Chapter 18

Purification and Characterization of the Total Cellulase Activities (TCA) of Cellulolytic Microorganisms

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Abstract

Cellulose is the earth's most abundant plant polysaccharide containing a large array of glucose units linked through β (1 → 4) linkages by existing in both crystalline and amorphous forms. Cellulose is widely distributed in plants, constituting up to 40–50% overall dry weight of the plant biomass. Majorly, microorganisms secrete three types of enzymes such as endoglucanases, exoglucanases, and beta-glucosidase for the hydrolysis of cellulose, contributing to the total cellulase activity. Industrially, the cellulolytic microorganisms are assessed based on their total cellulolytic activities. Similarly, total cellulase activity can also be used for the isolation and characterization of the cellulolytic microorganisms. In this chapter, we have specifically discussed about the methods used for the purification and characterization of the total cellulase activities of the microorganisms such as filter paper assay and cellulase zymogram assay. Our present chapter can be used as primer for characterizing cellulolytic abilities of cellulose-degrading microorganisms.

Key words Cellulose, Total cellulolytic ability, Endoglucanase, Exoglucanases and β-glucosidases

1 Introduction

Over the past few decades, studies on the production of plant biomass-based biofuels and biorefineries have significantly increased. Especially much attention was given to cellulose-based production of ethanol. Microbial degradation of cellulose (and other plant biomass units) is considered an environmentally friendly, cost-effective, and potentially efficient method. Majorly cellulose degrading enzymes secreted by microorganisms can be classified as endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4) which are involved in random fragmentation of amorphous cellulose units resulting in oligosaccharides units of varying lengths. Exoglucanases which includes cellodextrinases or 1,4-β-D-glucan glucanohydrolases (EC 3.2.1.74) and cellobiohydrolases or 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91) these set of enzymes act on reducing and nonreducing ends of cellulose or microcrystalline cellulose and β-glucosidases or β-glucoside

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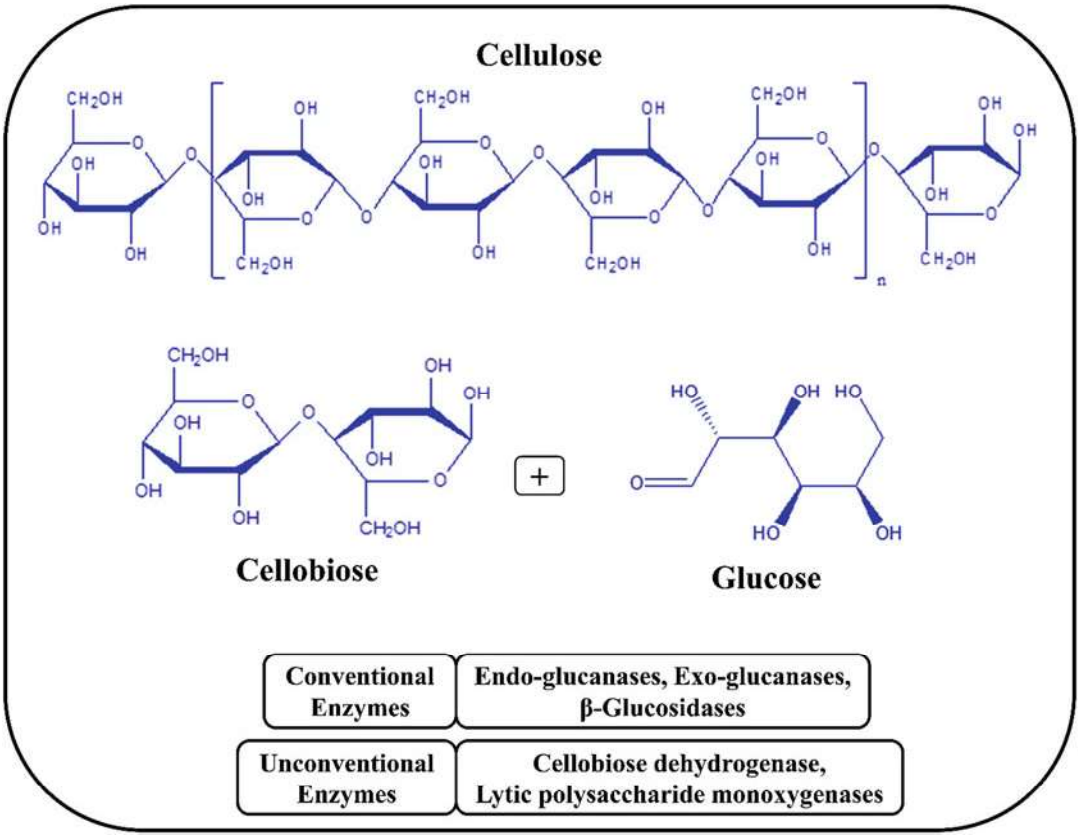

