Environment & Ecology 37 (1) : 62—70, January—March 2019
Website: environmentandecology.com ISSN 0970-0420

A Search for Cellulose Degraders in the Indian Soils

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Received 9 July 2018; Accepted 12 August 2018; Published on 1 September 2018

Abstract Microbial cellulases can be used to convert the lignocellulosi cbiomass to chemicals, but this technology is not commercially viable because of low conversion efficiency of the currently available enzymes. This work aimed at isolating bacteria capable of efficiently degrading cellulose under highly alkaline conditions. Bacteria were isolated from soil obtained from different climatic zones. Low osmolarity media containing either Carboxymethyl cellulose or Avicel at pH 10 were used for screening of cellulase producers. The isolates were identified based on the 16S rDNA sequences. The cellulolytic activities of the isolates at different temperatures and pH were studied. Five isolates from 39 were shortlisted for further investigation. Their ability to degrade different types of cellulosic substrates was assessed. Molecular weights of the endoglucanases secreted by the isolates were determined using SDS- PAGE. These studies suggest that they have potential for industrial or environmental applications.

Keywords Alkaline cellulase, Plant biomass, Alkali pretreatment, Saccharification.

Introduction

Hydrolysis of lignocellulosic biomass can be achieved by chemical breakdown of glycosidic bonds at high temperatures, or by microbes at lower temperatures through a concerted action of several enzymes including cellulases (Obeng et al. 2017). Though fungi do secrete large amounts of cellulases, bacteria have their own advantages with smaller growth times and robust enzymes. Microbial enzyme systems from fungi have been found to complement those from bacteria, increasing biomass degradation efficiency (Resch et al. 2013).

To meet the growing demand for cellulases and to realize their full potential in a variety of industrial applications, continued search for enzymes with novel properties is necessary. Cellulose swells under alkaline conditions and at high temperatures, thus making the strands more accessible to breakdown by enzymes (Horikoshi K 1999). Hence thermophilic and alkalophilic microorganisms would show promise in industrial applications. This present work aimed to isolate cellulolytic bacteria capable of growing in extreme alkaline conditions.

(This research was partially supported by funding

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from University Grants Commission, Government of India).

Materials and Methods

Isolation, characterization and selection of cellulase producers

Soil samples from the black-soil region of Hulkoti, Gadag, Karnataka State wete collected (at about $15^0$25´40´´ N, $75^0$31´50 ´´ E). The pH of the soil samples determined in the laboratory was found to be 4.0-4.5. Soil samples were also collected from a traditional organic compost spread on a rice field in Yerlapady, Karkala, Karnataka State (at about $13^0$18´ 03´´ N, 74^0 56´49´´ E). The pH of this soil was found to be 6.5-7. Soil sample from Vadodara, Gujarat (at about $22^0$18´0´´ N, $73^0$12´0´´ E) was also used. The texture of soil was granular as desert soil. The pH of this soil was found to be 5.5-6.

Low osmolarity medium (LOM) contained yeast extract 5g/L, tryptone 5g/L, NaCl 200 mg/L, Na_2CO_3 200 mg/L, Agar 20g/L. Avicel Congo red agar (ACR) contained peptone 2g/L, $MgSO_4$ 0.25g/L, K_2HPO_4 0.3g/L, Na_2CO_3 0.2g/L, Avicel 2 g/L, Congo red 0.2 g/L. Carboxymethyl cellulose (CMC) Congo red agar (CCR) contained the same ingredients as above, except that Avicel (Sigma) was replaced by CMC (ICN chemicals). A 1:10 dilution of soil samples in sterile distilled water was spread on sterile LOM-Avicel agr, LOM-CMC agar, ACR and CCR agar plates at pH 7 and 10 and incubated at 25^0C, 37^0C and 50^0C for 24 to 48 h.

