Chapter 14

Cellobiohydrolase (CBH) Activity Assays

Hem Kanta Sharma, Wensheng Qin, and Chunbao (Charles) Xu

Abstract

Cellulosic biomass is the most abundant biopolymer on the earth. It has great potential to quench the thirst of liquid energy by producing biofuels and thus help to mitigate human reliance on fossil fuels. Although several cellulase activity assay methods have been used to disintegrate the glycosidic bonds, the appropriate selection of substrates and synergistic involvement of multiple enzymes in hydrolytic activity is not yet fully understood. The proper quantification of hydrolytic enzymes and hydrolysates is challenging because of the heterogeneity of cellulose, changes in enzyme-substrate ratio and the presence of some inhibitory compounds like cellobiose and cellodextran. In the glycosyl hydrolase (GH) family, cellobiohydrolase (CBH) is expected to disrupt the crystalline cellulose and release the sugar molecules. Several methods have been proposed for CBH assay with slight modification in substrate and quantification of hydrolysates. However, the Avicel method is still considered as the most promising and efficient hydrolytic technique so far. The most commonly used CBH assays including Avicel and other recent methods for proper quantification are outlined in this chapter. Also a qualitative screening of CBH producing bacteria using carboxymethyl cellulose (CMC) agar plates is described.

Key words Cellobiohydrolase, Cellulose, Enzyme assay, Glucose, Avicel, ELISA

1 Introduction

Cellulose is the most abundant natural biopolymer, composed of D-glucose subunits linked by β-1,4 glycosidic bonds. The crystalline nature of cellulose and the protective covering of lignin in the lignocellulosic biomass are the main hurdles for its efficient hydrolysis [1–3]. The crystallinity of cellulose can be degraded into monomeric sugar units by synergistic action of hydrolytic enzymes collectively called as cellulase. Cellulase consists of 1,4-β-endoglucanase, 1,4-β-exoglucanase or cellobiohydrolase (CBH), and 1,4-β-glucosidase, all these enzymes belong to glycosyl hydrolase (GH) family [4]. Among the 128 GH families, the CBH can be found in GH families 5, 6, 7, 9, 48, and 74 [5]. Two major types of cellobiohydrolase are CBHI and CBHII, which effectively degrade the crystalline cellulose, presumably by peeling the microcrystalline structure of cellulose chain, whereas endoglucanase

Mette Lübeck (ed.), *Cellulases: Methods and Protocols*, Methods in Molecular Biology, vol. 1796,
https://doi.org/10.1007/978-1-4939-7877-9_14,

typically acts on more soluble amorphous region of cellulose, showing high degree of synergism and thus releasing the sugar molecules [5, 6]. There has been increasing interest in the hydrolysis of cellulose for sustainable and renewable biofuels production, a promising alternative to fossil fuels. Various bacteria and fungi are known to secrete endo or exo-acting cellulases that act on cellulose, resulting in the release of glucose and cellobiose. There have been extensive studies into the cellulolytic system of *Trichoderma reesei*, which is composed of two cellobiohydrolases (CBHI and CBHII) [7, 8]. Miettinen-Oinonen and Suominen [7] quantified the CBHI and CBHII in *T. reesei* by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA). However, Brook et al. [9] isolated three cellobiohydrolases named CBHI-A, CBHI-B, and CBHII from crude extracts of *Talaromyces emersonii* liquid cultures. Recently, four cellobiohydrolase I enzymes named as CBHI-A, CBHI-B, CBHI-C, and CBHI-D have been purified from the growth of *Penicillium decumbens* JU-A10. The enzyme activity was tested against *p*-nitrophenyl-β-D-cellobioside (*p*NPC) [10]. The cellodextrin and cellobiose have their inhibitory activities during cellulose hydrolysis, thus β-glucosidase is essential to break the final glycosidic bonds of cellobiose so as to produce sufficient glucose molecules and reduce or eliminate cellobiose inhibition [11, 12]. The CBH assay is more difficult than endoglucanase and β-glucosidase assays due to lack of proper substrates and hindrances from cellulase components [13]. Although there is no such single standard assay method for CBH activity, the Avicel method [12, 13] has been repeatedly used. Some other substrates and quantitative methodologies are also described in this chapter along with a qualitative screening method.

1.1 Qualitative Assay

Qualitative screening of CBH producing bacteria can be carried out by comparing the relative diameter of cellulase hydrolytic activity using carboxymethyl cellulose (CMC) agar plates.