Fig. 1 Schematic representation of enzymatic breakdown of cellulose by the microbial enzyme systems

glucohydrolases (EC 3.2.1.21) are required for solubilizing cellodextrins and cellobiose to simple glucose residues [1]. Several extensive and informative reviews are already available on microbial degradation of cellulose units [1–7] (Fig. 1).

Thus, it is important to understand different methods and techniques involved in the isolation and characterization of cellulose degrading microorganisms, various studies have already developed various easy and significant methods for microbe isolation. In this chapter, we have clearly explained about various quantitative and qualitative methods used for the measurement of cellulases secreted by the microorganisms.

2 Materials

2.1 Filter Paper Assay

1. DNS Reagent: 3,5-dinitro salicylic acid, sodium hydroxide (19.3 N), deionized water, sodium potassium tartrate $3H_2O$, double distilled H_2O.
2. Citrate buffer: citric acid monohydrate 210 g, deionized water, NaOH.

3. Filter paper: cut approximately 1.0 × 6.0 cm of Whatman No.1 filter paper, weight must be 50 mg (*see* **Note 1**).
4. Glucose standard: glucose 0.4 g dissolved in 200 ml of ddH_2O, citrate buffer (pH 4.8) solutions.

2.2 Purification of Cellulase

1. Fresh and desiccated ammonium sulfate $[(NH_4)_2SO_4]$.
2. Magnetic stirrer, 1 L graduated cylinder, hot air oven, and centrifuge.

2.2.1 Ammonium Sulfate Precipitation and Dialysis

3. Dialysis tubing and the elution buffer (100 mM sodium phosphate buffer pH 7.0).

2.2.2 Ion Exchange Chromatography Using DEAE-Sephadex A-50

1. Take a glass column with the dimensions 1.5 × 40 cm.
2. DEAE (diethyl-aminoethyl) Sephadex A-50.
3. 100 mM sodium phosphate buffer with pH 7.0.
4. Elution buffer: 10 ml each of sodium chloride in increasing concentrations from 0.1 to 0.5 M.

2.2.3 Gel Filtration Chromatography Using Sephadex G-75

1. Take a glass column with the dimensions of 1.0 × 60.0 cm.
2. Sephadex G-75 commercial grade (Sigma-Aldrich Pvt. Ltd., USA, 1.5 × 40 cm).
3. 100 mM sodium phosphate buffer with pH 7.0.

2.3 Lowry's Method

1. Stock solution A: 2% (w/v) Na_2CO_3 (sodium carbonate) in distilled water.
2. Stock solution B: 1% (w/v) $CuSO_4{\cdot}5H_2O$ (Copper (II) sulfate pentahydrate) in distilled water.
3. Stock solution C: 2% (w/v) $KNaC_4H_4O_6{\cdot}4H_2O$ (sodium potassium tartarate) in distilled water.
4. 2 N NaOH (sodium hydroxide).
5. Folin's reagent (commercially available): Use at 1 N concentration.
6. Bovine serum albumin or any other standard protein is used for standard protein stock solution.
7. Stock solutions of standard protein (2 mg/ml) is dissolved in distilled water and stored at −20 °C.
8. Standard solutions of different concentrations can be prepared by diluting the stock solution with distilled water. Increasing concentrations of standard protein can be achieved by mixing different proportions of contents as shown below (Table 1) [8].