Colonies from LOM-Avicel and -CMC agar plates were gridded on to fresh media plates of the same type. After incubation, the growths were scraped off the plate under gentle flow of water. The plate devoid of any growth was stained with 0.5% Congo red solution for 15 minutes and then destained with 1.5M NaCl till a clearance surrounding the colony was seen (Mazeau and Wyszomirski 2012). Cellulolytic colonies were picked and subcultured to obtain pure cultures. Screening for cellulose degraders was also carried out on CCR and ACR plates. The isolates with a clear zone around them were streaked on fresh CCR plates to obtain single cell colonies. Stock cultures of the cellulolytic isolates were made in SOB (Super Optimal Broth : Yeast extract extract 5 g/L, tryptone 20 g/L, NaCl 500 mg/L, KCL 160 mg/L, $MgSO_4$ 2.46 g/L, Agar 20g/L) medium containing 50% glycerol (v/v) and stored at -20^0C.

Morphological and biochemical characterization of the isolates

The isolates were studied for their Gram nature, morphology and presence of spores. Biochemical characterization of the isolates was carried out using Biomerieux Vitek 2 cards.

Amplification of 16S rRNA, sequencing and analyses

Genomic DNA was isolated from the organisms using Marmur's method (Marmur 1961). The amplification of 16S rRNA was performed using universal 16S rDNA primers – OF 5´-GTGTAGCGGTGAAATG-CG-3´, and OR 5´-GGTGGAGCATGTGGTTTA-3´ (Sauer et al. 2005). PCR amplification was performed with a thermocycler (Eppendorf) using the following conditions : An initial denaturation at 94^0C for 5 min, followed by 40 cycles of denaturation (30 sec at 94^0C), annealing (1 min at 55^0C) and extension (1 min at 72^0C). The final extension was at 72^0C for 10 min. The PCR product was gel purified by GeneJet Gel Extraction kit (Fermentas) and analyzed by agarose gel electrophoresis. The purified PCR product was sequenced and compared with NCBI database.

Temperature and pH tolerance of the cellulolytic isolates

The ability of the isolates to grow and produce cellulases at different pH and temperatures was investigated. The cultures were inoculated as grids on sterile CCR and LOM-CMC agar plates at different pH values from 3 to 12 and incubated at temperatures ranging from 23^0C to 50^0C. The CCR plates were observed for growth and zones of clearance for up to 48 h. After incubation of the LOM=CMC plates for 48 h, they were stained with 0.5% Congo red solution and destained with 1.5M NaCl until clear zones around the colonies was seen.

Assay for cellulases

Avicel binding assay

This qualitative assay was designed to compare the affinity of the cellulases from different isolates to the substrate. Selected isolates were grown in sterile SOB-CMC medium pH 10 for 5 days at 37^{0}C. Cultures were spun to separate the pellet. The supernatant was incubated with 10 mg and 50 mg Avicel for 10 min at 50^{0}C. After incubation, the mixture was spun to pellet down the Avicel Supernatant of these assay sets (5µL), along with untreated supernatant, were spotted on CMC assay plates at pH 5, 7 and 10, incubated at 55^{0}C for an hour. The plates were stained with 0.1% Congo red, followed by destaining with 1M NaCl.

Endoglucanase assay

CMCase activity was determined (Ghose 1987). All the isolates were grown in sterile SOB medium at pH 10.0 containing 0.5% CMC for 3 days on shaker at 37^{0}C. Thereafter the cultures were spun to separate the cells and the cell-free supernatant was used for endoglucanase assay. The endoglucanase activity of the cultures was quantified using 2% CMC made in buffers. The following buffers at 50mM concentrations were used: Na-citrate buffer pH 4.8, Na-phosphate buffer pH 7 and glycine-NaOH buffer pH 9. The supernatants (0.5ml) were mixed with the substrate (0.5mL) in buffers at different pH. Release of reducing sugars in 1 h at 50^{0}C was measured by DNSA method (Miller 1959). A unit of activity is defined as the amount of enzyme required to liberate 1µmol of glucose per min under assay conditions. Protein content of culture filtrates was determined by Folin- Lowry method (Lowry et al. 1951) using Bovine Serum Albumin (BSA) as standard.

