



Endoglucanase (EG) Activity Assays

**M. Shafiqur Rahman, Sheran Fernando, Brian Ross, Jiangning Wu,
and Wensheng Qin**

Abstract

Cellulosic biomass, the most common organic compound of primary energy source on earth, is a network of interwoven biopolymers of plant cell walls. Degradation of cellulose is important for global carbon recycling. Moreover, biofuel, a renewable fuel whose energy can be derived from cellulosic biomass by enzymatic hydrolysis of cellulases. Among cellulases are endoglucases that act synergistically for subsequent hydrolytic reactions to break down the polymeric cellulose. However, in cellulolytic enzyme activity endoglucanase plays a prominent role in initiating and sustaining the hydrolytic process. Endoglucanase randomly cleaves the cellulose polymer into smaller sugar and oligomeric polysaccharides. Characterization and quantification of endoglucanase activity is important for industry and in the overall study of cellulose degradation. All assays including those for endoglucanase fall into two broad categories either qualitative or quantitative. Quantitative assays can tell if the enzyme is present, how much and its activity. Measurement can be done indirectly using a secondary colorimetric product like (1) molybdenum blue, (2) 3-amino-5-nitrosalicylic acid, (3) bicinchoninic acid (BCA), and (4) 2-cyanoacetamide or directly using an antibody in an ELISA. In this chapter, we discuss several common protocols for the measurement of endoglucanase activity.

Key words Endoglucanase, Cellulolytic enzyme, Cellulose hydrolysis, Reducing sugar, CMC

1 Introduction

Endoglucanases are a subgroup of a larger family of enzymes referred to as cellulases. Cellulases collectively refer to three broad groups of enzymes: endoglucanases, exoglucanases, and β -glucosides. Moreover, cellulases are part of a superfamily of enzymes called hydrolases, which use water to break apart larger molecules. All three types of cellulases are involved in and are required to effectively degrade cellulose, an important structural component of plant cell walls. Exoglucanases or cellobiohydrolases target cellulose chains from the ends, endoglucanases randomly cleave within the chain, and β -glucosidases hydrolyze cellobiose, the products of exoglucanases. There are many subtypes of endoglucanases (e.g., endoglucanase I, II) each with its own molecular

topology. Indeed, some organisms are capable of producing several different subtypes of endoglucanases. As a whole these endoglucanases are grouped into glycoside hydrolase families (glycoside hydrolase 5, 6, etc.) [1]. Endoglucanases vary in their substrate specificity being able to cleave differing glycosidic bonds (β -1-4, β -1-6, etc.) [2]. β -1-4-endoglucanases are the most common and are what is referred to by default. They cleave the internal β -1-4-glycosidic bonds of the cellulose chain. The exact nature and energetics of the reaction are controversial but it is believed that the mechanical sheering in the enzyme's active domain causes the breaking of the bonds [3]. Cellulose hydrolysis through enzymes occurs in two steps: (1) the physical disruption of the crystalline ultrastructure of cellulose and (2) hydrolysis of the disrupted cellulose glycosidic linkages. The hydrolysis is carried out by the two carboxylic acid groups, where one acts like an acid and the other as the base [4]. However, for the purposes of endoglucanase, the assays have relatively the same sensitivity but this is not the case for other enzymes [5]. Moreover, in this chapter, we will provide an overview of assays used to either: (1) prove or disprove the presence of the enzyme (qualitative assays) and/or (2) determine the amount of enzyme in the sample (quantitative assays).

There are many endoglucanase activity assays, and we have presented only the most popular assays. It is our hope that we have given you an idea of the general categories of assays possible and the underlying principles. Qualitative assays based on viscosity or dye decolorization will tell you whether endoglucanase is present or not. Quantitative assays based on the generation of a colorimetric product, which will indirectly measure endoglucanase levels, or ELISA assays will directly measure endoglucanase levels. For most purposes, indirect measurement is sufficient unless you are looking into a specific subtype of endoglucanase, which would require an ELISA. All the quantitative assays mentioned in the chapter have similar sensitivities for endoglucanase within a range of hydrolysis. Whichever assay you decide to use it is important to keep in mind sources of error, the assay operating conditions, and what exactly you are measuring.

