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ORIGINAL PAPER

# Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA

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**Abstract** The present study investigated the cultivable mesophilic (37°C) and thermophilic (60°C) cellulose-degrading bacterial diversity in a weathered soil-like sample collected from the deep subsurface (1.5 km depth) of the Homestake gold mine in Lead, South Dakota, USA. Chemical characterization of the sample by X-ray fluorescence spectroscopy revealed a high amount of toxic heavy metals such as Cu, Cr, Pb, Ni, and Zn. Molecular community structures were determined by phylogenetic analysis of 16S rRNA gene sequences retrieved from enrichment cultures growing in presence of microcrystalline cellulose as the sole source of carbon. All phylotypes retrieved from enrichment cultures were affiliated to *Firmicutes*. Cellulose-degrading mesophilic and thermophilic pure cultures belonging to the genera *Brevibacillus*, *Paenibacillus*, *Bacillus*, and *Geobacillus* were isolated from enrichment cultures, and selected cultures were studied for enzyme activities. For a mesophilic isolate (DUSELG12), the optimum pH and temperature for carboxymethyl cellulase (CMCase) were 5.5 and 55°C, while for a thermophilic isolate (DUSELR7) they were 5.0 and 75°C, respectively. Furthermore, DUSELG12 retained about 40% CMCase activity after incubation at 60°C for 8 h. Most remarkably, thermophilic isolate, DUSELR7 retained 26% CMCase activity at 60°C up to a period of 300 h. Overall, the present work revealed the presence of different cellulose-degrading bacterial lineages in the unique deep subsurface environment of the mine. The results also have strong implications for biological conversion of cellulosic agricultural and forestry wastes to commodity chemicals including sugars.

**Keywords** Cellulose-degrading bacteria · DUSEL · Deep subsurface · Thermostable enzymes · Gold mine

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## Introduction

Cellulosic waste-materials including agricultural, forestry, and municipal wastes are among the Earth's most abundant and available renewable resources. Several federal agencies including the National Science Foundation, the US Department of Energy (DOE), and the US Department of Agriculture (USDA) are strongly expanding the role of biomass (all plants and plant-derived materials) as an alternative energy source to reduce the need for oil and gas imports; as a way to promote the growth of agriculture, forestry, and rural economies; and to foster biorefineries for generating biofuels and other value-added chemicals. In April 2005, a joint study conducted by DOE and USDA reported 1.3 billion dry tons per year of biomass potential which is enough to produce biofuels to meet more than one-third of the current demand for transportation fuels [39]. However, the heterogeneous and recalcitrant nature of cellulosic wastes present a major obstacle for conventional conversion processes in energy generation and environmental restoration

[25]. One of the best strategies for rapid release of fermentable sugars from cellulosic waste-materials is to develop thermostable enzyme systems. The operation of a thermophilic reactor consumes more electrical energy than a low temperature reactor; however, a high temperature reactor enables much improved hydrolysis of cellulosic substrates in a shorter retention period [37, 42]. Thus thermophilic cellulose-degrading bacteria (CDB) and their enzymes for bioenergy conversion processes are key targets in the development of alternative fuels including sugars and bioethanol.

CDB have been isolated from various environments such as compost systems [22, 43], soils [1, 21], wastewaters [40], and thermal springs [19]. However, to date there is no report of CDB from the deep terrestrial subsurface environments such as those exemplified by ultra-deep mines which represents an emerging area for exploring the microbial populations with bewildering arrays of metabolic capabilities [10]. These mines provide unique extreme environments for microorganisms, both natural and anthropogenic, including extreme temperature, pressure, low oxygen concentration, toxic metals, and pH. In fact culture-dependent and -independent surveys of gold mines in Japan [15], South Africa [31] have shown active microbial populations composed of diverse groups of microorganisms with unusual physiological properties. These reports also showed the existence of numerous novel and yet uncultured microbes representing a hitherto unidentified diversity.

The Homestake gold mine (44°35′2074′′N, 103°75′ 082′′W) is the deepest mine (2.4 km deep) in North America and had the largest gold deposit ever found in the Western Hemisphere. A full description of the mine is located at the Lawrence Berkeley National Laboratory, CA, website (http://www.lbl.gov/nsd/homestake/Reference.html). The mine was closed in December 2001 after more than 125 years of mining. On 10 July 2007, the National Science Foundation, USA selected this mine as a Deep Underground Science and Engineering Laboratory (DUSEL) site. This gold mine offers a unique opportunity for direct exploration of the deep subsurface environment. During active mining-operations for over 125 years, exogenous (surface) microbes and lignocellulosic substrates were introduced into the extreme deep subsurface environment of the mine. It would be interesting to speculate that interactions between the exogenous and indigenous microbes might have induced gene alterations through fortuitous mutations and lateral gene transfers resulting in highly stable enzymes and proteins capable of significant geochemical functions in situ and potentially adaptable to biotechnological applications. Thus, it is believed that the Homestake gold mine represents a most promising source for high-value microbes and microbial enzymes including those capable of degrading lignocellulosic biomass. Therefore, the objectives of the present study were the molecular characterization of mesophilic and thermophilic cellulose-degrading enrichment cultures and isolation and characterization of isolates and their enzymes involved in cellulose degradation from the unique settings of the Homestake gold mine.

## Materials and methods

### Sample collection and chemical characterization

Weathered soil-like samples were aseptically obtained along the Yates shaft of the Homestake gold mine at a depth of 1.5 km. These samples were collected directly from the build up on the shafts that were not disturbed for a long time. Similar method has been used earlier for collecting the rock samples in South African gold mines [31]. Care was taken to minimize any possible exogenous microbial contamination during sampling and post-sampling processing. The samples were transported to the laboratory in sterile polypropylene tubes on ice and stored at –80°C until analysis. Elemental composition of the weathered soil-like sample was characterized at the Geo Analytical Laboratories (Washington State University, Pullman, WA, USA) by X-ray fluorescence spectroscopy (XRF) using the method as described earlier [33].

### Enrichment and isolation of mesophilic (37°C) and thermophilic (60°C) cellulose-degrading bacteria

Weathered soil-like materials were used to isolate cellulose-degrading mesophilic and thermophilic bacteria. The medium contained (per liter): 0.1 g nitrilotriacetic acid, 1-ml $FeCl_3$ solution (0.03%), 0.05 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.01 g NaCl, 0.01 g KCl, 0.3 g $NH_4Cl$, 1.8 g of 85% $H_3PO_4$, 0.005 g methionine, 0.05 g yeast extract, 0.01 g casamino acids, and 1-ml of Nitsch's trace element solution [44]. The medium was supplemented with cellulose (microcrystalline; 20 μm particle size, 0.5 g/l) as sole source of carbon. Eighteen grams of nutrient-free agar were added per liter of medium to prepare agar plates for isolation of pure cultures. The pH of the medium was adjusted to 7.0 before autoclaving using 10 M NaOH. One gram of soil samples was inoculated in 125-ml serum bottles containing 50-ml of pre-sterilized minimal growth medium. The bottles were sealed with butyl rubber stoppers, and crimped with aluminum seals. The enrichments were performed by incubating the serum bottles at 37°C and 60°C in an incubator shaker (120 rpm) for 6 days. Triplicate serum bottles were used for each enrichment experiment, and controls included were: (1) soil samples

autoclaved at 121°C, (2) soil-free controls, and (3) cellulose-free controls. Periodically, 1-ml samples were aseptically removed by syringe and needle and analyzed for total cell protein to measure the bacterial growth using a quantitative colorimetric Coomassie assay as described previously [36] and cultures showing growth were transferred into fresh medium. This process was repeated five times prior to initiating the isolation of pure cultures.