Total cellulase assay

Total cellulase assay was carried out for five selected isolates on filter paper, acid-treated paddy straw and alkali-treated coir. Paddy straw and coir were soaked in 1.0N HCl and 1.5% NaOH for a day, respectively, then washed with water and dried. Three-day-old culture supernatants of the isolates were used for the assay. Whatman filter paper (50mg) suspended in 1 mL phosphate buffer pH 7 was mixed with 0.5mL supernatant and incubated at 50^{0}C for an hour. Treated coir and paddy straw (50-100mg) were incubated in separate tubes with 1mL of the culture supernatants. To assess synergism between the cellulases, substrates were incubated with a mixture containing 1mL of all the five supernatants. Following incubation at 50^{0}C for 1 h, 1 mL sample was removed from the assay tubes and mixed with 3ml DNSA. The tubes were then heated in a boiling water bath for exact 5 minutes followed, by immediate chilling on ice. The reaction mixture was diluted as per the protocol and the absorbance was read at 540nm.

Concentration and enrichment of the enzyme

The concentration of the cellulase from the culture supernatants was attempted with organic solvents. Selected isolates were grown in SOB-CMC broth, pH 10 at 37^{0}C on shaker for 72 h, after which the broth was centrifuged to separate the pellet. Supernatants were mixed with different organic solvent to get final solvent concentrations between 20 and 90%. The precipitation was carried out on ice with chilled supernatants and solvents. After the treatment, the precipitated proteins were separated by centrifugation at 4^{0}C. The pellets were dried at -20^{0}C and reconstituted in 50mM Na-acetate buffer at pH 4.8. The supernatant left after precipitation and the reconstituted pellet were spotted on CMC assay plates at pH 5, 7 and 10. These CMC assay plates were incubated at 37^{0}C for 3 h or overnight, stained with 0.1% Congo red and destained with IM NaCl.

Stability studies of the cellulases in the supernatants

Qualitative studies were carried out to evaluate the stability of the enzymes at high temperatures and in the presence of ionic detergent. Isolates were grown in SOB containing 0.2% CMC pH10.0 for 48 h at 37^{0}C. The cultures were spun at 14,000 rpm for 15 min. Aliquots of the supernatants were incubated at RT, 40^{0}C, 60^{0}C, 80^{0}C and 100^{0}C for 10 min. In separate tubes, SDS was added to the supernatants to get a final concentration of 0.1% and incubated at the same temperatures for 10 min. All treated samples were

spotted on CMC assay plates. Untreated supernatant acted as a control.

Effect of additives on MI-41 and 7A-31 supernatant (qualitative assay)

Qualitative assay was carried out with ethanol-concentrated culture supernatants of M1-41 and 7A-31. Concentrated enzyme (10 µL) was mixed with appropriate amount of the effect or compound and the volume was made up to 50µL with 10mM Na acetate buffer pH 5.0. This mixture was incubated at 37^{0}C for 10 min, centrifuged and 5µL of the supernatant was spotted on CMC assay plates at pH 5, 7 and 10. The compounds tested were $CaCl_2$, $MgSO_4$, $K_2Cr_2O_4$, $CdSO_4$, $CoSO_4$, $CuCl_2$, $MnCl_2$,$HgCl_2$, Pb $(NO_3)_2$, $ZnSO_4$, Citric acid, EDTA, DTNB and DTT. The final concentrations used were between 1mM to 100mM.

Determination of molecular weight and pI of the cellulases

Three-day-old cultures of 50-01, 7A-31, M1-41, B3-51 and 50-09 grown in SOB-CMC broth were spun to separate the pellet. The supernatants were run on SDS-PAGE with and without 0.2% CMC. Pre-stained molecular weight ladder was run along side the samples. After the run was complete the CMC containing gel was washed with 50mM phosphate buffer and then incubated at 50^{0}C for 3-4 h followed by staining with 0.1% Congo red and destaining with IM NaCl. The other get was stained for proteins with Coomasie blue R-250.

