Environment and Ecology 36 (3) : 828—834, July—September 2018
Website: environmentandecology.com ISSN 0970-0420

Rapid and Effective Method for Exploring Cellulase-Producing Potential of Bacterial Strains

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Received 7 February 2018; Accepted 8 March 2018; Published on 23 March 2018

Abstract Cellulolytic bacteria were isolated from waste dumping site, Bhopal, India. Bacterial strains were identified using the molecular technique. Qualitative and quantitative cellulolytic potential of these bacterial strains were evaluated. Indices of relative enzyme activity (I_{CMC}) was observed highest (3.10) by *Achromobacter xylosoxidans* B-26 with modified carboxy methyl cellulose (CMC) media and Gram's iodine. *Bacillus amyloliquefaciens* B-16 (KF479460) showed maximum (0.00379 IU mL^{-1}) CMC ase activity whereas, maximum β-glucosidase enzyme activity was expressed by isolate *Bacillus subtilis* B-23 (KF479462) as 0.245 IU mL^{-1}. It was concluded that Gram's iodine dye method in combination with modified CMC media (as sodium nitrate was replaced by ammonium tartarate) is rapid, safe and efficient in comparison to congo red dye method.

Keywords Cellulase, β-glucosidase, CMC agar, Congo red, Gram's iodine.

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Introduction

Cellulose is considered as the most abundant carbohydrate in nature and its annual biosynthesis by both plants and marine algae occurs at a rate of 0.85×10^{11} tonnes per annum (Nowak et al. 2005, Niranjane et al. 2007). The major structural component in the plant cell wall is cellulose. It is a linear homo-polysaccharide consisting of anhydrous glucose units (500–15,000) that are linked by β-1, 4-glycosidic bonds, with cellobiose as the smallest repetitive unit. The β-1, 4 orientation of the glucosidic bonds results in the potential formation of intra-molecular and inter-molecular hydrogen bonds, which make native cellulose highly crystalline, insoluble, and resistant to enzyme attack. As cellulose is the most abundant renewable natural product in the biosphere (Bakare et al. 2005, Feng et al. 2007), cellulolytic microorganisms are fundamental for the transformation of cellulose into sugars. Moreover, the degradation pathway of cellulose by microorganisms is a major component of the carbon and energy flux in soil. Ligno cellulosic crop residues, such as cereal straw, provide the principal input of cellulose to arable soils (Ahmad et al. 2013). Additionally, since the annual production of cellulose is estimated to be 4.0×10^7 tons (Bakare et al. 2005), large quantities of industrial, agricultural and municipal cellulosic waste have been accumulated due to inefficient transformation process or due to high cost of their utilization processes (Lee et al. 2008). Therefore, it has become of considerable economic interest to develop processes for the effective treat-

ment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen et al. 2005). Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic substrates. The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulase, endoglucanase (1,4-β-D-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-β-D-glucan glucohydrolase; EC 3.2.1.74) and β-glucosidase (β-D-glucoside glucohydrolase; EC 3.2.1.21). The endoglucanase randomly hydrolyzes the β-1,4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Lastly, the cellobiose is converted to glucose by β-glucosidase. Various research studies have documented the biotechnological potential of cellulases in many industries, such as textiles, detergent, food, animal feed, bio-fuel, paper and pulp, pharmaceutical, and waste management (Kuhad et al. 2011). Moreover, immobilized cellulase enzyme or efficient cellulose producing microbes or microbial consortia could also be instrumental for utilizing vast quantity of agricultural, industrial and urban wastes. Although, most of the cellulase research was mainly focused on fungi, but now a days there has been increasing interest in cellulase production by bacteria because of their fast growth rate and secretion of a complete multi-enzyme system for lignocellulose degradation. For selection of potential cellulolytic microbial strains from a large population or from a new source, quick and reliable screening of extracellular cellulase is very essential. Thus, the most crucial step to address this challenge is to isolate the potential strains. An assay based on better visualization techniques needs to be evolved as a pre-requisite for screening and isolation of promising cellulose producers. The present study was therefore concentrated on developing an easy, fast, and eco-friendly qualitative method for screening of cellulase producing microorganisms on agar plate.

