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Laboratory Analytical Procedure (LAP)

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B. Adney and J. Baker

Technical Report
NREL/TP-510-42628
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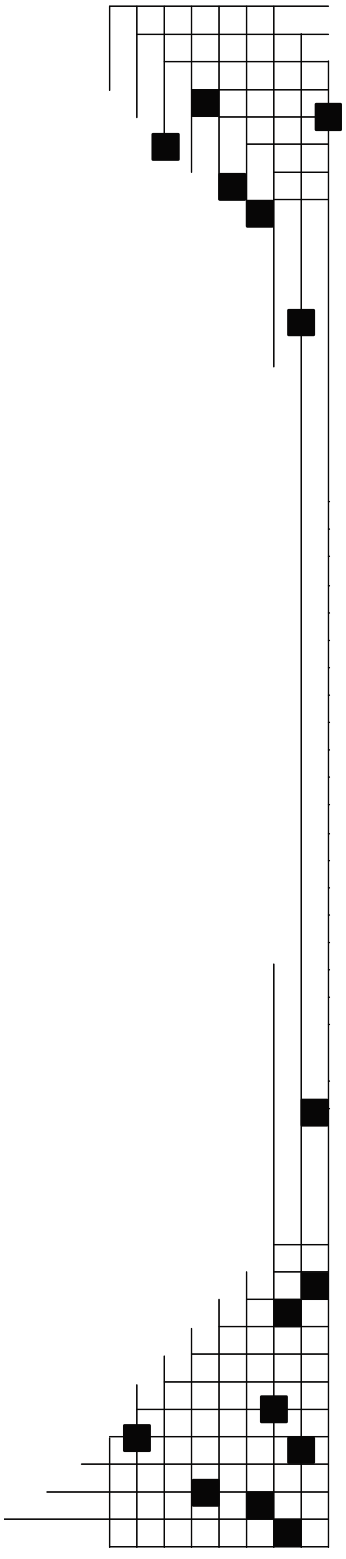
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Measurement of Cellulase Activities

Laboratory Analytical Procedure

1. Introduction

1.1 The following method describes a procedure for measurement of cellulase activity using International Union of Pure and Applied Chemistry (IUPAC) guidelines (1). The procedure has been designed to measure cellulase activity in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. For quantitative results the enzyme preparations must be compared on the basis of significant and equal conversion. The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC.

1.2 It is extremely important to keep in mind that the FPU is defined only at this extent of conversion. Reducing sugar yield is not a linear function of the quantity of enzyme in the assay mixture; as discussed by Ghose (1987), twice the amount of enzyme would not be expected to yield twice the reducing sugar in equal time. The assay procedure therefore involves finding a dilution of the original enzyme stock such that a 0.5 mL aliquot of the dilution will catalyze 4% conversion in 60 minutes (or, in practical terms, finding two dilutions that bracket the 4%-conversion point so closely that the required dilution can be obtained, with reasonable accuracy, by interpolation) and then calculating the activity (in FPU/mL) of the original stock from the dilution required. Further comments on the required calculations, and their significance, are to be found in the Appendix.

1.3 Assay mixtures may in some cases contain reducing sugars unrelated to hydrolysis of substrate glycosidic bonds by the enzyme. Culture filtrates to be assayed for cellulase may contain nutrient sugars, and the reducing ends of the cellulose polymers of the substrate may sometimes be measurable as glucose equivalents before any enzyme attack. For this reason, controls consisting of (a) enzyme without substrate and b) substrate without enzyme are included with all enzyme assays and sample values are corrected for any blank values.

2. Scope

2.1 This procedure is only appropriate for the determination of FPU activity in a cellulase preparation as defined by the IUPAC procedure as outlined above.

3. References

3.1 Ghose, T.K. 1987. "Measurement of Cellulase Activities." *Pure & Appl. Chem.* 59: 257-268.

3.2 Miller, G.L. 1959. "Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar." *Anal. Chem*. 31:426-428.

4. Significance and Use

4.1 This procedure follows IUPAC guidelines and determines enzyme activity as filter paper units in a cellulase preparation.

5. Apparatus

5.1 Water bath capable of maintaining 50°C ± 0.1°C.
5.2 Spectrophotometer suitable for measuring absorbance at 540 nm.

6. Reagents and Materials

6.1 **DNS Reagent**

Mix:		
	Distilled water	1416 mL
	3,5 Dinitrosalicylic acid	10.6 g
	Sodium hydroxide	19.8 g

Dissolve above, then add:

Rochelle salts (sodium potassium tartrate)	306 g
Phenol (melt at 50°C)	7.6 mL
Sodium metabisulfite	8.3 g

Titrate 3 mL sample with 0.1 N HCl to the phenolphthalein endpoint. It should take 5-6 mL of HCl. Add NaOH if required (2 g = 1 mL 0.1 N HCL).