For determination of pI, 1% agarose gels containing 0.2% medium viscosity CMC with centrally located wells were made in 50mM Na acetate buffer

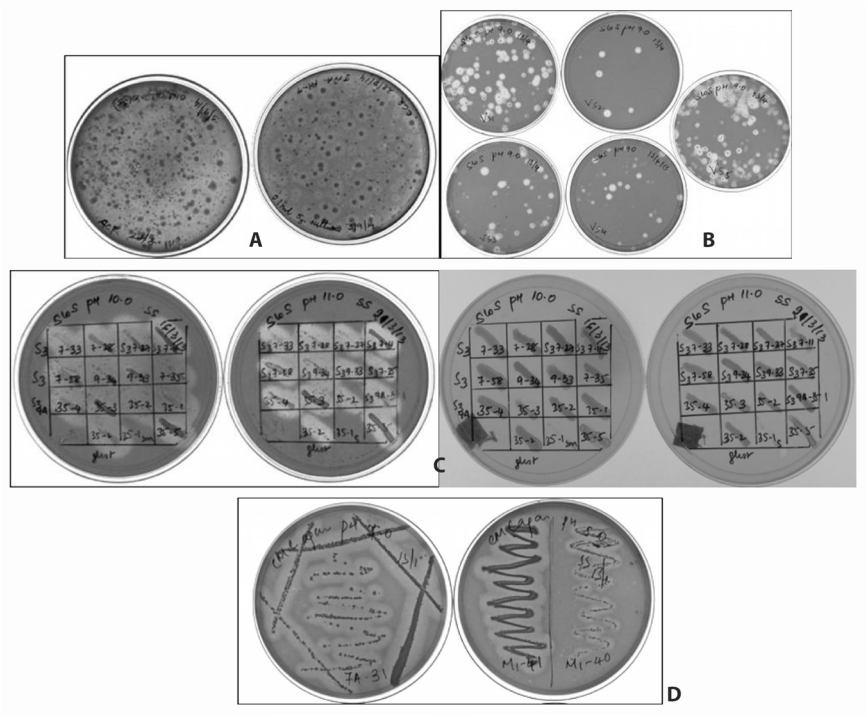

Fig. 1.A. Gadag soil suspension plated on ACR pH 9. **Fig. 1. B.** Screening of the Vadodara soil sample. **Fig. 1. C**. Positive isolates from Gadag sample gridded on LOM-CMC media at pH 10 and 11. LHS-after staining with Congo red. RHS- Before staining with Congo red. The cellulolytic isolates were gridded on LOM-CMC media and after 24 h of growth, the plate was scrapped free of growth and stained with 0.2% CMC. Destaining was done with 1M NaCl till yellow to colorless transparent zones appeared. Some zones as seen in LHS pic is much more diffused than the streak as compared to others. **Fig. 1. D**. Growth of M1-40, 41 on CCR plate pH 7 (cellulase activity seen as clearing on the plate).

