Chapter 4

Isolation and Screening of Cellulose-Degrading Microorganisms from Different Ecological Niches

Ayyappa Kumar Sista Kameshwar and Wensheng Qin

Abstract

Increased interest in developing cellulose-based ethanol over the last few years was the main reason behind inflated research to find cellulose-degrading microorganisms. Several methods have been developed in the past for efficient isolation and characterization of cellulolytic microorganisms. However, it is critical to choose a specific method from a list of qualitative methods for the characterization of cellulose degrading microorganisms. In this chapter, we have extensively listed various qualitative methods used for the isolation and characterization of the cellulolytic microorganisms isolated from different ecological niches such as soil, decaying wood, gut, and rumen.

Key words Cellulosic ethanol, Cellulolytic microorganisms, Qualitative methods, Soil, Decaying wood, Rumen, Gut

1 Introduction

Cellulose is the most abundant organic polysaccharide (linear chain of glucose units joined by β (1→4) glycosidic bonds) on the earth's surface, and thus cellulolytic microorganisms play a key role in maintenance of the global carbon cycle [1]. Studies conducted in the past have revealed that majority of cellulolytic bacteria belongs to Actinobacteria (aerobic bacteria) and Firmicutes (anaerobic bacteria), while the entire kingdom of filamentous fungi including Ascomycetes, Basidiomycetes, Deuteromycetes (aerobic fungi) and Chytridiomycetes (anaerobic fungi) contain larger groups of cellulolytic fungal species [1, 2]. Cellulose-degrading microorganisms majorly exhibit two types of cellulose hydrolysis patterns such as synchronized extracellular synergistic system, e.g., *Trichoderma* species, cellulosomes-based cellulose hydrolysis, e.g., *Clostridium* species and Neocallimastigomycetes fungi (anaerobic microbes) [3, 4].

In the past, several studies have reported a wide range of methods and assays for the efficient isolation and characterization

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of cellulolytic microorganisms. Traditionally, cellulose degrading microorganisms are isolated and characterized using cellulose (insoluble in growth media), which is highly reliable and carboxymethyl cellulose (CMC) (soluble in growth media) degraded by many microorganisms which can produce endoglucanase, will give a positive result. Several reports have used filter paper, ball-milled cellulose, acid-treated cellulose, dewaxed cotton string, bacterial cellulose, Avicel, carboxymethyl cellulose, cellobiose, xylan, wood fractions, plant biomass and wood pulp as cellulose substrates for testing the cellulolytic ability of microorganisms [1]. In this chapter, we have outlined the detailed protocols of qualitative and semiquantitative assays for isolation and characterization of cellulose degrading microorganisms, developed over the last few years, and discussed the advantages and disadvantages of the methods, as shown in Table 1. This chapter can act as a consolidated (one-stop) document harboring the qualitative assays for the isolation of cellulose-degrading microorganisms from different habitats such as soil, decaying wood, gut, and rumen.

Table 1
Advantages and drawbacks of different quantitative methods used for the estimation of cellulases

Quantitative method	Drawbacks and advantages
Filter paper degradation	The ambiguity caused due to visual interpretation of filter paper degradation is a major disadvantage of this assay, and the data obtained by this method cannot be used for comparative studies [7]
Cellulose agar plate	Recording the cellulose clearance zones in the growth medium is difficult, especially with fungal strains with dense and dark hyphae [8–10]
Esculin agar plate	Formation of black color in the esculin agar medium clearly indicates the breakdown of cellobiose to glucose by β-glucosidase. Dark hyphal growth by test fungal strain might ambiguity in the results [7]
CMC-Congo red	Compared to other qualitative methods, the CMC-Congo red agar plate method is easy and clear for screening cellulase producing microbial strains. The Congo red staining method can create ambiguity when used for screening fungal strains with dark hyphal growth [6, 7]
CMC-1% HAB	The clearance zones obtained from CMC-1% HAB are hazy and is costly and time expensive study [6]
CMC-Gram's iodine	When compared to CMC-Congo red and CMC-1% HAB methods CMC-Gram's iodine method is easier, cheap and rapid. Clear and sharp clearance zones were developed by this method [6, 7]
Dye diffusion method	Though this method is time-consuming it can be simultaneously used for both cellulolysis and lignin modifying activities. This study must be performed carefully as there are good chances of contamination [6, 7]

2 Materials

Analytical grade reagents must be used, with all the solutions prepared using the ultrapure water. All the reagents and solutions can be stored at room temperature, until and unless specified otherwise in the text.