6.2 **Citrate Buffer**: For *Trichoderma reesei*, cellulase assays are carried out in 0.05 M citrate buffer pH 4.8. For other cellulase enzymes, the pH and the assay temperature may be different. The assay conditions must be defined when reporting results.

Citric acid monohydrate	210 g
DI water	750 mL
NaOH - add until pH equals 4.3	50 to 60 g

Dilute to 1 L and check pH. If necessary add NaOH until the pH is 4.5. When the 1 M stock citrate buffer stock is diluted with water to 50 mM the pH should be 4.8. After diluting the citrate buffer check and adjust the pH if necessary to pH 4.8.

7. ES&H Considerations and Hazards

7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.
7.2 Care must be taken when working with phenol, which is toxic and corrosive.

8. Procedure for the Filter Paper Assay for Saccharifying Cellulase

8.1 The detection of glycosidic bond cleavage by this method involves the parallel and identical treatment of three categories of experimental tubes (assay mixtures, blanks and controls, and glucose standards), prepared as detailed below. The substrate is a 50 mg Whatman No. 1 filter paper strip (1.0 x 6.0 cm).
8.2 Enzyme assay tubes:
8.2.1 Place a rolled filter paper strip into each 13 x 100 test tube.

8.2.2 Add 1.0 mL 0.05 M Na-citrate, pH 4.8 to the tube; the buffer should saturate the filter paper strip.

8.2.3 Equilibrate tubes with buffer and substrate to 50°C.

8.2.4 Add 0.5 mL enzyme diluted appropriately in citrate buffer. At least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (absolute amount) and one slightly less than 2.0 mg of glucose. Target 2.1 and 1.9 mg glucose, respectively, for these two dilutions. Depending on the enzyme these targets may be hard to achieve and additional dilutions must be run.

8.2.5 Incubate at 50°C for exactly 60 min.

8.2.6 At the end of the incubation period, remove each assay tube from the 50°C bath and stop the enzyme reaction by immediately adding 3.0 mL DNS reagent and mixing.

8.3 Blank and controls:

8.3.1 Reagent blank: 1.5 mL citrate buffer.

8.3.2 Enzyme control: 1.0 mL citrate buffer + 0.5 mL enzyme dilution (prepare a separate control for each dilution tested).

8.3.3 Substrate control: 1.5 mL citrate buffer + filter-paper strip.

8.4 Glucose standards:

8.4.1 A working stock solution of anhydrous glucose (10 mg/mL) should be made up. Aliquots of this working stock should be tightly sealed and stored frozen. The standard should be vortexed after thawing to ensure adequate mixing.

8.4.2 Dilutions are made from the working stock in the following manner:
1.0 mL + 0.5 mL buffer = 1:1.5 = 6.7 mg/mL (3.35 mg/0.5 mL).
1.0 mL + 1.0 mL buffer = 1:2 = 5 mg/mL (2.5 mg/0.5 mL).
1.0 mL + 2.0 mL buffer = 1:3 = 3.3 mg/mL (1.65 mg/0.5 mL).
1.0 mL + 4.0 mL buffer = 1:5 = 2 mg/mL (1.0 mg/0.5 mL).

8.4.3 Glucose standard tubes should be prepared by adding 0.5 mL of each of the above glucose dilutions to 1.0 mL of citrate buffer in a 13 x 100 mm test tube.

8.4.4 Blanks, controls and glucose standards should be incubated at 50°C along with the enzyme assay tubes, and then "stopped" at the end of 60 minutes by addition of 3.0 mL of DNS reagent.

8.5 Color development (Miller, 1959):

8.5.1 Boil all tubes for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by the reaction mixture plus reagent. All samples, controls, blanks, and glucose standards should be boiled together. After boiling, transfer to a cold ice-water bath.

8.5.2 Let the tubes sit until all the pulp has settled, or centrifuge briefly. Dilute all tubes (assays, blanks, standards and controls) in water (0.200 mL of color-developed reaction mixture plus 2.5 mL of water in a spectrophotometer cuvette works well, use the pipettor to mix by drawing the mixture into the pipettor tip repeatedly). Determine color formation by measuring absorbance against the reagent blank at 540 nm. With this dilution the glucose standards described above should give absorbance in the range of 0.1 to 1.0 A.

9. Calculations

9.1 Construct a linear glucose standard curve using the absolute amounts of glucose (mg/0.5 mL) plotted against A_{540}. The data for the standard curve should closely fit a calculated straight line, with the correlation coefficient for this straight line fit being very near to one. Verify the standard curve by running a calibration verification standard, an independently prepared solution of containing a known amount of glucose which falls about midpoint on the standard curve.