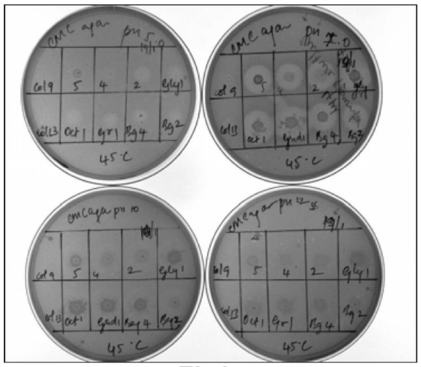

Fig2

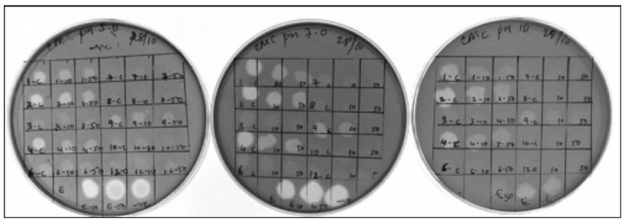

Fig3

Fig. 2. Growth and cellulolytic activity of some isolates on CCR at pH 5.7, 10, 12 incubated at 45^{0}C for 24 h. At pH 12, though the growth was not very prominent, cellulolytic activity (as diffused doscolored zones) could be easily seen on the CCR plates. **Fig. 3.** Avicel binding assay with isolates 50-1, -12 on CMC assay plates at pH 5,7,10. 1-C indicates isolate 50-01 crude sup as control, 1-10 indicates isolate 50-01 supernatant with 10 mg Avicel and 1-50 indicates supernatant with 50 mg Avicel and so on. E-10, -50, indicates *A. niger* enzyme (1 mg/mL, Sigma) with 10 mg, 50 mg Avicel and -ve is enzyme without Avicel. For isolate 50-01, as the amount of Avicel increased the residual enzyme activity reduced.

pH 5, and Tris buffer pH 8.8, separately. Concentrated supernatants (10 µL) mixed with 2µL of gel-loading dye (prepared by mixing 250µL of saturated bromophenol blue with 750µL of glycerol) were loaded in the central wells. The gels were run at 100V, 50mA for 1 h in 50mM respective buffers. After the run, they were incubated at 37^{0}C overnight, followed by staining with 0.2% Congo red and destaining with 1M NaCl.

Results

Isolation, characterization and selection of cellulase producers

Screening of one of the soil samples from Mangalore (M1) yielded nine interesting cellulolytic bacterial isolates, whereas one of the Gadag soil samples (S_3) yielded 12 isolates (Fig. 1). Similarly, a search for organisms capable of cellulose catabolism at high pH and high temperature using another Mangalore sample yielded 18 isolates. All of these isolates were assessed for their ability to grow at different pH and temperature.

Temperature and pH tolerance of the cellulolytic isolates

All the 39 cellulolytic isolates grew at 37^{0}C, in media of pH between 5 to 10. Twenty isolates could grow upto 50^{0}C in media at pH 10. Some isolates could grow at extreme alkaline conditions (pH 12) as shown in the Fig. 2.

Morphological, biochemical characterization of the isolates and 16S rRNA analyses

On the basis of morphology,Gram staining,Vitek 2 results (Biomerieux) and 16S rRNA analyses, it was concluded that majority of the isolates were Gram positive rods and belonged to the genus *Bacillus.* Of the five isolates selected for further study, 50-09 and 7A-31 belonged to Genus *Bacillus.* M1-40 and M1-41, which were highly motile Gram-negative rods belonged to the genus *Cupriavidus.* Whereas 50-01, highly motile, sporulating, Gram-positive short rods was found to be *Paenibacillus.*

PCR amplification of the 16S rRNA gene using partial sequence primers yield a DNA fragment of 705 bp. The PCR products were purified and sent for sequencing. The sequences obtained were com-

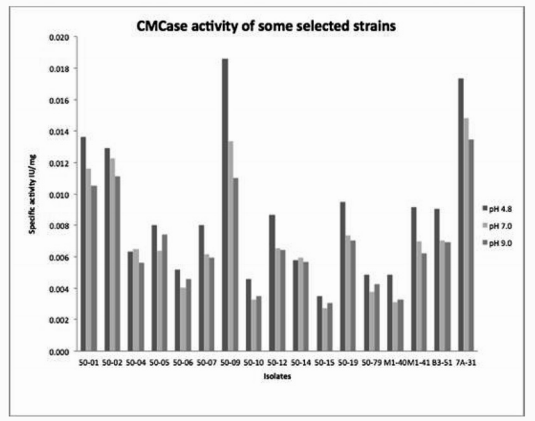

Fig. 4. Endoglucanse activity of the isolates were carried out with crude supernatants and 2% CMC made in 50mM buffers at three different pH namely 4.8, 7 and 9.

pared with the database in the NCBI repository. The GenBank accession numbers for the 16S rDNA sequences are: isolate 50-01 – MH675921; M1-40 – MH715291; M1-41 – MH715396-MH715397; B3-51 –MH715398-MH715399; and 7A-31–MH715296.