1.1 Qualitative Endoglucanase Assays

Based on the methods described by Zhang et al. [6] and Hendricks et al. [7], dye decolorization assays can be used to detect endoglucanase qualitatively and semiquantitatively on agar or agarose containing media. This assay is suitable for screening a large number of samples, with differences in endoglucanase activity that are visible to the eye. In the assay, the polysaccharides (CMC in this case) are stained with a dye and forming noncovalent complexes with cellulose. Degradation of cellulose by endoglucanase results in a halo or zone of clearance caused by the simultaneous degradation of the dye. Figure 1 is an example of a dye decolorization assay. The halo can be used qualitatively and as a semiquantitative assessment using



Fig. 1 Degradation of cellulose by endoglucanase results in a halo or zone of clearance caused by the degradation of the dye Congo Red [8]

the following formula, which linearly relates endonuclease activity with halo diameter:

$D = K \times \log(A) + N$, where the D is the diameter, A is the enzyme activity, and K and N are parameters determined by the constructing a standard curve with known solutions having known enzyme activities.

However, another method, enzyme-linked immunosorbent assay (ELISA) can be used for endoglucanase assays [9]. This is an ELISA assay used to measure antibody binding to antigen, in this case monoclonal antibody EG-1-2 (mAb EG-1-2) binding to endoglucanase I (EG I) produced by *T. reesei*. *T. reesei* produces many types of endoglucanases—endoglucanase II, IV, etc.; mAb EG-1-2 was made specifically for a nonconserved epitope unique to EG-1 (see **Note 1**). EG-1 is produced by a wide range of cellulolytic organisms. Although this mAb is highly specific to EG-1, it is possible to create a less specific mAb by targeting epitopes common to multiple endoglucanases.

1.2 Quantitative Endoglucanase Assays

1.2.1 Endoglucanase Activity Assays Using Reducing Agents

The principle behind using reducing agents such as sugars is that within a limited amount of hydrolysis, the production of these sugars such as glucose from the intact polymeric cellulose is largely controlled by endoglucanases. As endoglucanases generate reducing sugars, it results in a measurable change in the solution, either a change in viscosity or a change in color depending on the assay. Water-soluble cellulose derivatives such as Carboxymethyl Cellulose (CMC) or Hydroxyethyl Cellulose (HEC) are used instead of native cellulose because underivatized cellulose has a very low accessible fraction of β -glucosidic bonds making the reaction activity rate too slow to measure.

There are three popular colorimetric reducing sugar assays: (1) Nelson-Somogyi (NS) method, (2) 3,5-dinitrosalicylic acid (DNS) method, and (3) 2-cyanoacetamide method. All these assays achieve in large similar results but differ in their reagents, methodology and operating conditions (temp., pH etc.). Therefore, it is important to choose the correct assay for your own particular operating parameters. The BCA method is an interesting exception because the reducing agent is the endoglucanase itself, which directly produces the colorimetric product unlike the other three assays above, which rely on reducing sugars.

1.2.2 Endoglucanase Assay Using CMC by the DNS Method

Endoglucanase assay (CMCase) estimates a fixed amount of glucose (about 0.5 mg) produced by the substrate (CMC). Enzymatic activity is estimated by the DNS method. For quantitative results, enzymes must be diluted, or assay reaction time decreased until the amount of product plotted against enzyme concentration is reasonably linear. A shortcoming and important point to note is that although CMC is widely used for the assay of endoglucanase activity there are some problems with its use since the enzyme is being measured over a range of pH values. As an ionic substance like CMC, its properties will change with pH, requiring the use of mildly acid assay conditions. Nonionic substrates, such as HEC, can be used as a substitute if particular applications require more different levels of acidity or alkalinity [6, 10].

The DNS method is an estimation of reducing sugars. Reducing sugars like glucose and other oligosaccharides convert DNS (yellow) to 3-amino,5-nitrosalicylic acid (orange-red) [11]. This change in color can be measured by a spectrophotometer as an absorbance, thereby used as an estimation of endoglucanase activity, which produces the reducing sugars. Another shortcoming of this method and other methods that use reducing sugars is that citrate buffer and other substances might affect the DNS test [11]. Furthermore, the dissolved oxygen and the acidic buffer can also reduce the colorimetric agent and further confound the results [11].

1.2.3 Endoglucanase Assay Using CMC by Nelson-Somogyi Method

The principle behind this assay is similar to the CMC or BCA method except in this case it is not a peptide bond that reduces Cu^{2+} to Cu^+ , instead it is a reducing sugar. Reducing sugars when heated with alkaline copper tartrate reduce Cu^{2+} to Cu^+ resulting in the formation of Cu_2O . When Cu_2O interacts with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue take place [12, 13]. For the purposes of endoglucanase, the sensitivity of this test is comparable to the DNS method [5].