1.2 Quantitative Assays

1.2.1 CBH Assay Using Avicel as a Substrate

Avicel is a microcrystalline substrate and in comparison, to carboxymethyl cellulose (CMC), it is easily hydrolyzed by CBH showing high enzyme activity. Thus, Avicel has been routinely used as a suitable substrate in CBH assays. Most of the CBHs release cellobiose and a small amount of glucose from the cellulose. The reducing sugars produced after hydrolysis can be estimated by dinitrosalicylic acid (DNS) and Nelson Somogyi method whereas the total sugar is estimated by phenol-sulfuric acid method [14]. Centrifugation (14,000 × *g* for 3 min) of host culture is a simple step to collect the crude enzyme as a supernatant. The appropriate dilution of enzyme may be required.

1.2.2 CBH Assay Using Regenerated Amorphous Cellulose as a Substrate

Native cellulose has crystalline structure, comparatively more stable than regenerated cellulose due to network of intramolecular and intermolecular hydrogen bonds. Regenerated amorphous cellulose (RAC) is made from the chemical modification of microcrystalline cellulose or Avicel. Several solvents such as SO_2-amine solvent system using SO_2, diethylamine, and dimethylsulfoxide [15, 16] and acidic treatment using sulfuric acid [17], phosphoric acid [18–21] are being used for preparation of RAC. The treatment on cellulose crystals is used to disrupts the hydrogen bonds and thus provide larger (about 20-fold) surface area for enzymatic hydrolysis [20, 21].

1.2.3 CBH Assay Using p-Hydroxybenzoic Acid Hydrazide Method

The cellobiohydrolase assay on different polysaccharides were measured by Takahashi et al. [22], following the *p*-hydroxybenzoic acid hydrazide (PAHBAH) method discovered by Lever, M [23]. The activity of *Magnaporthe oryzae* GH-6 family cellobiohydrolase (MoCel6A) catalyzed the hydrolysis of amorphous and water-soluble polysaccharides. Since the acid hydrazides react with reducing sugars in alkaline solution, the PAHBAH can be used to detect less than 1 μg of sugars [22, 23]. There are different choices for polysaccharide selection. It can be cellulose, Avicel, CMC, hydroxyethyl cellulose, or other polysaccharides. However, very high concentration of protein and calcium are the impeding factors [23], and a strong agitation is also required if water-insoluble polysaccharides are selected as substrates [22].

1.2.4 CBH Assay Using Lytic Polysaccharide Monooxygenase

Lytic polysaccharide monooxygenase (LPMO) is a relatively newly developed method for depolymerization of recalcitrant polysaccharide chains in their crystalline regions based on the principle of oxidative disintegration so as to release oxidized oligosaccharides [24, 25]. The LPMO was initially discovered for its activity on chitin degradation [2, 25] however it also degrades cellulose [2]. The LPMO belongs to the auxiliary activities (AA) enzyme class. It is a copper-dependent monooxygenase [26, 27] and works slowly in association with hydrolytic enzymes. LPMO can strongly boost up the saccharification process and enhance the soluble sugar yield from lignocellulosic biomass [28].

1.2.5 CBH Assay Using Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) technique is aimed at detecting and quantifying substances such as antigen, antibodies, peptides, proteins, and hormones. The quantification of CBHI in crude cellulase enzyme can be done by using double-antibody sandwich enzyme-linked immunosorbent assay. At least two antibodies (either monoclonal or polyclonal antibodies) are required to act in the sandwich. The polyclonal antibodies (PAb) have been used in characterization of CBHI and CBHII. This is a highly specific and direct procedure, used to quantify the CBHI at its range of 0.1–0.8 μg/mL [29].

2 Materials

Use analytical grade chemicals, sterilized/autoclaved instruments and protective wearing (gloves/protective clothing/eye protection/face protection) throughout all the experiments. Use laminar air flow/biosafety cabinet during any microbial handling and transfer with high level of caution to prevent possible contamination. Prepare all reagents in autoclaved distilled water and store at room temperature unless otherwise indicated. Autoclave (at 121 °C for 30 min) all the contaminated growth medium and other used materials such as petri discs, centrifuge tubes, pipette tips, glass wares, gloves etc. before its effective disposal following the regulation.

2.1 Qualitative Assay

1. Gram's iodine solution (300 mL): Take 300 mL distilled water into a 500-mL glass bottle. Add 2 g potassium iodide and 1 g iodine (*see* **Note 1**) into it by continuous steering with magnetic bar.
2. LB broth (100 mL): Take 100 mL distilled water into a 250-mL conical flask. Add 1 g peptone, 0.5 g yeast extract, and 0.5 g NaCl into the flask with continuous steering (*see* **Note 2**).
3. CMC Agar plate (100 mL): Take 100 mL distilled water into a 250-mL conical flask. Add 0.5 g CMC, 0.1 g $NaNO_3$, 0.1 g K_2HPO_4, 0.1 g KCl, 0.05 g $MgSO_4$, 0.05 g yeast extract, and 1.5 g agar into the flask with continuous steering. Autoclave the mixture before use to make agar plate (*see* **Note 3**).

