Pure & Appl. Chem., Vol. 59, No. 2, pp. 257–268, 1987.
Printed in Great Britain.
© 1987 IUPAC

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

APPLIED CHEMISTRY DIVISION
COMMISSION ON BIOTECHNOLOGY*

MEASUREMENT OF CELLULASE ACTIVITIES

Prepared for publication by
T. K. GHOSE
Biochemical Engineering Research Centre,
Indian Institute of Technology, New Delhi-110016, India

*Membership of the Commission during the preparation of the report (1981–1985) was as follows:

Chairman: 1981–83 H. Dellweg (FRG); 1983–85 C. L. Cooney (USA); *Vice-Chairman*: 1981–83 C. L. Cooney (USA); 1983–85 M. Ringpfeil (GDR); *Secretary*: 1981–83 R. C. Righelato (UK); 1983–85 G. G. Stewart (Canada); *Titular and Associate Members*: H. T. Blachère (France; Titular 1981–83); V. K. Eroshin (USSR; Associate 1981–83); A. Fiechter (Switzerland; Associate 1981–83); T. K. Ghose (India; Titular 1981–85); P. P. Gray (Australia; Associate 1983–85); J. Holló (Hungary; Titular 1981–83); A. E. Humphrey (USA; Associate 1981–83); M. Linko (Finland; Associate 1983–85); R. C. Righelato (UK; Associate 1983–85); G. G. Stewart (Canada; Associate 1981–83); J. Takahashi (Japan; Titular 1981–83); J. E. Zajic (USA; Associate 1981–83); *National Representatives*: R. J. Ertola (Argentina; 1981–85); P. P. Gray (Australia; 1981–83); H. J. G. Wutzel (Austria; 1981–85); W. Borzani (Brazil; 1981–85); M. Moo-Young (Canada; 1983–85); B. Sikyta (Czechoslovakia; 1981–85); K. Von Meyenburg (Denmark; 1981–85); H. Dellweg (FRG; 1983–85); M. Linko (Finland; 1981–83); L. Penasse (France; 1983–85); M. Ringpfeil (GDR; 1981–83); J. Holló (Hungary; 1983–85); V. Jagannathan (India; 1983–85); L. Goldstein (Israel; 1983–85); F. Parisi (Italy; 1983–85); S. Fukui (Japan; 1981–85); B. G. Yeoch (Malaysia; 1983–85); O. Ilnicka-Olejiniczak (Poland; 1981–83); E. Galas (Poland; 1983–85); A. Fiechter (Switzerland; 1983–85); V. Johanides (Yugoslavia; 1981–85).

Republication of this report is permitted without the need for formal IUPAC permission on condition that an acknowledgement, with full reference together with IUPAC copyright symbol (© 1987 IUPAC), is printed. Publication of a translation into another language is subject to the additional condition of prior approval from the relevant IUPAC National Adhering Organization.

Measurement of cellulase activities

Fermentation Commission, IUPAC, (changed to Commission on Biotechnology, IUPAC, July 1980) put in a considerable effort and time since July 1976 to prepare a document prescribing standard assay procedures for cellulase enzyme system. The subject area was considered significantly important because the number of groups engaged in the studies of enzymatic break-down of cellulose of various sources were on steady increase. At least nine Commission meetings held during the last several years (Berlin, July 1976; Warsaw, June 1977; Munich, Sept. 1978; Davos, Sept. 1979; London (Ontario), July 1980; Leuven, Aug. 1981; MIT (Boston), Aug. 1982; Lyngby, Aug. 1983 & New Delhi, Feb.1984) gave considerable attention to this project. Several aspects of the proposed standard procedures available to workers for enzyme and substrate evaluation were considered. The detailed text of the draft prepared during mid 1980 and partially modified in early 1982 were discussed by the Commission at the MIT workshop in 1982. At the Lyngby meeting the Commission gave its final recommendations on the manuscript which after some additional review was adopted at the New Delhi meeting in February 1984 for release. Several procedures were suggested in the draft for consideration of the members of the Commission and other experts in the field. The final version of the Commission's draft is expected to be useful as a document of standard procedures for assay and evaluation of cellulase enzyme system.

Although for a subject area so complicated and involved like the assay procedures of various enzymes associated with cellulose-cellulase system, it was expected that the concerned groups and individuals might find it difficult to agree to a common draft. However, the members of the Commission as well as others who collaborated in the preparation of the document felt the need for a working document to form the basis of exchange of ideas and comparison of results. With this in view the New Delhi meeting of the Commission finally approved the recommendations for circulation. Necessarily, the Commission will review the assay procedures as and when some new information is available.

Contents

Preface
I. Introduction
II. General Information
III. Folin Protein Determination
IV. Cellobiase Assay
V. Filter Paper Assay for Saccharifying Cellulase
VI. Carboxymethyl Cellulase Assay for Endo-β-1, 4-glucanase
VII. Additional Assay Procedure for Endoglucanase
VIII. Evaluation of Cellulase under Process Conditions
IX. General Remarks
References
International Collaborators

I. INTRODUCTION

The characterization of cellulase enzymes poses special problems to the enzymologist, which are rarely encountered in the study of other enzymes. He is presented with a situation where kinetic studies are difficult since the natural substrate is both insoluble and structurally variable, and thus relatively undefined with respect to concentration and chemical form; where often a multitude of endo- and exo-glucanases act in synergy and in a complex manner still poorly understood; and where a variety of end-products and transglycosylation species are frequently formed, involving various mechanisms of feedback control. The presence of β-glucosidase or other enzymes, such as cellobiose phosphorylase, which are required for cellobiose metabolism and to enhance cellulose hydrolysis but which are not, strictly speaking, cellulases, further complicates the picture. Moreover, there has been comparatively little elucidation of the differences in the modes of action of the cellulase system of various organisms, and especially between eucaryotes and procaryotes.