After 6 days of incubation, the fifth generation growing mixed cultures were used to isolate pure mesophilic and thermophilic CDB by serial dilution method using sterilized saline water (0.85% NaCl). An aliquot (100-µl) of each dilution was spread on agar medium in duplicate, containing cellulose as sole source of carbon, and incubated at 37°C and 60°C for 5–6 days. The plates were sealed with a polyvinylchloride electrical insulation tape to prevent evaporation during high temperature (60°C) incubation. The isolates were streaked repeatedly on agar plates to ensure purity. An uninoculated control was included to check for any microbial contamination.

## Molecular characterization of mixed enrichment cultures and isolates

### DNA extraction, PCR, and clone library construction

For molecular characterization of mixed cellulose-degrading enrichment cultures and isolates grown at 37°C and 60°C, 16S rDNA cloning and sequencing analyses were performed. DNA extraction was performed using the method described by Zhou et al. [47]. Total DNA extracted from the enrichment cultures growing at 37°C and 60°C was used to establish clone libraries using universal 16S rRNA gene primers 530F (5′-GTCCCAGCMGCCGCGG) and 1490R (5′-GGTTACCTTGTTACGACTT) with cycling conditions as described earlier [26]. Identification of isolates was performed by amplifying almost full-length 16S rRNA genes using bacteria-specific primers 8F (5′-AGAG TTTGATCCTGGCTCAG) and 1492R (5′-GGTTACC TTGTTACGACTT) with cycling conditions as described earlier [34]. The 50-µl polymerase chain reaction (PCR) mixtures were composed of 1.5 mM $MgCl_2$, 0.4 mM of each dNTPs, 25 pmol of each primer, ~100 ng of template DNA, 0.5 U of *Taq* DNA polymerase (New England Biolab, Ipswich, MA, USA), and 10x reaction buffer supplied by the manufacturer. A control PCR without DNA was set up to check for nonspecific amplification. PCR products of expected size (~950 bp) obtained from enrichment cultures were cloned in pGEM-T Easy Vector and transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA) as per manufacturer's protocol. Plasmids were isolated using a plasmid extraction kit (Qiagen, Valencia, CA, USA) and nucleotide sequences of cloned genes were determined in an automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) for all the correct-sized clones in a library. PCR products obtained from isolates were sequenced and analyzed directly without cloning.

### Phylogenetic and statistical analyses of clone libraries

The 16S rRNA gene sequences were checked for the presence of any anomaly by Mallard at 99.9% cut-off line [2]. The similarity searches for sequences were carried out using the BLAST (N) program of the National Center of Biotechnology Information, MD, USA, and alignment was carried out using the CLUSTALW program available at European Molecular Biology Laboratory, Cambridge, UK. A Jukes-Cantor corrected distance matrix was constructed by DNADIST program of PHYLIP [11] which was used to assign sequences in various operational taxonomic units (OTU) using DOTUR at 97% sequence similarity cut off [38]. The phylogenetic trees were constructed using neighbor-joining method by MEGA v 3.1 [20]. Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies. Statistical parameters e.g. randomized rarefaction curves and Shannon-Weaver ($H'$) index, with 95% confidence intervals (CI) were calculated to estimate the diversity of phylotypes using DOTUR.

### Nucleotide sequence accession numbers

The sequences generated in this study were deposited in GenBank. The 16S rDNA sequences retrieved from enrichment cultures were assigned accession numbers EU008334-EU008394 (37°C enrichment) and EU008395-EU008468 (60°C enrichment). The accession numbers of DUSEL isolates are FJ428200 (DUSELG12), EU010241 (DUSELG16), EU010242 (DUSELR7), and EU010244 (DUSELR13).

### Scanning electron microscopy of DUSEL isolates

The morphology of the isolates was observed using a scanning electron microscope (SEM) (model 3500 N; Hitachi, Tokyo, Japan) using the method as described earlier [8].

### Growth of DUSEL isolates on different carbon sources

DUSEL isolates were grown in liquid medium containing carbon sources (each [0.5 g/l]) such as cellobiose, sawdust (extruder-treated fine powder of pine wood chips), microcrystalline cellulose, carboxymethylcellulose (CMC) sodium

salt (low viscosity; molecular weight: 90 kDa, degree of polymerization: 400, degree of substitution: 0.65–0.90), KimWipes (Kimberly-Clark Co., Neenah, WI, USA), and catalog papers (coated). Pine wood chips were tested as it is a major cellulosic material available especially in South Dakota e.g. 300,000 tons/yr pinewood waste (R. Kramer, personal communication, KL Energy, Rapid City, SD, USA). For these experiments, the isolates were first grown in a 10-ml volume of medium containing a particular carbon source. After the exponential phase of growth, the cells from seed cultures were re-inoculated in 100-ml of medium in 500-ml Erlenmeyer flasks containing the same carbon sources. Control flasks were included with no carbon source for each experiment. The inoculated flasks were incubated at respective temperatures under shaking conditions (120 rpm) for a period of 10–12 days. Evaporation at 60°C was checked everyday by weighing the flasks and corrected when necessary by adding a corresponding amount of pre-sterilized water. Furthermore, cellulose-utilization by isolates was studied under microaerophilic conditions. For this experiment, isolates were inoculated into 50-ml of medium in 125-ml serum bottles sealed with butyl rubber stoppers, and crimped with aluminium seals.

### Analysis of soluble end products produced by DUSEL isolates

The culture of DUSEL isolates (10–12 days old) growing in Erlenmeyer flasks or serum bottles on carbon sources (e.g. cellulose/sawdust/CMC/KimWipes/catalog paper) were analyzed for soluble end products (e.g. sugars, acids, and alcohols). The analysis was done using high-pressure liquid chromatography (HPLC). Samples were prepared by centrifuging the 1-ml cultures at 10,000 rpm and supernatants obtained were further filtered using membrane filters (Gelman Acrodisc; pore diameter, 0.2 μm). The appropriate dilution of cell-free filtrate (10-μl) was injected onto a heated Aminex ion exclusion column (HPX-87H; Bio-Rad, Hercules, CA, USA) on a HP 1100 Series HPLC system equipped with a refractive index detector (Agilent Technologies, Palo Alto, CA, USA). The samples were eluted with flow rate of 0.6 ml/min using 5 mM $H_2SO_4$ as mobile phase.

### Measurement of enzyme activities of DUSEL isolates

In our study, all enzyme activities were measured from cell-free culture supernatants of DUSEL isolates. Two types of enzyme activities were measured throughout the growth periods (1) CMCase (2) filter paper cellulase, hereafter referred to as cellulase. For CMCase activity, the enzyme assay mixture (0.5-ml) contained 375-μl of an appropriate dilution of enzyme and 125-μl of 1% (w/v) CMC solubilized in 200 mM Tris–HCl buffer (pH 7.0). Measurement of cellulase activity was performed in an enzyme assay mixture (2-ml) that contained ~50 mg of Whatman No. 1 filter paper (Whatman, Inc., Florham Park, NJ, USA), 1-ml of 100 mM phosphate buffer (pH 7.0), and 1-ml of an appropriate dilution of enzyme [12]. Appropriate enzyme and substrate controls were also included in all assays. The assay mixtures were incubated for 20 min at 37°C and 60°C for mesophilic and thermophilic isolates, respectively. The reaction was stopped by the addition of 3,5-dinitrosalicylic acid (DNS) solution, boiled for 5 min, and then cooled in water for color stabilization [30]. The optical absorbance was measured at 540 nm and the amounts of liberated reducing sugars (glucose equivalents) were estimated against a glucose standard curve. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of glucose per minute under the assay conditions. Each experiment was repeated three times and average values were plotted for each experiment.