2.2 Quantitative Assays

2.2.1 CBH Assay Using Avicel as a Substrate

1. Avicel suspension in sodium acetate buffer (pH 4.8): Prepare 0.1 M sodium acetate buffer pH 4.8 (*see* **Note 4**). Add 1.25% (w/v) Avicel and mixed well to make the Avicel suspension in sodium acetate buffer.
2. Enzyme dilution: Dilute a series of enzyme solutions (one of which release less than and another release more than 0.5 mg of glucose) by using 0.1 M sodium acetate buffer (optional) or collect the crude enzyme from supernatant (*see* **Note 5**) of bacterial culture.
3. Reaction mixture (2 mL): Prepare the reaction mixture by adding 1.6 mL of Avicel suspension in 0.1 M sodium acetate buffer (pH 4.8) and 0.4 mL of diluted enzyme or crude enzyme samples in a 5 mL glass test tube.
4. Other reagents: Avicel, 5% phenol solution (*see* **Note 6**), ~98% sulfuric acid (*see* **Note 7**), 0.1 M sodium acetate buffer (pH 4.8).

2.2.2 CBH Assay Using RAC as a Substrate

1. Reaction mixture (1 mL): Prepare a reaction mixture containing 0.5 mL of 1% (w/v) RAC (*see* preparation of RAC in Subheading 3.2.2), 0.05 mL of 1 M citrate buffer pH 4.5 (*see* **Note 8**), 0.25 mL of distilled water, 0.2 mL of diluted enzyme solution or crude enzyme from host culture.
2. Enzyme dilution (optional): Dilute a series of enzyme solutions by using 50 mM acetate buffer.
3. Other reagents: Regenerated amorphous cellulose (RAC) 1% (w/v), phenol solution (5%), sulfuric acid (~98%), phosphoric acid solution (86%), 2 M sodium carbonate, 0.2% (w/v) sodium azide solution (*see* **Note 9**).

2.2.3 CBH Assay Using PAHBAH

1. Reaction mixture (100 μL) composition: Prepare a reaction mixture of 100 μL final volume by adding 100 mM sodium phosphate pH 6.0, 0.1 μg of CBH, and 0.5–5 mg of polysaccharide (*see* **Note 10**) in distilled water.
2. 1% w/v of *p*-hydroxybenzoic acid hydrazide (PAHBAH).

2.2.4 CBH Assay Using LPMO

1. Cellulosic substrate: Prepare the substrate containing 0.1% (w/v) cellulose in 50 mM sodium phosphate buffer pH 6.0 (*see* **Note 11**).
2. Other reagents: Purified LPMO (9 μg LPMO/mg of cellulosic substrate), 0.1% (w/v) Avicel or microcrystalline cellulose, 0.1% (w/v) amorphous cellulose (or, follow the RAC preparation Subheading 3.2.2), 7.5 μM ascorbic acid, hydrolytic enzymes (see below).
3. Hydrolytic enzymes (μg of protein/mg cellulosic substrate):
   (a) Complete cellulase: 25 μg cellulase/mg of cellulosic substrate.
   (b) CBHI: 100 μg CBHI/mg of cellulosic substrate.
   (c) CBHII: 100 μg CBHII/mg of cellulosic substrate.
   (d) Endoglucanase: 100 μg endoglucanase/mg of cellulosic substrate.
   (e) β-glucosidase: 5 μg β-glucosidase/mg of cellulosic substrate.
   (f) The crude enzymes extracted from host culture can also be used.

2.2.5 CBH Assay Using ELISA

1. Immunoglobulin (IgG): Rabbit anti-mouse IgG or goat anti-rabbit IgG.
2. Other reagents: 1 μg/mL polyclonal antibodies (PAb), 5–10 μg/mL monoclonal antibodies (MAb), 50 mM carbonate buffer pH 9.5 (*see* **Note 12**), 0.1 M phosphate-buffered saline (PBS) pH 7.4 (*see* **Note 13**), CBHI, 1× PBS containing

0.1% Tween 20 (1× PBS Tween 20) (*see* **Note 13**), 2% bovine serum albumin, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS).

3 Methods

3.1 Qualitative Assay of CBH

The bacterial strain grown overnight in 1.5 mL Luria–Bertani liquid nutrient medium (LB broth) at 30 °C could be a better choice to inoculate in CMC agar plates, however the slower growing strains may require 2–3 days of incubation in plate along with positive (*Cellulomonas xylanilyticus*) and negative controls (*E. coli*) to measure and compare the clear zone or halo size, representing the cellulase activity [30, 31]. The qualitative assay can be done with following procedures.