Avicel binding assay

Culture supernatants from the isolates that grew at 50^{0}C in alkaline medium were incubated with 10mg and 50mg of Avicel. The supernatants after Avicel incubation were spotted on CMC assay plates at pH 5,7, and 10. There was reduced activity seen in the supernatant after incubation with Avicel. The reduction in the activity correlated with the amount of Avicel, i.e., more the Avicel, less was the enzyme activity left in the supernatant. Isolates 50-01, 2, 4, 5 and colony 13 showed considerable decrease in activity with increasing substrate presence (Fig. 3), indicating good binding ability or affinity of the enzyme.

Endoglucanase assay

Based on the endocellulase activity of the isolates at pH 4.8, 7 and 9, five promising isolates were selected namely 50-01, 50-09, 7A-31, M1-41 and B3-51 for further studies (Fig. 4).

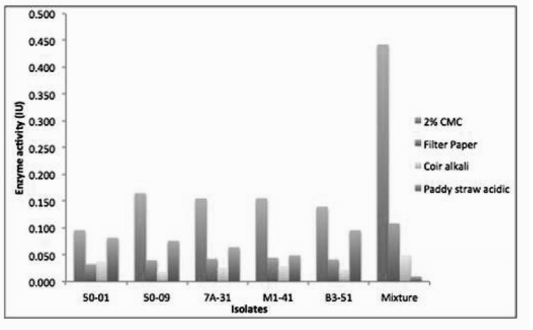

Fig. 5. The crude supernatants of isolates were assayed on 2% CMC, Whatman filter paper, alkali treated coir and acid treated paddy straw. One unit of enzyme activity (IU) is the amount of enzyme required to liberate 1 µmol of glucose per min at 55^{0}C.

Total cellulase assay

The enzyme from culture supernatants of the five selected isolates were assayed on four different substrates namely CMC, filter paper, alkali-treated coir, acid-treated paddy straw. The cellulases from all the isolates showed good endoglucanase activity on CMC. Acid-treated paddy straw acted as a good substrate as well and the total cellulase activities for the isolates (in IU/ml/min) were as follows : 50-01– 0.081; 50-09 – 0.076; 7A-31 – 0.064; M1-41 – 0.049 and B3-51 – 0.096. There was a notable increase in the sacharification (Fig. 5) when supernatants from all these five isolates were used together indicating good synergism between these enzymes.

Stability studies of the cellulases in the supernatants (qualitative)

Cellulase from isolate 50-01 was found to be stable at 60^{0}C for 10 min with and without 0.1% SDS. With increasing temperature, there was reduction in activity. M1-41 cellulases were found to be stable at 40^{0}C for 10 min even in the presence of 0.1% SDS. At 60^{0}C and 0.1% SDS, there was a major loss in M1--41 enzyme activity (Fig. 6).

Concentration of the enzyme from the supernatants

Precipitation or concentration of the enzyme from culture supernatants was attempted using organic