1.2.4 Endoglucanase Assay Using CMC by 2-Cyanoacetamide Method

It is an accurate, rapid and nontoxic spectrophotometric quantitative assay method where 2-cyanoacetamide can detect D-glucose in a linear fashion under a wide range of pH (4.0–8.0). As sensitive as

the DNS test, 2-cyanoacetamide is also detecting endoglucanase activity using CMC as a substrate [14]. This assay was developed as a safer alternative to the DNS-method since many of the reagents in the DNS method are known highly toxic, carcinogens and corrosive [14].

1.2.5 Endoglucanase Assay Using CMC and Viscosity

This is a qualitative assay based on the reduction in specific viscosity of CMC as sugars are released by endoglucanase. A viscometer should be used when performing this assay. It is important to maintain a constant temperature in this assay, as viscosity varies greatly with temperature. Both endoglucanase and exoglucanase can reduce the specific viscosity. But within a limited degree of hydrolysis, endoglucanases activity alone can be selected for because they decrease the specific viscosity faster than exoglucanases, which decrease it more slowly [6, 15].

2 Materials

Microbial culture transfer and preparation of all the reagents should be performed under aseptic conditions (*see Note 2*). Prepare all reagents and buffers using deionized, double distilled or milli-Q water at room temperature and store the reagents at room temperature (unless indicated otherwise). However, for microbial media preparation you can use distilled water. Always use analytical or reagent grade chemicals.

2.1 Qualitative Assays

2.1.1 Endoglucanase Assay on Agar Medium

- 1 g/L Congo Red solution: Dissolve 100 mg Congo Red in 99.9 mL water containing 1% ethanol.
- 1 M NaCl solution: Dissolve 58.44 g of NaCl into distilled water in a final volume of 1 L.
- 0.1 M sodium phosphate buffer (pH 6.5): Prepare 0.5 M sodium phosphate monobasic stock by dissolving 30.0 g of anhydrous sodium phosphate monobasic in a final volume of 500 mL of H₂O.
- 1% w/v, low viscosity CMC agar medium: Take 0.5 g CMC, 0.1 g NaNO₃, 0.1 g K₂HPO₄, 0.1 g KCl, 0.05 g MgSO₄, 0.05 g yeast extract, 1.5 g agar, and 100 mL of distilled water in a 250 mL conical flask. Autoclave (sterilized) the medium before use to make agar plates (*see Note 3*).

This is an example of a possible CMC based minimal salt agar for growing bacteria, formulation can be varied as needed.

2.1.2 Endoglucanase Assay on Agarose Gel

- 1 g/L Congo Red solution: For preparation refer to Subheading 2.1.1.
- 1 M NaCl solution: For preparation refer to Subheading 2.1.1.

3. 0.1 M sodium phosphate buffer pH 6.5: For preparation refer to Subheading 2.1.1.
4. 1% w/v, low viscosity CMC in 0.8% agarose: For preparation refer to Subheading 2.1.1. The only difference is to use 0.8 g agar.

2.1.3 *Endoglucanase Assay on Polyacrylamide Gel*

This method can separate the protein components of a sample by electrophoresis and then detects endoglucanase activity on polyacrylamide gels via dye staining and zones of clearance. Refer to Fig. 2 for an example of how the assay looks.

1. 1 g/L Congo Red solution: For preparation refer to Subheading 2.1.1.
2. 1 M NaCl solution: For preparation refer to Subheading 2.1.1.
3. 0.1 M sodium phosphate buffer pH 6.5: For preparation refer to Subheading 2.1.1.
4. 1% w/v CMC in sodium phosphate buffer whose pH is chosen depending on the specific cellulase: For preparation refer to Subheading 2.1.1.

2.1.4 *Enzyme-Linked Immunosorbent Assay (ELISA)*

1. Monoclonal Antibody (mAb) EG-I against Endoglucanase I.
2. Phosphate buffered saline with Tween-20 (PBST): 20 mM sodium phosphate buffer (pH 7.2) containing 150 mM sodium chloride, 0.02% sodium azide, and 0.05% Tween 20. Or, mix 3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 150 mM NaCl, 0.05% Tween[®] 20, pH 7.4. Sterilize by autoclaving, and store in room temperature.

