# Chapter 14

# Cellulase Assays

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### Summary

Cellulose is a heterogeneous polysaccharide, and its enzymatic hydrolysis requires endoglucanase, exoglucanase (cellobiohydrolase), and β-glucosidase to work together. We summarize the most commonly used assays for individual enzymes and cellulase mixture.

**Key words:** β-Glucosidase, Cellobiase, Cellobiohydrolase, Cellulose, Cellulase assay, Endoglucanase, Sugar assay

## 1. Introduction

Cellulose, which is the most abundant renewable biological resource, is produced mainly by plant photosynthesis. Cellulose biodegradation mediated by cellulases or cellulolytic microorganisms releases organic carbon in plant, animal, and microbial sediments back to the atmosphere as carbon dioxide and methane. Complete enzymatic crystalline cellulose hydrolysis requires three types of enzymes (endoglucanase, exoglucanase or cellobiohydrolase (CBH), and β-glucosidase) to work together *(1–4)*.

Physical heterogeneity of the cellulosic materials and the complexity of cellulase enzyme systems (synergy and/or competition) on solid enzyme-accessibility-limited substrate surfaces present some challenges for cellulase activity assays *(5–8)*. A number of cellulase activity assays have been summarized *(5, 6)*. In this chapter, we describe the common cellulase activity assays including the total cellulase assays, β-glucosidase assays, endoglucanase assays, and exoglucanase (CBH) assays. This chapter will provide some useful guidance, especially in **Subheading 4**.

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## 2. Materials

### 2.1. Total Cellulase Assays

#### 2.1.1. Filter Paper Activity Assay

DNS (3,5-dinitrosalicylic acid) reagent. Dissolve 10.6 g of DNS and 19.8 g of NaOH in 1,416 ml of distilled water. After complete dissolution, add 360 g of Rochelle salts (sodium potassium tartrate), 7.6 ml of melted phenol (at 50°C) (*see* **Note 1**), and 8.3 g of sodium metabisulfite, and then mix well. Titrate 3 ml of the DNS reagent using 0.1 M HCl using the phenolphthalein endpoint pH check. It should take 5–6 ml of HCl for a transition from red to colorless. Add NaOH if required (2 g of NaOH added = 1 ml of 0.1 M HCl used for 3 ml of the DNS reagent) (*see* **Note 2**).

Citrate buffer (1 M, pH 4.5). Dissolve 210 g of citric acid monohydrate in 750 ml of distilled water, and add 50–60 g solid NaOH until pH is 4.3. Dilute the solution to nearly 1,000 ml and check the pH. If necessary, add NaOH to adjust the pH to 4.5.

Citrate buffer (50 mM, pH 4.8). Dilute 1 M citrate buffer (pH 4.5) by adding 19 times distilled water.

Filter paper strip (50 mg, 1.0 × 6.0 cm). Cut 1.0 × 6.0 cm Whatman No. 1 paper strips with a paper cutter (*see* **Note 3**).

Glucose standard stock solution – 10 g/l (*see* **Note 4**).

#### 2.1.2. Anaerobic Cellulase Assay Using Avicel

1. Tris–HCl buffer (0.5 M Tris, pH 7.0, 0.1 M $CaCl_2$, and optional 1.5% $NaN_3$). Prepare 0.5 l of 1 M Tris–HCl buffer (pH 7.0), dissolve 11.1 g of $CaCl_2$ and 15 g $NaN_3$, and add distilled water to make up to 1 l.
2. Dithiothreitol (DTT, 0.5 M). The DTT solution can be stored at 4°C for at least a half year. Less costly cysteine can replace DTT *(9)*.
3. Avicel suspension solution (24.4 g/l). Suspend 20 g of completely dry Avicel (FMC 105 or Sigmacell 20) in 820 ml of distilled water with a magnetic stirrer.
4. Glucose standard solution – 1 g/l.
5. Phenol aqueous solution (5% w/v). Store at 4°C in darkness.
6. Sulfuric acid ~98% w/w.

### 2.2. β-Glucosidase Assays

#### 2.2.1. β-Glucosidase Assay Using p-Nitrophenyl-β-D-Glucoside (pNPG)

1. Sodium acetate buffer, 0.1 M, pH 4.8.
2. *p*NPG (5 mM) in acetate buffer. Dissolve 0.1576 g of *p* NPG in 100 ml acetate buffer.
3. Clycine buffer (0.4 M) pH 10.8. Dissolve 60 g of glycerin in 1,500 ml of distilled water, add 50% w/v NaOH until the pH is 10.8, and then dilute to 2 l.
4. *p*-Nitrophenol (pNP; 20 g/l) in acetate buffer (*see* **Note 5**).

*2.2.2. β-Glucosidase Assay Using Cellobiose*

1. Cellobiose (15 mM) in citrate buffer (freshly made substrate solution).
2. Citrate buffer (50 mM, pH 4.8).

### 2.3. Endoglucanase Assays

*2.3.1. Endoglucanase Assay Using Carboxymethylcellulose (CMC)/DNS*

1. Citrate buffer (50 mM, pH 4.8).
2. CMC (2% w/v) in citrate buffer (above).
3. DNS reagent (above).
4. Glucose standard (2 g/l).