In the face of these difficulties, and in view of the applied nature of most cellulase work, it is understandable that investigators in different laboratories have each developed a series of empirical assay procedures. While a common approach is shared, a situation has nonetheless resulted where comparison of cellulase activities between laboratories is not readily made in a quantitative manner. These recommendations for standard assays are intended to alleviate to some extent the present lack of uniform procedures with, however, certain reservations.

First, these procedures have received common acceptance primarily for the evaluation of the cellulase system of *Trichoderma*, and possibly other mesophilic fungal species. Studies with cellulolytic bacteria, such as *Clostridium thermocellum* or *Thermomonospora fusca*, suggest that these cellulase systems may have a different mode of action. Moreover, pH and temperature conditions for optimal cellulase activity from these organisms are different than those for most fungi. Thus, the assay procedures will often need to be adapted to other cellulase systems by linearizing with respect to time, substrate and enzyme concentration, and by proper specification of pH and temperature. However, the criteria of assaying FPA employing filter paper as insoluble substrate must be based on equal conversion (in the present recommendations—4%) since the rates are not likely to be linear and representative of true filter paper activity (see Section II. General Information).

Second, the specific assays which have been recommended are not meant to be inclusive, but were chosen on the basis of common usage and procedural ease. Other assays in the literature will undoubtedly continue to find limited application. Some notable examples are:

(1) viscosimetric assay[1] using soluble, derivatized cellulose, which is a very sensitive, but somewhat difficult method of measuring endoglucanase (EC 3.2.1.4) activity;
(2) cellulose azure assay[2] using laboratory prepared or commercially available dyed cellulose, a convenient method of measuring primarily cellobiohydrolase (EC 3.2.1.91) activity by dye release; and
(3) filter paper degrading (FPD) assay[3], a simple, useful, but somewhat time consuming method, requiring no sophisticated equipment.

Some investigators prefer to use alternative substrates, e.g. microcrystalline cellulose in the form of Avicel instead of filter paper, or different forms of soluble substituted cellulose. Where these alternative procedures appear to be justified, their use will undoubtedly continue. Again, the empirical nature of the recommended assays will more likely be of continued value to biotechnologists than to enzymologists, who will find these methods lacking in theoretical definition. The need yet remains to develop accurately defined cellulosic substrates for precise assay procedures.

It is well known that the level of β-glucosidase in an enzyme preparation may affect the result of cellulase assays, in particular the assay of FPA[4]. In order to overcome this problem, it would be important in the future either to modify the FPU assay of Mandels *et al.*, or else to adopt a method which is not affected by the level of β-glucosidase in the enzyme preparation. One such method is the Dyed Avicel method of Leisola and Linko[2], which was not, however, adopted in the present recommendations. Modfication of the FPU assay would involve either addition of excess β-glucosidase so that all of the product of the cellulolytic enzyme (s) would be measured as glucose or alternatively removal or inhibition of the β-glucosidase component of the cellulase enzyme so that the reaction product measured would be produced solely by the action of the truly cellulolytic enzyme (s).

Finally it must be remembered that assay activities may not reflect potential saccharification performance. Other considerations become of great importance to commercial cellulose hydrolysis, such as end-product inhibition, the addition of cellobiase activity, or reactor and process configuration. These factors will vary with different cellulase systems and significantly affect conversion efficiency.

II. GENERAL INFORMATION

1. For soluble enzyme, filter or centrifuge culture sample to remove solids and analyze supernatant or filtrate. For cell bound enzymes, homogenize cells in appropriate buffer such as 0.05 M citrate, pH 4.8. Dissolve enzyme powders at 1-5 mg per ml in buffer. Dilute enzyme solutions in buffer.

2. Blanks of enzyme without substrate and substrate without enzyme are included with all enzyme assays and sample values are corrected for any blank value.

3. For quantitative results, enzyme must be diluted or assay reaction time decreased until the amount of product plotted against enzyme concentration is reasonably linear. For the assay procedures described here, this would be when about 0.5 mg (or less) of glucose is produced from carboxymethyl cellulose, or cellobiose; or 2.0 mg of glucose (or less) is produced from filter paper, or other insoluble substrates. For insoluble cellulose, initial rates are of little value since there is always some amorphous cellulose which is readily hydrolyzed, but rates fall off rapidly to zero if the cellulase is incomplete. For quantitative results enzyme preparations should be compared on the basis of significant and equal conversion. Twice as much enzyme will give equal sugar in half the time, but it will not give twice as much sugar in equal time. An arbitrary 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.

4. **Citrate Buffer:** For *Trichoderma reesei* cellulase assays are carried out in 0.05 M citrate buffer pH 4.8.

   210 g Citric Acid Monohydrate $C_6H_8O_7 \cdot H_2O$
   750 ml distilled water
   NaOH—add until pH equals 4.3 (50-60 g)

Dilute to 1000 ml and check pH. If necessary add NaOH until pH = 4.5. This is 1 M citrate buffer pH 4.5. When diluted to 0.05 M, pH should be 4.8.

5. **Reducing Sugar Estimation by Dinitrosalicylic Acid (DNS) Method**[5]

DNS Reagent

Mix:	Distilled Water	1416 ml
	3,5-Dinitrosalicylic acid	10.6 g
	NaOH	19.8 g
Dissolve above, then add:		
	Rochelle salts (Na-K tartarate)	306 g
	Phenol (melt at 50°C)	7.6 ml
	Na metabisulfite	8.3 g

Titrate 3 ml sample with phenolpthalein with 0.1 N HCl. Should take 5-6 ml HCl. Add NaOH if required (2 g = 1 ml 0.1 N HCl).