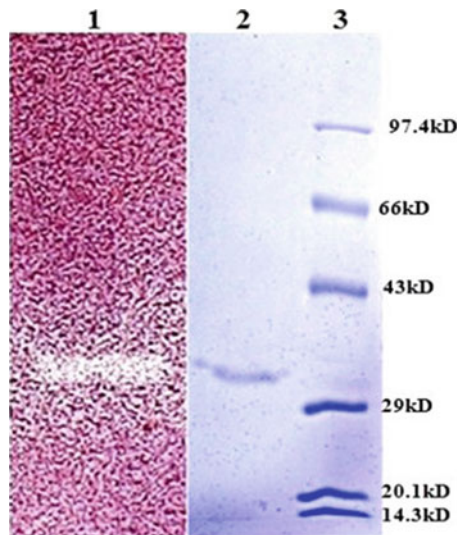


Fig. 2 Separation of protein mixtures by SDS-PAGE [16]

3. 1.5 M Tris-HCL buffer (pH 8.8): Dissolve 18.15 g of Tris base in 80 mL distilled water. Adjust pH to 8.8 using 6 M HCl. Afterward add 100 mL distilled water to bring volume up to 150 mL.
4. Bovine serum albumin (BSA) from Sigma-Aldrich.
5. 200 μ L of a 1-mg/mL *p*-nitrophenyl phosphate (PNPP) solution: Mix 1 mg PNPP salt with 1 mL of distilled water.
6. 2 M sodium hydroxide: Dissolve 79.99 g NaOH in 1 L of distilled water.
7. Alkaline phosphatase-conjugated anti-rabbit immunoglobulin G produced in swine and alkaline phosphatase-conjugated anti-mouse IgG (heavy and light chains) produced in rabbits such as that made by Orion Diagnostics, Espoo, Finland and other companies.

2.2 Quantitative Assays

2.2.1 Endoglucanase Assay Using CMC by the DNS Method

1. 50 mM Citrate buffer pH 4.8: Add 210 g citric acid monohydrate to 750 mL distilled water. Then add 1.0 M NaOH (39.997 g/L distilled water) until pH equals 4.3 (around 50–60 g). Dilute the solution to 1000 mL and check pH. If necessary add 1.0 M NaOH until pH = 4.8.
2. 2% w/v CMC in 0.05 M citrate buffer: Add 2.0 g of CMC per 100 mL of distilled water. Heat and dissolve, centrifuge to remove any residue if needed.
3. 500 mL DNS reagent: Add 3.15 g DNS and 10.48 g NaOH into 250 mL of distilled water. Dissolve the above ingredients, then add 91 g Rochelle salts (Na-K tartrate), 2.5 g phenol, and 2.5 g sodium metabisulfite. Add distilled water up to 500 mL.
4. Glucose standards (GS):
Preparation of known glucose standards. For example:
GS1–0.125 mL of 2 mg/mL glucose + 0.875 mL of 0.05 M citrate buffer.
GS2–0.250 mL of 2 mg/mL of glucose + 0.750 mL of 0.05 M citrate buffer.
GS3–0.330 mL of 2 mg/mL of glucose + 0.670 mL of 0.05 M citrate buffer.
GS4–0.500 mL of 2 mg/mL of glucose + 0.500 mL of 0.05 M citrate buffer.
GS5–1.000 mL of 2 mg/mL of glucose.
Note: concentrations and preparation volumes can vary as appropriate.
5. Prepare enzyme dilution series/standards:
EZ1–0.1 mL enzyme/crude enzyme extract + 0.9 mL 0.05 M citrate buffer.
EZ2–0.1 mL of EZ1 + 0.9 mL 0.05 M citrate buffer.

2.2.2 *Endoglucanase Assay Using CMC and Bicinchoninic Acid (BCA)*

1. 0.05 M Citrate buffer pH 4.8: Prepare 0.05 M solutions of both 10.51 g/L citric acid and 14.71 g/L sodium citrate in double distilled water. Adjust the pH of the 0.05 M citrate solution to 4.8 with the 0.05 M citric acid solution (may require about 667 mL of citric acid solution per 1 L of sodium citrate solution). Store in refrigerator.
2. 0.05% w/v CMC solution in 0.05 M citrate buffer: Add 0.05 g CMC per 100 mL of 0.05 M citrate buffer.
3. Working BCA reagent. Mix equal volumes of following reagents A and B. The reagent should be used immediately after being made.
 - (a) BCA reagent A: Dissolve 97.1 mg disodium 2,2-bicinchoninate 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.
 - (b) BCA Solution B: Dissolve 62.4 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 63.1 mg L-serine in 50 mL of water. Solution B will remain stable for 4 weeks at 4 °C in darkness.
4. Glucose standard (GS) solution 1 mL of 5 mM glucose diluted to 50 mM using citrate buffer. Prepare the sugar standards (GS) as below:
 - GS1–0.4 mL of 50 mM glucose + 1.6 mL of buffer.
 - GS2–0.8 mL of 50 mM glucose + 1.2 mL of buffer.
 - GS3–1.2 mL of 50 mM glucose + 0.8 mL of buffer.
 - GS4–1.6 mL of 50 mM glucose + 0.4 mL of buffer.
 - GS5–2.0 mL of 50 mM glucose.
5. Prepare enzyme blank and substrate blank:
 - Substrate blank: 1.8 mL of CMC solution + 0.2 mL of citrate buffer.
 - Enzyme blank: 1.8 mL of CMC solution + 0.2 mL of dilute enzyme solutions. Treat blanks identically as the experimental samples.

