RESEARCH LETTER

An untapped bacterial cellulolytic community enriched from coastal marine sediment under anaerobic and thermophilic conditions

Shiqi Ji1, Shian Wang1, Yang Tan1, Xiaohua Chen1, Wolfgang Schwarz2 & Fuli Li1

1Shandong Provincial Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, China; and 2Lehrstuhl für Mikrobiologie, Technische Universität München, Freising, Germany

Correspondence: Fuli Li, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, 189 Songling Road, Qingdao 266101, China. Tel.: +86 532 80662655; fax: +86 532 80662778; e-mail: lifl@qibebt.ac.cn

Received 22 May 2012; revised 28 June 2012; accepted 9 July 2012. Final version published online 30 July 2012. DOI: 10.1111/j.1574-6968.2012.02636.x

Editor: Marco Moracci

Keywords
marine; anaerobe; thermophilic bacteria; cellulose degradation; cellulase.

Abstract
A bacterial community with strong cellulose [filter paper (FP) and microcrystalline cellulose] degradation ability was isolated from the coastal marine environment. They were isolated under thermophilic (60 °C) and anaerobic cultivation conditions. The library of 16S rRNA gene clones revealed a total of 16 operational taxonomic units after 50 clones were surveyed. Sixty percent of the clones were most related to the type strain of Clostridium thermocellum with 16S rRNA gene identity around 87–89%. All of them showed extremely low sequence similarities and were novel at least in species level. The gene clone libraries of glycosyl hydrolase family 48 showed low gene and amino acid sequence similarities around 70–72%. The results indicated that the cellulose degradation systems in the specific environment have not been well studied. The enrichment could disrupt FP within 3 days in a basal medium. The cellulase activity of the community was comparable to that of C. thermocellum LQR1. The main fermentation products were ethanol, acetic acid and butyric acid. This work identified a novel microbial resource with a potential in lignocellulose conversion and biofuel production.

Introduction
Lignocellulose is one of the most abundant polysaccharides on the earth. The prospect of using lignocellulose as biofuel source has increased interest in identifying new lignocellulose-degrading microorganisms. Complex enzyme components, such as beta-1,4-endoglucanases (EC 3.2.1.4), beta-1,4-exoglucanases or cellobiohydrolases (EC 3.2.1.91), beta-glucosidases (EC 3.2.1.21) and xylanase (EC 3.2.1.8), have been shown to be involved in the digestion of lignocellulose. A few cellulolytic systems have been intensively studied, for example in the anaerobic bacterium Clostridium thermocellum (Zverlov et al., 2002) and aerobic fungi Phanaerochaete chrysosporium (Martinez et al., 2004). Cellulolytic communities have been identified in a wide variety of sources such as bio-compost, soil, decaying lignocellulose materials, and the feces of ruminants (Maki et al., 2009; Izquierdo et al., 2010). Although the digestion of lignocellulose by terrestrial microorganisms has been widely studied, cellulolytic microorganisms in marine environments have received less attention.

Early studies indicated that bacteria were the predominant degraders of lignocellulose in marine ecosystems, with the exception of marine animals such as teredinid bivalves (Benner et al., 1986; Distel, 2003). Recently, an aerobic and mesophilic bacterium Saccharophagus degradans has been intensively studied (Taylor et al., 2006). However, few bacteria with strong cellulolytic activities have been isolated and characterized, especially anaerobic species. Given the diversity of habitats of the ocean, there exists the possibility of some efficient cellulose enzymatic digestion system in the marine ecosystems. For example, mangroves have been considered to be an important location for lignocellulose decomposition (Pointing & Hyde, 2000). The exploration of novel cellulose-degrading microbial communities is of particular importance in the identification of novel microorganisms. Because of its high salinity (3%), the marine environment is likely to have evolved different cellulose-degrading microorganisms.
than the terrestrial environment. Studies of lignocellulose degradation under saline conditions have a great potential in the search for enzymes with novel catalytic properties and microorganisms with novel metabolic pathways.

In this paper, an anaerobic and thermophilic cellulosytic community was enriched from a coastal marine sediment sample. To explore the community members of the unusual consortium, libraries of 16S rRNA gene and functional gene glycosyl hydrolase family 48 (GHF48) gene were constructed and analyzed.

Materials and methods

Enrichment protocol

Samples collected from marine sediment of a coastal region of the Yellow Sea (36°5′N, 120°32′E), China, in July 2011, were used as inocula in 100 mL of basal medium containing 1 g Avicel (PH-101; Sigma Aldrich, Shanghai, China) or a piece of filter paper (FP) (No. 1, Whatman) as the carbon source. The medium consisted of 0.1 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.1 g L\(^{-1}\) K\(_2\)HPO\(_4\), 1 g L\(^{-1}\) NaHCO\(_3\), 2 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 0.5 g L\(^{-1}\) L-cysteine, and 0.0001 (w/v) resazurin. Vitamins were added in the following concentrations (in mg L\(^{-1}\)): pyridoxamine dihydrochloride, 1; p-amino-benzoic acid (PABA), 0.5; d-biotin, 0.2; vitamin B12, 0.1; thiamine-HCl-2 \(\times\) H\(_2\)O, 0.1; folic acid, 0.2; pantothenic acid calcium salt, 0.5; nicotinic acid, 0.5; pyridoxine-HCl, 0.1; thiotic acid, 0.5; riboflavin, 0.1. The samples were incubated under thermophilic (60 °C) and anaerobic conditions. Samples showing FP degradation were selected for further transfers.