Glucose Standards: 0.2 – 5.0 mg of glucose per ml or per 0.5 ml as appropriate.

Procedure: Place 1-2 ml sample in a test tube and add 3 ml DNS Reagent. Place in boiling water for 5 minutes. Cool to room temperature. Dilute samples if necessary so that light transmittance in the colorimeter will be between 20% and 80%. Include glucose standard made up and diluted like samples. Read % transmittance at 540 nm with a water blank for 100% T. Plot the standards on semilog paper (log % T versus concentration). This should give a straight line intersecting abscissa at 0.04 mg of glucose. The 0.04 mg represents the glucose lost by oxidation. For accurate determination of low concentration of glucose add 0.1 mg of glucose to each sample. Three ml of DNS Reagent will react with about 10 mg of glucose. Therefore concentrated sugar solutions should be diluted so that samples for analysis will contain 5 mg of reducing sugar or less.

Enzyme Assays: Standards should be made up and diluted after boiling like the assay unknowns. For example, for the filter paper assay use 0.5 ml of standards containing 0.2 – 5.0 mg glucose per 0.5 ml. Add 1 ml 0.05 M buffer and 3 ml DNS Reagent.

Comments:
Color develops only under alkaline conditions, so acidic samples should be neutralized.
This method is non-specific and measures any reducing compound. If glucose is used as the standard, values for cellobiose will be 15% low and values for xylose will be 15% high on a weight basis.
Boiled samples may be left a reasonable time before reading. Unboiled samples gradually deteriorate.

III. FOLIN PROTEIN DETERMINATION (ref. 6)

1. **Reagents**

Reagent A	20 g Na_2CO_3 4 g NaOH distilled water to make 1000 ml
Reagent B-1	1 g $CuSO_4 \cdot 5H_2O$ water to make 100 ml
Reagent B-2	2 g Na K Tartarate water to make 100 ml
Reagent C	(Keep only one day) 1 ml Reagent B-1 1 ml Reagent B-2 100 ml Reagent A Mix in this order
Phenol Reagent—1N	Dilute Folin Ciocalteau Reagent (2 N) with an equal volume of water.

10% Trichloroacetic acid in water.

2. Soluble protein. Filter or centrifuge culture to remove solids. Use supernatant.

3. Precipitation. Place 2 ml in conical centrifuge tube (15 ml). Add 2 ml 10% trichloroacetic acid. Mix. Incubate 30-60 minutes in refrigerator. Centrifuge 25 minutes at 2000 RPM. Discard supernatant. Dissolve pellet in 2 ml Reagent A.

4. Protein may also be precipitated by adding 4 ml acetone to 2 ml sample. In this case the pellet is dissolved in 0.05 M citrate buffer of pH 4.8 and enzyme determination can also be made. This procedure is used to measure enzyme in samples containing so much sugar that it interferes with the FP assay.

5. Place 0.5 ml sample (0.05 — 1.0 mg protein/ml) in a test tube. Add 5 ml Reagent C and mix well. Wait 10 minutes. Add 0.5 ml 1 N Phenol Reagent and mix at once. Wait 30 minutes and read % transmittance at 750 nm.

6. Look up protein values on a calibration curve made using Bovine Serum Albumin as standard (plot log % T vs protein concentration).

IV. CELLOBIASE ASSAY (ref. 7)

Substrate: 15.0 mM cellobiose (e.g. Fluka AG, puriss, p.a., product 22150) in 0.05 M citrate buffer pH 4.8. Fresh cellobiose solution should be prepared daily.

Method

1. Add 1.0 ml of enzyme, diluted in citrate buffer, to a small test tube. At least two dilutions must be made of each enzyme sample investigated. One dilution should release slightly more and one slightly less than 1.0 mg (absolute amount) of glucose in the reaction conditions.
2. Temperate to 50°C.
3. Add 1.0 ml substrate solution, mix.
4. Incubate at 50°C for exactly 30 min.
5. Terminate the reaction by immersing the tube in boiling water for exactly 5.0 min.
6. Transfer the tube to a cold water bath and determine glucose produced using a standard procedure (e.g. using a kit based on the glucose oxidase reaction).

Cellobiose blank 1.0 ml cellobiose substrate solution
1.0 ml citrate buffer
30 min, 50°C
Boil 5.0 min, cool.

Use in the GOD reaction and subtract absorbance from that of the sample. Note that a single cellobiose blank can be used for a whole series of activity determinations for which an enzyme blank is not necessary.

Enzyme blank 1.0 ml citrate buffer
1.0 ml enzyme dilution
30 min, 50°C
Boil 5.0 min, cool.

Use in the GOD reaction and subtract absorbance from that of the sample, along with the absorbance of the cellobiose blank. Enzyme blanks are necessary only when glucose is present in the enzyme preparation and/or when small dilutions are used.