(We would like to express our appreciation to ICAR-Indian Institute of Soil Science, Bhopal and Dr Udai B. Singh and Dr Renu from ICAR-National Bureau of Agriculturally Important Microorganisms, Mau for providing necessary facilities to carry out the experiments.)

Materials and Methods

Culture source and isolation of bacterial strains

Soil samples were taken from Bhanpur dumping site (23°17′51.74′′N, 77°26′9.10′′E), Bhopal, India (Fig. 1). Physico-chemical parameters for each study soil

Fig. 1. View of Bhanpur dumping site.

Table 1. PCR primers used in this study.

Primer	Sequence (5′–3′)	Target gene	Reference
pA-F	AGA GTT TGA TCC TGG CTC AG	16S rDNA	Edwards et al. (1989)
pH-R	AAG GAG GTG ATC CAG CCG CA		

sample were determined (data not shown). Serial dilutions of soil samples were made and plated in the following culture media: 0.5 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NH_4NO_3, 0.02 g $FeSO_4.7H_2O$, 0.05 g Ca $(NO_3)_2.4H_2O$, 15 g agar, and 1% carboxy methyl cellulose (CMC) per liter of distilled water with pH 7.0 by spread-plate technique. The plates were incubated for 3 days at 30°C. Bacterial colonies were then isolated and sub cultured to obtain single strain. The purified cultures were maintained at 4°C for further analysis.

Taxonomic characterization of isolated strains

For bacterial identification, the genomic DNA was extracted by the Ultra Clean Microbial DNA Isolation Kit (MO Bio, USA) and amplified using universall 6S rRNA primers (Edwards et al. 1989) as mentioned in Table 1. The obtained sequences were compared to sequences in NCBI GenBank.

Comparative study of qualitative assay of cellulolytic activity

The isolated bacterial strains were tested and screened for their potential cellulolytic activity on two different media viz. medified CMC agar (5g $C_4H_{12}N_2O_6$, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.001 g $CaCl_22H_2O$, 0.1g yeast extract, 18g agar supplemented with 1% CMC with high viscosity; Himedia, India) (Pointing 1999) and Cellulose powder agar (5g $C_4H_{12}N_2O_6$, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.001 g $CaCl_2.2H_2O$, 0.1 g yeast extract, 18 g agar supplemented with 2% cellulose; Himedia, India) media by flooding them with congo red and Gram's iodine dye. The novelty of this study lies in the selection of dye with modified media. As in normal CMC agar $NaNO_3$ is generally used whereas here in modified CMC agar, ammonium tartarate ($C_4H_{12}N_2O_6$) has been used as basal media along with Gram's iodine dye.

Three sets of culture plates (in triplicates) were prepared for each bacterial strain

Modified CMC agar with congo red dye: The first set of culture plates were inoculated with 5 μl of 24 h old culture on modified CMC agar. The inoculated culture plates were incubated at 30°C for 72 h. Once the colonies had grown, 5 mL of 0.1% (w/v) congo red was added. After 15 minutes of incubation, excess congo red was removed, and 5 mL of 1M NaCl was added. It was followed by another 15–20 minutes of incubation. After that, the excess NaCl was removed, and halo zones produced due to hydrolysis of cellulose were measured.

Cellulose powder agar with Gram's iodine (GI) dye: The second set culture plates were inoculated with 5 μl of 24 h old culture on cellulose powder agar. Culture plates with bacterial colonies (grown at 30 °C for 72 h) were flooded with Gram's iodine (2 g KI, 1 g iodine in 300 ml distilled water). After 5 minutes incubation, the excess iodine was removed and hydrolysis halo zones were measured.