2.4 SDS-PAGE

1. Analytical grade gel preparation reagents: Sodium dodecyl sulfate (SDS), TEMED (4-4-tetraethylenemethylene diamine), ammonium per sulfate, acrylamide, bis-acrylamide,

Table 1
Increasing dilutions of the standard protein samples

Stock solution (μl)	0	2.5	5	12.5	25	50	125	250	500
Water (μl)	500	498	495	488	475	450	375	250	0
Protein concentration (μg/ml)	0	10	20	50	100	200	500	1000	2000

Bromophenol Blue, Tris base, Glycine, EDTA, Glycerol, Isopropanol, Tris–HCl (pH 6.8), β-mercaptoethanol, and Coomassie Blue.

2. Protein molecular weight marker (Bio-Rad Laboratories, USA).
3. Prepare 30% acrylamide stock solution by adding acrylamide–bis-acrylamide at a ratio of 37.5:1.
4. Prepare (10×) electrophoresis running buffer by adding 30.3 g of Tris base, 144.0 g D-glycine, 10.0 g SDS. Dissolve the above ingredients in 880 ml of double distilled H_2O after attaining a clear solution make it up to 1000 ml using ddH_2O. The pH of the electrophoresis running buffer is maintained at 8.3 and no pH adjustment is required further [9].
5. Prepare (2×) protein sample buffer solution by adding 1.25 ml 1 M Tris–HCl (pH 6.8), 4.0 ml 10% (w/v) SDS, 2.0 ml glycerol, 0.5 ml 0.5 M EDTA, 4 mg Bromophenol Blue, 0.2 ml β-mercaptoethanol (14.3 M). Dissolve the above ingredients in 10 ml ddH_2O [9].

2.5 Cellulase Zymogram Assay

1. Prepare CMC zymogram by adding 0.1% of carboxymethyl cellulose (CMC) to 12% separating gel (SDS-PAGE gel can be prepared as described in Subheading 2.4).
2. Prepare wash buffer containing sodium citrate buffer (50 mmol/L) pH 5.5, 1% Triton X-100.
3. 0.1% Congo Red.
4. 1 mol/L NaCl.

3 Methods

3.1 Filter Paper Assay (FPA)

It is one of the highly studied and used enzyme assay recommended by International Union of Pure and Applied Chemistry (IUPAC) for the determination of cellulase enzyme. FPA is based on the conversion of defined amounts of substrates (2 mg of glucose released from 50 mg of filter paper measured using DNS assay at given time of 60 min). The wide availability and reasonable susceptibility of the filter paper towards cellulase activities are major

advantages of FPA method. The detailed protocol of the filter paper assay was clearly and extensively explained earlier [10]. In this chapter, we have discussed about the microplate method of the filter paper assay explained by Xiao et al. [11]. FPA is highly used for the determination of cellulases secreted by the aerobic microorganisms, whereas for the determination of cellulases and cellulosomal enzyme activities Avicel assay is highly preferred. The avicel assay protocol for anaerobic cellulases was clearly and extensively explained earlier [10].

DNS Reagent

1. Mix distilled water 400 ml with 3,5-dinitrosalicylic acid 10.0 g and 19.3 N (50% w/w) sodium hydroxide 20.75 ml; slightly heat to help dissolving.
2. Add Rochelle salts (sodium potassium tartrate tetrahydrate) 300 g; make sure that all the salts are dissolved; add deionized water up to 1 L.
3. Store the above prepared reagents in dark conditions (in amber-colored bottles or by covering the bottles using aluminum foil) (*see* **Note 2**).

Citrate Buffer

1. Mix citric acid monohydrate 210 g with DI water 750 ml.
2. Dilute to 1 L and check pH.
3. Adjust by adding NaOH until pH equals 4.5; the solution above should be 1 M citrate buffer.
4. Dilute 1 M buffer to 0.05 M by adding 1: 19 folders DI water.

Glucose Standard

1. Prepare working stock solution of anhydrous glucose (2 mg/ml) by dissolving 0.4 g glucose up to 200 ml pH 4.8 citrate buffer, use immediately or tightly seal and store frozen (store at 4 °C will not last long) (*see* **Note 3**).
2. Vortex the solution before use to ensure adequate mixing.
3. Dilute the working stock solution to different concentration by the following manner: 0.5 ml + 0 ml buffer = 1.0 mg/0.5 ml, 400 μl + 100 μl buffer = 0.8 mg/0.5 ml, 300 μl + 200 μl buffer = 0.6 mg/0.5 ml, 200 μl + 300 μl buffer = 0.4 mg/0.5 ml, 100 μl + 400 μl buffer = 0.2 mg/0.5 ml.
4. Add 0.5 ml of each of the above glucose dilutions to 1.0 ml citrate buffer in test tube with cap, incubate at 50 °C for exactly 60 min.
5. Remove each assay tube from the 50 °C bath; add 3.0 ml DNS reagent to each tubes and mixing.