Note

Before continuing with the glucose determination, it is important to appreciate the following:

Commercial glucose oxidase (GOD) preparations contain small amounts of β-glucosidase as an impurity. This enzyme usually has only slight activity against the residual cellobiose from the cellobiase reaction during the subsequent GOD reaction (unfavorable temperature and pH). However, it does cause an appreciable background reading, which must be taken into account. This is done using a cellobiose blank (see above).
Another consequence of the contaminating β-glucosidase in the GOD preparation is that the color in the glucose oxidase reaction does not reach a stable value in the incubation time recommended by the manufacturer. Color intensity continues to increase because new glucose (substrate) for the GOD reaction is continuously produced by the action of the contaminating β-glucosidase on the residual cellobiose substrate. For this reason it is recommended that the GOD reaction should be terminated by acidification, which halts the activity of both the GOD and the contaminating β-glucosidase enzyme. A suitable acid addition is 0.2 ml 72% H_2SO_4 to a reaction volume of 2-20 ml. Of course, acid should be added to all samples, zeros, blanks and standards, regardless of whether they contain cellobiose. If the reaction is *not* terminated by addition of acid, it is necessary to read the absorbances of all the samples after *exactly* the same time from the start of the GOD reaction.

Unit Calculation

1. Determine the glucose concentrations (mg ml^{-1}) in the cellobiase reaction mixtures obtained using at least two different enzyme dilutions.

2. Multiply by 2 to convert glucose concentrations into absolute amounts (mg).

3. Translate enzyme dilutions into concentrations:

$$\text{concentration} = \frac{1}{\text{dilution}} \left(= \frac{\text{volume of enzyme sample in dilution}}{\text{total volume of dilution}}\right)$$

4. Estimate the concentration of enzyme which would have released exactly 1.0 mg of glucose by plotting glucose liberated (2) against enzyme concentrations (3) on semilogarithmic graph paper.

5. Calculate cellobiase activity:

$$\text{CB} = \frac{0.0926}{\text{enzyme concentration to release 1.0 mg glucose}} \text{ units ml}^{-1}$$

Derivation of the Cellobiase Unit

The unit of cellobiase (CB) is based on the International Unit (IU)

1 IU = 1 μmol min^{-1} of substrate converted
= 2.0 μmol min^{-1} of glucose formed in the case of the CB reaction

The absolute amount of glucose released in the CB assay at the critical dilution is 1.0 mg:

1.0 mg glucose = 1.0/0.18 μmol glucose
= 0.5/0.18 μmol cellobiose converted

This amount of cellobiose was converted by 1.0 ml enzyme in 30 min, i.e., 1.0 mg glucose = 0.5/0.18 × 1.0 × 30 μmol min^{-1} ml^{-1} cellobiose converted = 0.0926 μmol min^{-1} ml^{-1}. Therefore, the estimated amount of enzyme which releases 1.0 mg glucose in the CB reaction contains 0.0926 units, and

$$\text{CB} = \frac{0.0926}{\text{enzyme concentration to release 1.0 mg glucose}} \text{ units ml}^{-1}$$

Note: cf end of FPU unit derivation.

V. FILTER PAPER ASSAY FOR SACCHARIFYING CELLULASE (FPU Assay) (ref. 8)

Substrate: Whatman No. 1 filter paper strip, 1.0 × 6.0 cm (≃ 50 mg).

Method

1. Add 1.0 ml 0.05 M Na-citrate, pH 4.8, to a test tube of volume at least 25 ml.

2. Add 0.5 ml enzyme, diluted in citrate buffer. At least two dilutions must be made of each enzyme sample investigated. One dilution should release slightly more and one slightly less than 2.0 mg (absolute amount) of glucose (= reducing sugars as glucose) in the reaction conditions.

3. Temperate to 50°C, add one filter paper strip, mix (NB! it does not matter if a small part of the paper is above the liquid surface, but if the paper "winds" up the tube it must be pushed down again).

4. Incubate 50°C, 60 min.

5. Add 3.0 ml DNS, mix. Transfer tube to a rack on the table.

6. Boil for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks, glucose standards and the spectro zero should be boiled together. After boiling, transfer to a cold water bath.

7. Add 20 ml deionized or distilled water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion (NB. This is important!).

8. When the 'pulp' has settled well, i.e., after at least 20 min, the color formed is measured against the spectro zero at 540 nm. If the paper pulp does not settle, it will do so after stirring with a glass rod. (The necessity for stirring can be seen after only a few minutes of settling time).

Spectro Zero

1.5 ml citrate buffer
3.0 ml DNS
5 min boil, 20 ml H_2O, etc.
Spectro zero is used to set the spectrophotometer at zero absorbance.

Enzyme blank

1.0 ml citrate buffer
0.5 ml enzyme
3.0 ml DNS
Boil, H_2O, etc.
Color measured against spectro zero and subtracted from the value of the appropriate reaction tube.

Standards

0.5 ml standard
1.0 ml citrate buffer
3.0 ml DNS
Boil, etc. and measure against spectro zero.

Glucose Stock solution

10 mg ml^{-1} anhydrous glucose.
Aliquots of 5-10 ml can be stored frozen.
Remember to stir well after thawing.

Dilutions:

1 ml + 0.5 ml buffer = 1:1.5 = 6.7 mg ml^{-1} (3.35 mg/0.5 ml)
1 ml + 1.0 ml buffer = 1:2 = 5.0 mg ml^{-1} (2.5 mg/0.5 ml)
1 ml + 2.0 ml buffer = 1:3 = 3.3 mg ml^{-1} (1.65 mg/0.5 ml)
1 ml + 4.0 ml buffer = 1:5 = 2.0 mg ml^{-1} (1.0 mg/0.5 ml)

Unit Calculation

1. Construct a linear glucose standard using the absolute amounts of glucose (mg/0.5 ml) plotted against A_{540}.
2. Using this standard, translate the absorbance values of the sample tubes (after subtraction of enzyme blank) into glucose (= mg glucose produced during the reaction).
3. Translate the dilutions used into enzyme concentrations:

$$\text{concentration} = \frac{1}{\text{dilution}} \left(= \frac{\text{volume of enzyme in dilution}}{\text{total volume of dilution}}\right)$$

4. Estimate the concentration of enzyme which would have released exactly 2.0 mg of glucose by plotting glucose liberated (2) against enzyme concentration (3) on semilogarithmic graph paper.
5. Calculate FPU:

$$\text{FPU} = \frac{0.37}{\text{enzyme concentration to release 2.0 mg glucose}} \text{ units ml}^{-1}$$

N.B. 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 product is formed at the same rate during each and every minute of the reaction. Therefore, it is recommended that the results are expressed simply as units per milliliter, as in the given example.