2.1 Isolation and Collection of Samples

2.1.1 From Insect Gut Samples

1. 70% ethanol solution.
2. Sterilized distilled water.
3. Phosphate buffer solution (1×PBS): 1.44 g Na_2HPO_4, 0.2 g KCl, 8 g NaCl, and 0.24 g KH_2PO_4, dissolve all the salts in 800 mL, followed by adjusting the pH to 7.4 using HCl further water is added appropriately to make the total volume to 1 L.
4. 1.5 mL microcentrifuge tube.
5. Sonicator, vortex.
6. 250 mL Erlenmeyer flasks, plastic pestle.
7. Tryptic soy broth: 3 g of tryptic soy broth, 15 g of agar, 1 L of distilled water with pH maintained at 7.0.
8. Luria–Bertani broth: 10 g Bacto tryptone, 5 g Bacto yeast extract, 5 g NaCl with pH maintained at 7.0.

2.1.2 From Rumen Samples

1. Collecting bottle with 250 mL capacity.
2. Cheesecloth.
3. −86 °C freezer.
4. Two types of growth mediums can be used for the isolation of cellulose-degrading microorganisms from the rumen:
5. Growth medium A
   (a) *15 mL Mineral Solution I*: 3.0 g KH_2PO_4; 6.0 g $(NH_4)_2SO_4$; 6.0 g NaCl; 0.6 g $MgSO_4$; 0.795 g $CaCl_2{\cdot}2H_2O$ per L.
   (b) *15 mL Mineral Solution II*: 3 g K_2HPO_4, 0.25 g yeast extract, 1 g tryptone, 0.1 mL 0.1% resazurin, 0.2 mL 0.05% hemin, 0.5 g microcrystalline cellulose, 0.1 g cellobiose, 0.4 g sodium carbonate, 20 mL clear rumen fluid, 50 mL distilled water, and 50 mg cysteine hydrochloride.
6. Growth medium B
   (a) Scott and Dehority agar growth medium supplemented with clarified rumen fluid 5% (v/v), 0–3% (w/v) glucose, cellobiose, and 0–6% (w/v) starch can be used for the culture of microbes.
7. The cultures must be incubated in an anaerobic chamber, which can maintain 90% of carbon hydroxide and 10% hydrogen.

8. Growth medium must be freed from oxygen gas by bubbling carbondioxide gas into the anaerobic culture/Hungate tubes.
9. Please refer the cited state-of-the-art review on preparation of culture mediums for isolating the anaerobic microorganisms [1].

2.1.3 Collection of Soil and Degrading Wood Samples

1. Soil samples.
2. Decaying wood samples.
3. Test tubes, 250-mL Erlenmeyer flasks.
4. Phosphate buffer saline solution (1×PBS) composition is same as above.
5. Shaking incubator.
6. Potato dextrose broth: potato infusion 4 g and dextrose 20 g dissolved in 1 L of distilled water.
7. Nutrient broth: peptone 10 g, beef extract 10 g, NaCl 5 g, dissolved in 1 L of distilled water.

2.2 Methods for Isolation of Cellulase Producing Bacteria and Fungi

To achieve good results, it is necessary to employ uniform inoculation procedures. The test microbial strains (fungi) must be inoculated on minimal salt medium supplemented with 0.4% w/v glucose and 1.6% w/v agar. The test strains inoculated on minimal salt mediums can be used for the qualitative assays, as it does not carry over nutrients and does not interfere with result interpretations. Single agar disc with the test strains can be used further for inoculating the assay medium; the test strains can also be cultured using the cellulose basal medium, see below.

2.2.1 Filter Paper Degradation Method

1. Cellulose basal medium (CBM): 5 g of cellulose, 1 g KH_2PO_4, 0.5 g $MgSO_4.7\ H_2O$, 0.1 g yeast extract, and 0.001 g $CaCl_2.2H_2O$ are dissolved in 1 L distilled water.
2. 200-mL or 250-mL Erlenmeyer flasks.
3. Whatman's No. 1 filter paper (25 × 5 mm) or sterile filter paper (almost 100% cellulose).