*2.3.2. Endoglucanase Assay Using CMC/Bicinchoninic Acid (BCA)*

1. Citrate buffer (50 mM, pH 4.8).
2. CMC solution (0.05% w/v) in the citrate buffer.
3. BCA Solution A. Dissolve disodium 2,2′-bicinchoninate (97.1 mg) in a solution of 2.714 g of $Na_2CO_3$ and 1.21 g of $NaHCO_3$ with a final volume of 50 ml. Solution A will remain stable for 4 weeks at 4°C in darkness.
4. BCA Solution B. Dissolve $CuSO_4.5H_2O$ (62.4 mg) and L-serine (63.1 mg) in 50 ml of water. Solution B will remain stable for 4 weeks at 4°C in darkness.
5. Working BCA reagent. Mix equal volumes of solution A and B. The reagent is to be made immediately before use.
6. Glucose standard solution (0.9 g/l, 5 mM).

*2.3.3. Endoglucanase Assay Using CMC/Viscosity*

1. Sodium acetate buffer (50 mM, pH 5.0).
2. CMC solution (0.5% w/v, medium viscosity, degree of substitution of 0.5–0.7) in acetate buffer.

*2.3.4. Semiquantitative Endoglucanase Assay Based on Dye Release*

1. Congo red solution (1 g/l) prepared by dissolving 100 mg Congo red in 99 ml water and 1% ethanol.
2. NaCl (1 M) solution.
3. Sodium phosphate buffer (0.1 M, pH 6.5).

Microbe-Secreted Endoglucanase Assay on Agar Medium

1. CMC (1% w/v, low viscosity) in 1.5% agar medium. Dissolve CMC before adding agar and autoclave.

Endoglucanase Assay on Agarose Gel

1. CMC (1% w/v, low viscosity) in 0.8% agarose. Dissolve CMC completely before adding agarose.

Endoglucanase Assay on Polyacrylamide Gel

1. CMC (1% w/v) in sodium phosphate buffer whose pH is chosen depending on the specific cellulase.

### 2.4. Exoglucanase Assays

*2.4.1. Exoglucanase Assay Using Avicel*

1. Avicel (FMC PH 101 or PH 105 or Sigmacell 20).
2. Sodium acetate buffer (0.1 M, pH 4.8).
3. Phenol (5%) solution.
4. Sulfuric acid, ~98%.

*2.4.2. Exoglucanase Assay Using Regenerated Amorphous Cellulose (RAC)*

1. Sodium acetate buffer (1 M, pH 4.5).
2. Phenol (5%) solution.
3. Sulfuric acid (~98%).
4. RAC (1% w/v). RAC preparation is given below.

## 3. Methods

### *3.1. Total Cellulase Assays*

A total cellulase system consists of three enzymatic activities: endoglucanases, exoglucanases, and β-D-glucosidases. Total cellulase activities are always measured using insoluble substrates, including pure cellulosic substrates such as Whatman No. 1 filter paper, cotton linter, microcrystalline cellulose, bacterial cellulose, algal cellulose, as well as cellulose-containing substrates such as dyed cellulose, α-cellulose, and pretreated lignocellulose *(2)*. The two most common assays (filter paper assay and anaerobic cellulase assay) are described here.

*3.1.1. Filter Paper Assay (FPA)*

FPA is the most common total cellulase activity assay recommended by the International Union of Pure and Applied Chemistry (IUPAC) *(6)*. IUPAC recommends a filter paper activity (FPA) assay that differs from most enzyme assays based on soluble substrate for initial reaction rates. This assay is based on a fixed degree of conversion of substrate, i.e. a fixed amount (2 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper (i.e., both amorphous and crystalline fractions of the substrate are hydrolyzed) within a fixed time (i.e., 60 min). In part due to the solid heterogeneous substrate, reducing sugar yield during hydrolysis is not a linear function of the quantity of cellulase enzyme in the assay mixture. That is, twice the amount of enzyme does not yield two times the reducing sugar within equal time. Total cellulase activity is described in terms of "filter-paper units" (FPU) per milliliter of original (undiluted) enzyme solution. The strengths of this assay are that (1) the substrate is widely available and (2) the substrate is reasonably susceptible to cellulase activity. However, the FPA has long been recognized for its complexity and susceptibility to operator errors *(10)*.

Procedure

1. Place a rolled filter paper strip into each 13 × 100 test tube.
2. Add 1.0 ml of 50 mM citrate buffer (pH 4.8) to the tubes; the paper strip should be submerged in the buffer.
3. Prepare the enzyme dilution series, of which at least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (~2.1 mg) and one slightly less than 2.0 mg of glucose (1.9 mg) (*see* **Note 6**).