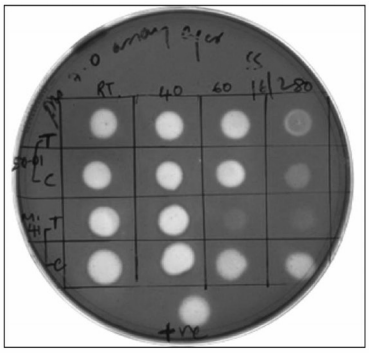

Fig6

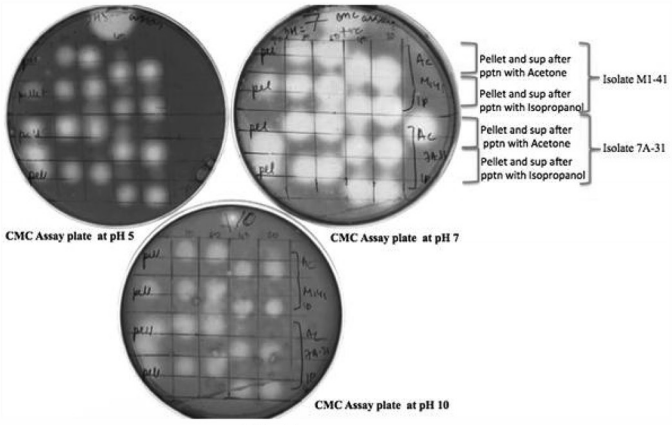

Fig7

Fig. 6. Stability of crude enzyme made by isolates 50-01 and MI-41 to high temperature in the presence of 0.1% SDS. The stability of the isolates to high temperatures in presence of 0.1% SDS differed, as can be seen in the picture. Isolate 50-01 was stable at 60^0C whereas for M1-41, some enzyme activity is lost at 60^0C with and without the SDS."+" indicates 3µL of *A. niger* cellulase (1 mg/mL).
Fig. 7. Precipitation of 7A-31 and M1-41 enzymes in the crude extract with Acetone and isopropanol.

solvents. The enzyme activity in the supernatant and pellets at increasing organic solvent concentrations were assayed qualitatively on CMC assay plates. At lower concentrations of ethanol (20%), all the cellulase activity was seen in the supernatant. As the concentration of ethanol increased, more and more cellulase activity was found in the precipitates. The enzyme secreted by M1-41 was seen to precipitate between 60-80% concentration of ethanol, whereas the cellulases made by 7A-31 precipitated at the ethanol concentration between 80-90% . The cellulases made by 50-01 precipitated at the ethanol concentration between 80-90%. The final pH of the culture broth did not affect the concentration of ethanol needed for precipitation and concentration of the cellulases. Acetone and isopropanol precipitated the enzymes in both 7A-31 and M1-41 supernatants at 60% concentration (Fig. 7).

Effect of additives on M1-41 and 7A-31 supernatant (qualitative assay)

Preliminary studies showed that the cellulases made by isolates 7A-31 and M1-41 are very study. As shown divalent ions like Ca^{+2}, Mg^{+2} (upto 100mM), Mn^{+2} did not have much effect on the enzyme activity. Whereas, Cu^{+2}, Cr^{+2}, Zn^{+2} (10mM) did show a moderate inhibition of enzyme activity. Heavy metal Hg^{+2} (1mM) inhibited the enzyme completely whereas Pb^{+2} at the same concentration did not affect both the enzymes. A summary of the effect of individual additives on the enzymes is given in Table 1.

Determination of molecular weight and pI of the cellulases

Zymogram analyses of supernatants of the isolates 50-01, 50-09, M1-41, B3-51 run on a 10% SDS-PAGE gel containing CMC indicated that the approximate molecular weights were 50-01; 54-70 kDa, 50-09: 90-125 kDa, 7A-31, M1-41 and B3-51 : 70-90 kDa (Fig. 8).

Supernatants from the two isolates, 7A-31 and M1-41, show the presence of two cellulases when run on CMC-agarose gels at pH 5 and 8.8 (Fig. 9). They both have a Component 1, which migrates towards anode at pH 8.8 and therefore has a pI of more than 8.8. Component 2 migrates towards cathode at pH 5.0 and therefore has a pH of less than 5.0. Component 1 is active at alkaline pH, while component 2 is active at acidic pH for both the isolates.