3.2 Filter Paper Assay Using Microplate

1. Whatman No.1 filter paper (using a paper cutter cut into 1.0 × 6.0 cm strips for 1.5 ml filter paper assay and for 96-well plate assay cut the paper into 7.0 mm using a punch machine) (*see* **Notes 1** and **3**) [11].
2. Prepare 50 mM NaAc (Sodium acetate buffer) with pH 4.8.
3. DNS solution preparation is same as described above Subheading 2.2.1.
4. The 7 mm diameter paper disk with an area of 38.5 mm^2 is used in the 96-well plate assay. Where as in the standard FPA assay one filter paper strip of 600 mm^2 (1 × 6 cm) is used as the substrate.
5. The final reaction volume contains 32 μl aliquot of the diluted enzyme extract, 64 μl of 50 mM NaAc buffer (pH 4.8).
6. Incubate the 96 μl-well plates at 50 °C in a temperature cycler after 60 min of incubation, transfer 50 μl of the above reaction volume is transferred to the corresponding well of second 96-well plate, containing 100 μl of DNS.
7. Incubate the 96-well plate at 95 °C for 5 min in a temperature cycler.
8. Once the color is developed, transfer 36 μl of sample solution to the flat bottomed 96-well plate containing 160 μl of H_2O.
9. Monitor the reaction using a spectrophotometer or a plate reader, at an absorbance of 540 nm.
10. According to Xiao et al., in relation to the amounts of glucose equivalents expected to be produced from standard FPA assay, amount of enzyme used in 96-well plate should release around 128 μg of glucose equivalents in each well [11].
11. Xiao et al. also proposed a 60 μl format FPA assay, where the reaction mixture contains 20 μl aliquot of diluted enzyme, 40 μl of 50 mM NaAc buffer and a filter paper disk of 7 mm diameter [11].
12. After adding all the contents of reaction mixture mentioned above, 96-well plate is incubated in a temperature cycler at 50 °C for 60 min.
13. After the incubation, 120 μl of DNS solution is added to each well, and further incubated at 95 °C for 5 min in a temperature cycler.
14. Finally, transfer 36 μl of aliquot of each sample to a flat bottomed 96-well plate containing 160 μl of H_2O.
15. Monitor the reaction using a spectrophotometer or a plate reader, at an absorbance of 540 nm (*see* **Note 4**).

16. Calculation of filter paper units (FPU), one filter paper unit is defined as average of 1 μmol of glucose equivalents released per minute in the assay reaction (*see* **Note 5**).

$$\text{FPU/ml} = \frac{(\text{Sample } A_{540})}{(\text{Glucose solution } A_{540}/\text{mg})} \quad (5.55\ \mu\text{mole/mg}) \times \left(\frac{1}{60\ \text{min}}\right)\left(\frac{1}{0.02\ \text{ml}}\right)$$

3.3 Purification of Cellulase

3.3.1 Ammonium Sulfate Precipitation

1. Measure the culture filtrate obtained from the CMC broth cultures using a graduated cylinder.
2. Transfer the filtrate to a beaker with twice the capacity of the measured filtrate solution.
3. Place the beaker in a bed of ice pack.
4. Use fresh, desiccated ammonium sulfate and dry it overnight in a hot air oven at 120 °C in a large beaker.
5. Transfer dried ammonium sulfate to a clean mortar and grind the ammonium sulfate to fine powder using the pestle (*see* **Note 5**).
6. To achieve 80% saturation, use 53.2 g of ammonium sulfate is added for 100 ml of filtrate.
7. Add small amounts of ammonium sulfate powder to the filtrate solution and constantly mix with the help of a stirrer (or a magnetic stirrer).
8. Do not stir vigorously as it might result in denaturation and avoid the foam formation.
9. Continue stirring for some time even after the addition of ammonium sulfate to ensure complete precipitation (*see* **Note 6**).
10. Transfer the filtrate protein solution to a polycarbonate centrifuge tubes by using a balancing tube filled with water centrifuge at 10,000 × *g* for 15 min at 4 °C (*see* **Notes 7** and **8**).
11. Collect the supernatant from the centrifuge tube, and resuspend the protein pellet in the minimum volume of (1–2 pellets volume) 0.02 M sodium phosphate buffer (pH 7.0).
12. Repeat the **step 11** for 2–3 times to ensure maximum protein collection [12, 13] (*see* **Notes 8** and **9**).