9.2 Using this standard curve determine the amount of glucose released for each sample tube after subtraction of enzyme blank.

9.3 Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by means of a plot of glucose liberated against the logarithm of enzyme concentration (refer to the example in Appendix B, which uses semilogarithmic graph paper). To find the required enzyme concentration take two data points that are very close to 2.0 mg and draw a straight line between them, use this line to interpolate between the two points to find the enzyme dilution that would produce exactly 2.0 mg glucose equivalents of reducing sugar. Appendix B presents an example.

> Note: In this plot, and in the equation below for calculating FPU, the term "enzyme concentration" refers to the proportion of the original enzyme solution present in each enzyme dilution (i.e., the number of mL of the original solution present in each mL of the dilution).

9.4 Calculate FPU:

$$Filter\ Paper\ Activity = \frac{0.37}{[enzyme]\ releasing\ 2.0\ mg\ glucose}\ units/ml$$

Where [enzyme] represents the proportion of original enzyme solution present in the directly tested enzyme dilution (that dilution of which 0.5 mL is added to the assay mixture). For the derivation of the FPU see Ghose (1987) and Appendix A.

9.5 Refer to Appendix B for an example for calculating IUPAC-FPU.

10. Precision and Bias

10.1 Precision can be measured only by the closeness of repeated measurements of the same quantity of enzyme. This procedure, if carefully followed, should give the same approximate numerical readings as obtained by other laboratories using the same procedure. Precision in filter paper assays may be affected by the inherent physical properties of cellulase preparations.

11. Quality Control

11.1 *Reported significant figures:* Typically results are reported as whole integers along with the standard deviation. The assay conditions must be defined when reporting the results.
11.2 *Replicates:* Run each dilution in triplicate.
11.3 *Blank:* As described in the section "Blank and controls".
11.4 *Relative percent difference criteria:* Not defined; dependent on the enzyme being tested.
11.5 *Method verification standard:* Not available since enzymes change over time.
11.6 *Calibration verification standard:* A calibration verification standard shall be independently prepared and analyzed as described in the section "Calculations".
11.7 *Sample size:* Dependant upon enzyme concentration.
11.8 *Sample storage:* Dependant upon source of enzyme. Manufacturer's instructions should be followed.
11.9 *Standard storage:* Store frozen at -20^{o}C or prepare fresh batch; shake vigorously prior to use.
11.10 *Standard preparation:* As described in the section "Glucose standards".
11.11 *Definition of a batch*: Run all standards, blanks, and samples together in one batch. The size of the batch may be limited by instrument constraints and should not be larger than what is practical to handle together.
11.12 *Control charts:* Not applicable.
11.13 *Others:* Not applicable.

12. Appendix A: Numerical Values in Equation Used to Calculate Filter Paper Activity

The practical bottom line is that if the assays are set up according to the instructions, and the calculations are carried out using the equation presented in the calculations section, the results obtained will correspond to the generally accepted activities in "filter paper units" that would be obtained by other laboratories around the world, were these other laboratories to test the same enzyme solution. For those workers interested in the derivation of this equation, and of the "filter paper unit", the following comments may be helpful in conjunction with Ghose (1987).

The numerator (0.37) in the equation is derived from the factor for converting the 2.0 mg of "glucose-equivalents" generated in the assay to mmoles of glucose (2.0 ÷ 0.18016), from the volume of the enzyme being tested that is used in the assay (0.5 mL), and from the incubation time (60 minutes) required for generation of the reducing equivalents.

Thus,

$$\frac{(2.0\ mg\ glucose\ /\ 0.18016\ mg\ glucose/\mu mol)}{(0.5\ mL\ enzyme\ dilution\ x\ 60\ minutes)} = 0.37\ \mu mol/\ minute\ -\ mL$$

Because the "enzyme concentration" in the denominator of the equation is a dimensionless number (equal to the ratio of the enzyme concentration in the 0.5 mL of enzyme dilution added to the assay to the enzyme concentration in the original solution, for which FPU values are desired), the right side of equation therefore winds up with units (mmol $min^{-1}mL^{-1}$) that look like "International Units per mL" (I.U./mL). Ghose himself points out, however, that "because the FPU assay is non-linear, the use of the International Unit *per se* is incorrect as this unit is based on initial velocities, i.e., linear reactions in which the product is produced at the same rate during each and every minute of the reaction." The author goes on to recommend that FPU values for a given cellulase solution be given simply as "units/mL".