4. Prepare the dilute glucose standards (GSs) as below:
   GS1: 1.0 ml of glucose standard + 4.0 ml buffer = 2 mg/ml (1.0 mg/0.5 ml).
   GS2: 1.0 ml of glucose standard + 2.0 ml buffer = 3.3 mg/ml (1.65 mg/0.5 ml).
   GS3: 1.0 ml of glucose standard + 1.0 ml buffer = 5 mg/ml (2.5 mg/0.5 ml).
   GS4: 1.0 ml of glucose standard + 0.5 ml buffer = 6.7 mg/ml (3.35 mg/0.5 ml).
   Add 0.5 ml of GS1–4 solutions to 13 × 100 mm test tubes, and add 1.0 ml of 0.050 M citrate buffer.
5. Prepare the blank and controls.
   Reagent blank (RB): 1.5 ml of 50 mM citrate buffer.
   Enzyme controls (EC1–5): 1.0 ml of 50 mM citrate buffer + 0.5 ml enzyme dilution series whose enzyme concentrations are the same as those from E1 to E5 (*see* **Note 7**).
   Substrate control (SC): 1.5 ml of 50 mM citrate buffer + filter paper strip.
6. Prewarm the enzyme solutions, blank, and controls until equilibrium.
7. Add 0.5 ml of the enzyme dilution series to the tubes with filter paper substrate (E1–5); add 0.5 ml of the enzyme dilution series to the tubes without filter paper substrate (EC1–5).
8. Incubate the tubes of E1–5, GSs, RB, EC1–5, and SC in a 50°C water bath for exactly 60 min.
9. Add 3.0 ml of the DNS reagent to stop the reaction, and mix well.
10. Boil all tubes for exactly 5.0 min (*see* **Note 8**).
11. Transfer the tubes to an ice-cold water bath.
12. Withdraw ~0.5 ml of the colored solutions into 1.5-ml microcentrifuge tubes and centrifuge at ~10,000 g for 3 min.
13. Add 0.200 ml of the supernatant into 3-ml spectrophotometer cuvette tubes, add 2.5 ml of water, and mix well by using a pipette or by inversion several times.
14. Measure absorbance at 540 nm, where the absorbance of RB is used as the blank.

#### Calculation

1. Draw a standard sugar curve (sugar along the $x$-axis vs. absorbance at 540 along the $y$-axis), as shown in **Fig. 1**.
2. Calculate the delta absorbance of dilute enzyme solutions (ΔE1–4) for E1–5 by subtraction of the sum of the absorbance of EC1–5 and SC.
3. Calculate the real glucose concentrations released by E1–5 according to a standard sugar curve.

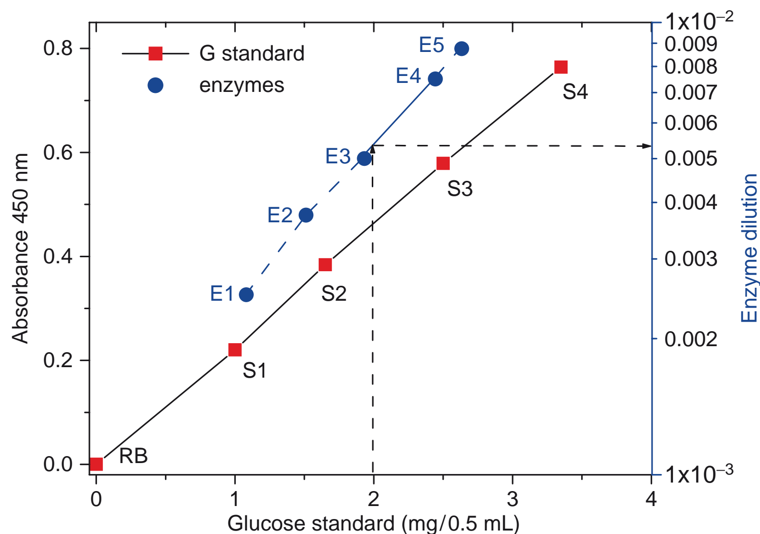


Fig. 1 The relationship of absorbance at 540 nm for the DNS assay and EDRs in terms of glucose concentration.

4. Draw the relationship between the real glucose concentrations and their respective enzyme dilution rates (EDRs) **(Fig. 1)**.
5. Link the points less than 2 mg and greater than 2 mg by a line, and identify the EDR by using the point for 2-mg glucose based on the line **(Fig. 1)**.
6. Calculate the FPA of the original concentrated enzyme solution in terms of FPU/ml:

$$\mathrm{FPA} = \frac{0.37}{\mathrm{EDR}}$$

where 2 mg glucose = 2 mg/(0.18 mg/μmol) × 0.5 ml × 60 min = 0.37 μmol/min/ml (*see* **Notes 9, 10**).

*3.1.2. Anaerobic Cellulase Assay Using Avicel*

Some cellulases or cellulosomes isolated from anaerobic environments need the presence of a reducing agent and some metal ions, such as calcium, to exert maximal hydrolysis ability, for example, the cellulosome from the thermophilic anaerobic bacterium *Clostridium thermocellum (11)*. Johnson et al. *(11)* established a turbidometric method based on the change of 0.6 g/l Avicel (FMC RC-591), which is a blend of microcrystalline cellulose and sodium carboxymethylcellulose, but the results often suffer from large variations. The anaerobic cellulosome assay was modified on the basis of the soluble sugar release during the initial hydrolysis period *(12, 13)*.