Bacterial strain isolation

Cultures showing FP degradation were transferred five times to ensure their cellulose degradation ability. Thereafter, the culture was plated out on anaerobic agar plate containing 1% microcrystalline cellulose and 0.1% yeast extract in the basal medium with 1% agar after step dilutions. Individual colonies were collected from the plate and inoculated in the basal medium containing 0.5% cellobiose and 0.1% yeast extract.

Evaluation of fermentation products and crude enzyme activity

After five consecutive transfers, a fermentation experiment was carried out using the 200 mL basal medium containing 2 g of FP as the carbon source. The concentrations of fermentation products were analyzed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The detected fermentation products included acetate, ethanol, butyric acid, butanol, cellobiose and glucose.

The fermentation broth was taken at 72 h to determine the crude enzyme activities. The sample was centrifuged at 10 000 g for 5 min, and the supernatant was used as crude extract. Clostridium thermocellum LQR1 was used as control, which was cultivated in CM3 medium with 1% FP as the carbon source (Weimer & Zeikus, 1977), and the crude enzyme was taken after 72 h cultivation. All assays were performed at 60 °C in 20 mM PIPES [piperazine-N,N-bis (2-ethanesulfonic acid)] buffer (pH 7) under static conditions for 60 min. FP hydrolysis activity (FPase) was determined using Whatman No. 1 FP and was expressed in filter paper units (FPU) (Wood & Kellogg, 1988). One FPU was defined as the amount of enzyme capable of producing 1 μmol of reducing sugars in 1 min. Endoglucanase and xylanase activities were measured using carboxymethylcellulose (CMC) and birch wood xylan (Sigma-Aldrich), respectively, with a 1% solution of CMC or xylan as the substrate. The β-glucosidase, β-xylosidase and pNPCase activities were determined using p-nitrophenyl-β-D-glucoside, p-nitrophenyl-β-D-xyloside and p-nitrophenyl-cellobioside (Sigma-Aldrich) as the substrate, respectively (Wood & Kellogg, 1988). One unit of enzyme releases 1 μmol equivalent of glucose, xylose or p-nitrophenol per minute. The release of reducing sugars was measured by the dinitrosalicylic colorimetric (DNS) method (Miller, 1959). Protein concentrations were determined with the Bradford assay kit (Biomed, Beijing, China) with bovine serum albumin as the standard.

DNA extraction and PCR amplification of the 16S rRNA gene and GHF48 gene

After five consecutive transfers using basal medium with Avicel as the growth substrate, total DNA of enrichment culture was extracted using the E.Z.N.A. TM Soil DNA Kit (Omega Bio-Tek). The DNA obtained from each set of triplicate extractions was pooled. Using the universal oligonucleotide primers 27F and 1492R, the extracted DNA was used in triplicate PCR amplifications targeting the 16S rRNA gene. PCR amplifications were prepared with 25 μL 2× Taq PCR Master Mix (Biomed), and 1 μM of each primer for a final volume of 50 μL, using 30 cycles of 94 °C (30 s), 50 °C (45 s), and 72 °C (90 s), with an initial denaturation at 95 °C (5 min) and a final extension at 72 °C (5 min). The PCR amplifications targeting the GHF48 gene used two degenerate primers, GH48F (5'-GGATTCCCTAYCAYTCHATGAAA-3') and GH48R (5'-CATGCGCCTGVAVWCCRAACCA-3') (Izquierdo et al., 2010). Optimal PCR conditions utilized 30 cycles of 94 °C
(30 s), 52 °C (30 s), and 72 °C (30 s), with an initial denaturation at 95 °C (5 min) and a final extension at 72 °C (5 min), and the same concentrations of reagents as used for 16S rRNA gene PCR.

**Clone library generation**

Clone libraries based on the 16S rRNA gene and the GHF48 gene were constructed by pooling amplicon DNA, purifying from PCR and cloning into a pMD18-T vector (TaKaRa Biotechnology Co. Ltd., Dalian, China). Two vector-specific primers were used for the amplification of the DNA inserts: M13-47 (5′-CGCCAGGGTTTTCCGAGTCACGAC-3′) and RV-M (5′-GACGGATAAATTACACAGG-3′). PCR amplification was using 30 cycles of 94 °C (30 s), 54 °C (45 s), and 72 °C (2 min), with an initial denaturation at 95 °C (5 min) and a final extension at 72 °C (10 min). Clones were screened by agarose gel electrophoresis to check the inserts were the correct size. The PCR products were purified using the TaKaRa agarose gel DNA purification kit (TaKaRa Biotech Co.) and were sequenced by Shanghai Biosune (Shanghai, China) with an Applied Biosystems automatic sequencer (ABI3730). A total of 50 clones from each clone library were screened.