1. Transfer 1.5 mL of LB broth (culture medium) into a 5 mL test tube.
2. Inoculate a pure single strain bacterial colony into a test tube with LB broth (*see* **Note 14**).
3. Keep the tube in shaking incubator at 30 °C and 200 rpm for 24 h (*see* **Note 15**).
4. After incubation, inoculate 5 μL of bacterial culture on center of the CMC agar plate and incubate at 30 °C for 48 h.
5. Gently pour the Gram's iodine solution on the CMC agar plates until the plates are covered. Observe the halo or zone size of clearance after 1 min.
6. Measure the diameter of halo regions to judge the hydrolytic ability. The larger the size of the halo the higher the hydrolytic activity of bacterial strains.

3.2 Quantitative Assay of CBH

Following are some of the widely used quantitative methods for CBH assay.

1. CBH assay using Avicel as substrate [12–14, 22].
2. CBH assay using amorphous cellulose as substrate [13].
3. CBH assay using *p*-hydroxybenzoic acid hydrazide method [22].
4. CBH assay using lytic polysaccharide monooxygenase (LPMO) for oxidative degradation of cellulosic substrate [2, 24].
5. CBH assay using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) [29].

3.2.1 CBH Assay Using Avicel as a Substrate

1. Transfer 2 mL of reaction mixture (*see* Subheading 2.2.1) into the microcentrifuge tubes.
2. Incubate the reaction mixture at 50 °C for 2 h.

3. Quench the reaction by placing the tubes in ice-cold water for 10 min.
4. Centrifuge the mixture at 13,000 × *g* for 3 min and collect the supernatant.
5. Analyze the supernatant by DNS method (see below) or Nelson Somogyi method for reducing sugars [14, 32]; and phenol–sulfuric acid method for total sugar [13] (make sure to have triplicates of each sample).
6. Prepare the blanks:
   (a) Enzyme blank: 0.4 mL of diluted enzymes or crude enzyme + 1.6 mL of 0.1 M acetate buffer.
   (b) Substrate blank: 0.4 mL of 0.1 M acetate buffer + 1.6 mL of 1.25% (w/v) Avicel suspension buffer.

Procedure for DNS method: The following procedures for DNS method consist of larger volume of sample solution and other chemicals. However, it can be scaled down by using minimal volume (*see* **Note 16**).

1. Take 1 mL of supernatant in a 25 mL test tube.
2. Add 3 mL of DNS solution (*see* **Note 17**) into the tube and mix well by gently vortex for 3–5 s.
3. Put the tube in boiling water bath for 5 min.
4. Remove the tube from hot water bath and cool down the tube by keeping it in ice-cold water for 10 min.
5. Add 20 mL of distilled water into the tube and mix it properly.
6. Measure the absorbance at 540 nm in spectrophotometer with three replicates for each sample.
7. Subtract the absorbance values of enzyme blank from substrate blank and analyze the enzyme activity.
8. The standard calibration graph for known amount of cellobiose is used to determine reducing sugars, whereas the graph of glucose is used to estimate the total sugars.
9. The linear relationship between sugar released and enzyme dilution helps to estimate the enzyme activity. One unit of CBH corresponds to the release of 1 μM of glucose equivalent per minute from Avicel.

3.2.2 CBH Assay Using RAC as a Substrate

Preparation of RAC [13, 20].

1. Add 0.2 g of microcrystalline cellulose or Avicel and 0.6 mL distilled water into a regular 50 mL centrifuge tube (cellulose slurry will form).
2. Gently pour 10 mL of ice-cold (86%) phosphoric acid into the tube with continuous stirring so that the cellulose suspension solution will thoroughly mixed and turns transparent.

3. Add another 40 mL of ice-cold water (10 mL per addition for four times) with vigorous stirring produce a white cloudy precipitate.
4. Centrifuge the precipitated cellulose at 5000 × *g* for 1 min and keep it in water bath at 4 °C for 20 min.
5. Discard the supernatant and keep the pellet for further wash with ice-cold water followed by centrifugation.
6. Suspend the cellulose pellet by adding 45 mL of ice-cold water in the tube and centrifuge at 5000 × *g* for 1 min to remove the supernatant containing phosphoric acid (repeat this step four times).
7. Add 0.5 mL of 2 M Na_2CO_3 to neutralize the residual phosphoric acid.
8. Suspend the cellulose pellet in 45 mL of ice-cold distilled water and centrifuge at 5000 × *g* for 1 min (repeat this step two times).
9. Collect the cellulose pellet and make 1% (w/v) of RAC suspension ready to use and store (*see* **Note 18**).