Modified CMC agar with Gram's iodine (GI) dye: The third set culture plates with bacterial colonies (grown at 30°C for 72 h on modified CMC agar were flooded with Gram's iodine (2 g KI, 1 g iodine in 300 ml distilled water). After 5 minutes incubation, the excess iodine was removed and hydrolysis halo zones were measured (Kasana et al. 2008).

Quantification of cellulolytic activity

The microorganisms that grew in selective media and tested positive for congo red and/or iodine were cho-

sen for determination of β-glucosidase and carboxy methyl cellulase activities. The bacteria were grown on solid media containing 1% CMC and incubated for 24 h at 30°C. For β-glucosidase activity, bacteria were replicated on solid media containing 1% powdered cellulose and incubated for 7 days. The microorganisms were subsequently inoculated in 5 mL of liquid media containing 1% powdered cellulose. Carboxy methyl cellulase (CMCase) activity was determined by inoculating the loopful of bacterial culture in 1% CMC broth medium. The cultures were incubated without shaking for 15 days at 30°C. The cultures were subsequently centrifuged at 10,000 rpm for 10 min. at 4°C, and enzymatic activities were determined by measuring released p-nitrophenol with several modifications. For this experiment, 180 μL of 1 mg/mL p-nitrophenyl-β-D-glucopyranoside (pNPG) in Phosphate buffer (pH 7.0) was added to 20 μL of culture supernatant. After incubation at 40°C for 1 h, 80 μL of 2% sodium carbonate was added, and the reaction was stopped by refrigeration for 10 min. The absorbance was measured at 405 nm. The calibration curve was generated with 0–5 μmol of p-nitrophenol (Zhou et al. 2008).

The CMCase activity was determined by the 3,5-dinitrosalicylic acid method (Ghose 1987) with several modifications. It was determined by adding 0.5 mL of 1% CMC to 0.5 mL of culture supernatant. The mixture was incubated at 50°C for 30 min, then 3 mL DNS reagent was added. The sample mixtures were boiled for 5 min then transferred immediately to the cold water bath to stop the reaction. Dilute the mixture with distilled water, measure the formed color at 540 nm spectrophotometrically. The enzymatic activity was expressed as the specific enzymatic activity, where I IU (international unit) of activity is defined as the amount of enzyme required to liberate 1 μmol of glucose equivalent per minute under the assay conditions.

Table 2. Identification of bacterial isolates with their accession number.

Code	Bacterial isolates	Accession number
B-02	*Bacillus licheniformis*	KF479458
B-07	*Bacillus pumilus*	KF479459
B-16	*Bacillus amyloliquefaciens*	KF479460
B-23	*Bacillus subtilis*	KF479462
B-26	*Achromobacter xylosoxidans*	KF479463
B-UI	*Bacillus amyloliquefaciens*	KF479464

Results and Discussion

Isolation and identification of celluloytic bacteria

The six bacterial isolates with potential cellulase producing capacity were identified as *Bacillus licheniformis, Bacillus pumilus, Bacillus amyloliquefaciens, Becillus subtilis, Achromobacter xylosoxidans* and *Bacillus amyloliquefaciens* based on 16s rDNA/RNA gene sequencing. The obtained 16S rDNA sequences of isolated bacteria were compared with those of other known species deposited in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN 2.2.24 program. The nucleotide sequences were deposited in GenBank with following accession numbers (Table 2).

Plate assay for estimation of cellulolytic activity

The cellulose clearance zone/hydrolysis halo zone development was best observed in CMC agar media with Gram's iodine dye as compared with CMC agar media with congo red dye and cellulose agar media with Gram's iodine dye. As cellulose being the insoluble substance, Carboxy Methyl Cellulose (CMC) was used as the cellulosic substrate in the medium. It is an amorphous and esterified form of cellulose and considered to be the perfect analogue for carrying out the experiments related to cellulase enzymes (Ponnambalam et al. 2011). It was observed that the intensity and the time taken for development of clearance zone were in the following order: CMC agar with Gram's iodine (3–5 minutes) > Cellulose agar with Gram's iodine (8–10 minutes) > CMC agar with congo