Procedure

1. Add 4.10 ml of the well-suspended Avicel solution into 16 × 125 mm Hungate tubes, and add 0.50 ml of Tris–HCl buffer (each sample needs triplicate tubes).
2. Add the rubber stopper and seal the tubes.
3. Vacuum and flush the headspace gas by ~5 psi (ultra) pure nitrogen at least three times.
4. Add 0.10 ml of 0.5 M DTT solution using a syringe with a 23G needle before enzyme activity assay.
5. Prewarm the tubes in a water bath at 60°C.
6. Prepare the enzyme solution.
7. Add 0.30 ml of the dilute enzyme solution series into the tube using a syringe with a 23G needle.
8. After the first 10 min of adsorption and reaction, withdraw ~0.5 ml of well-suspended sample using a syringe with a 21G needle as the starting point for enzymatic hydrolysis. The larger gauge needle is needed for homogeneous sampling of cellulose slurry.
9. Shake the tubes continuously or manually mix them every 10–15 min.
10. Withdraw another 0.50 ml of well-mixed Avicel suspension every 1 h using a syringe with a 21G needle into the precooled 1.5-ml microcentrifuge tubes or stop the reaction after 1 h by transferring to an ice-cooled water bath.
11. Centrifuge the samples in 1.5-ml microtubes at 13,000 g for 3 min.
12. Measure total soluble sugars in the supernatants by the phenol–sulfuric acid assay.
13. Calculate the net soluble sugar release during the hydrolysis process by subtraction of the sugar at the starting point.
14. Determine enzyme activity at a linear range between sugars released and enzyme concentrations.

Phenol–Sulfuric Acid Assay (A Linear Range from Sugars in the Samples from 0.005 to 0.1 g/l)

1. Add 0.7 ml of sugar-containing solution to 13 × 100 mm disposable glass tubes, and mix with 0.7 ml of 5% phenol solution.
2. Add 3.5 ml of concentrated sulfuric acid with vigorous mixing (*see* **Note 11**).
3. Read absorbance at 490 nm after cooling to room temperature (e.g., 20–30 min).

### *3.2. β-Glucosidase Assays*

β-Glucosidase can cleave β-1,4-glucosidic bonds of soluble substrates, including cellobiose, longer cellodextrins with a DP from 3 to 6, and chromogenic substrates, such as *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl β-D-1,4-glucopyranoside,

β-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside, and 4-methylumbelliferyl-β-D-glucopyranoside *(2)*. The term "cellobiase" is often misleading because of this key enzyme's broad substrate specificity.

*3.2.1. β-Glucosidase Assay Using pNPG*

This *p*NPG method is an initial reaction rate assay *(6)*.

Procedure

1. Add 1.0 ml of *p*NPG solution and 1.8 ml of acetate buffer into test tubes.
2. Equilibrate at 50°C.
3. Prepare the enzyme dilution series.
4. Add 0.2 ml of diluted enzymes into the tubes containing the substrate, and mix well.
5. Enzyme blanks: Add 0.2 ml of diluted enzymes into the tubes containing 2.8 ml of acetate buffer, and mix well; Substrate blank: Add 1.0 ml of pNPG solution and 2.0 ml of acetate buffer into test tubes.
6. Incubate all tubes at 50°C for 15 or 30 min.
7. Add 4.00 ml of glycine buffer to stop the reaction.
8. Measure the absorbance of liberated products of *p*-nitrophenol at 430 nm based on the substrate blank.
9. Read the net absorbance of the enzyme solutions by subtracting readings of the enzyme blanks.
10. Determine *p*-nitrophenol release on the basis of the known concentration of *p*-nitrophenol diluted by glycine at 430 nm.
11. Calculate the enzyme activity on the basis of the linear range between absorbance and enzyme concentrations.

*3.2.2. β-Glucosidase Assay Using Cellobiose*

The β-glucosidase based on cellobiose assay recommended by IUPAC is based on a fixed amount (1 mg) of glucose formation from cellobiose *(6)*. The glucose concentrations in the cellobiose reaction mixture are determined using at least two different enzyme dilutions. One dilution should release slightly more and one slightly less than 1.0 mg (absolute amount) of glucose in the reaction conditions.

Procedure

1. Dilute the enzyme solution by citrate buffer in a series. 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 (i.e., 0.5 mg glucose released/ml).
2. Add 1 ml of diluted enzyme solution (DES) to the tubes.
3. Equilibrate the enzyme solutions and substrate solutions at 50°C.
4. Add 1.0 ml of substrate solution into the tubes containing the enzyme solution.

5. Incubate at 50°C for exactly 30 min.
6. Immerse the tubes in boiling water for exactly 5.0 min to stop the reaction.
7. Transfer the tubes to a cold water bath.
8. Substrate Blank: A mixture of 1.0 ml of cellobiose solution and 1.0 ml of citrate buffer. Enzyme Blanks: A mixture of 1.0 ml of cellobiose solution and 1.0 ml of DESs. Treat substrate and enzyme blanks identically as the experimental tubes (i.e., equilibrate at 50°C, heat, boil, and cool).
9. Determine glucose release using a commercial glucose oxidase kit (GOD) or a glucose hexose kinase and glucose-6 phosphate dehydrogenase kit (HK/G6PDH) or HPLC.
10. Measure the absorbance of all solutions based on the substrate blank.