2.2.2 Cellulose Agar Plate Method

1. 4% (w/v) cellulose is supplemented to the cellulose basal medium along with 1.6% (w/v) agar.
2. Autoclave at 121 °C for 15 min.
3. Petri plates.

2.2.3 Esculin-Iron Agar Plate Method

1. Cellulose basal medium is supplemented with 0.5% (w/v) esculin and 1.6% (w/v) agar.
2. 1 mL of 2% (w/v) aqueous ferric sulfate solution for 100 mL CBM growth medium.
3. Petri plates.

2.2.4 Cellulose Agar Staining Methods

Method a (CMC-Congo Red)

1. Composition of carboxymethyl cellulose sodium: 0.05% K_2HPO_4, 0.025% $MgSO_4$, 0.188% CMC sodium salt, 0.02% Congo Red, 1.5% agar, 0.2% gelatin.
2. Petri plates.
3. For Congo-Red staining method: use the CMC agar plates same composition as mentioned above except Congo Red.
4. 1 M NaCl, distilled water.

Method B (Hexadecyltrimethyl Ammonium Bromide)

1. 2% (w/v) carboxymethyl cellulose (CMC), 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.02% peptone, and 1.7% (w/v) agar.
2. 1% hexadecyltrimethyl ammonium bromide (HAB).
3. 1 M NaCl and distilled water.
4. Petri plates.

Method C (Gram's Iodine Staining)

1. 2% (w/v) carboxymethylcellulose, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.02% peptone, and 1.7% (w/v) agar.
2. Gram's Iodine complex (2.0 g KI and 1.0 g iodine dissolved in 300 mL distilled water).
3. Petri plates.

Method D (Dye Diffusion Method):

1. Cellulose basal medium, 1% (w/v) cellulose azure (or) cellulose dyed with Remazol Brilliant Blue R and Brilliant Blue R, 1.6% (w/v) agar.
2. 10-mL glass culture bottle.
3. Distilled water.

3 Methods

3.1 Isolation and Collection of Samples

3.1.1 Isolation of Gut from Insect Samples

1. Sterilize the surface of the test insect samples using 70% ethanol for 1 min.
2. Rinse the insect samples using sterile distilled water.
3. Following sterilization, suspend the insect samples in 10 mM sterile phosphate buffer saline (1× PBS) solution.
4. Dissect the above insect samples inside a sterile laminar air flow chamber using a dissection scissors and fine tipped forceps.
5. Using dissection scissors, detach the head and last abdominal segments of the larva.
6. To release the gut, apply pressure on the anterior region of the crop.

7. Held the thorax portion of the insect with the help of forceps, and pull the head from the thorax to stretch the gut out from the insect crop.
8. Separate the gut from the insect samples by detaching the extremities in a drop of sterilized PBS solution.
9. To avoid possible contamination from other tissues, rinse the gut samples using sterile PBS solution.
10. Gut samples can be either pooled or transferred individually to 1.5 mL micro centrifuge tube containing PBS solution (50, 100, or 500 μL).
11. Finally, sonicate the gut samples for 30 sec at 50/60 Hz, 117 V, 1.0 Amps, macerate with a plastic pestle and vortex at medium speed for 10 s for the separation of bacterial cells from the insect gut wall.

3.1.2 Isolation of Rumen Samples

1. Collect the rumen fluid from the ruminating animals, e.g., from the local slaughterhouse.
2. Place the rumen fluid immediately on dry ice and store further at −50 °C.
3. Clarify the obtained rumen samples initially by straining rumen fluid using two-layered cheesecloth.
4. Autoclave the filtered rumen samples using the standard conditions followed by centrifuging the samples at 6000 × *g* [5] (*see* **Notes 1** and **2**).
5. Later the rumen samples are ice thawed and used for further screening experiments.

3.2 Methods for Characterization of Cellulolytic Microorganisms

All the experiments must be carried out at room temperature unless the temperature conditions are specified otherwise.