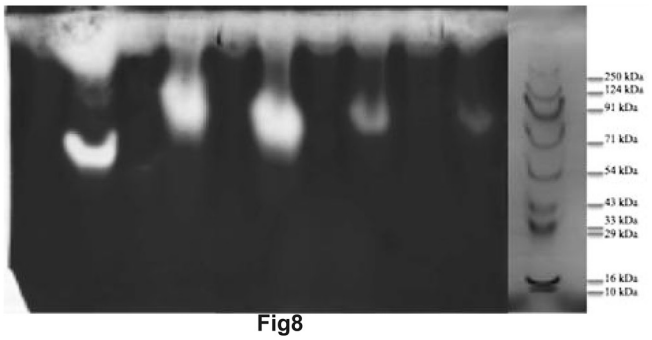

Fig8

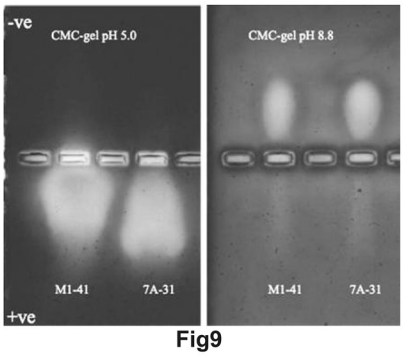

Fig9

Fig. 8. Crude enzymes run on 10% SDS PAGE. L2 :50-01. L4 : 50-09, L6: 7A-31, L7: Ladder, L8: M1-41, L10: B3-51. **Fig. 9.** Isoelectric pH of M1-41 (lane 2) and 7A-31 (lane 4) cellulases.

Discussion

Microbial cellulases have found numerous applications in the industry including pulp and paper, textile, food and renewable energy (Horikoshi 1999, Yeoman et al. 2010, Ben Hamad and Gargouri 2017). This work presents isolation of bacteria that grow and produce cellulases at alkaline pH and high temperatures.

Table 1. Summary of the effect of additives, heavy metals, chelators and detergent on the cellulase from isolates 7A-31 and M1-41 (qualitative assay). The table is a summary of the enzyme activity on CMC assay plates with different effectors at the concentrations indicated. Maximum enzyme activity is marked as 5. Complete inhibition as 0. Range of 1 to 4 indicates intermediate level of inhibition of enzyme activity as compared to the untreated enzyme control.

Additive	Concentration	7A-31	M1-41
$CaCl_2$	100mM	4	4
	500 mM	4	1
$MgSO_4$	100mM	5	5
	500 mM	5	5
$K_2Cr_2O_4$	1mM	4	4
	10mM	3	4
$HgCl_2$	1mM	0	0
	5mM	0	0
$Pb(NO_3)_2$	1mM	5	5
	5mM	5	5
$MnCl_2$	10mM	5	5
$CuCl_2$	10mM	4	4
Citric acid	100mM	5	5
$CdSO_4$	50mM	2	2
DTNB	2mM	5	5
DTT	100mM	4	5
EDTA	100mM	4	5
$ZnSO_4$	100mM	3	3
$CoSO_4$	100 mM	1	0

The screening of soil samples in this study yielded some isolates that could grow at pH 12. The isolates were identified by morphology, Gram staining and 16S rRNA sequencing. Some of these isolates included strains from genus *Cupriavidus, Bacillus, Paenibacillus.* The cellulases made by the isolates were found to be extracellular. All the five isolates possess good endoglucanase activity as seen by its activity on CMC. The isolates are truly cellulolytic as they showed good saccharification of acid-treated paddy straw. Most bacterial species that are used for cellulase production show growth and synthesis of enzyme only up to pH 9. Kim et al. (2005) reported cellulolytic *Bacillus subtilis* strains capable of growing upto pH 10. Acharya (2012) isolated thermophilic *Aneurinibacillus thermoaerophilus* WBS2 capable of growing at pH 9 and 65^{0}C.

Bhattacharya et al. (2015) had proposed that one of the ways of making lignocellulosic technology cost effective would be incorporation of novel enzymes in to cellulosomes, making designer cellulosomes and a synergy between bacterial and fungal cellulases. The highlight of this study was that the crude cellulases made by the bacterial isolates act synergistically in a mixture giving three-fold more saccharification than that achieved individually. Inhibition of M1-41 and

71-31 cellulases with Hg^{+2} indicates that they possess an -SH group at the active site. Having enzymes which can resist high concentrations of divalent ions, lead, chelators and detergents certainly indicates novelty of the isolates and should be candidates for further extensive study.

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