Derivation of the FPU Unit

The unit of FPU is based on the International Unit (IU)

1 IU = 1 μmol min^{-1} of substrate converted
= 1 μmol min^{-1} of "glucose" (reducing sugars as glucose) formed during the hydrolysis reaction
= 0.18 mg min^{-1} when product is glucose

The absolute amount of glucose released in the FPU assay at the critical dilution is 2.0 mg:

2 mg glucose = 2/0.18 μmol

This amount of glucose was produced by 0.5 ml enzyme in 60 min, i.e., in the FPU reaction:

2 mg glucose = 2/0.18 × 0.5 × 60 μmol min^{-1} ml^{-1}
= 0.37 μmol min^{-1} ml^{-1} (IU ml^{-1})

Therefore, the estimated amount of enzyme (= critical enzyme concentration = ml ml^{-1}) which releases 2.0 mg glucose in the FPU reaction contains 0.37 units, and:

$$\text{FPU} = \frac{0.37}{\text{enzyme concentration to release 2.0 mg glucose}} \text{ units ml}^{-1}$$

Note to the FPU, CMC and Cellobiase Methods

The assays of FPU, CMC and cellobiase are all based on the same principle of estimating a fixed (rather large) amount of glucose from the relevant substrate. As a result, workers performing these assays for the first time may be surprised by the very high spectrophotometer absorbances required in attaining release of the critical amount of glucose. If, depending on the light path of the colorimeter used, it is found that the color formed is so dense that it cannot be measured, it is permissible to dilute the color before measurement (e.g. by adding 1.0 ml distilled water to 1.0 ml of the colored solution, mixing and multiplying the glucose obtained by 2). However, because the enzymatic reaction is nonlinear, it is *not* permissible to use results from more dilute enzyme dilutions and extrapolate to the critical release of glucose. The instruction appearing in all three assay methods to the effect that one dilution should release more and one less than the critical amount of glucose *must* be observed in all cases.

When assaying low levels of activity, it may be found that even the undiluted enzyme releases less than the critical amount of glucose. In this case calculate the activities from the amounts of glucose (absolute amounts) released by the undiluted enzymes as follows:

1. FPU= mg glucose released × 0.185

   Derivation

   1.0 mg glucose = 1.0/0.18×0.5×60 μmol min^{-1} ml^{-1} substrate cleavage
   = 0.185 units ml^{-1}

2. CMC = mg glucose released × 0.37

   Derivation

   1.0 mg glucose = 1.0/0.18 × 0.5 × 30 μmol min^{-1} ml^{-1} substrate cleavage
   = 0.37 units ml^{-1}

3. CB = mg glucose released × 0.0926

   Derivation

   1.0 mg glucose = 0.5/0.18 × 1.0 × 30 μmol min^{-1} ml^{-1} substrate cleavage
   = 0.0926 units ml^{-1}

In all three cases, if accurate results are desired, undiluted enzyme should be used *only* when the amount of glucose released is below or equal to the critical amount of glucose in the reaction concerned.

VI. CARBOXYMETHYL CELLULASE ASSAY FOR ENDO-β-1, 4-GLUCANASE (ref. 8)

Substrate

2% Carboxymethyl cellulose CMC 7L2 (degree of substitution = 0.7) (Hercules Inc., Wilmington, Delaware 19899, USA) in 0.05 M sodium citrate buffer, pH 4.8.

Method

Add 0.5 ml enzyme, diluted in citrate buffer, to a test tube of volume at least 25 ml. At least two dilutions must be made of each enzyme sample investigated. One dilution should release slightly more and one slightly less than 0.5 mg (absolute amount) of glucose (= reducing sugars as glucose) in the reaction conditions.

2. Temperate to 50°C.
3. Add 0.5 ml substrate solution, mix well and incubate at 50°C for 30 min.
4. Add 3.0 ml DNS, mix. Transfer to a rack on the table.
5. Boil for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks, glucose standards and the spectro zero should be boiled together. After boiling, transfer immediately to a cold water bath.
6. Add 20 ml deionized or distilled water. Mix by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion (N.B. This is important!).
7. Measure the color formed against the spectro zero at 540 nm. When necessary (i.e., when small dilutions are used), the color formed in the enzyme blank (see below) is subtracted from that of the sample tube.
8. Translate the absorbance of the sample tube (corrected if necessary by subtraction of the enzyme blank) into glucose production during the reaction using a glucose standard curve (see below).