Calculation

1. Calculate the delta absorbance of dilute enzyme solutions by subtracting absorbance of the respective enzyme blanks.
2. Calculate the real glucose concentrations released according to a standard glucose curve by the enzyme kit.
3. Link the points less than 1 mg and greater than 1 mg by a line, and determine the EDR by using the point that is supposed to produce 1 mg glucose.
4. Calculate cellobiase solution activity (IU/ml) (*see* **Note 12**):

$$\text{Cellobiase} = \frac{0.0926}{\text{EDR}}.$$

#### 3.2.3. β-Glucosidase Assay Using Cellobiose

β-Glucosidase activity can be measured on the basis of initial reaction rates of cellobiose by combining the methods of **Subheading 3.2.1** and **3.2.2**. The hydrolysis product – glucose – can be measured by the glucose HK/G6PDH kit *(14)*.

### 3.3. Endoglucanase Assays

Endo-β-1,4-D-glucanase (EC 3.2.1.4) randomly cleaves accessible intermolecular β-1,4-glucosidic bonds on the surface of cellulose. Because insoluble cellulose has very low accessible fractionation of β-glucosidase bonds to cellulase *(3, 8, 15)*, water-soluble cellulose derivatives such as CMC and hydroxyethylcellulose (HEC) are commonly used for endoglucanase activity assays. The hydrolysis can be determined by measuring the changes in reducing sugars or viscosity or color. Since CMC is an anionic substrate, its properties change with pH. Nonionic substrates such as HEC are recommended sometimes.

#### 3.3.1. Endoglucanase Assay Using CMC/DNS

The IUPAC-recommended endoglucanase (CMCase) assay is a fixed conversion method, which requires 0.5 mg of absolute glucose released under the reaction condition *(6)*. The reducing end concentration is measured by the DNS method.

Procedure

1. Prepare the enzyme dilution series, of which at least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 0.5 mg of glucose and one slightly less than 0.5 mg of glucose.
2. Add 0.5 ml of the DESs into test tubes with a volume of at least 25 ml.
3. Equilibrate the enzyme solution and substrate solution at 50°C.
4. Add 0.5 ml of the CMC solution to the test tubes and mix well.
5. Incubate at 50°C for 30 min.
6. Add 3.0 ml of DNS solution and mix well.
7. Boil for exactly 5.0 min in vigorously boiling water.
8. Place the tubes in an ice-cooled water bath to quench the reaction.
9. Add 20 ml of distilled water and seal with parafilm or by a similar method. Mix by inverting the tubes several times.
10. Read the absorbance at 540 nm based on the substrate blank.
11. Prepare the substrate blank (0.5 ml of CMC solution + 0.5 ml of citrate buffer) and the enzyme blanks (0.5 ml of CMC solution + 0.5 ml of dilute enzyme solutions). Treat substrate and enzyme blanks identically as the experimental tubes.
12. Prepare the glucose standards:
    GS1 – 0.125 ml of 2 mg/ml glucose + 0.875 ml of buffer.
    GS2 – 0.250 ml of 2 mg/ml glucose + 0.750 ml of buffer.
    GS3 – 0.330 ml of 2 mg/ml glucose + 0.670 ml of buffer.
    GS4 – 0.500 ml of 2 mg/ml glucose + 0.500 ml of buffer.
13. Calculate the glucose released by the enzyme solutions with deduction of the enzyme blank absorbance based on the glucose standard curve.
14. Draw the relationship between the real glucose concentrations and their respective EDRs.
15. Link the points less than 0.5 mg and greater than 0.5 mg by a line, and identify the EDR by using the point for 0.5 mg glucose.
16. Calculate the CMCase activity of the original concentrated enzyme solution in terms of IU/ml:

$$\text{CMCase} = \frac{0.185}{\text{EDR}}$$

*3.3.2. Endoglucanase Assay Using CMC/BCA*

This initial reaction rate enzymatic assay can be conducted at a very low enzyme concentration. The reducing end concentration

is measured by the BCA method, in which the color development reaction is conducted at 75°C in order to avoid β-glucosidic bond cleavage during the color-development process *(16)*.

Procedure

1. Dilute the enzyme solution extensively (e.g., 1,000-fold) using the 50 mM citrate buffer and prepare the dilute enzyme solution series.
2. Add 1.8 ml of CMC solution into 13 × 100 mm test tubes.
3. Equilibrate at 50°C water bath.
4. Add 0.2 ml of DES and mix well.
5. Incubate at 50°C for 10 min.
6. Add 2 ml of working BCA reagents and mix well.
7. Incubate at 75°C for 30 min.
8. Read absorbance at 560 nm after subtracting the readings for the enzyme blanks and the substrate blank.
9. Calculate the enzyme activity based on a linear range between reducing end concentrations and enzyme concentrations.

Substrate blank: 1.8 ml of CMC solution + 0.2 ml of citrate buffer; enzyme blanks: 1.8 ml of CMC solution + 0.2 ml of dilute enzyme solutions. Treat blanks identically as the experimental samples.