"Definition" of the "Filter Paper Unit":

As a result of the above choice of numerical values, the "Filter Paper Unit" is not actually explicitly defined. What is defined is the quantity 0.1875 FPU, which is that quantity of enzyme activity that, when assayed according to the instructions contained herein, will produce reducing sugar equivalent to 2.0 mg of glucose. One can verify this from the equation presented in the calculations section by assuming that the enzyme solution being tested needs no dilution to yield reducing sugar equivalent to 2.0 mg of glucose (i.e., the "enzyme concentration" ratio in the denominator is equal to 1.0), in which case the activity of the solution being tested is measured as 0.37 filter paper units per mL. Inasmuch as 0.5 mL of this solution was used in the assay, the absolute quantity of enzyme activity that is present in the assay (and to which the observed effect can be ascribed) is 0.1875 FPU.

To put it another way, we have a defined method for measuring the activity of a cellulase solution containing 0.1875 filter paper units per 0.5 mL assay aliquot (0.37 filter paper units per mL of enzyme solution) but we do not have method for measuring the filter paper activity of solutions with any other value. Solutions containing more than 0.37 "units" per mL must therefore be diluted to this standard value to be measured, and solutions containing less than 0.37 "units" per mL (reducing sugar produced in 60 minutes is less than that equivalent to 2.0 mg of glucose) cannot be assigned "filter paper unit" activities at all. These latter "sub-2.0-mg" solutions either must be concentrated before assay, or the activities should not be reported as "filter paper units" at all, but should be reported instead as "mmoles glucose equivalents released per minute averaged over 60 minutes."

Ghose (1987) explains the special circumstances involved in measurement of "filter paper activity", and workers are urged to pay close attention to the text of the paper (especially the text surrounding the equations on page 263 of the reference) rather than just "lifting" the equations themselves.

13. Appendix B: Example for calculating IUPAC-FPU

13.1 Determination of cellulase activity in a *T. reesei* enzyme preparation using the methods outlined by IUPAC. All enzyme dilutions were made in citrate buffer, pH 4.8, as indicated in the following table from a working enzyme stock solution that had been diluted 1:20 in citrate buffer.

Dilution #	Citrate buffer (ml)	1:20 Enzyme (ml)	Concentration*
1	1650	350	0.00875
2	1700	300	0.00750
3	1800	200	0.00500
4	1850	150	0.00375
5	1900	100	0.00250

*The term "concentration" is used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture. For example a 1:10 dilution of the 1:20 working stock of enzyme will have a "concentration" of 0.005.

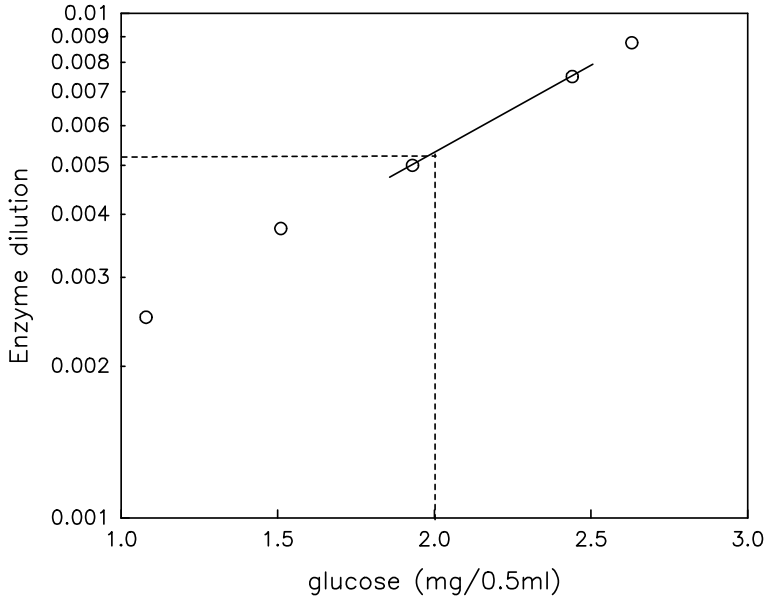
13.2 Dilution of glucose standards and construction of standard curve.

Glucose stock (mL)	Citrate buffer (mL)	Dilution	Concentration	Abs. 540 nm
1.0	0.5	1:1.5	3.35 mg/0.5 mL	0.765
1.0	1.0	1:2	2.50 mg/0.5 mL	0.579
1.0	2.0	1:3	1.65 mg/0.5 mL	0.384
1.0	4.0	1:5	1.00 mg/0.5 mL	0.220

13.3 Glucose concentration of samples as determined from standard curve.

Dilution #	Abs 540 nm	Glucose (ml/0.5 mL)
1	0.603	2.63
2	0.567	2.44
3	0.442	1.93
4	0.346	1.51
5	0.248	1.08

13.4 Determination of the concentration of enzyme which would have released exactly 2.0 mg of glucose by plotting glucose liberated against enzyme concentration.



13.5 Calculation of FPU from graph of dilution vs. glucose concentration.

$$\frac{0.37}{0.0053} = 70\ FPU/mL$$