3.3.2 Ion Exchange Chromatography Using DEAE-Sephadex A-50

1. Take column ranging 1.5 × 40 cm, thoroughly clean and dry the column.
2. Use fresh and hot air oven dried DEAE-Sephadex A-50 to pack the vertically mounted column.
3. The column is equilibrated using 100 mM sodium phosphate buffer of pH 7.0 and is allowed to pass through the column at 30 ml/h flow rate.

4. The enzyme filtrate obtained after ammonium sulfate precipitation is subjected to dialysis by transferring the enzyme filtrate to a dialysis tubing.
5. Dialysis is performed against 100 mM sodium phosphate buffer (pH 7.0) overnight kept at a constant stirring using a magnetic stirrer.
6. The buffer used for the dialysis is replaced twice at constant time interval with the fresh buffer.
7. After dialysis, the enzyme filtrate is run through on the vertical column by constantly adding 100 mM sodium phosphate buffer (pH 7.0).
8. The unbound fractions are constantly collected from the column and further analyzed for cellulase activity and total protein content.
9. Later the bound enzymes are eluted using the elution buffer containing 100 mM sodium phosphate buffer (pH 7.0) with increasing concentration of 0–0.5 M NaCl.
10. The bound filtrates are continuously collected using test tubes and tested for the total cellulase activity and total protein activity [12, 13].

3.3.3 Gel Filtration Chromatography Using Sephadex G-100

1. Take column ranging 1.0 × 60.0 cm, thoroughly clean and dry the column (*see* **Notes 10** and **11**).
2. Use fresh and hot air oven dried Sephadex G-100 slurry is packed into the vertically mounted column.
3. The column is equilibrated using 100 mM sodium phosphate buffer of pH 7.0 and is allowed to pass through the column at 10 ml/h flow rate.
4. The enzyme filtrates exhibiting highest cellulase activities from the ion exchange chromatography are applied on to the Sephadex G-100 column.
5. The 3 ml of the eluted fractions are constantly collected at a flow rate of 10 ml/h in clean and dry test tubes.
6. These collected enzyme filtrates are tested for the total protein concentration using spectrophotometer at 280 nm wavelength.
7. Eluted fractions with higher protein activity are pooled and collected together in test tubes.
8. The enzyme filtrates are also tested for the total cellulase activities [12, 13].

3.4 Protein Estimation Using Lowry's Method

1. Prepare 1 mg/ml stock solution of bovine serum albumin.
2. Prepare solution A containing 2% (w/v) sodium carbonate (Na_2CO_3) in distilled water.

3. Prepare solution B containing 1% (w/v) copper sulfate ($CuSO_4 \cdot 5H_2O$) in distilled water.
4. Prepare solution C containing 2% (w/v) sodium potassium tartarate in distilled water.
5. Prepare alkaline copper sulfate reagent by mixing 100 ml of solution A and 1 ml of solution B and 1 ml of solution C.
6. Take a clean and dry test tube add different dilutions of BSA stock solutions from (0.25–5 ml) to prepare the standard curve.
7. Take a clean and dry test tube add 0.1 ml of test sample and 0.1 ml of 2 N NaOH.
8. Place the test tube in a heating block or boiling water bath at 100 °C temperature for 10 min to hydrolyze the protein.
9. Allow the test tubes to attain room temperature.
10. Add 1 ml of alkaline copper sulfate reagent and allow the test tubes at room temperature for 10 min.
11. Add 0.1 ml of Folin–Ciocalteau reagent using a vortex mixer and incubate the test tubes for 30–60 min at room temperature under dark conditions.
12. Read the absorbance at 750 nm using a spectrophotometer or calorimeter.
13. Plot the standard curve based on the values obtained from the BSA stock solution, use these values for finding out the concentration of test protein sample [8].