### Characterization of crude CMCases of DUSEL isolates

The pH optimum of crude CMCases, secreted by DUSELG12 and DUSELR7 was estimated in the pH range of 3.0–11.0 using different assay buffers. Enzyme assays were conducted in 0.5-ml reaction volumes containing 375-μl of an appropriate dilution of enzyme and 125-μl of 1% (w/v) CMC solubilized in 200 mM of sodium citrate (pH 3.0–5.5) or sodium phosphate (pH 6.0–7.5), or Tris–HCl (pH 8.0–9.0), or glycine-NaOH (pH 9.5–11) buffer. The assay mixture was incubated for 20 min at 37°C or 60°C for mesophilic and thermophilic isolates, respectively. Maximum activity obtained at a particular pH was used to calculate the relative percentage enzyme activity at other pHs. The optimum pH determined from the above experiments for DUSELG12 and DUSELR7 was used to determine the optimum temperature ($T_{opt}$) for crude CMCases. The experiment was carried out in the temperature range of 5–100°C using the similar assay conditions.

The thermal stability (50–100°C) of crude CMCase produced by DUSELG12 and DUSELR7 was further evaluated and residual activities were determined under optimum pH and temperature conditions using the DNS method as described above. Thermal stability of CMCase secreted by DUSELG12 was tested by determining the enzyme activity remaining after incubation of the crude CMCase at different temperatures (50–70°C) for a period of 84 h. In case of DUSELR7, activity was determined by incubating the CMCase up to 540 h at different temperatures (60–100°C). In all cases the initial activity at a particular temperature was assumed to be 100% and used to

calculate the percentage relative activities during the incubation period.

## Results and discussion

### Chemical characteristics of weathered soil-like sample

The chemical characteristics of the sample used for the isolation of mesophilic and thermophilic CDB are presented in Table 1. Significant amounts of toxic metals including Cu, Cr, Ni, Pb, and Zn were observed. Although cellulose-degradation was not studied in the presence of these toxic metals, isolation of CDB from soils containing such elevated levels of toxic metals indicates that these bacteria would be adapted to tolerate toxic metal concentrations and hence of particular biotechnological interest such as in the bioremediation of wood treated with preservatives.

**Table 1** X-ray fluorescence spectroscopic analysis of major and trace elements of soil sample collected from the Homestake gold mine

| Major elements | Amount (% weight) |
|---|---|
| $SiO_2$ | 42.22 |
| $TiO_2$ | 0.39 |
| $Al_2O_3$ | 8.24 |
| FeO | 21.21 |
| MnO | 0.45 |
| MgO | 6.23 |
| CaO | 19.09 |
| $Na_2O$ | 0.47 |
| $K_2O$ | 1.29 |
| $P_2O_5$ | 0.41 |
| **Trace elements** | **(mg/kg dry soil)** |
| Ni | 205.8 |
| Cr | 904.5 |
| Sc | 6.5 |
| V | 125.7 |
| Ba | 507.7 |
| Rb | 43.7 |
| Sr | 215.1 |
| Zr | 160.4 |
| Y | 29.7 |
| Nb | 15.6 |
| Ga | 16.3 |
| Cu | 198.2 |
| Zn | 678.3 |
| Pb | 81.6 |
| La | 18.7 |
| Ce | 95.1 |
| Th | 13.4 |
| Nd | 19.3 |

### Bacterial community structures of mixed cellulose-degrading enrichment cultures

Bacterial community structures of mixed enrichment cultures were studied to provide greater knowledge of the mechanisms of effective cellulose degradation by cooperation amongst bacteria. A total of 61 and 74 clone sequences were included in phylogenetic analysis that generated 22 and 9 OTUs, respectively, from mesophilic and thermophilic enrichment cultures (Figs. 1 and 2). Thus, mesophilic enrichment had more species richness compared to the thermophilic. The Shannon diversity index further pointed to relatively higher diversity in mesophilic enrichment ($H' = 2.31$) compared to the thermophilic ($H' = 1.46$). The lower diversity in thermophilic enrichment culture was most likely due to the higher temperature (60°C) of incubation that selected only specialized bacterial populations adapted to tolerate high temperatures. Rarefaction analysis, which plots the number of clones sequenced versus the number of OTUs generated showed that in the mesophilic clone library the diversity was less exhaustively captured compared to that of thermophilic clone library. Rarefaction plot for mesophilic clone library was more non-asymptotic ($\theta = 18°$) than the thermophilic clone library ($\theta = 5°$) (Fig. 3). All the OTUs retrieved from mixed enrichment cultures were affiliated to phylum *Firmicutes.*

In mesophilic enrichment cultures (Fig. 1), OTUs were distributed in two clusters belonging to order *Clostridiales* (62.3% of total clones) and *Bacillales* (37.7% of total clones) spanning within the *Firmicutes.* The genus *Clostridium* includes obligately anaerobic bacteria; however, most clostridia are aerotolerant and do not resume growth if oxygen is present [13]. The presence of *Clostridium*-related OTUs in the mesophilic clone library indicated that enrichment of CDB in serum bottles crimped with aluminum seals caused the deprivation of oxygen after a certain time of growth period. It can be, however, speculated that during the initial period of growth, available dissolved and head space oxygen in the serum bottles favored the growth of aerobes and facultative anaerobes belonging to genus *Bacillus*. Once the oxygen was consumed by these bacilli, the oxygen-limiting conditions favored the growth of clostridia. It can be seen from the Fig. 1 that several OTUs including OTU-1 to 5, and OTU-22 spanning within *Clostridiales* showed phylogenetic position distinct from those of known species and might represent novel lineages. Previous studies have shown the presence of CMCase and cellulase activities in *Clostridium* strains [5]. Phylotypes falling in *Bacillales* formed several monophyletic clusters