3.2.1 Filter Paper Degradation

1. Prepare cellulose basal medium (CBM) and transfer 10 mL of the CBM solution into glass bottles followed by autoclaving at 121 °C and 15 psi conditions (*see* **Notes 1** and **3**).
2. Transfer aseptically 25 × 5 mm sterile filter paper strips to the above glass bottles.
3. Inoculate the test strain into the glass bottles and incubate at corresponding temperatures (fungi 28 °C and bacteria 37 °C).
4. Use uninoculated glass bottles as control.
5. Assess the degradation of filter paper based on the physical degradation and increased opacity in the inoculated samples.
6. Compare inoculated samples with the uninoculated samples to observe the difference.

3.2.2 Cellulose Agar Plate

1. Prepare growth medium with CBM supplemented with 4% (w/v) cellulose and 1.6% (w/v) agar (*see* **Note 4**).
2. After standard sterilization (gently mix the contents of the medium for uniform distribution) transfer appropriate amounts of medium to petri plates (*see* **Note 3**).
3. In a laminar air flow inoculate the test strain (fungi/bacteria) and incubate the plates at 27 °C and 37 °C respectively.
4. Observe the plates for the development of clearance zones around the test colonies on the opaque agar.

3.2.3 Esculin Agar Plate Method

1. Prepare growth medium with CBM supplemented with 0.5% (w/v) esculin and 1.6% (w/v) agar dissolved in 1 L distilled water, and add 1 mL of 2% (w/v) aqueous ferric sulfate for every 100 mL of CBM solution added to the sterilized medium (*see* **Note 1**).
2. Gently mix the medium contents, until uniform distribution is attained.
3. Aseptically transfer appropriate amounts of growth medium to sterile petri plates.
4. Inoculate the test strain on the petri plates under laminar airflow cabinet, and uninoculated plates can be used as control.
5. Incubate these petri plates at 27 °C in darkness, and monitor the plates for the development of black color, which indicates the production of β-glucosidase.

3.2.4 Dye Diffusion Based Methods

Method a (CMC-Congo Red Plate)

1. For Congo-Red staining method, prepare CMC-Congo Red agar growth medium based on the abovementioned composition dissolved in 1 L of distilled water and autoclave at standard conditions (*see* **Notes 1** and **5**).
2. Aseptically transfer the growth medium into sterile petri plates.
3. Inoculate 5 μL of test microbial strain on to the center of the plate, uninoculated plates are used as controls.
4. Incubate the inoculated plates at 27 °C for fungi and 37 °C for bacteria respectively.
5. Monitor daily for the development of clearance zones around the growing colony.
6. For Congo-Red staining method: flood the CMC agar plates with 0.1% Congo Red and incubate for 15–20 min, followed by 1 M NaCl for 15–20 min (*see* **Note 5**).
7. Observe the development of clearance zones around the growing colonies (Fig. 1).

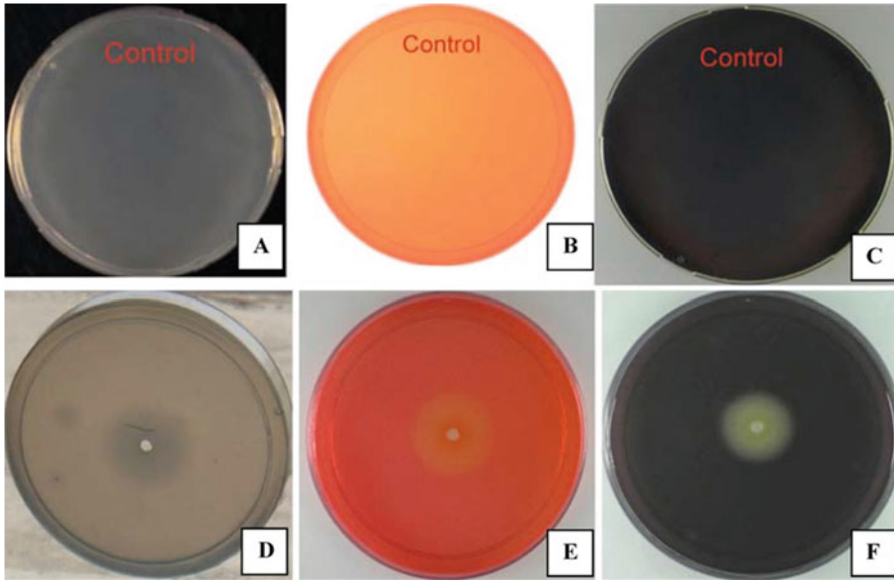

Fig. 1 Qualitative assays for the estimation of cellulases using the methods CMC-1% HAB (**a**) control, (**d**) test strain, CMC-Congo Red (**b**) control, (**e**) test strain and CMC-Gram's Iodine (**c**) control, (**f**) test strain (Reprinted with permission from [6])