Glucose standard: 1 ml of 5 mM glucose diluted by 50 mM citrate buffer by 100-fold to 50 μM glucose standard solution; prepare the sugar standards as below:

GS1 – 0.4 ml of 50 μM glucose + 1.6 ml of buffer.
GS2 – 0.8 ml of 50 μM glucose + 1.2 ml of buffer.
GS3 – 1.2 ml of 50 μM glucose + 0.8 ml of buffer.
GS4 – 1.6 ml of 50 μM glucose + 0.4 ml of buffer.
GS5 – 2.0 ml of 50 μM glucose.

*3.3.3. Endoglucanase Assay Using CMC/Viscosity*

This initial reaction rate assay is based on the reduction in specific viscosity of soluble cellulose derivatives such as CMC and HEC *(2)*. Both endoglucanase and exoglucanase can release new reducing sugar ends from soluble substrates. Within a limited degree of hydrolysis, endoglucanase can decrease specific viscosity greatly, and exoglucanase can decrease specific viscosity slowly *(7)*.

Procedure

1. Add 6.0 ml of prewarmed CMC solution in a water bath at 30°C into an Ostwald viscometer (water flow time of 15 s at 30°C) (*see* **Note 13**).
2. Add 1.0 ml of the prewarmed DES (*see* **Note 14**).
3. Determine the flow rates every 5 or 10 min.
4. Calculate specific viscosity ($\eta_{sp}$):

$$\eta_{sp} = \frac{t - t_0}{t_0}$$

where $t$ is the effluent time of the buffer (s) and $t_0$ is the efflux time of the buffer (s).

5. Plot the increasing rate of the reciprocal of the specific viscosity against the enzyme concentration; a linear relation should be obtained.
6. Calculate unit of activity *arbitrarily* from the linear relationship between enzyme concentration/rate of increase of reciprocal of the viscosity of the CMC solution (*see* **Note 15**).

*3.3.4. Semiquantitative Endoglucanase Assay Based on Dye Release*

Endoglucanase activity can be detected semiquantitatively on solid supports by staining polysaccharides with various dyes because these dyes are adsorbed only by long chains of polysaccharides. These methods are suitable for monitoring large numbers of samples but differences in enzyme activity levels of less than twofold are difficult to detect by eye. A linear relationship between the halo diameter and the logarithm of endoglucanase activity can be established as $D = K \times \log(A) + N$, where the $D$ is the diameter, $A$ is the enzyme activity, and $K$ and $N$ are parameters determined by the standard curve of the known enzyme activity solutions. The activity of unknown samples can be calculated on the basis of the standard curve. Three procedures are described involving in vivo as well as in vitro endoglucanase detection.

Microbe-Secreted Endoglucanase Assay on Agar Medium

*Procedure*

1. Inoculate the endoglucanase-secreted microorganisms on the solid CMC medium. The growth time depends on the growth rate of the microorganism and enzyme activity (*see* **Note 16**).
2. Stain a 9-cm Petri dish by adding 20 ml of Congo red solution at room temperature for 30 min.
3. Rinse the residual dye on the dish using distilled water.
4. Destain Congo red with ~20 ml of 1 M NaCl for 30 min. If the halos are not clear, destain the dish by another ~20 ml of NaCl solution.
5. Detect the clear, weak yellow halos for endoglucanase activity with the red background.
6. Option: In order to increase halo contrast, add ~20 ml of 5% acetate acid or 1 M HCl to the plate at room temperature for 10 min, and pour off. The background of the plate will turn blue.

Endoglucanase Assay on Agarose Gel

*Procedure*

1. Pour ~20 ml of the melted CMC agarose solution (~50°C) into a 9-cm Petri dish.
2. Drill wells on the solidified agarose gel with a cork borer, and remove the agarose particles in the wells by suction or a pair of tweezers (*see* **Note 17**).
3. Add 10–20 μl of the enzyme solution into the holes.

4. Put the plate in the incubator (37°C or desired temperature) for several hours or even overnight.
5. Wash the plate with distilled water.
6. Add 10 ml of the Congo red solution and incubate at room temperature for 30 min.
7. Wash the residual dye on the plate by distilled water.
8. Destain the dye by using 20 ml of 1 M NaCl solution at room temperature for 30 min, and decant the destained solution (*see* **Note 18**).
9. Detect the clear yellow halo with the red background.

#### Endoglucanase Assay on Polyacrylamide Gel

This method can separate mixed protein components by electrophoresis and then detect endoglucanase activity on polyacrylamide gels (SDS PAGE or native PAGE). If SDS-PAGE is used, cellulase activity must be detected after SDS removal and protein re-naturation.

*Procedure*

1. Separate the protein mixtures by native or SDS PAGE.
2. Rinse the gel in distilled water for 5 min.
3. Soak the gel in the sodium phosphate buffer with gentle shaking for 20 min, and repeat the washing procedure three times to remove the SDS.
4. Transfer the gel into the CMC/phosphate buffer for 30 min.
5. Rinse the gel with distilled water.
6. Incubate the gel in 0.1 M sodium phosphate buffer at 40°C for 1 h.
7. Stain the gel with the Congo red solution at room temperature for 30 min.
8. Wash the gel with distilled water, and destain the gel in 1 M NaCl solution at room temperature for 30 min (*see* **Note 19**).