2.2.3 *Endoglucanase Assay Using CMC by Nelson-Somogyi Method*

1. Alkaline copper Tartrate:
 - Solution A: Dissolve 2.5 g anhydrous sodium carbonate, 2.0 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20.0 g anhydrous sodium sulfate in 60 mL distilled water and dilute to 100.0 mL.
 - Solution B: Dissolve 15.0 g copper sulfate in 80.0 mL distilled water. Add one drop of sulfuric acid and dilute to 100.0 mL. Mix 4 mL of Solution B and 96 mL of solution A before use.
2. Arsenomolybdate reagent: Dissolve 2.5 g ammonium molybdate in 45 mL water. Add 2.5 mL sulfuric acid and mix well.

Then add 0.3 g disodium hydrogen arsenate dissolved in 25 mL water. Mix well and incubate at 37 °C for 24–48 h.

3. Standard glucose solution (Stock): Dissolve 100 mg glucose in 100 mL distilled water. From this stock solution, make seven working standards. For example, 10 mL of stock diluted to 100 mL with distilled water (100 µg/mL).

2.2.4 Endoglucanase Assay Using CMC by 2-Cyanoacetamide Method

1. 1% 2-cyanoacetamide (w/v): Dissolve 0.1 g of 2-cyanoacetamide in 10.0 mL of distilled water.
2. 100 mM borate (pH 9.0): Dissolve about 6.2 g of boric acid in 800 mL distilled water, adjust pH by adding 1.0 M NaOH (39.997 g/L distilled water), and then top up to 1 L. Adjust pH as needed. Heat a little to dissolve all ingredients.

2.2.5 Endoglucanase Assay Using CMC/Viscosity

1. 50 mM sodium acetate buffer pH 5.0: Mix 3 g of acetic acid and 0.5 g of sodium acetate in 1 L of distilled water. Adjust to desired pH with 1.0 M NaOH or 1.0 M HCl (*see Note 4*).
2. 0.05% CMC solution in acetate buffer: Add 0.05 g of CMC to 100 mL of acetate buffer.

3 Methods

3.1 Qualitative Assays

3.1.1 Endoglucanase Assay Using Agar Medium

1. Inoculate a NanoDrop (6 µL) of fungal spore or bacterial suspension (*see Note 5*) or a tiny amount of fungal mycelial growth onto a CMC agar plate (*see Note 6*), incubate for 2–3 days.
2. Stain the culture medium with the microorganism by adding 5–10 mL of Congo Red solution at room temperature for 20–30 min.
3. Rinse the residual dye on the plate using distilled water.
4. Destain Congo Red with 5–10 mL of 1 M NaCl for 20–30 min.
 - (a) This will kill the bacteria, so make sure that you do not need them anymore.
 - (b) If the halos are not clear, destain again using another 5–10 mL of 1 M NaCl solution.
5. Measure the clear or halo zone diameter in millimeters, and yellow halos for endoglucanase activity with the red background (*see Note 7*).

3.1.2 Endoglucanase Assay on Agarose Gel

1. Pour CMC agarose solution into a Petri dish to cover and let it set.
2. Make several wells at equidistance using a sterilized cork borer (*see Note 8*) in the solidified agarose gel, and remove the agarose particles from the wells by a sterilized forceps (*see Note 8*).

3. Add 0.1–0.2 mL of the enzyme solution into the holes.
4. Put the plate at room temperature for several hours or even overnight (*see Note 9*).
5. Wash the plate with distilled water.
6. Add 5–10 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 5–10 mL of 1 M NaCl solution at room temperature for 20–30 min, and pour away the destain solution.
9. Measure the clear yellow halo zone in mm in diameter with the red background.

3.1.3 *Endoglucanase Assay on Polyacrylamide Gel*

1. Separate the protein mixtures by native PAGE or SDS-PAGE: Perform electrophoresis at room temperature at 60 V until samples migrate into resolving gel and then at 80 V until the dye front reaches toward end of the resolving gel.
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. (If using native PAGE one soak is enough because the sodium will precipitate.)
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.
9. Detect the clear yellow halo with the red background.