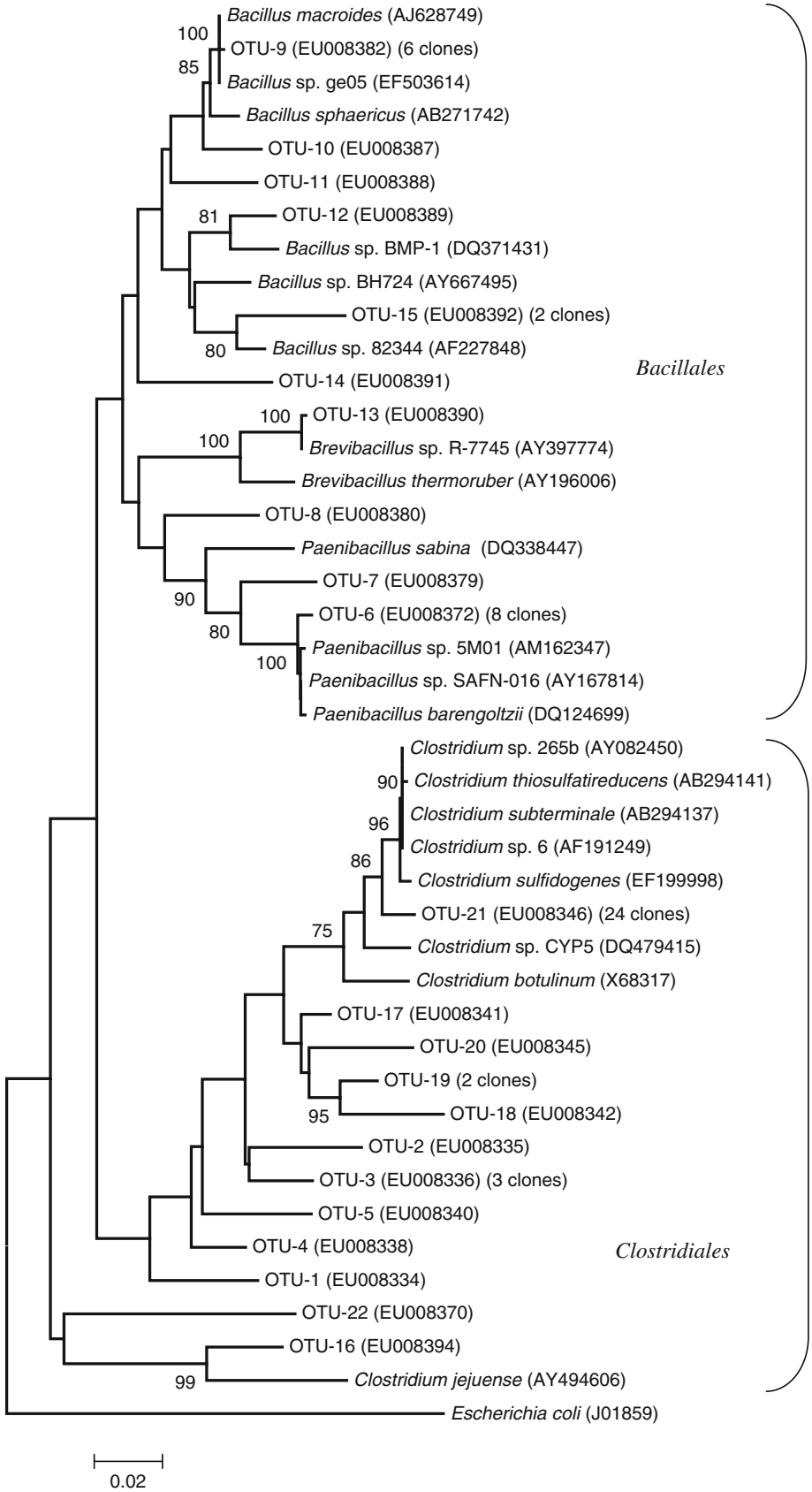


**Fig. 1** Phylogenetic dendrogram showing the relationship between 16S rRNA gene sequences retrieved from mesophilic (37°C) cellulose-degrading enrichment with reference sequences in GenBank. For OTUs representing multiple clones, the number of additional clones is given in parentheses along with the accession number. *Escherichia coli* (J01859) was selected as out-group to root the tree. The tree was constructed as described under phylogenetic and statistical analyses of clone libraries section in text. The *scale bar* represents 0.02 substitutions per nucleotide position. Numbers at the node are the bootstrap values (%). Bootstrap values which were <75% were not shown

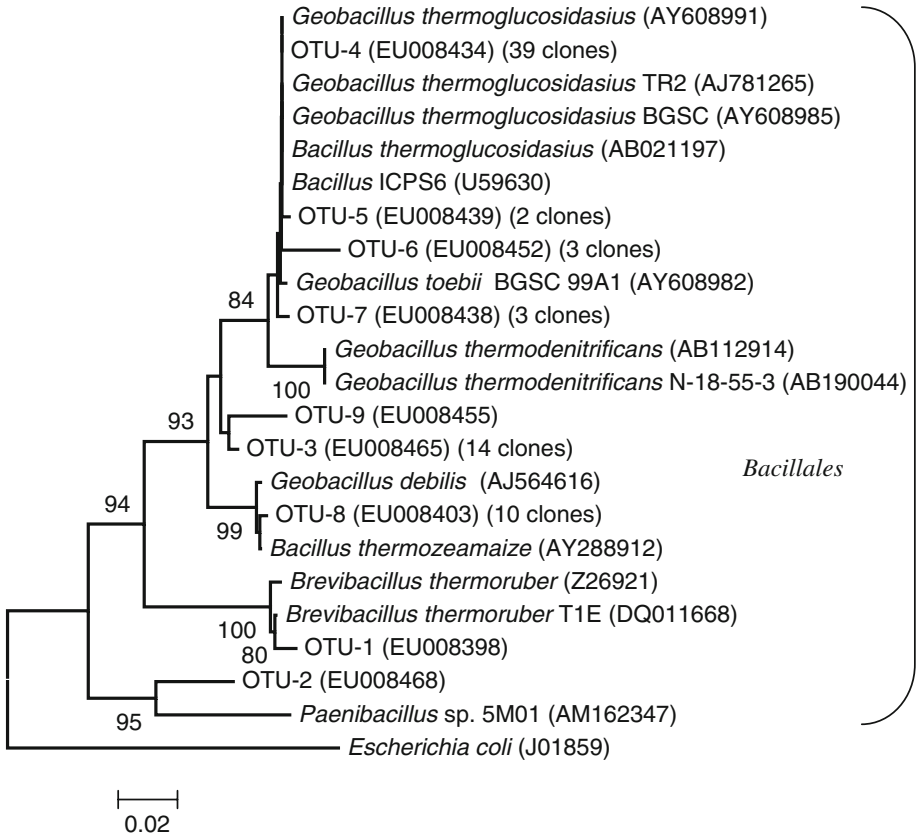


**Fig. 2** Phylogenetic dendrogram showing the relationship between 16S rRNA gene sequences retrieved from thermophilic (60°C) cellulose-degrading enrichment with reference sequences in GenBank. The tree was constructed as described in the legend to Fig. 1

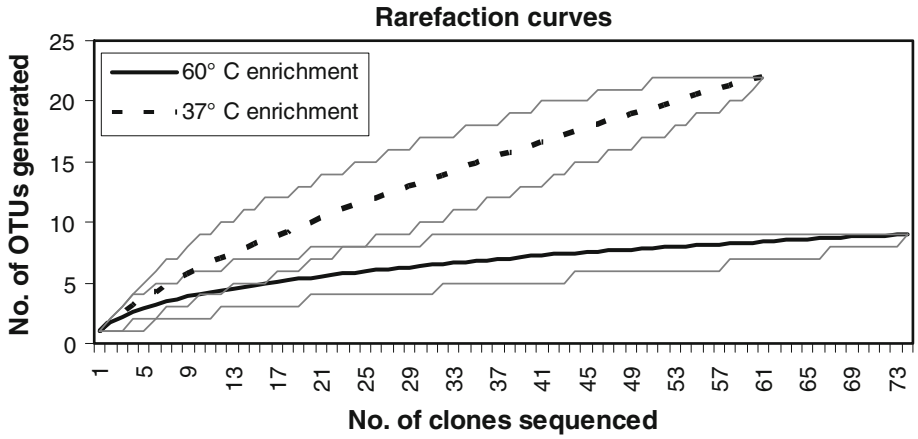


**Fig. 3** Rarefaction curves of 16S rRNA gene library of mesophilic (*dashed line*) and thermophilic (*smooth line*) enrichments, illustrating the relationship between number of clones sequenced and the phylotype richness. Sequences were grouped in to OTUs based on 97% sequence similarity and the curves were established by DOTUR. The *wavy lines* indicate 95% CI The steepness ($\theta$) of each curve was calculated from equation $y = mx + c$ where '$x$' and '$y$' are the coordinates of the points, $m$ is the slope, and $c$ is the "*y intercept*" of the *straight line graph*. The $\theta$ for mesophilic and thermophilic library were 18º and 5º, respectively. In a molecular inventory where 100% diversity is captured $\theta$ reaches to 0 i.e., the curve takes the form of plateau

with known lineages belonging to genera *Bacillus, Brevibacillus,* and *Paenibacillus*.