Assay procedures

1. Take 1 mL of reaction mixture (*see* Subheading 2.2.2) in 1 mL centrifuge tubes.
2. Incubate the reaction mixture at 50 °C for 30 min.
3. Quench the reaction by submerging the tubes in ice-cooled water bath.
4. Centrifuge the mixture at 10,000 × *g* for 3 min and the collect the supernatant.
5. Prepare the blanks:
   (a) Enzyme blank: 0.2 mL of diluted enzymes or crude enzyme + 0.05 mL of 1 M citrate buffer + 0.75 mL of distilled water.
   (b) Substrate blank: 0.5 mL of 1 M citrate buffer + 0.5 mL of 1% (w/v) RAC + 0.45 mL of distilled water.
6. Analyze the total soluble sugars in the supernatants by the phenol–sulfuric acid method (*see* below) at 490 nm absorbance.
7. Subtract the absorbance values of enzyme blank from substrate blank and analyze the enzyme activity.

Procedure for phenol–sulfuric acid method

1. Transfer 0.7 mL of supernatant into a 5 mL glass tubes.
2. Add 0.7 mL of 5% phenol solution and 3.5 mL of sulfuric acid with continuous mixing (*see* **Note 19**).

3. Allow 20–30 min time to cool down the exothermic reaction to room temperature and measure the absorbance at 490 nm.
4. Use the standard calibration graph of known amount of glucose to determine the total sugars.
5. A linear range gives 0.005–0.1 g/L sugars in the samples. It helps to analyze the enzyme activity which is one unit of exoglucanase or CBH corresponds to the release of 1 μmol of glucose equivalent per minute from Avicel.

3.2.3 CBH Assay Using PAHBAH Method

1. Transfer 100 μL of reaction mixture (*see* Subheading 2.2.3) into 1 mL centrifuge tubes.
2. Incubate the tubes at 30 °C for up to 18 h.
3. Centrifuge the tubes at 22,000 × *g* for 5 min.
4. Take 50 μL of supernatants into microcentrifuge tubes and mix with 150 μL of PAHBAH solution (1% w/v).
5. Keep the microcentrifuge tubes in a boiling water bath for 5 min.
6. Remove the tubes from water bath and wait till cooled down to room temperature.
7. Measure the absorbance at 410 nm in spectrophotometer.
8. Use standard calibration graph of glucose to estimate the reducing sugars.
9. The linear relationship between sugar released and enzyme dilution help to estimate the enzyme activity.
10. The liquid chromatography-mass spectrometry (LC-MS) or high pressure liquid chromatography-mass spectrometry (HPLC-MS) of the hydrolysates is useful in product analysis.

3.2.4 CBH Assay Using LPMO

Method I (involving pretreatment of substrate)

1. For the pretreatment in the initial step, transfer 450 μL of cellulosic substrate into Eppendorf tubes (make triplicate of each sample).
2. Add LPMO (9 μg LPMO/mg of cellulosic substrate) into the tubes.
3. Cover each tube with oxygen-permeable parafilm and incubate at 25 °C for 5 h.
4. After 5 h of pretreatment, add 50 μL of one of the hydrolytic enzyme (from the list of hydrolytic enzymes, Subheading 2.2.4). Again, incubate the tube at 50 °C for 12 h.
5. Remove one of reaction tube (for sampling) from incubation in every 15 min for up to 1 h (total four tubes).

6. Stop the enzyme action by keeping the tubes at 95 °C for 10 min.
7. Lower the temperature to 4 °C by keeping in cold (4 °C) water
8. Centrifuge the tubes at 9000 × *g* for 1 min.
9. Collect the supernatant for final product analysis using high performance anion exchange chromatography (HPAEC).
10. For control (*see* **Note 20**).

Method II (without pretreatment of substrate)

1. Transfer 500 μL of cellulosic substrate plus LPMO, CBHI and β-Glucosidase into the Eppendorf tubes (*see* **Note 21**)
2. Cover the tubes with oxygen-permeable parafilm.
3. Incubate the tubes at 50 °C for up to 96 h.
4. Remove one reaction tube (for sampling) from incubation in 5 h and another tube in 96 h for product analysis.
5. Stop the enzyme reaction by keeping tubes at 95 °C for 10 min.
6. Lower the temperature to 4 °C by keeping the tubes in 4 °C cold water.
7. Centrifuge the tubes at 9000 × *g* for 1 min and collect the supernatant.
8. Use the supernatant for final product analysis by using HPAEC.
9. For control (*see* **Note 20**).