### 3.4. Exoglucanase Assays

Exoglucanase (CBH, EC 3.2.1.91) can release either glucose and/or cellobiose from ends of cellulose chains. *Trichoderma reesei* CBH1 and CBH2 cleave cellobiose units from the reducing end and the non-reducing end of cellulose chains, respectively. In contrast to endoglucanase and β-D-glucosidase, exoglucanases are difficult to measure due to the lack of specific substrates and interference from other cellulase components. Accordingly, exoglucanases have to be assayed in the purified form. The activity of purified exoglucanases is often estimated using Avicel. Avicel is a good substrate for exoglucanase activity assay because of its highest ratio of end/accessibility *(3, 7)*. To some extent, Avicelase is regarded as synonymous with exoglucanase or CBH. In addition, amorphous cellulose can be used for determining of exoglucanase activity.

*3.4.1. Exoglucanase Assay Using Avicel*

Procedure

1. Suspend 1.25% (w/v) Avicel in acetate buffer (*see* **Note 20**).
2. Add 1.6 ml of Avicel suspension solution into the tubes.
3. Dilute a series of enzyme solutions by acetate buffer.
4. Equilibrate the substrate and enzyme solutions in a water bath at 50°C.
5. Add 0.4 ml of the dilute enzyme solutions to the Avicel substrate and mix well.
6. Incubate at 50°C for 2 h.
7. Stop the reaction by submerging the tubes in ice-cooled water bath.
8. Withdrew ~1 ml of hydrolysate into microcentrifuge tubes and centrifuge the sample at 13,000 g for 3 min.
9. Prepare enzyme blanks (0.4 ml of diluted enzymes and 1.6 ml of 0.1 M acetate buffer) and substrate blank (0.4 ml of 0.1 M acetate buffer and 1.6 ml of 1.25% (w/v) Avicel suspension buffer).
10. Determine the total soluble sugars in the supernatant by the Phenol–Sulfuric Acid assay where the absorbance of the substrate blank is used as the blank (*see* the Phenol-Sulfuric Acid assay in **Subheading 3.1.2**).
11. Calculate the enzyme activity on the basis of a linear relationship between the total soluble sugar release and enzyme dilution. One unit of exoglucanase activity is defined as the amount of enzyme that releases one micromole of glucose equivalent per minute from Avicel.

*3.4.2. Exoglucanase Assay Using RAC*

RAC Preparation Procedure (*17*)

1. Microcrystalline cellulose (0.2 g) is added to a 50-ml centrifuge tube, and 0.6 ml distilled water is added to form a suspended cellulose slurry.
2. Ten milliliters of ice-cold 86.2% $H_3PO_4$ is slowly and carefully added to the slurry with vigorous stirring. Before adding the last 2 ml of phosphoric acid, the cellulose suspension solution must be thoroughly mixed. The cellulose mixture turns transparent within several minutes, and should be held for ca. 1 h on ice with occasional stirring.
3. Approximately 40 ml of ice-cold water is added at the rate of approximately 10 ml per addition with vigorous stirring between additions, resulting in a white cloudy precipitate.
4. The precipitated cellulose is centrifuged at ~5,000 g and 4°C for 20 min.
5. The pellet is suspended in about 45 ml ice-cold water followed by centrifugation to remove the supernatant containing phosphoric acid; this step is repeated four times.

6. Approximately 0.5 ml of 2 M $Na_2CO_3$ is added to neutralize the residual phosphoric acid, and then 45 ml of ice-cold distilled water is used to suspend the cellulose pellet.
7. After centrifugation, the pellet is suspended in distilled water and centrifuged twice or until the pH reaches 5–7.
8. The carbohydrate concentration of RAC is calibrated by the Phenol-Sulfuric Acid method and diluted to 1%.
9. Addition of 0.2%, w/v sodium azide is optional for extended RAC storage at 4°C (*see* **Note 21**).

Assay Procedure

1. 0.5 ml of 1% (w/v) RAC and 0.05 ml of 1 M citrate buffer plus 0.25 ml water in the tubes.
2. Dilute the enzyme solution series with 50 mM acetate buffer.
3. Equilibrate the tubes containing the enzyme and substrate solutions at 50°C.
4. Add 0.2 ml of the DESs and mix well.
5. Incubate at 50°C for 10–30 min.
6. Place the tubes in an ice-cold water bath.
7. Centrifuge the hydrolysate sample at 10,000 g for 3 min.
8. Prepare enzyme blanks (0.2 ml of the DES, 0.05 ml of 1 M citrate buffer, and 0.75 ml of distilled water) and substrate blanks (0.5 ml of 1% w/v RAC, 0.45 ml of distilled water, and 0.5 ml of 1 M citrate buffer).
9. Measure the total soluble sugar concentration in the supernatants by the Phenol–Sulfuric Acid method and measure the absorbance at 490 nm using the absorbance of the substrate blank as the blank; (*see* the Phenol–Sulfuric Acid assay in **Subheading 3.1.2**).
10. A linear relationship between the total soluble sugar release and enzyme dilution is used for calculating the enzyme activity. One unit of exoglucanase activity is defined as the amount of enzyme that releases one micromole of glucose equivalent per minute from Avicel.