In thermophilic enrichment cultures (Fig. 2), all OTUs were affiliated to *Bacillales* and none of them was found related to *Clostridiales*. A closer look at the *Clostridium*-related lineages identified in mesophilic enrichment cultures such as those represented by *C. thiosulfatireducens* (AB294141), *C. sulfidogenes* (EF199998), and *C. jejuense* (AY494606), show that these species have been reported as mesophilic in literature, hence this could be the most likely reason that we did not retrieve any *Clostridium*-related OTUs in thermophilic enrichment cultures. Within the

*Bacillales,* phylotypes displayed strong similarities with known species of genera *Bacillus*, *Geobacillus*, and *Paenibacillus*.

## Molecular and phenotypic identification of DUSEL isolates

Four isolates were selected, two from each of the enrichment cultures growing at 37°C and 60°C. The phylogenetic affiliations of mesophilic (DUSELG12 and DUSELG16) and thermophilic (DUSELR7 and DUSELR13) isolates are shown in Fig. 4. Evolutionary distance and bootstrap analyses showed that mesophilic isolates were phylogenetically clustered with *Paenibacillus* sp. and *Brevibacillus* sp. and tentatively identified belonging to these genera. It has been suggested that bacteria with ≥97% similarity in their 16S rRNA gene sequences are likely to be the members of same species [38]. DUSELG12 showed 99% similarity with *Brevibacillus* sp. (unpublished EU383032; isolated from soil). Furthermore, microscopic observation using SEM showed typical spherical- to rod-shaped cells characteristics of genus *Brevibacillus* (images are not shown) [3]. Several species belonging to *Brevibacillus* have been reported to produce cellulase [17, 46]. DUSELG16 was 99% related to *Paenibacillus siamensis* (AB295645) a xylanase producing bacteria reported from soil [18]. Morphologically cells were almost rod-shaped, a typical characteristic of genus *Paenibacillus* (images are not shown). Genus *Paenibacillus* has also been widely reported to utilize heteropolysaccharides, cellulose, and xylan as sole sources of carbon [32, 35, 45]; however, so far there has been no report of cellulose-degrading *Paenibacillus siamensis*.

Among thermophilic isolates, the 16S rDNA sequence of DUSELR7 was phylogenetically grouped with sequences belonging to *Geobacillus* sp. (AY603071; a thermophile isolated from a volcano island) [29] and displayed 100% DNA similarity with it. It has been reported that members belonging to genus *Geobacillus* are rod-shaped, chemo-organotrophic, aerobic or facultative anaerobic and obligatorily thermophilic with an optimum growth temperature range of 55–65°C [28]. Similar morphological features were observed in the cells of DUSELR7 (images are not shown). *Geobacilli* have been isolated from sugar refinery

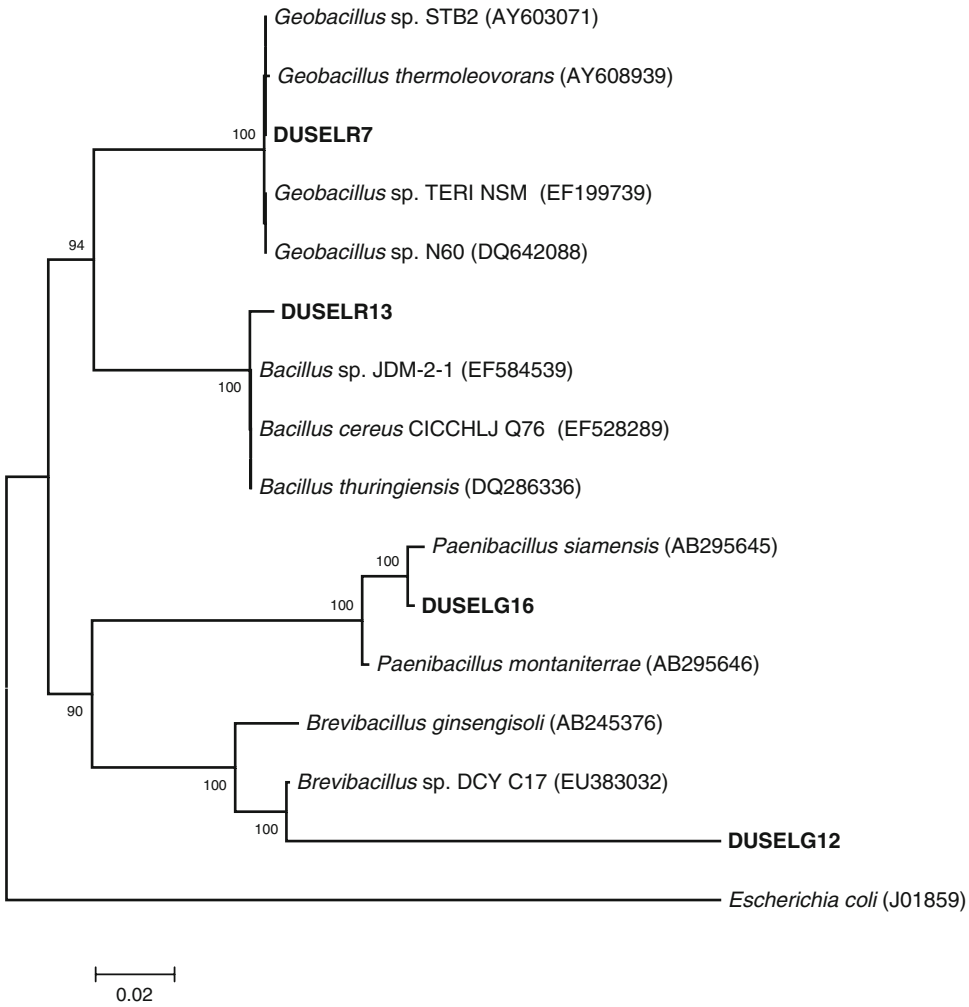


**Fig. 4** Phylogenetic dendrogram showing the relationship between 16S rRNA gene sequences retrieved from cellulose-degrading DUSEL isolates (*bold letters*) growing at 37°C and 60°C with reference sequences in GenBank. GenBank accession numbers of DUSEL isolates are given in *parentheses*. The tree was constructed as described in the legend to Fig. 1

wastewater and soil and have been shown to produce CMCases [1, 40]. More interestingly, *Geobacillus* spp. have been isolated from water collected from an ultra-deep South African gold mine and have been to shown to utilize wide variety of carbon sources including cellobiose, hydrocarbons, and lactate but not cellulose [8]. DUSELR13 displayed 99% DNA similarity with *Bacillus thuringiensis* (unpublished DQ286336), and formed a monophyletic cluster with sequences of other known *Bacillus* sp. Genus *Bacillus* includes aerobic or facultative aerobic, rod-shaped bacteria with a wide diversity with respect to carbon source utilization, temperature, pH, and salinity [14]. Morphological observation of whole-cells of DUSELR13 showed typical rod-shaped morphology of genus *Bacillus* (images are not shown). *Bacillus thuringiensis* has been shown to degrade chitin but so far there is no report of cellulase production from this species [41].