3.2.5 CBH Assay Using ELISA

1. Pour 100 μL of PAb (1 μg/mL) in 50 mM carbonate buffer (pH 9.5) or purified MAb (5–10 μg/mL) in 1× PBS for coating into the ELISA plates wells.
2. Incubate the plates at 4 °C for 12 h.
3. Wash the wells with 1× PBS Tween-20.
4. Block the nonspecific spots with 2% bovine serum albumin at 37 °C for 2 h.
5. Add 100 μL of CBHI or diluted crude cellulase (from host culture) into the wells.
6. Incubate the plates at 37 °C for 1 h.
7. Wash the wells with 1× PBS Tween 20 and then add the second antibody. It may be MAb or PAb (*see* **Note 22**).
8. Incubate the wells at 37 °C for 30 min to 1 h (*see* **Note 23**).
9. If unlabeled antibody is used in second time, then a peroxidase-labeled third antibody is used in detection. It may include either rabbit anti-mouse or goat anti-rabbit immunoglobulin (IgG) and incubate at 37 °C for 30 min.

10. Wash the plate with 1× PBS Tween-20 and add 100 μL of ABTS substrate into the wells.
11. Keep the plates in room temperature for 30 min to observe the color.
12. Measure the absorbance values in microplate reader at 410 nm.
13. Analyze the absorbance values by plotting standard curve against the concentration of CBHI standards.

3.3 Optimization of Cellobiohydrolase Assay

Higher yield of cellobiohydrolase is feasible only after its optimization. Generally, optimization is to analyze the hydrolytic activities of enzyme at various temperatures, pH, agitation speed, incubation time, the source of carbon/polysaccharides, source of nitrogen, and other inorganic salts' concentration. Replication of reaction mixtures for various substrates is required for the optimization. It may vary with different methods, however generally the following "one at a time" factors are taken into consideration.

1. The optimization of temperature for hydrolysis can be analyzed by incubating the reaction mixture at different temperatures (20–60 °C).
2. The optimization of incubation time for hydrolysis can be analyzed by varying the time of incubation in hot water bath. It may range from 1 to 25 h.
3. The optimization of pH on the hydrolytic activity of CBH can be measured by equilibrating the reaction mixture at different pH (ranges from pH 3.5 to 11.0), for example, with sodium acetate (pH 3.5–5.5), sodium phosphate (pH 5.5–7.5), Tris–HCl (pH 7.5–9.0), or CAPS (N-cyclohexyl-3-aminopropanesulfonic acid; pH 9.0–11.0) [22].
4. Determination of the optimum agitation speed for hydrolysis can be measured by incubating in shaking incubator at 120–220 rpm.
5. The optimum CBH production can be analysed by using various sources of carbon (such as starch, CMC, Avicel, RAC, xylose, and glucose), nitrogen (such as yeast extract, peptone, urea, and ammonium sulfate) and metal ions (such as Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+}).

3.4 Concluding Remarks

There are several methods developed for cellulase assays. The CBH plays a significant role in production of cellobiose and some other sugars from crystalline cellulose. However, the specific substrate and suitable CBH enzyme assay protocols need to be further developed. Recent CBH assays are slightly varied on the types of substrate used and detection methods for hydrolyzed products. Some of the direct methods like high pressure liquid chromatography (HPLC) and cellobiose oxidoreductase assay are complicated due

to production of cellobiose during CBH activities [29]. The ELISA is gaining its popularity in better quantification of CBH. However, Avicel and RAC are widely used for CBH enzyme assay because these can act as a substrate for exoglucanases and endoglucanases due to some amorphous cellulose and soluble cellodextrans [14, 33].

4 Notes

1. Iodine takes a long time to dissolve in distilled water. So, continuous 1–2 h of stirring may require. Keep in mind that iodine also has volatile tendency and should be kept in air tight bottle and better to cover with aluminum foil.
2. Autoclave (121 °C for 30 min) the LB broth before use and it can be stored at 4 °C for few days. LB broth is a good nutrient medium for bacterial growth. So, precaution should be made while transferring the bacterial strain to avoid the contamination. Use biosafety cabinet during all bacterial inoculation.
3. Autoclave (121 °C for 30 min) the agar mixture. Gently shake the flask and pour the agar mixture into the sterilized/disposable plastic petri discs. Use biosafety cabinet to make the agar plates. Leave the plate for 20 min in the cabinet to become solidified. The agar will be solidified at room temperature. The plate sizes can vary with its availability and researcher's interest.
4. Take 0.82 g of sodium acetate in a beaker and add distilled water till up to 100 mL. Adjust the desired pH of sodium acetate buffer by using 5 N NaOH and 1 N HCl.
5. Take 1 mL of overnight cultured broth medium in a 1.5 mL microcentrifuge tube. Centrifuge the tube at 12,000 × *g* for 3 min and collect the crude enzyme from supernatant.
6. Phenol is a strongly corrosive and combustible solid. It can severely affect the skin, eyes, and mucosal membrane. Use personal protective wear/equipment while handling. Since it is also toxic to plants and animals, do not release into environment and always follow the local, regional, and or national laws and regulations for disposal. Store in a cool, dry, and well-ventilated room.
7. The 98% sulfuric acid is highly corrosive in nature. It can cause serious damage to the skin upon contact by chemical burning and dehydration, can lead to blindness if splashed onto eyes. Use of protective wearing is highly recommended.
8. Dissolve 19.21 g of citric acid in a final volume of 100 mL distilled water. Adjust the pH 4.5 by dissolving solid NaOH.