Spectro Zero

0.5 ml substrate solution
30 min, 50°C
3.0 ml DNS
0.5 ml citrate buffer, mix
Boil 5.0 min, add 20 ml H_2O
Zero at 540 nm

Enzyme Blank

0.5 ml substrate solution
30 min, 50°C
3.0 ml DNS
0.5 ml enzyme dilution
Boil 5.0 min, add 20 ml H_2O
Measure at 540 nm

Standards

0.5 ml substrate solution
30 min, 50°C
3.0 ml DNS
0.5 ml standard
Boil 5.0 min, add 20 ml H_2O
Measure at 540 nm

Glucose Stock Solution

2 mg ml^{-1} anhydrous glucose
Aliquots of about 5 ml can be stored frozen
Remember to stir well after thawing

Undiluted	= 2.0 mg ml^{-1}	(1.0 mg/0.5 ml)
1.0 ml + 0.5 ml buffer = 1:1.5	= 1.33 mg ml^{-1}	(0.67 mg/0.5 ml)
1.0 ml + 1.0 ml buffer = 1:2	= 1.0 mg ml^{-1}	(0.5 mg/0.5 ml)
1.0 ml + 3.0 ml buffer = 1:4	= 0.5 mg ml^{-1}	(0.25 mg/0.5 ml)

Unit Calculation

1. Construct a linear glucose standard using the absolute amounts of glucose (mg/0.5 ml) plotted against A_{540}.

2. Using this standard, translate the absorbance values of the sample tubes (after subtraction of enzyme blank) into glucose (= mg glucose produced during the reaction).

3. Translate the dilutions used into enzyme concentrations

$$\text{concentration} = \frac{1}{\text{dilution}} \left(= \frac{\text{volume of enzyme in dilution}}{\text{total volume of dilution}} \right)$$

4. Estimate the concentration of enzyme which would have released exactly 0.5 mg of glucose by plotting glucose liberated (2) against enzyme concentration (3) on semilogarithmic graph paper.

5. Calculate CMC

$$\text{CMC} = \frac{0.185}{\text{enzyme concentration to release 0.5 mg glucose}} \text{ units ml}^{-1}$$

Derivation of the CMC Unit

The unit of CMC is based on the International Unit (IU) and its calculation is analogous to that of the unit of FPU.

$1 \text{ IU} = 1\ \mu\text{mol min}^{-1}$ of liberated hydrolysis product
$= 0.18 \text{ mg min}^{-1}$ when the product is glucose

The critical amount of glucose in the CMC assay is 0.5 mg:

$0.5 \text{ mg glucose} = 0.5/0.18\ \mu\text{mol}$

This amount of glucose was produced by 0.5 ml in 30 min, i.e., in the CMC reaction

$$0.5 \text{ mg glucose} = \frac{0.5}{0.18 \times 0.5 \times 30}\ \mu\text{mol min}^{-1}\text{ ml}^{-1}$$
$$= 0.185\ \mu\text{mol min}^{-1}\text{ ml}^{-1}\ (\text{IU ml}^{-1})$$

Therefore, the estimated amount of enzyme (= critical enzyme concentration, ml.ml^{-1}) which releases 0.5 mg glucose in the CMC reaction contains 0.185 IU, and:

$$\text{CMC} = \frac{0.185}{\text{critical enzyme concentration}}\ \frac{\text{IU ml}^{-1}}{\text{ml ml}^{-1}}$$
$$= \frac{0.185}{\text{critical enzyme concentration}}\ \text{units ml}^{-1}$$

NB. Because the CMC assay is non-linear ... (see end of FPU derivation).

VII. ADDITIONAL ASSAY PROCEDURE FOR ENDOGLUCANSE (HEC Assay) (ref. 9)

Substrate

1.0% HEC (Hydroxyethylcellulose, medium viscosity, Fluka AG pract., product 54290, DP 450, DS 0.9-1.0) in 0.05 M citrate buffer, pH 4.8. The HEC powder is dissolved using a magnetic stirrer for at least one hour, after which it must stand for a further 1 h to clarify. The solution may be stored for 2 weeks at +4°C.

Method

1. Add 1.8 ml substrate solution to a 15 ml test tube, preferably using an automatic pipette.
2. Temperate to 50°C.
3. Add 0.2 ml of enzyme solution diluted in citrate buffer, mix.
4. Incubate 50°C, 10 min.
5. Add 3.0 ml DNS, mix. Transfer to a rack on the table.
6. Boil for exactly 5.0 min in a vigorously boiling water bath containing sufficient water. All samples, enzyme blanks, glucose standards and the spectro zero should be boiled together. After boiling, transfer to a cold water bath.

7. Measure the color formed against the spectro zero at 540 nm, after correction for the enzyme blank (see below) when necessary (low dilutions).

Spectro zero

1.8 ml HEC
10 min, 50°C
3.0 ml DNS
0.2 ml citrate buffer
Boil 5 min, cool and zero at 540 nm

Enzyme Blank

1.8 ml HEC
10 min, 50°C
3.0 ml DNS
0.2 ml enzyme dilution
Boil 5 min, cool and measure color at 540 nm

Standards

1.8 ml HEC
10 min, 50°C
3.0 ml DNS
0.2 ml glucose standard
Boil 5 min, cool and measure color at 540 nm

Glucose Stock Solution

10^{-2} M (e.g. 0.18 g in 100 ml citrate buffer) anhydrous glucose of high purity.

Aliquots of 5-10 ml can be stored frozen.

Stir well after thawing.