## Utilization of different carbon sources by DUSEL isolates

Results showed that all DUSEL isolates utilized a variety of cellulosic forms (including microcrystalline cellulose and sawdust) as sole source of carbon (data are not shown). In most of the carbon sources during growth period, total protein concentration reached 40–50 mg/l which showed that isolates grew actively utilizing these sole carbon sources. Literature search indicated that *Brevibacillus* sp. [43, 46], *Paenibacillus* sp. [45], *Geobacillus* sp. [1, 40], and *Bacillus* sp. [21, 43] have been shown to degrade CMC, cellulose, lignocellulose, and filter paper (crystalline cellulose); however, CMC was hydrolyzed more efficiently than cellulose. More interestingly, an anaerobic *Paenibacillus* sp. has also been shown to degrade pineapple wastes [32]. *Geobacillus* sp. [40] has been shown to ferment glucose (but not cellulose); however, fermentation end products have not been reported. Our data on the utilization of carbon sources such as cellulose, CMC, sawdust, and catalog paper by DUSEL isolates are generated under unoptimized conditions, and are not quantitative therefore; more detailed comparisons were not made with published literature. Furthermore, a closer look at the literature reveals that only fungi and a few anaerobic bacteria such as *Clostridium thermocellum* and *C. cellulolyticum* have been reported to degrade crystalline cellulose, with their multi-enzyme complex, the cellulosome [25]. Even hyperthermophilic marine archaea ($T_{opt}$ 100°C) belonging to genera *Pyrococcus*, *Thermococcus*, *Thermotoga*, and *Sulfolobus*, are not capable of utilizing crystalline cellulose as growth substrate [7]. Although some aerobic *Bacillus* species have been shown to produce endoglucanases that can degrade amorphous cellulose, most of them cannot degrade crystalline cellulose efficiently [6, 16]. Thus, DUSEL isolates could be used in biorefineries to convert a variety of biomass into target products including sugar and ethanol.

The soluble end products analysis of supernatants collected from stationary phase cultures of DUSEL isolates grown in Erlenmeyer flasks revealed glucose and cellobiose as major end products after active growth periods. However, under microaerophilic conditions, in addition to glucose and cellobiose, all DUSEL isolates except DUSELG16 produced ethanol and acetate as fermentation end products. This showed that under oxygen-limiting conditions, cellulose or cellulose-degradation products were fermented to ethanol by the DUSEL isolates. This was an interesting observation because DUSEL isolates were capable of fermenting cellulose into ethanol in one step. There are only a few reports on converting cellulose into ethanol in one step using pure mesophilic or thermophilic culture [4, 23]. Although, *Geobacillus* sp. [40] has been shown to ferment glucose (but not cellulose), fermentation end products have not been reported.

The use of thermophilic microbes and their enzymes for lignocellulose hydrolysis has the potential to provide unique advantages over existing approaches in bioprocessing [42]. If lignocellulose hydrolysis, saccharification, fermentation, and separation of products can be achieved under similar conditions, the consolidation of several unit operations into one step would lower costs and increase efficiency. In addition, the elevated temperature and thus increased compound vapor pressures could be used to at least partially separate the volatile products (such as ethanol) from the bioreactor leading to lower distillation cost and reducing the potentially inhibitory effects of ethanol production during fermentation. Such consolidated bioprocessing (CBP—simultaneous saccharification and fermentation process) for lignocellulose conversion would be highly desirable and is one of the recommendations made by several federal agencies [24, 42]. In the near future, a modified consolidated bioprocess could be established using DUSEL isolates or their thermostable enzymes for faster hydrolysis rates and thermophilic fermentative microbes (ethanologens) for sugars fermentation. Furthermore, thermophilic ethanol fermentations are of interest to industrial alcohol production; however, more fundamental and applied research is needed before practical economic assessments can be made.

## Growth patterns and enzyme production of DUSEL isolates

On the basis of our data presented above on DUSEL mesophiles and thermophiles (phylogenetic characterization, growth on various cellulosic substrates, and end-product analysis), one mesophilic (DUSELG12; *Brevibacillus* sp.) and one thermophilic (DUSELR7; *Geobacillus* sp.) isolates were selected for further studies. Total cell protein and

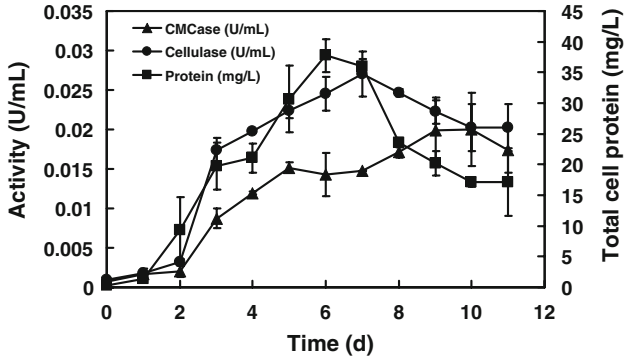


**Fig. 5** Cellulase and CMCase activities of mesophilic DUSELG12 during the growth in a medium containing cellulose as sole source of carbon. Total cellular protein and enzyme activities were measured as described in "Materials and methods" section. *Error bars* smaller than the symbols are not shown

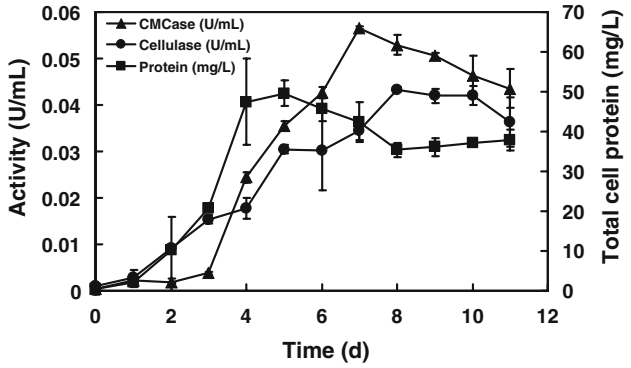


**Fig. 6** Cellulase and CMCase activities of thermophilic DUSELR7 during the growth in a medium containing cellulose as sole source of carbon. Total cellular protein and enzyme activities were measured as described in "Materials and methods" section. *Error bars* smaller than the symbols are not shown

production of enzymes in DUSELG12 and DUSELR7 grown on cellulose as a sole source of carbon are shown in Figs. 5 and 6. In general enzyme activity was associated with an increase in total cellular protein, thus indicating that isolates utilized cellulose actively during the course of their growth. DUSELG12 produced maximum CMCase activity (0.02 U/ml) on day 10 when the culture reached death phase (Fig. 5), while maximum cellulase activity (0.027 U/ml) was observed on day 7 at the end of exponential growth phase, after which cellulase activity decreased with increase in time. There are only few reports on cellulose degradation by *Brevibacillus* sp. Kato et al. [17] showed cellulose-degrading capabilities in *Brevibacillus* sp. in a mixed culture. Previous report on *Brevibacillus* sp. isolated from compost piles showed that it degraded lignocellulosic wastes with high efficiency as evident from a decrease in cellulose-to-lignin ratio [43]. In an another report *Brevibacillus* strains isolated from termite gut were examined for CMCase activity in CMC-congo red agar medium and have also shown for using filter paper as sole source of carbon [46]. However, the data were not directly comparable with our study therefore comparisons were not possible with our findings on DUSELG12.