### 3.5. Summary

A number of cellulase activity assays have been developed over several decades, but we have presented only the most popular cellulase activity assays here. Heterogeneity of insoluble cellulose, complicated synergy/competition among endoglucanase and exoglucanase, and changes in ratio of enzyme/substrate pose formidable challenges in developing cellulase activity assays *(2, 7, 8)*. Keeping special properties of insoluble substrates (such as limited accessibility to enzyme, degree of polymerization (DP), etc.) in mind *(8)*, there is no clear relationship between the hydrolysis rates obtained on soluble and insoluble substrates,

mainly because of large variations in limited solid substrate accessibility to cellulase *(7)*. A functionally based model has been developed to suggest the complexity among endoglucanase, exoglucanase, their ratio, cellulose accessibility, DP, enzyme concentration, and reaction time *(7)*. The model suggests the challenges in applying the results of total cellulase activity assay measured on one solid substrate to a different solid substrate. Researchers must state clearly all parameters of their assay conditions and resist the temptation to compare their results to those of other researchers using different substrates, experimental conditions, etc. An understanding of enzymatic cellulose hydrolysis mechanisms among substrate characteristics is urgently needed, as well as development of enzyme activities to evaluate cellulase performance on insoluble cellulosic materials, especially for pretreated lignocellulosic materials.

## 4. Notes

1. Be careful to handle the phenol safely.
2. The DNS reagent can be stored in darkness at 4°C for at least 1 month. It could lose its reducing ability after long storage *(18)*. The freshness of the DNS reagent is often ignored *(18)*.
3. It is important to check each paper strip weight to ensure that the weight variation is less than 1 mg per strip because FPA is subject to the filter paper weight. Handle the paper with forceps or gloved hands.
4. Aliquots of the standard glucose solution can be tightly sealed and stored frozen. The solution should be mixed well after thawing.
5. 4-Methylumberliferyl-β-glucoside can replace *p*NPG, which results in an assay with higher sensitivity.
6. Take commercial concentrated cellulase solution as an example. Dilute the enzyme solution 20-fold using 50 mM citrate buffer DES, and then prepare a series of dilutions from E1 to E5 with different dilution rates as below:

   E1: 0.10 ml of DES + 1.90 ml of citrate buffer (dilute rate = 0.0250).

   E2: 0.15 ml of DES + 1.85 ml of citrate buffer (dilute rate = 0.0375).

   E3: 0.20 ml of DES + 1.80 ml of citrate buffer (dilute rate = 0.0500).

E4: 0.30 ml of DES + 1.70 ml of citrate buffer (dilute rate = 0.0750).
E5: 0.35 ml of DES + 1.65 ml of citrate buffer (dilute rate = 0.0850).

7. Commercial enzyme solutions can contain a significant amount of reducing sugars (19).
8. The boiling condition should be severe, and the volume of the boiling water bath should be maintained above the level of the total liquid volume of the test tubes to promote full color development.
9. International Unit (IU) is defined as 1 μmol/min, based on the initial hydrolysis rate, and is different from FPU assay, which is a fixed conversion assay.
10. The β-D-glucosidase level present in the cellulase mixture greatly influences the FPA assay (2, 5, 6, 20) because the DNS readings are strongly influenced by the reducing end ratio of glucose, cellobiose, and longer cellodextrins (16).
11. The Phenol–Sulfuric Acid assay is an extremely exothermic reaction; so be cautious not to spill the liquid.
12. 0.5 mg of glucose produced/ml × 2 ml volume = 1 mg of glucose produced = 5.56 μmol of glucose produced = 2.78 μmol of cellobiose consumed. Since reaction time is 30 min, 0.0926 IU of β-glucosidase can produce 1 mg of glucose from cellobiose within 30 min.
13. A convenient viscometer such as capillary tubing could be used to replace the Ostwald viscometer.
14. Constant temperature during the viscosity measurement is important because viscosity is greatly influenced by temperature change.
15. Exact endoglucanase activity (μmol bond cleavage/min) based on changes in specific viscosity can be calculated through a relationship between the viscosity and the DP of CMC. Viscosity of the substrate is strongly associated with substrate DP, and medium-viscosity CMC is recommended.
16. If CMC inhibits microorganism growth, a second layer of CMC solid medium can be applied to the primary medium containing other carbon sources or nutrients.
17. Strong enzyme activity or the short distance between the wells results in the fused halos, which may be difficult to differentiate or measure.
18. Often, the halo can be observed in 5 min; if the halo is not clear, destain again by adding 20 ml of the NaCl solution. If the band is not clear, destain the gel by using the NaCl solution again.

19. For native PAGE, a one-time soak is enough. Do not use a potassium phosphate buffer because potassium precipitates in the gel.
20. Since Avicel powder could contain approximately 4–8% moisture, weight adjustment is needed.
21. RAC, different from phosphoric acid-swollen cellulose (PASC), is a homogeneous amorphous cellulose that is precipitated from dissolved cellulose. RAC has a constant quality because it is regenerated from homogeneous dissolved cellulose, has easy handling and transferring properties, is a homogeneous substrate, and has high substrate reactivity *(17)*. Take care if azide is used because of both toxicity and the explosive nature of powders.

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