9. Sodium azide is soluble in water, and very toxic (comparable to alkali cyanides) and has a severe poisonous effect. Even an exposure to small quantities through skin contact or swallowing may be fatal. The lethal dose for an adult human is about 0.7 g.
10. The amount of polysaccharide depends upon its types, such as 5 mg for Avicel, 5 mg for cellulose, or 0.5 mg for other polysaccharides.
11. Prepare the 50 mM sodium phosphate buffer pH 6.0 by dissolving 0.599 g of monosodium phosphate to the final volume of 100 mL distilled water. Adjust the pH by using NaOH and phosphoric acid.
12. 50 mM carbonate buffer pH 9.5 by dissolving 1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ to a final volume of 1000 mL deionized water. Adjust the pH 9.5.
13. Prepare 0.1 M phosphate-buffered saline (PBS) pH 7.4 by using 10.9 g anhydrous Na_2HPO_4, 3.2 g anhydrous NaH_2PO_4, and 90 g NaCl in a final volume of 1000 mL deionized water. Make sure to adjust the pH 7.4. Add 1 mL of Tween 20 to make 1× PBS Tween-20.
14. A separate culture of positive control (*Cellulomonas xylanilyticus*) and negative control (*E. coli*) is highly desirable for comparative qualitative assay with other bacterial strains.
15. The hours of incubation vary according to bacterial growth rate. The slower growing bacterial strain may require an additional 24–48 h of incubation to get comparatively equivalent biomass growth as that of faster growing strains.
16. The DNS method can be scale down with minimal volume of enzymes and reagents. Say for example 50 or 100 μL of diluted enzyme or crude enzyme from supernatant and 100 or 200 μL of DNS can use in 1 mL microcentrifuge tube for incubation. However, make sure to use equivalent proportion of volume and concentration of reagents during experiment and estimation/calculation of enzyme activity.
17. Preparation of DNS reagent (500 mL): Take a 250 mL conical flask. Put 3.15 g of DNS and 10.48 g of sodium hydroxide into the flask containing 250 mL of distilled water and make a homogeneous mixture with the help of magnetic steering. Take another 250 mL conical flask. Put 91 g sodium-potassium tartrate, 2.5 g phenol and 2.5 g sodium-metabisulfite into the flask containing 250 mL of distilled water and make a homogeneous mixture with the help of magnetic steering. Gently mix the reagent solution (from first and second flask) into a separate 500 mL glass bottle, wrap it with aluminum foil and store at 4 °C.

18. RAC suspension can be stored for about 1 year at 4 °C when mixed with 0.2% (w/v) sodium azide solution (*see* **Note 8**).
19. The phenol and sulfuric acid reaction is highly exothermic. So be cautious and use all protective wearing while handling.
20. The negative control does not contain LPMO or contains LPMO lacking ascorbic acid.
21. Make triplicate of sample and put all three enzymes in each tube.
22. Use of antibodies MAb or PAb depends on which antibody was used as the coating antibody. If PAb is used first as coating antibody then MAb would serve as the second antibody and vice versa.
23. Incubation time depends on the types of second antibody. Incubation time for labeled antibody (horseradish peroxidase) is 1 h, whereas if unlabeled antibody is used the incubation time is 30 min.