In case of DUSELR7, maximum CMCase (0.058 U/ml) activity was found on day 7 in the death phase and after that it decreased linearly with time (Fig. 6). Like CMCase profiles of DUSELR7, similar cellulase activity profiles were observed. Unlike DUSELG12, DUSELR7 produced higher CMCase activity than cellulase activity (0.043 U/ml on day 8). It is known that the cellulase systems consist of endoglucanases, exoglucanase, and β-glucosidase. The synergy of all these enzymes makes possible the hydrolysis of cellulose to glucose [25]. CMC is an example of amorphous cellulose which is generally used as a substrate for the study of endoglucanases. On the other hand, exoglucanases can degrade microcrystalline cellulose efficiently. In our study, DUSELR7, even grown on cellulose, had greater catalytic affinity for CMC; therefore DUSELR7 cellulases could be categorized as endoglucanases which posses lesser exoglucanase activity. There is very scarce information available on the production of cellulase/CMCase from *Geobacillus* sp. A *Geobacillus* sp. (isolated from soil) grown at 55°C has been reported to produce 0.074 U/ml cellulase under highly optimized conditions [1]. Tai et al. [40] showed that *Geobacillus* sp. grown at 60°C had maximum CMCase activity (0.0113 U/ml) at 70°C in 0.1 M sodium phosphate buffer (pH 7.0).

The low levels of enzyme activities (<1 U/ml) detected in DUSEL isolates were most likely due to the fact that cell-free supernatants were assayed for enzyme activities. It is well known that some of the cellulase activity is cell-bound [25] therefore use of cell-free supernatants in assay reactions may have underrepresented enzymatic activity produced by DUSEL isolates. Other factors include the use of unoptimized minimal medium and culture conditions used for the growth and enzyme production of DUSEL isolates. Nonetheless, *Geobacillus* sp. has been shown in literature to produce maximum enzyme activity of 0.074 U/ml under optimized conditions [1]. In another study even much lower cellulase activity (0.0113 U/ml) was observed under optimized conditions from *Geobacillus* sp. [40]. Thus, in our study considering the unoptimized medium and culture conditions and analysis of cell-free enzymes, the amount of enzyme produced especially by DUSELR7 (*Geobacillus* sp.) was significant. The other problem associated with enzyme production by thermophiles is low cell yield associated with their growth. This has stimulated a lot of research to improve thermophilic cell yield and consequently increasing the enzyme yield. Special equipment, process configuration, medium compositions, and culture optimizations have been designed to increase cell yield in thermophilic bacteria [37, 42]. For example, it has been

shown in *Geobacillus* sp. that optimizing the culture conditions, and factorial designs and additions of yeast extract and ammonium sulfate resulted in twofold increase in cellulase production [1].

## Effect of pH and temperature on enzyme activity

The effect of pH on the crude CMCase activity of DUSEL isolates was examined at various pHs ranging from pH 3.0 to 11.0 (Fig. 7). DUSELG12 and DUSELR7 showed maximum enzyme activity in sodium citrate buffer. The activity was found to increase with increasing pH but beyond pH 6.0, enzymes showed a linear decrease in their activities with increase in pH. DUSELG12 and DUSELR7 showed maximum CMCase activity at pH 5.5 and 5.0, respectively. It was interesting to note that in contrast to mesophilic DUSELG12, thermophilic DUSELR7 maintained almost a similar level of activity in a broad pH range (pH 4–6).

The effect of temperature on the CMCase activity of DUSEL isolates under optimum pH conditions are shown in Fig. 8. DUSELG12 showed maximum CMCase activity at 55°C while DUSELR7 showed at 75°C (Fig. 8). In case of DUSELG12, enzyme activity increased linearly with increasing temperature up to 55°C, after which it decreased sharply with no activity at 90°C. However, with DUSELR7, activity was increased up to 75°C and the enzyme was presumably denatured at 100°C. Figure 8 further showed that DUSELR7 retained almost a similar level of activity in a broad temperature range (70–85°C). Thus our data on pH and temperature showed that DUSELR7 CMCase was more resistant to change in pH and temperature, and hence better suited for harsh process conditions involved in lignocellulosic bioprocessing.

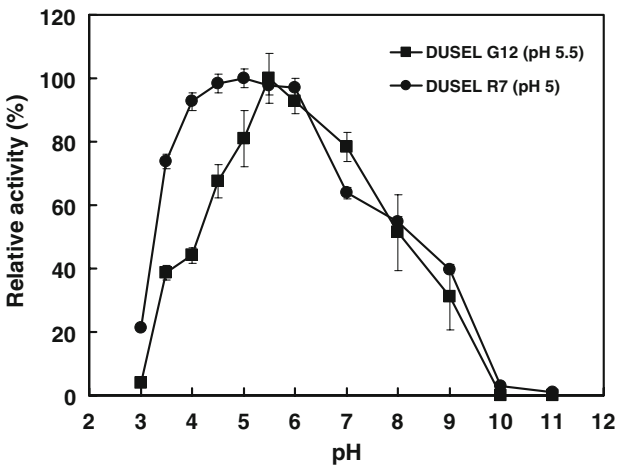


**Fig. 7** Determination of optimum pH for the maximum activity of crude CMCases of DUSELG12 and DUSELR7 isolates. The enzyme activities were expressed as percentages of the maximum activity. *Error bars* smaller than the symbols are not shown

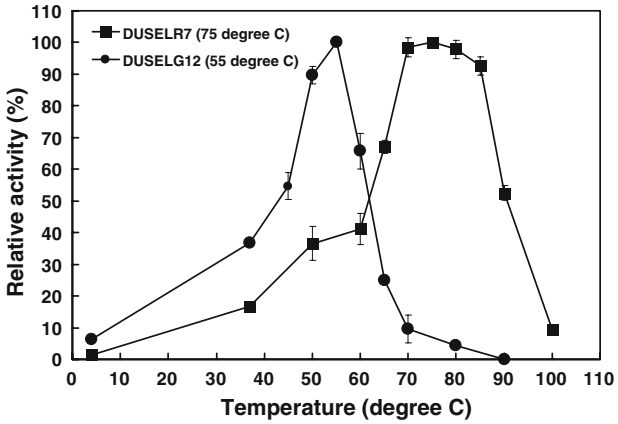


**Fig. 8** Determination of optimum temperature for the maximum activity of crude CMCases of DUSELG12 and DUSELR7 isolates. The enzyme activities were expressed as percentages of the maximum activity. *Error bars* smaller than the symbols are not shown

## Thermal stability of crude CMCase of DUSELG12 and DUSELR7 isolates

Results shown in Fig. 9 indicated that CMCase of DUSELG12 was optimally stable at 50°C over 1 h without any loss of activity, and about 48% activity was retained after incubation of 8 h. At 50°C all the activity was lost in 84 h of incubation most likely due to complete denaturation of enzyme. At 60°C about 40% residual activity was detected at 8 h of incubation and complete activity was lost in about 45 h. DUSELG12 crude CMCase was very unstable at temperatures ≥70°C as more than 98% activity was lost within 5 min (data not shown). Further incubation of the enzyme at higher temperatures (80, 90, and 100°C) even for 2 min caused the loss of about 98% activity (data not shown).