Method B (CMC-HAB Method)

1. Prepare CMC agar medium based on the abovementioned compositions and autoclave at standard conditions (*see* **Notes 1** and **5**).
2. Aseptically transfer the growth medium into sterile petri plates.
3. Inoculate 5 μL of test microbial strain on to the center of the plate, uninoculated plates are used as controls.
4. Incubate the inoculated plates at 27 °C for fungi and 37 °C for bacteria respectively.
5. Plates are flooded with 1% HAB for 30–40 min (*see* **Note 5**).
6. Observe the development of clearance zones around the growing colonies (Fig. 1).

Method C (CMC-Iodine Stain)

1. Prepare CMC agar medium based on the abovementioned compositions and autoclave at standard conditions (*see* **Notes 1** and **5**).
2. Aseptically transfer the growth medium into sterile petri plates.
3. Inoculate 5 μL of test microbial strain on to the center of the plate, uninoculated plates are used as controls.
4. Incubate the inoculated plates at 27 °C for fungi and 37 °C for bacteria respectively.
5. Prepare Gram's Iodine solution by adding 2.0 g KI and 1.0 g iodine in 300 mL of distilled water (*see* **Note 5**).

6. Flood the plates with Gram's Iodine solution and incubate the plates for 3–5 min.
7. Observe the development of clearance zones around the growing colonies (Fig. 1).

Method D (Dye Diffusion Method)

1. Prepare CBM growth medium supplemented with 1.6% (w/v) agar dissolved in distilled water and autoclaved at standard conditions (*see* **Notes 1** and **3**).
2. Prepare 1% (w/v) cellulose azure supplemented with CBM and 1.6% (w/v) agar dissolved in distilled water and autoclaved at standard conditions (*see* **Note 1**).
3. Transfer the CBM agar solution prepared in the **step 1** to 10 mL to glass culture bottles.
4. Mix the cellulose azure agar prepared in **step 2** gently to attain uniform distribution and transfer 0.1 mL aseptically on to the surface (as an overlay) of the solidified CBM agar.
5. Uninoculated glass culture bottles can be used as controls.
6. Incubate the inoculated glass culture bottles at 27 °C and 37 °C for fungi and bacterial strains respectively.
7. Monitor the cultures carefully for the migration of dye from upper layer (cellulose azure agar) to lower layer (CBM agar), which indicates cellulolysis. The subsequent dye degradation of color in the upper layer indicates the presence of lignin-modifying activity.

4 Notes

1. Please refer to the appropriate laboratory manual before working with autoclave and other standard microbiology lab equipment mentioned in our chapter.
2. Centrifugation is performed to remove the particulate material present in the ruminal fluid. Be careful when you are using the autoclave, please read and understand the functioning of the autoclave.
3. Mix CMC thoroughly and ensure that no lumps are formed.
4. The cellulose source used in this assay can be ball-milled cellulose, acid-swollen and microcrystalline cellulose thus making the medium appear opaque. Cellulolysis leads to development of clearance zones around the colonies.
5. Avoid physical contact (by wearing gloves, lab coat, and eye gear) while using synthetic chemical dyes with Congo Red, Iodine solution, and hexadecyltrimethyl ammonium bromide.

5 Summary

In the past, several studies have reported a wide range of methods and assays for the efficient isolation and characterization of cellulolytic microorganisms. In this chapter, we have discussed the detailed protocols of qualitative and semi-quantitative assays for isolation and characterization of cellulose degrading microorganisms, developed in the last few years. This chapter can act as a consolidated (one-stop) document harboring the protocols using qualitative and semi-quantitative assays for the isolation of cellulose-degrading microorganisms from different habitats such as soil, decaying wood, gut, and rumen samples.

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