3.1.4 *Enzyme-Linked Immunosorbent Assay (ELISA)*

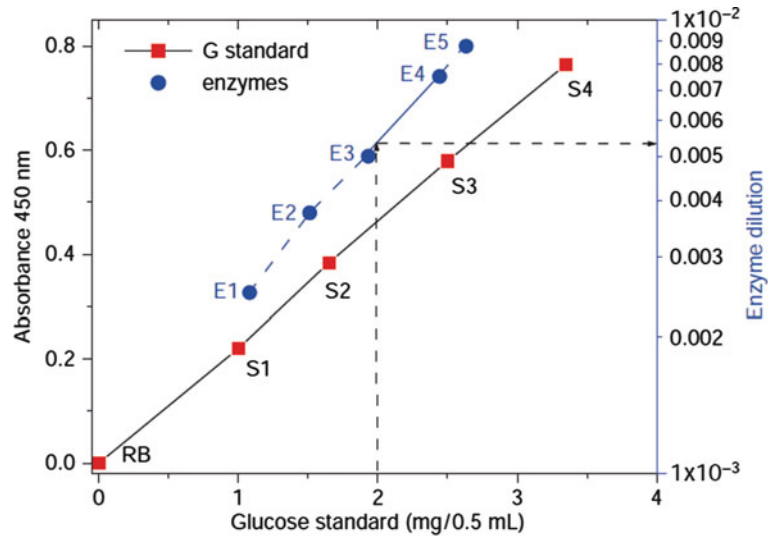
1. Dilute 100 µL of 1 µg/mL antigen in 30 mM Tris-HCl pH 8.8. Incubate overnight at 4 °C.
2. Wash the plates with PBST by emptying and filling the plates three times.
3. Incubate with 200 µL of a 1% BSA solution in PBST per well for 1 h at 37 °C to block the remaining binding sites on the plastic surface.
4. Empty plates. Then add 100 µL of antiserum, hybridoma culture supernatant or another solution containing the mAb diluted in 1% BSA in PBST, incubate for 2 h at 37 °C and then wash three times with PBST.

5. To detect bound polyclonal antibodies, incubate plates for 2 h at 37 °C with 100 μ L of anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBST. To detect bound monoclonal antibodies, incubate the plates with anti-mouse IgG-alkaline phosphatase conjugate.
6. After three final washes with PBST, add 200 μ L of a 1-mg/mL *p*-nitrophenyl phosphate solution in substrate buffer to the wells, and develop the color for 30 min at room temperature. Stop the reaction by the addition of 50 μ L of 2 M sodium hydroxide, and measure the A405 nm.
7. The detection limit is defined as the concentration at the intersection of a standard dilution curve with the mean blank A405 plus 4 standard deviations. There are six blank values per plate and one standard curve.

3.2 Quantitative Assays

3.2.1 Endoglucanase Assay Using CMC by the DNS Method

1. Prepare enzyme dilution series, with the aim that at least one dilution will release slightly more than 0.5 mg of glucose and one releasing slightly less than 0.5 mg of glucose.
2. Mix 0.5 mL of CMC solution and 0.5 mL of each diluted enzyme solution (DES) from the dilution series in a test tube (s). Maintain the temperature of the solution at approximately 50 °C.
3. Incubate at 50 °C for 30 min.
4. Add 3.0 mL of DNS reagent and mix.
5. Heat in boiling water for 5 min. Then put into ice or in ice water bath to stop the reaction.
6. Add 20 mL of distilled water and mix homogeneously.
7. Prepare an enzyme blank without CMC, and contains 0.1 mL enzyme solution + 0.9 mL 0.05 M citrate buffer, and substrate blank without DNS containing 0.1 mL CMC 2% w/v + 0.9 mL 0.05 M citrate buffer. These blanks should also be boiled for 5 min and then cooled.
8. Take about 200 μ L from each sample plus blanks and put into the microplate/96 well plate in triplicate.
9. Read absorbance at 540 nm.
10. Subtract the absorbance values of enzyme blank from substrate blank to adjust for background and analyze the enzyme activity.
11. Draw the relationship between the glucose concentration and their respective EDRs. Figure 3 below is an example of such a graph.
12. Use standard glucose curve (standard calibration graph) for known concentrations of glucose to determine reducing sugars. Whereas the graph of glucose is used to estimate the total sugars. Graph the relationship between absorbance, glucose concentration and enzyme dilution rates (EDRs).