Undiluted = 1:1 = 10.0 μmol ml^{-1} (16.67 nkat ml^{-1}*)
1.0 ml + 1.0 ml buffer = 1:2 = 5.0 μmol ml^{-1} (8.33 nkat ml^{-1})
1.0 ml + 2.0 ml buffer = 1:3 = 3.33 μmol ml^{-1} (5.56 nkat ml^{-1})
1.0 ml + 3.0 ml buffer = 1:4 = 2.5 μmol ml^{-1}(4.17 nkat ml^{-1})

*A glucose concentration of 10 μmol ml^{-1} in the standard is equivalent to 16.67 nkat ml^{-1} in the enzyme reaction:

$10\ \mu\text{mol ml}^{-1} \times 1/600\ \text{s} \times 1000 = 16.67\ \text{nmol s}^{-1}\ \text{ml}^{-1}\ (\text{nkat ml}^{-1})$

Unit Calculation

1. Construct a linear glucose standard by plotting absorbances at 540 nm (ordinate) against glucose concentration converted to nkat ml^{-1} as in the example above (abscissa). Use this glucose standard to read enzyme activities directly as nkat ml^{-1} after determining the extent of linearity of the enzyme reaction as described below

2. Perform the enzyme assay procedure using a suitable dilution series of enzyme. Measure against the spectro zero and use enzyme blanks if necessary (i.e., if small dilutions are used). Plot absorbance at 540 nm against enzyme concentration (dilution^{-1}). Only enzyme dilutions yielding results within the linear region can be read from the linear glucose standard as nkat ml^{-1}.

In practice, it is usually necessary to determine the extent of linearity of the HEC reaction only once for enzyme from any given organism. The range of linearity for *Trichoderma* enzyme is at least 5-15 nkat ml^{-1}. When working with other organisms, it may be found that linearity of the enzyme reaction continues beyond this level, in which case a glucose standard extending to higher absorbances may be constructed.

VIII. EVALUATION OF CELLULASE UNDER PROCESS CONDITIONS

Enzyme assays are carried out on pure cellulose or cellulose derivatives and are normally of short duration. A practical process will usually be based on crude or waste cellulose. Enzyme unit values are based on limited conversion of the substrate. Hydrolysis rates decline with time due to depletion of the more amorphous substrate, product inhibition, and enzyme inactivation. Therefore, it is often desirable to evaluate an enzyme preparation under process conditions. It has been known for some time that a plot of sugar produced in a given time versus the log of enzyme concentration gives a straight line. This relationship can be generalized and applies when the percent saccharification at any given time is plotted against the log of enzyme units per gram initial substrate. Under favorable hydrolysis conditions, including adequate mixing, this straight line relationship usually applies from about 10% to 60% or more conversion, and such plots can be used to predict the quantity of enzyme (and substrate) required to produce the desired sugar concentration within a selected time. Addition of supplemental β-glucosidase or other favorable change in conditions will increase values to generate an analogous linear plot. As a result of these kinetics very large quantities of enzyme are required if high conversions and concentrated sugar syrups are desired.

Procedure (refer to Figure 1)

1. Select a suitable substrate and give it the required pretreatment.

2. Carry out saccharification in 50 or 100 ml volume in shake flasks using 2 or 3 substrate concentrations, for example 5, 10 and 15%, and a few enzyme concentrations, for example 0.125, 0.25, 0.5 and 1.0 Filter Paper Cellulase units/ml to give initial enzyme/substrate ratios of about 1-20. Temperature and pH should be optimum, 50° and 4.8 for *Trichoderma* cellulase. Incubate on shaker for 24-48 hours, sample, and estimate total reducing sugar in the supernatant by DNS or other procedure.

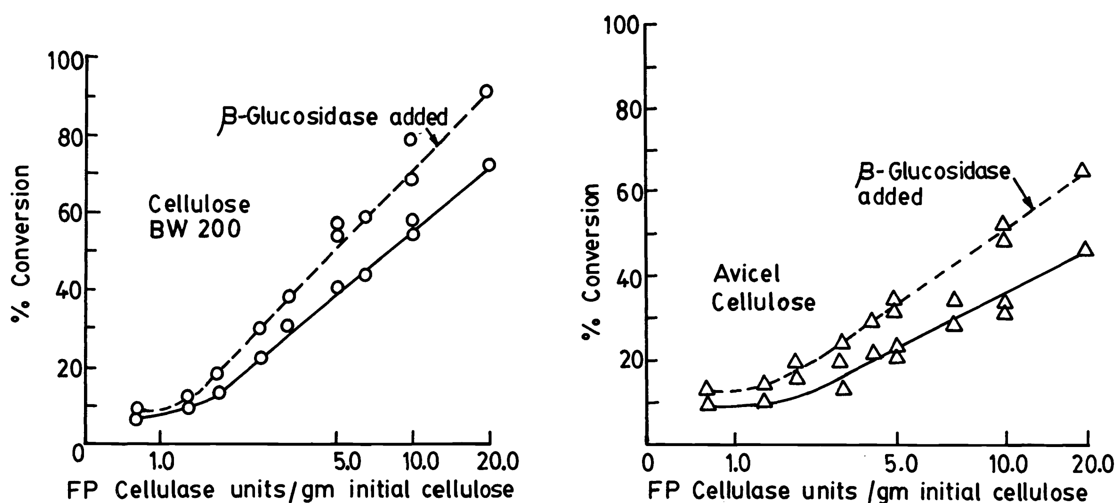

Figure 1: Effect of enzyme substrate ratio and added β-glucosidase on percent conversion.

3. Calculate percent saccharification at each time as

$$\frac{\text{reducing sugar (mg/ml)} \times 0.9 \times 100}{\text{initial substrate (mg/ml)}}$$

4. Plot percent saccharification vs logarithm of initial enzyme/substrate ratio. This should give a straight line.

5. If supplemental cellobiase is added it should be at a level to give one cellobiase unit (including cellobiase in cellulase preparation) per filter paper cellulase unit.

Note:

For evaluation of cellulase under process conditions, all important parameters like pretreatment of the lignocellulosic substrate (if done), cellulose/hemicellulose concentrations, source, size and water contents of the substrate, pH, temperature, time of saccharification, nature of agitation, addition of supplemental enzyme (if any) and the composition of sugars released should be provided in each case of evaluation.