3.5 SDS-PAGE for Cellulase Size Estimation

(A) SDS-PAGE Gel Preparation

1. Whole SDS-PAGE units containing glass plates, combs, and spacers are thoroughly clean and dried.
2. Based on the manufacturer instructions assemble the gel cassette appropriately.
3. Prepare 10% separating gel by adding the following reagents appropriately 2 ml double distilled H_2O, 1.67 ml 30% acrylamide–bis, 1.25 ml 1.5 M Tris (pH 8.8), 25 µl 20% SDS, 25 µl 10% ammonium per sulfate (freshly prepared and stored at 4 °C), 2.5 µl TEMED, total volume of the separating gel is 5 ml (*see* **Notes 12–15**).
4. Mix the above solution thoroughly and transfer it between the glass plates present in the casting chamber using 1 ml micropipette.
5. Fill the separating gel up to 0.7 cm below the bottom of the comb, by arranging the comb in its place.

6. Add few microliters of isopropanol to the top of the separating gel to aid in polymerization and straightening the level of the gel.
7. Once the separating gel is polymerized, using a filter paper remove the isopropanol on the top of the separating gel.
8. Prepare the 5% stacking gel by adding the following solutions 2.088 ml of double distilled H_2O, 0.506 ml of 30% acrylamide–bis, 0.375 ml 1 M Tris (pH 6.8), 15 µl 20% (w/v) SDS, 15 µl 10% ammonium per sulfate, and 1.5 µl of TEMED, total volume of the stacking gel is 3 ml (*see* **Notes 12–15**).
9. After adding TEMED, the stacking gel solution is immediately transferred to the casting chamber using 1 ml micropipette (*see* **Notes 16** and **17**).
10. Slowly insert the appropriate comb on to the casting chamber.
11. Allow the gel to solidify, once the gel is solidified carefully remove the comb.
12. The above prepared gels can be stored for 1–2 weeks at 4 °C, by plastic wrapping the whole casting chamber along with combs and placing it on a second chamber containing paper towel moistened with double distilled sterile water.

(B) Cellulase Sample Preparation

1. Prepare 0.1 ml of purified protein sample solution.
2. To the purified protein sample solution, add same amount of 2× protein sample buffer to each protein sample prepared.
3. Transfer the sample solutions to 1 ml Eppendorf tubes.
4. Mix the protein samples thoroughly and boil the samples at 95 °C in a heating block module for 10 min.
5. Using a table top centrifuge spin the sample tubes at maximum speed for 1 min.
6. Leave the sample tubes at room temperature until they are loaded to the gel.
7. The above sample tubes can be stored at −20 °C and can be loaded on to the gel by reheating the sample tubes at 95 °C for 5 min.

(C) Electrophoresis

1. Carefully, remove the gel cassette from the casting chamber and place the gel cassette in the electrode assembly by leaving short plate inside.
2. Press down the electrode assembly while clamping the frame to secure the electrode assembly, and place the clamping frame in to the electrophoresis tank.

3. Slowly, add 1× electrophoresis running buffer into the casting frame opening and between the gel cassettes.
4. Buffer is added until the wells of the gels are filled; also fill the region outside the frame with 1× running buffer.
5. Add 10 µl if the final concentration is 2 mg/ml (if the well dividers are in good shape add 20 µl), add same volume of the protein samples into each well.
6. Add 10 µl of protein molecular weight marker obtained from Bio-rad, USA, which includes β-galactosidase (120 kDa), Bovine Serum Albumin (91 kDa), Serum Albumin (66 kDa), Glutamic dehydrogenase (56 kDa), Ovalbumin (48 kDa) Carbonic anhydrase (4 kDa), Myoglobin (26 kDa), and Lysozyme (19 kDa).
7. After all the protein samples are added, connect the power supply.

(D) Detection of Protein

1. After the completion of run carefully dismantle the cassette and place the gel resolving gel in Coomassie Blue stain with a gentle agitation for 30 min.
2. After 30 mins pour of the Coomassie Blue stain and add destaining solution.
3. Repeat **step 2** for about 4–5 times.
4. To fasten the destaining process place a piece of paper towel or filter paper to absorb the stain.
5. Once the gel is destained, dry the gel by placing it on Whatman's no.1 filter paper (Fig. 2).