References

1. Chen H, Zhao X, Liu D (2016) Relative significance of the negative impacts of hemicelluloses on enzymatic cellulose hydrolysis is dependent on lignin content: evidence from substrate structural features and protein adsorption. ACS Sustain Chem Eng 4(12):6668–6679
2. Horn S, Vaaje-Kolstad G, Westereng B, Eijsink VG (2012) Novel enzymes for the degradation of cellulose. Biotechnol Biofuels 5(1):45
3. Zhang Y, Ding SY, Mielenz JR et al (2007) Fractionating recalcitrant lignocellulose at modest reaction conditions. Biotechnol Bioeng 97(2):214–223
4. Henrissat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol 7 (5):637–644
5. Annamalai N, Rajeswari MV, Sivakumar N (2016) Cellobiohydrolases: role, mechanism, and recent developments. In: Vijai Kumar G (ed) Microbial enzymes in bioconversions of biomass. Springer, New York, pp 29–35
6. Divne C, Ståhlberg J, Teeri TT, Jones TA (1998) High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. J Mol Biol 275 (2):309–325
7. Miettinen-Oinonen A, Suominen P (2002) Enhanced production of *Trichoderma reesei* endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. Appl Environ Microbiol 68(8):3956–3964
8. Geng A (2014) Genetic transformation and engineering of *Trichoderma reesei* for enhanced enzyme production. In: Geng A (ed) Biotechnology and biology of Trichoderma. Elsevier, Amsterdam, pp 193–200
9. Brooks MM, Tuohy MG, Savage AV et al (1992) The stereochemical course of reactions catalysed by the cellobiohydrolases produced by *Talaromyces emersonii*. Biochem J 283:31–34
10. Gao L, Gao F, Wang L et al (2012) N-glycoform diversity of cellobiohydrolase I from *Penicillium decumbens* and synergism of nonhydrolytic glycoform in cellulose degradation. J Biol Chem 287(19):15906–15915
11. Maki M, Leung K, Qin W (2009) The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int J Biol Sci 5(5):500–516
12. Dashtban M, Maki M, Leung KT et al (2010) Cellulase activities in biomass conversion: measurement methods and comparison. Crit Rev Biotechnol 30(4):302–309
13. Zhang YHP, Hong J, Ye X (2009) Cellulase assays. In: Mielenz JR (ed) Biofuels: methods and protocols. Springer, New York, pp 213–231

14. Wood TM, Bhat KM (1988) Methods for measuring cellulase activities. Methods Enzymol 160:87–112
15. Ciolacu D, Ciolacu F, Popa VI (2011) Amorphous cellulose – structure and characterization. Cellul Chem Technol 45(12):13–21
16. Isogai A, Atalla R (1991) Amorphous celluloses stable in aqueous media: regeneration from SO_2-amine solvent systems. J Polym Sci Part A Polym Chem 29(1):113–119
17. Ioelovich M (2012) Study of cellulose interaction with concentrated solutions of sulfuric acid. ISRN Chem Eng 2012:428974
18. Zhang Y, Cui J, Lynd L, Kuang L (2006) A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: evidence from enzymatic hydrolysis and supramolecular structure. Biomacromolecules 7(2):644–648
19. Hao X, Shen W, Chen Z et al (2015) Self-assembled nanostructured cellulose prepared by a dissolution and regeneration process using phosphoric acid as a solvent. Carbohydr Polym 123:297–304
20. Hong J, Wang Y, Ye X, Zhang YHP (2008) Simple protein purification through affinity adsorption on regenerated amorphous cellulose followed by intein self-cleavage. J Chromatogr A 1194(2):150–154
21. Jia X, Chen Y, Shi C et al (2013) Preparation and characterization of cellulose regenerated from phosphoric acid. J Agric Food Chem 61 (50):12405–12414
22. Takahashi M, Takahashi H, Nakano Y et al (2010) Characterization of a cellobiohydrolase (MoCel6A) produced by *Magnaporthe oryzae*. Appl Environ Microbiol 76(19):6583–6590
23. Lever M (1972) A new reaction for colorimetric determination of carbohydrates. Anal Biochem 47(1):273–279
24. Eibinger M, Ganner T, Bubner P et al (2014) Cellulose surface degradation by a lytic polysaccharide monooxygenase and its effect on cellulase hydrolytic efficiency. J Biol Chem 289(52):35929–35938
25. Vaaje-Kolstad G, Westereng B, Horn SJ et al (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 80(330):6001
26. Hemsworth GR, Taylor EJ, Kim RQ et al (2013) The copper active site of CBM33 polysaccharide oxygenases. J Am Chem Soc 135 (16):6069–6077
27. Aachmann FL, Sørlie M, Skjåk-Bræk G et al (2012) NMR structure of a lytic polysaccharide monooxygenase provides insight into copper binding, protein dynamics, and substrate interactions. Proc Natl Acad Sci U S A 109 (46):18779–18784
28. Patel I, Kracher D, Su M et al (2016) Salt-responsive lytic polysaccharide monooxygenases from the mangrove fungus *Pestalotiopsis* sp. NCI6. Biotechnol Biofuels 9(1):108
29. Riske FJ, Eveleigh DE, Macmillan JD (1990) Double-antibody sandwich enzyme-linked immunosorbent assay for cellobiohydrolase I. Appl Environ Microbiol 56(11):3261–3265
30. Paudel YP, Qin W (2015) Characterization of novel cellulase-producing bacteria isolated from rotting wood samples. Appl Biochem Biotechnol 177(5):1186–1198
31. Maki M, Broere M, Leung K, Qin W (2011) Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers. Int J Biochem Mol Biol 2(2):146–154
32. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428
33. Ozioko PC, Ikeyi Adachukwu IP, Ugwu OPC (2013) Review article cellulases their substrates activity and assay methods. Experiment 12 (2):778–785