IX. GENERAL REMARKS

1. It should be noted that the recommended methods apply to the estimation of activities of extracellular cellulase enzymes produced by the fungal species *Trichoderma* and not necessarily to other cellulase systems elaborated by the fungi *Phanerochaete, Penicillium, Fusarium,* etc. and the obligate anaerobes *Clostridium thermocellum.* It is likely that several of the recommended procedures will equally apply to the assay of other enzyme systems but these must be separately and adequately tested in each case before these become accepted standard assays.
2. More exactly defined substrates like higher cello-oligosaccharides should be developed for characterization of cellulase system and components from a biochemical point of view to help understand the mode of action of cellulases in cellulose hydrolysis.

REFERENCES

1. Almin, K.E., Eriksson, K-E. and Pettersson, L.G. (1975) Eur. J. Biochem., *51,* 207
2. Leisola, M. and Linko M., (1976) Anal. Biochem., *70,* 592
3. Toyama, N. and Ogawa, K., (1977) Proc. I. Bioconversion Symp., IIT Delhi, (T.K. Ghose, ed.), p. 305
4. Bailey, M.J. (1981) Biotechnol. Letters, *3,* 695
5. Miller, G.L. (1959) Analytical Chem., *31,* 426
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., *193,* 265
7. Sternberg, D., Vijaykumar, P., and Reese, E.T. (1977) Can. J. Microbiol., *23,* 139
8. Mandels, M., Andreotti, R. and Roche, C., (1976) Biotechnol. Bioeng. Symp. *6,* 17
9. Bailey, M.J. and Nevalainen, K.M.H., (1981) Enzyme Microb. Technol., *3,* 153
10. Mandels M., Medeiros J.E., Andreotti R.E., Bissett F.H. (1981) Biotechnol. Bioeng. *23,* 2009,

Acknowledgements

The MIT workshop where the recommendations were largely finalised could not have been completed if unreserved support and assistance were not received from colleagues like Mary Mandels of US Army Development Centre, MA; John Ferchak of Biological Energy Corporation of USA and Michael Bailey of Technical Research Centre, Espoo, Finland. Since appearance of the first draft (known as Green Book) released at the London, Ontario meeting of the Commission in July 1980, several other scientists were involved in carrying out recommended assay procedures using samples of enzymes and substrates supplied by Mary Mandels and Michael Bailey. The Commission expresses its sincere thanks to acknowledge all the support, comments, reviews and assay results provided by them. All these views were taken into consideration and discussed at the MIT workshop.

INTERNATIONAL COLLABORATORS

Dr. Michael J. Bailey
Technical Research Centre of Finland
Biotechnical Laboratory
Tietotie 2, SF-02150 Espoo 15
Finland

Dr. V.S. Bisaria
Biochemical Engineering Research Centre
Indian Institute of Technology, Delhi
Hauz Khas, New Delhi-110016
India

Prof. T.M. Enari
Technical Research Centre of Finland
Biochemical Laboratory
Tietotie 2, SF-02150 Espoo 15
Finland

Dr. D.E. Eveleigh,
Department of Biochemistry and Microbiology
Rutgers The State University of New Jersey
New Brunswick, NJ 08903
U.S.A.

Dr. Vladimir Farkas
Slovak Academy of Sciences
Institute of Chemistry
Dubravska Cesta
80933 Bratislava
Czechoslovakia

Dr. John D. Ferchak
Biological Energy Corporation
P.O. Box 766
2650 Eisenhower Avenue
Valley Forge, PA 19482
U.S.A.

Prof. E. Galas
Institute of Technical Biochemistry
Technical University
90 924 Lodz-Gdanska 162/168
Poland

Mr. V.K. Ghosh
Biochemical Engineering Research Centre
Indian Institute of Technology, Delhi
Hauz Khas, New Delhi-110016
India

Dr. G. Halliwell
Department of Microbiology and Botany
University College, Singleton Park
Swansea, SA2 8 PP
United Kingdom

Prof. J.M. Leabault
Departement de Genie Chimique
Universite de Technologie de Compiegne
60206 Compiegne Cedex
France

Dr. S. Levy
Biotechnology Research Department
SRI International
Life Sciences Division
333 Rovenswood Avenue
Menlo Park, CA 94025
U.S.A.

Dr. Matti Linko
Technical Research Centre of Finland
Biotechnical Laboratory
Tietotie 2, SF-02150 Espoo 15
Finland

Dr. Robert Longin
Institute Pasteur
28 Rue du doc. Roux
Paris, 75015
France

Dr. Mary Mandels
Enzyme and Biochemical Engg. Group
Environmental Sciences and Engg. Division
Science & Advanced Technology Laboratory
US Army Natick Research & Development Laboratories
Natick, MA 01760
U.S.A.

Prof. Bland Montenecourt
Department of Biological Sciences
Lehigh University
Bethlehem, PA 18015
U.S.A.

Dr. J.C. Sadana
Biochemistry Division
National Chemical Laboratory
Pune 411008
India

Prof. H. Sahm
Institut fur Biotechnologie der
Kernforschungsanlage
Julich GmbH, Postfach 1913
5170 Julich
FRG

Prof. C.R. Wilke
Lawrence Berkeley Laboratory
University of California, Berkeley
Berkeley, CA 94700
U.S.A.

Dr. Tom Wood
Carbohydrate Biochemistry Department
Rowett Research Institute
Aberdeen, AB2 9SB
United Kingdom