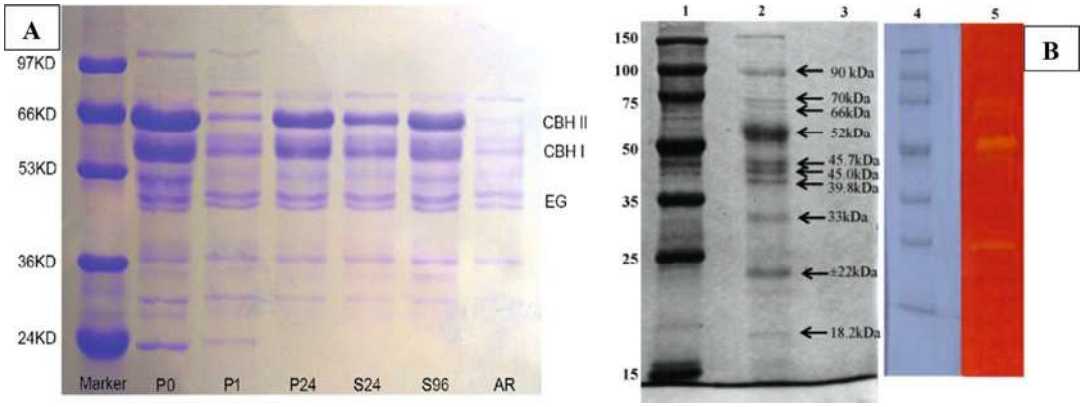

Fig. 2 Shows the separation of pure cellulase enzyme extracts (**a**) SDS-PAGE analysis of cellulase in supernatant during prehydrolysis for 0 (P0), 1 (P1), and 24 h (P24); SSF for 24 h (S24) and 96 h (S96); and after readsorption (AR). (Reprinted with permission from Ruoyu et al. [14].) (**b**) Lane 1—150 kDa protein marker; Lane 2—crude enzyme; Lane 3—medium; Lane 4—150 kDa protein marker; Lane 5—CMCase (CMC-Zymogram). (Reprinted with permission from Ang et al. [15])

3.6 Cellulase Zymogram Assay

1. CMC-zymogram prepare 12% separating gel by adding the following ingredients 3.2 ml of ddH_2O, 4 ml of 30% acrylamide–0.8% of bis-acrylamide, 1.5 M Tris base (pH 8.8), 10% (w/v) SDS, 0.1% of carboxy methyl cellulose (CMC), 10% (w/v) ammonium per sulfate and 10 μl TEMED, total volume of the separating gel is 10 ml (*see* **Notes 13–16** and **19**).
2. Prepare the 5% stacking gel by adding the following solutions 2.088 ml of double distilled H_2O, 0.506 ml of 30% acrylamide-bis, 0.375 ml 1 M Tris (pH 6.8), 15 μl 20% (w/v) SDS, 15 μl 10% ammonium per sulfate and 1.5 μl of TEMED, total volume of the stacking gel is 3 ml (*see* **Notes 16–18**).
3. For the assembly of gel cassette, arrangement of casting chamber, preparing protein samples and process of electrophoresis, the protocol is same as Subheading 2.4.
4. After the completion of SDS-PAGE run, take out the gel dismantle the assembly of gel cassette and carefully separate the gel.
5. The gel is transferred into a clean container at room temperature and further rinsed with sodium citrate wash buffer (pH 5.5) for 1 h.
6. Pour off the buffer solution from the container and add fresh sodium citrate wash buffer to the container (pH 5.5) and incubated at 50 °C for 4-h.
7. Pour off the solution from the container and stain the gels with 0.1% Congo red for 30 min.
8. After 30 min, destain the gels with 1 M NaCl (for 1 h) or until the zones of clearance around the enzymes are observed [16–19] (Fig. 3).

4 Summary

Increase in global temperatures and continuous depletion of fossil fuels to meet up the human needs are the major reasons behind inflated research in cellulose-based ethanol production. In this chapter, we have discussed various quantitative assays for the characterization of total cellulase activities among the isolated cellulolytic microorganisms. Also, we have discussed about methods used for the purification and estimation of cellulase activities and characterization of total cellulase activities using cellulase zymogram assays. Employing robust protocols for isolation, purification, and characterization of cellulose-degrading microorganisms will significantly help the growing lignocellulose-based biofuel and biorefining industries.