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

Fig. 3 Standard curve for conversion of absorbance to glucose concentration [6]

13. The linear relationship between sugar released and enzyme dilution help to estimate the enzyme activity. One unit (IU) of CMCase corresponds to the amount of enzyme consuming or forming 1 μmol substrate or 1 μmol product per *min* under standard conditions. Therefore, one unit of enzyme corresponds to the release of 1 μM of glucose equivalent per minute from the substrate.
14. Calculate the CMCase activity of the original concentrated enzyme solution in terms of IU/mL: $\text{CMCase} = 0.85/\text{EDR}$.

3.2.2 Endoglucanase Assay Using CMC and Bicinochoninic Acid (BCA)

1. Dilute enzyme solution (e.g., 1000 \times) using 50 mM citrate buffer and prepare DES.
2. Add 1.8 mL of CMC solution into the test tubes.
3. Heat to 50 $^{\circ}\text{C}$ in a water bath.
4. Add 0.2 mL of diluted enzyme solution (DES) and mix well.
5. Incubate at 50 $^{\circ}\text{C}$ for 10 min.
6. Add 2 mL of working BCA reagents and mix well.
7. Incubate at 75 $^{\circ}\text{C}$ for 30 min.
8. Place samples + enzyme blank + substrate blank into 96-well plate. Remember to place samples in triplicate.
9. Read absorbance at 560 nm.
10. Calculate the enzyme activity based on a linear relationship between reducing sugar concentrations and enzyme concentrations. Refer to Fig. 3 for an example graph.

3.2.3 *Endoglucanase Assay Using CMC by Nelson-Somogyi Method*

1. Dilute enzyme solution (e.g. 1000×) using 50 mM citrate buffer and prepare DES.
2. Add 1.8 mL of CMC solution into the test tubes.
3. Heat to 50 °C in a water bath.
4. Add 0.2 mL of diluted enzyme solution (DES) and mix well.
5. Incubate at 50 °C for 10 min.
6. Add 8 mL distilled water.
7. Pipette out aliquots of 0.1 or 0.2 mL to separate test tubes.
8. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 mL of the working standard solution into a series of test tubes.
9. Make up the volume in both sample and standard tubes to 2 mL with distilled water.
10. Pipette out 2 mL distilled water in a separate tube to set a blank.
11. Add 1 mL of alkaline copper tartrate reagent to each tube.
12. Place the tubes in boiling water for 10 min.
13. Cool the tubes and add 1 mL of arsenomolybolic acid reagent to all the tubes.
14. Make up the volume in each tube to 10 mL with water.
15. Read the absorbance of blue color at 620 nm after 10 min.
16. From the graph drawn, Calculate the amount of reducing sugars present in the sample (*see Note 10* for calculation).

3.2.4 *Endoglucanase Assay Using CMC by 2-Cyanoacetamide Method*

1. Dilute enzyme solution (e.g., 1000×) using 50 mM sodium acetate buffer pH 5.0 and prepare DES.
2. Add 1.8 mL of CMC solution into the test tubes.
3. Heat to 50 °C in a water bath.
4. Add 0.2 mL of diluted enzyme solution (DES) and mix well.
5. Incubate at 50 °C for 10 min.
6. Hundred microliters of glucose solution is added to 1 mL of 100 mM borate pH 9.0 and 200 µL of 1% 2-cyanoacetamide.
7. The solution is mixed by vortexing.
8. Heat to boil for 10 min and cool to room temperature.
9. Measure absorbance at 276 nm.
10. Calculate the enzyme activity based on a linear relationship between reducing sugar concentrations and enzyme concentrations. Refer to Fig. 3 for an example graph.

3.2.5 *S Viscometer (Water Flow Time of 15 s at 30 °C) or a Similar Device*

1. Add 1.0 mL of the prewarmed DES—keep temperature constant.
2. Determine the flow rates every 5 or 10 min.

3. Calculate specific viscosity (η_{sp}): $N_p = t - t_0/t_0$, where t is the effluent time of the buffer (s) and t_0 is the efflux time of the buffer (s).
4. Plot the increasing rate of the reciprocal of the specific viscosity against the enzyme concentration; a linear relation should be obtained.
5. Calculate unit of activity from the linear relationship between enzyme concentration/rate of increase of reciprocal of the viscosity of the CMC solution.

