer with appropriate membranes or equivalent glucose quantification method such as HPLC
6.7 200 µL and a 1000 µL Eppendorf Pipetman pipet with tips
6.8 20-mL glass scintillation vials equipped with plastic-lined caps

7. Reagents

7.1 Reagents
7.1.1 Tetracycline (10 mg/mL in 70% ethanol).
7.1.2 Cycloheximide (10 mg/mL in distilled water).
7.1.3 Alternate antibiotic – Sodium Azide (20 mg/ml in distilled water)
7.1.4 Sodium citrate buffer (0.1M, pH 4.80).
7.1.5 Cellulase enzyme of known activity, FPU/mL.
7.1.6 Beta-glucosidase enzyme of known activity, pNPGU/mL
7.1.7 (If necessary) Xylanase enzyme of known protein concentration, mg/ml

8. ES&H Considerations and Hazards

8.1 Cycloheximide, tetracycline and sodium azide are hazardous and must be handled with appropriate care.
8.2 Follow all applicable NREL chemical handling procedures

9. Sampling, Test Specimens and Test Units

None

10. Procedure

10.1 Perform LAP "Determination of Total Solids in Biomass" for all cellulose containing samples to be digested. Note: all lignocellulosic materials which have undergone some aqueous pretreatment must never be air-dried 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.
10.2 Weigh out a biomass sample equal to the equivalent of 0.1 g of cellulose or 0.15 g total biomass 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.
10.3 To each vial, add 5.0 mL 0.1 M, pH 4.8 sodium citrate buffer. To each vial, add 40 µL (400 µg) tetracycline and 30 µL (300 µg) cycloheximide to prevent the growth of organisms during the digestion. Since tetracycline and cycloheximide both pose reproductive hazards, 100 ul of a 2% sodium azide solution may be added as an alternate to the tetracycline/cycloheximide combination (Note: do not combine sodium azide with the tetracycline/cycloheximide combination).
10.4 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.

10.5 Bring the contents of each vial to 50°C by warming in the incubator set at 50° ± 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 *p*NPGU/g cellulose. Xylase may be added at the same time. 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.

10.6 Prepare a reaction blank for the substrate. The substrate blank contains buffer, water, and the identical amount of substrate in 10.00 mL volume.

10.7 Prepare enzyme blanks for cellulase, β-glucosidase, and xylanase with buffer, water, and the identical amount of the enzyme.

10.8 Close the vials tightly and place them in a scintillation 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 the release of soluble sugars from the sample(s) becomes negligible when measured by YSI, as described in the next step.

10.9 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. Use a 1-mL plastic syringe to draw a representative sample while constantly suspending the contents of the vial. Alternatively, this is accomplished by using a 1.0-mL 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 filtered through a 0.45 µm filter and subjected to glucose analysis using the YSI glucose analyzer or appropriate HPLC method.

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:

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

11.4 To report or calculate the relative percent difference (RPD) between two samples, use the following calculation:

$$RPD = \left(\frac{(X_1 - X_2)}{X_{mean}} \right) \times 100$$

Where:

X_1 and X_2 = measured values
X_{mean} = the mean of X_1 and X_2

11.5 To report or calculate the root mean square deviation (RMS deviation) or the standard deviation (st dev) of the samples, use the following calculations.
First find the root mean square (RMS), of the sample using

$$RMS = x_m = mean = \sqrt{\left(\frac{\sum_1^n x}{n} \right)^2}$$

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

$$RMS deviation = \sigma = stdev = \sqrt{\frac{\sum_1^n (x_i - x_m)^2}{n}}$$

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 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 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 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: None.

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: None.

14.10 Standard preparation: None.

14.11 Definition of a batch: Any number of samples which are 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

15.1 None.

16. References

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