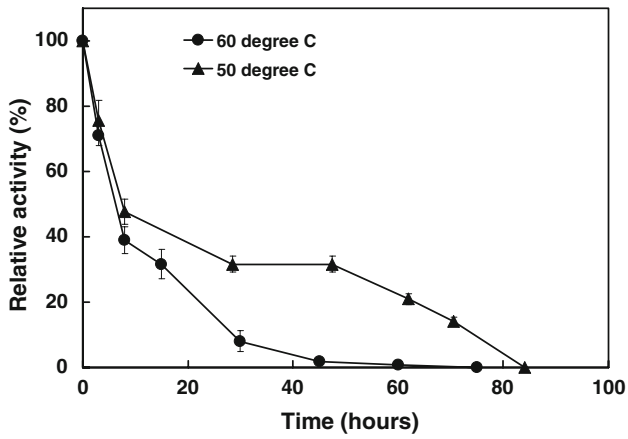


**Fig. 9** Thermal stability of crude CMCase activity produced by DUSELG12 isolate. The enzyme activities were expressed as percentages of the initial activity. *Error bars* smaller than the symbols are not shown

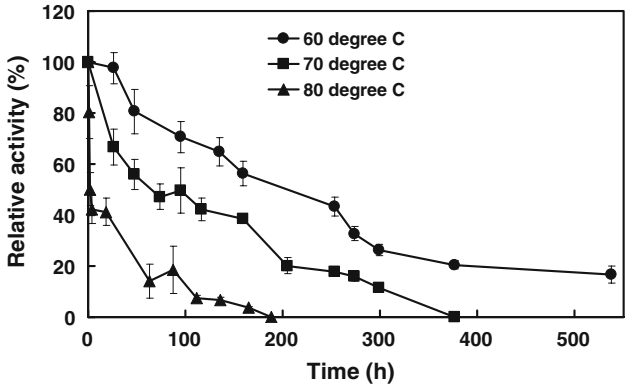


**Fig. 10** Thermal stability of crude CMCase activity produced by DUSELR7 isolate. The enzyme activities were expressed as percentages of the initial activity. *Error bars* smaller than the symbols are not shown

In contrast to mesophilic DUSELG12, thermophilic DUSELR7 showed remarkable residual CMCase activity after prolonged incubation at high temperatures. For example, at 60°C the enzyme was stable for 24 h, and only about 20% of activity was lost after incubation of 48 h (Fig. 10). With further increase in incubation temperature, enzyme denaturation was increased. At 70°C and 80°C, complete enzyme activity was lost in 376 and 188 h, respectively. At higher temperatures, e.g. 90°C and 100°C, enzyme activity was lost after 25 min (data not shown). Comparing our data on thermostability of CMCases with others reported in literature showed that CMCase secreted by DUSELR7 isolate was highly thermostable. For example, Tai et al. [40] showed that in *Geobacillus* sp. CMCase activity dropped immediately even with a slight increase of temperature beyond 70°C indicating that enzyme was highly sensitive to temperature >70°C. CMCase produced by *Geobacillus* sp. [40] retained 90% activity at 70°C after 1 h of incubation, and further incubation at higher temperatures for e.g. at 80°C for 1 h caused the loss of 40% activity. This indicated that CMCase secreted by *Geobacillus* sp. [40] was not highly thermostable.

Thermophiles and their thermostable enzymes are highly important in thermophilic bioprocessing [42]. With the latest developments in molecular genetic techniques it is possible to develop novel recombinant biocatalysts with desired properties. A number of biotechnology companies (e.g. Genencor International, Rochester, NY, USA and Novozymes, Davis, CA, USA) are prospecting novel thermostable enzymes by applying in vitro enzyme evolution strategies that includes modifying the thermostable cellulase genes in such a way that can diversify its substrate binding capabilities (for e.g. modification in carbohydrate binding module [27], or to increase its thermostability (by site-directed mutagenesis, [27]) or cloning in mesophilic organisms, such as *Escherichia coli* for over expression [9]. Thus our findings on significant thermostability of crude CMCases make DUSEL isolates good target for in vitro enzyme evolution strategies that could utilize the best thermostable gene from DUSEL isolates and engineer it to encode a single protein with overall desirable properties for industrial applications especially in biorefineries. However, more work remains before exploitation at large or industrial scale can be considered.

## Industrial implications of DUSEL isolates

Lignocellulosic biomass has long been recognized as a potential sustainable source of biomaterials and biofuels including sugars, ethanol, and hydrocarbons. For example, cellulosic feedstocks from agricultural and forest origins are regarded as the most abundant renewable resource. To date, however, only a relatively small percentage of crop and forestry wastes have been recycled for commercial use. The conversion of lignocellulose into value-added industrial products at greater rates and yields is highly desirable. This study examined the extremophilic microbes/enzymes from the unique environments of the deep subsurface of the Homestake mine for hydrolysis of lignocellulosic raw materials. The results presented in this study have the potential to suggest fundamental improvements in bioenergy conversion technologies through the characterization of thermophile cellulose-deconstruction enzymes. Our data suggested that the thermostable enzymes secreted by DUSELR7 would offer robust catalyst alternatives in biorefineries which require high temperatures to promote easy mixing, better substrate solubility and enzyme penetration, and cell-wall deconstruction of biomasses. Furthermore the highly thermostable nature of DUSELR7 could offer several potential advantages in the hydrolysis of lignocellulosic materials: higher specific activity (decreasing the amount of enzyme needed), and higher stability (allowing extended hydrolysis times), and increased flexibility for the process configuration.

In summary, cellulose-degrading bacteria were cultured for the first time from the unique deep subsurface environment of the Homestake gold mine. Although 16S rDNA sequences showed that isolates were very closely related to their cultured database neighbors, the characteristics of the enzymes showed the unique nature of DUSEL isolates. For example crude CMCase produced by DUSELG12 had an optimum temperature of 55°C, while DUSELR7 had 75°C. Crude CMCases of DUSEL isolates were optimally active between pH 5.0 and 6.0 and showed remarkable thermostability even at higher temperature for a prolonged period of time, which makes them good candidates for industrial applications. Our findings on the production of significant amounts of enzymes under unoptimized medium and culture conditions especially for DUSELR7, the remarkable

thermostable nature of CMCases, the broad temperature and pH range for activity, utilization of crystalline cellulose, and production of ethanol from cellulose in a single step under microaerophilic conditions far exceeds previous reports on cellulase production from other similar species. Even higher cellulose degradation rates can be achieved by optimizing physicochemical culture conditions and medium components, or mixing pure cultures via suitable grouping in order to complement their cellulase activity. Our long-term research will focus on developing economical bioprocesses for use of cellulosic waste materials as an alternative source of energy.

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