4 Notes

1. Aseptic technique is a set of principles and practices used for microbial culture preparation, and transferring culture to reduce the undesirable microbes. All the culture transfer work should be done under biosafety cabinet (laminar air flow) using sterilized materials, media, or reagents.
2. Autoclave (121 °C at 15 psi pressure for 20 min) the medium. Gently shake the flask to mix the agar homogeneously throughout the mixture, and pour the cooled and melted (Temp. 45–50 °C) agar medium into sterilized petri discs. Use biosafety cabinet (laminar air flow) for preparing the agar plates as well as transfer the microbial culture to prevent contamination. Leave the plates for 10–20 min under laminar air flow until solidified the medium. The plate sizes may vary with its availability and researcher's interest.
3. When producing buffer solution there are many ways to prepare it. A general approach would be: say you needed a 50 mM buffer—start with 50 mM of an appropriate base and 50 mM of appropriate acid.
4. Transfer one loop full of 20 h bacterial or 72–120 h fungal spore culture into 10 mL sterilized distilled water, mix thoroughly, diluted to 10^6 cell or spore per mL by adding sterilized water.
5. Transfer the same diameter of fungal mycelial growth from 72 to 120 h culture on Potato Dextrose or Sabouraud agar medium for each experiment.
6. Optional: In order to increase halo contrast, add 5–10 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.
7. Make wells in to agar plate using 4–6 mm in diameter cork borer. Use flame-sterilized cork borer before applying to make hole. Use flame sterilized forceps for removing medium from the wells.

8. Keep the plate at room temperature for several hours to diffuse the enzyme into gel.
9. Please note when you use this assay, if you wish to detect all endoglucanases you will need create your own antibody using a conserved epitope.
10. Calculation.
Absorbance corresponds to 0.1 mL of test = "x" mg of glucose.
10 mL contains = "x"/0.1 × 10 mg of glucose = % of reducing sugars.

References

1. Poidevin L, Feliu J, Doan A et al (2013) Insights into exo- and endoglucanase activities of family 6 glycoside hydrolases from *Podospira anserina*. *Appl Environ Microbiol* 79 (14):4220–4229
2. Wang D, Kim DH, Seo N et al (2016) A novel glycoside hydrolase family 5 β -1,3-1,6 endoglucanase from *Saccharophagus degradans* 2-40T and its transglycosylase activity. *Appl Environ Microbiol* 82(14):4340–4349
3. Zhang YH, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnol Bioeng* 88(7):797–824
4. Bhaumik P, Dhepe PL (2015) Conversion of biomass into sugars. In: Murzin D, Simakova O (eds) *Biomass sugars for non-fuel applications*. Green Chem series. Royal Society of Chemistry, London, pp 1–53
5. Gusakov AV, Kondratyeva EG, Sinitsyn AP (2011) Comparison of two methods for assaying reducing sugars in the determination of carbohydrate activities. *Int J Anal Chem* 2011:1–4
6. Zhang YH, Hong J, Ye X, Mielenz JR (2009) *Biofuels: methods and protocols*. Molecular Biology. Humana, New York, p 581
7. Hendricks CW, Doyle JD, Hugley B (1995) A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Appl Environ Microbiol* 61(5):2016–2019
8. Wang L, Yang Y, Cai B et al (2014) Coexpression and secretion of endoglucanase and phytase genes in *Lactobacillus reuteri*. *Int J Mol Sci* 15(7):12842–12860
9. Bühler R (1991) Double-antibody sandwich enzyme-linked immunosorbent assay for quantitation of endoglucanase I of *Trichoderma reesei*. *Appl Environ Microbiol* 57 (11):3317–3321
10. Ghose TK (1987) Measurement of cellulase activities. *Pure Appl Chem* 59(2):257–268
11. Marsden WL, Gray PP, Nippard GJ, Quinlan MR (1983) Evaluation of the DNS method for analysing lignocellulosic hydrolysates. *J Chem Tech Biotechnol* 32:1016–1022
12. Somogyi M (1952) Estimation of sugars by colorimetric method. *J Biol Chem* 200:245
13. Krishnaveni S, Balasubramanian T, Sadasivam S (1984) Sugar distribution in sweet stalk sorghum. *Food Chem* 15(3):229–232
14. Jurick WM, Whitaker BD, Gaskins VL, Janisiewicz WJ (2012) Application of the 2-Cyanoacetamide method for spectrophotometric assay of cellulase enzyme activity. *Plant Pathol J* 11(1):38–41
15. Zhang YH, Lynd LR (2006) A functionally-based model for hydrolysis of cellulose by fungal cellulase. *Biotechnol Bioeng* 94:888–898
16. Dave BR, Sudhir AP, Subramanian RB (2015) Purification and properties of an endoglucanase from *Thermoascus aurantiacus*. *Biotechnol Rep* 6:85–90