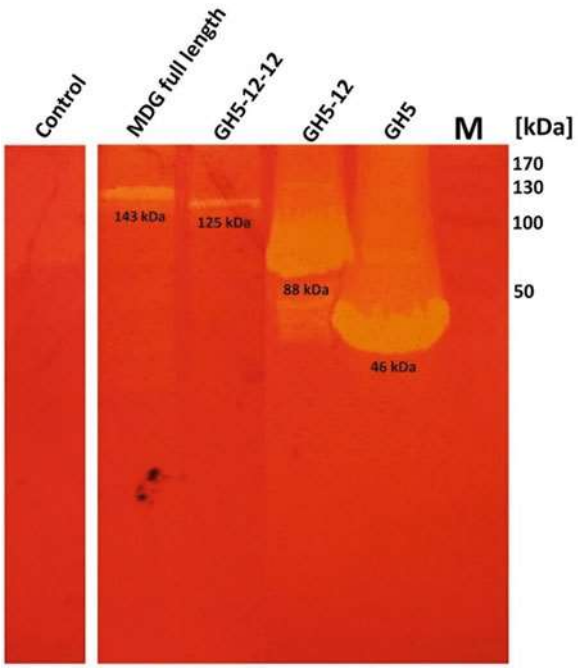

Fig. 3 Shows the CMC-Zymogram gels with multidomain glycosidase full length (MDG: Lane 1), glycoside hydrolase (GH5-12-12: Lane 2), (GH5-12: Lane 3) and GH5 on Lane 4. (Reprinted with permission from Gavrilov et al. [19])

5 Notes

1. It is important to make sure that weight of each paper strip do not vary more than 1 mg of weight. As filter paper assay (FPA) is subjected to the weight of filter paper.
2. The PBS and DNS (in darkness) reagents can be stored at 4 °C for at least 1 month. However, DNS reagents older than 1 month must be avoided for the quantitative assays as it could lose its reducing ability after long storage periods.
3. Handle the filter paper strips with forceps or gloved hands, do not use bear hands to transfer the paper strips.
4. The glucose standard solution is tightly sealed and stored frozen, the glucose solution is thawed and mixed well before usage.
5. International Unit (IU) is defined as 1 mmol/min, based on the initial hydrolysis rate, and is different from FPU assay, which is a fixed conversion assay.
6. For gel filtration chromatography pore size (matrix) plays a crucial role in separation of the protein mixtures.
7. The levels of β-D-glucosidase enzyme present in the cellulase mixture will significantly influence the FPA assay as the total

ratio of reducing ends (glucose, cellobiose and longer cellodextrins) will strongly impact on the DNS readings [10].

8. Use a face mask to avoid contact with ammonium sulfate powder.
9. Perform all the ammonium sulfate protein purification steps on ice at 4 °C, also store ammonium sulfate solution at 4 °C.
10. The protein samples must be kept at cold when adding ammonium sulfate. Slowly add the salt by constantly stirring to avoid the addition of excess than the desired concentration.
11. After resuspending the ammonium sulfate pellet, the sample will contain excess amounts of salt. Please remove the excess salt present in the samples using dialysis, if the protein samples are separated further using ion exchange chromatography.
12. Prior dialysis step is not required if the ammonium sulfate precipitated protein samples are subjected to gel chromatography.
13. If ammonium sulfate is settling at the bottom, stir the filtrate until it dissolves and then add ammonium sulfate powder again.
14. Wear a mask, lab coat and goggles when you weight acrylamide, as acrylamide is considered as a potential carcinogen and neurotoxic reagent (please refer MSDS sheet for further details).
15. Avoid exposure with acrylamide and cover the weighing boat containing acrylamide with another weighing boat and transport it to the fume hood.
16. Transfer the above weighed acrylamide to the cylinder inside the fume hood and mix with a glass rod/stirrer.
17. Avoid contact with unpolymerized acrylamide as it is a neurotoxin.
18. Gel solution should be quickly transferred to the casting chamber as to avoid the gel polymerization after the addition of TEMED.
19. Mix CMC thoroughly and ensure no lumps are formed.

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