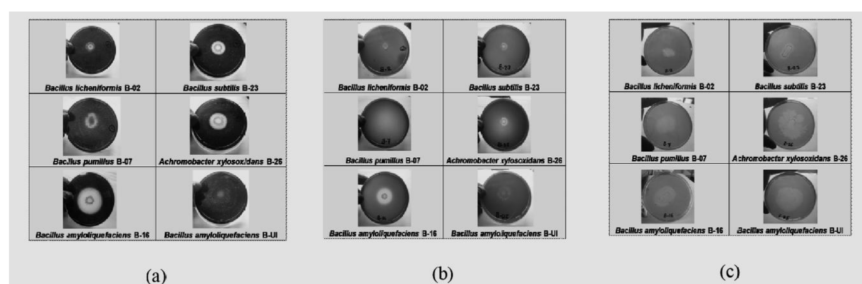

Fig. 2. Development of clearance zone (a) CMC agar with Gram's iodine dye; (b) Cellulose agar with Gram's iodine; (c) CMC agar with congo red dye.

red dye (30–40 minutes) (Figs. 2a, 2b, 2c). The plates flooded with Gram's iodine dye developed quick, prominent and distinct clearance zones around the bacterial colonies showing cellulase enzyme production with dark bluish coloration in the non-hydrolyzed portion of the medium, whereas, the plates flooded with congo red showed hazy and low intensity zone with less differentiation between hydrolyzed and non-hydrolyzed part. Further, congo red is a benzidine-based anionic diazo dye. It causes an allergic reaction and known to be metabolized to benzidine, a human carcinogen. It is a synthetic dye which is very difficult to remove because of its complex aromatic structure. Due to this structure it becomes physically, chemically, thermally and optically stable (Han et al. 2008). This may be the probable reason that this dye might have affected the growth of bacterial strains and thus its cellulase production potential. It is to specify the uniqueness of this work, that usually in CMC agar $NaNO_3$ is generally used but here ammonium tartarate ($C_4H_{12}N_2O_6$) has been used as nitrogen source, which is an organic source. Results indicate that the sources of nitrogen can greatly affect the production of cellulase enzyme. It is reported that good cellulase production can be obtained with the organic nitrogen sources such as $C_4H_{12}N_2O_6$ and yeast extract (Padmavathi et al. 2012). This supports the study that ammonium compounds are the most favorable nitrogen sources for cellulase synthesis (Rai et al. 2012, Rajoka 2004).

Table 3. Indices of relative enzyme activity (I_{CMC}).

Bacterial isolates	CMC agar with GI dye	CMC agar with CR dye	Cellulose agar with GI dye
B-2	1.89 ± 0.047	1.25 ± 0.002	0.95 ± 0.021
B-7	2.04 ± 0.035	1.09 ±0.017	1.00 ± 0.013
B-16	2.86 ± 0.062	2.76 ± 0.072	2.19 ± 0.028
B-23	1.75 ± 0.017	1.33 ± 0.013	1.33 ± 0.011
B-26	3.10 ± 0.120	1.75 ± 0.005	1.28 ± 0.009
B-UI	2.74 ± 0.072	1.15 ± 0.013	0.88 ± 0.017

Cellulase activities on screening media were recorded as the Indices of Relative Enzyme Activity, I_{CMC} i.e. clear or halo zone ratios, which is a measure of carboxy methyl cellulose hydrolyzing capacity (Table 3). I_{CMC} can be calculated as diameter of clearing or halo zone/colony diameter. The diameter of the halo zone is very useful for predicting the enzyme yield, as an aid to select strains with a high level of polysaccharide (such as cellulose) degrading activities. An even more accurate and convenient measure for *in vitro* cellulolytic potential is the clear zone ratio (I_{CMC} index) (Saini et al. 2012). The diameter of halo-zone is also a measure of cellulose digesting ability of bacterial strains (Tomova et al. 2014, Ahmad et al. 2013, Upadhyaya et al. 2012). The maximum cellulolytic activity was seen in the modified CMC agar plates with GI dye. Highest I_{CMC} was shown by B-26 (3.10) followed by B-16 (2.86) and B-UI (2.74), while the minimum cellulolytic activity was shown by B-23 (1.75).

Carboxy methyl cellulase (CMCase) assay of bacterial isolates

Bacterial isolates processed for quantitative analysis produced different levels of glucose concentration in

Table 4. Glucose concentration (CMCase) and enzyme units of bacterial strains by spectroscopy.

Sl. No.	Bacterial strains	Enzyme unit (IU/mL)
1.	B-2	0.00003
2.	B-7	0.00020
3.	B-16	0.00379
4.	B-23	0.00156
5.	B-26	0.00256
6.	B-UI	0.00247

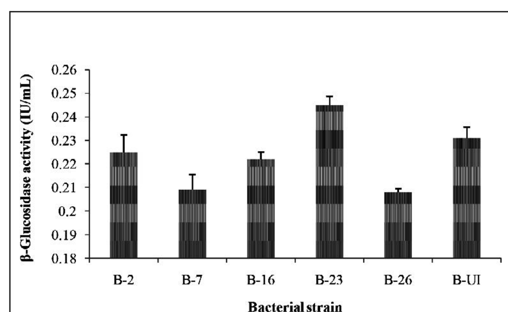

Fig. 3. β-glucosidase enzyme activity (IU/mL) of bacterial strains.

mg/0.5 mL which were determined from glucose standard curve. These glucose concentrations were translated into enzyme concentration in IU (Ghose 1987). All these isolates displayed activity in the range of 0.00003 to 0.00379 IU mL^{-1}. Out of 6 isolates, maximum amount of CMCase enzyme was released by isolate B-16 as determined to be 0.00379 IU mL^{-1} while minimum amount was released as 0.00003 IU mL^{-1} by isolate B-2 (Table 4). Some isolates displayed moderate cellulolytic activities while some showed considerably low activity in this method. By comparing the qualitative and quantitative results of cellulolytic activity of all the efficient isolates, it was found that B-16 isolate (*Bacillus amyloliquefaciens*) had displayed high zone ratio in Gram's iodine assay and maximum amount of enzyme in DNS method. In contrast to this result of B-16 isolate, isolate B-2 had displayed moderate zone ratio in qualitative assay while showed minimum amount of enzyme in quantitative assay. These variation in results may be due to fluctuations in experimental parameters such as pH changes in broth medium, incubation time and temperature because of which the bacteria was not able to release enzyme properly (Lynd et al. 2002).

β-glucosidase assay of bacterial isolates

All these isolates showed activity in the range of 0.208 to 0.245 IU mL^{-1}. Out of 6 isolates processed, the maximum amount of β-glucosidase enzyme was released by isolate B-23 as determined to be 0.245 IU mL^{-1} while minimum amount was released as 0.208 IU mL^{-1} by isolate B-26 as shown in Fig. 3. These are contradictory results with respect to their qualitative, quantitative CMCase activity, quantitative β-glucosidase activity. This variation in results may be due to fluctuations in experimental parameters such as pH changes in broth medium, incubation time, substrate and temperature because of which the bacteria was not able to release enzyme properly (Lynd et al. 2002). This finding is supported by previous reports that showed that small variations in carbon/nitrogen sources, pH, macro and micro- nutrients, temperature, and time of incubation induced differences in microorganism growth (Ahamed and Vermette 2008, Hanif et al. 2004).

Conclusion

The study can be concluded with two key points: (i) Using Gram's iodine dye method is rapid, safe and efficient in comparison to congo red dye method. (ii) Microorganisms favor organic nitrogen sources for cellulase synthesis. The present study confirmed that the use of Gram's iodine dye shows prominent clearance zone, and thus making simple, easy and rapid screening of cellulase-producing bacterial isolates. Further, it avoids the use of toxic chemicals. This research work also concluded that isolated bacterial species showed the remarkable ability to degrade cellulose in the modified CMC media containing organic nitrogen source.

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