**DNASTAR LASERGENE software** was used for manual editing of the amplified 16S rRNA and GHF48 gene sequences. Operational taxonomic units (OTUs) definition at 97% sequence similarity was determined using the DOTUR software package (Schloss & Handelsman, 2005). The rarefaction curve was generated by PAST software package with a confidence threshold of 95% (Hammer et al., 2001). The identification of phylodynamic neighbors and the calculation of pairwise 16S rRNA and GHF48 gene sequence similarities were achieved by blasting in EzTaxon-E database and NCBI (Kim et al., 2012). Sequences were classified into different bacterial taxa by RDP naive Bayesian rRNA classifier Version 2.4 with a confidence threshold of 80% (Cole et al., 2009). Phylogenetic analysis was performed with the software package MEGA version 4.0 (Tamura et al., 2007) after multiple alignment of data by CLUSTALX (Chenna et al., 2003). The phylogenetic trees were constructed using neighbor-joining (NJ) methods. Bootstrap values were calculated based on 1000 replicates.

**Nucleotide sequence accession numbers**

The nucleotide sequences of both the 16S rRNA genes and GHF48 genes from the clone libraries have been deposited in the GenBank database under accession numbers JQ741978–JQ741999.

---

**Results and discussion**

**Degradation properties of the cellulolytic community**

The isolated microbial community could degrade FP and Avicel under anaerobic conditions at 60 °C within 3 days, as shown in Fig. 1a. Initially, the FP became soft, then sticky, and eventually it dissolved completely. The phenomenon of the FP decomposition differed from that of C. thermocellum LQR1, in which the FP initially became thin and then dissolved. The fermentation products of the cellulolytic culture were detected by HPLC for 6 days. The results showed that the main products produced were ethanol, acetic acid and butyric acid (Fig. 1b). Ethanol was the primary fermentation product; and most of it was produced during the first day and the yield increased continuously. The content of acetic acid increased significantly, especially during the first 3 days. Like ethanol, butyric acid was mainly produced during the first day and thereafter maintained a constant level. Cellobiose was detected on the third day, and with a peak value of 0.02 g L⁻¹ on the fourth day. Glucose was only detected on the second day, with a concentration of 0.02 g L⁻¹. The low concentration of the cellobiose and glucose indicated their immediate
A minor proportion of butanol was detected on the 10th day, with a concentration of 0.016 g L\(^{-1}\). Normally, butanol is produced by mesophilic anaerobic bacteria such as *Clostridium acetobutylicum*; however, the thermophilic bacterial mixtures (60 °C) studied here also showed butanol production, indicating the presence of thermophilic butanol-producing species in the community. However, other fermentation products still remained to be determined. Note that FP degradation was not a secondary consequence of using L-cysteine and bicarbonate as primary carbon source. This was confirmed using a medium with FP as the sole carbon source (without L-cysteine and bicarbonate); the degradation of FP was not changed except that the decomposing rate was slower.

The enzyme activity of the fermentation supernatant was compared with that of *C. thermocellum* LQR1. The FPase and CMCase activities of the community were two times higher than that of *C. thermocellum* LQR1 and beta-xylosidase of the community was much more active. The activities of xylanase, beta-glucosidase and pNPCase of *C. thermocellum* LQR1 were also higher (Table 1).

### Table 1. Comparison of the crude enzyme glycoside hydrolase activities of the consortium and those of *Clostridium thermocellum* LQR1

<table>
<thead>
<tr>
<th>Glycoside hydrolases</th>
<th>Enzymatic activities (units mg(^{-1}) protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude enzyme</td>
</tr>
<tr>
<td>FPase</td>
<td>1.5 ± 0.22</td>
</tr>
<tr>
<td>Xylanase</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>CMCase</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>beta-Glucosidase</td>
<td>&lt; 0.002</td>
</tr>
<tr>
<td>pNPCase</td>
<td>&lt; 0.006</td>
</tr>
<tr>
<td>beta-Xylosidase</td>
<td>0.06 ± 0.02</td>
</tr>
</tbody>
</table>

*Values are means of three replications ± SD.

16S rRNA gene diversity

To identify the community members, a 16S rRNA gene library of the cellulolytic consortium was constructed. Diversity levels were determined with a cutoff value of 97% sequence similarity. A total of 16 OTUs were represented in the clone library after 50 clones were surveyed. Rarefaction analysis of the 16S rRNA clone library is shown in Fig. 2. The most abundant OTUs accounted for 42% and 18% of the clone library, and shared similarity with the type strain *C. thermocellum* ATCC 27405, which is known for its high cellulolytic ability due to cellulose formation. Although the 16S rRNA gene similarities of these clones were around 87–89%, we believe that they were mainly responsible for cellulose degradation. In other studies, *Clostridium straminisolvens*-like sequences accounted for a large portion of cellulolytic enrichments (Izquierdo et al., 2010). In our results, one OTU accounted for only 2% of the clone library and was most similar to *C. straminisolvens*. However, in contrast to other cellulolytic enrichments, all sequences from the OTUs represented novel species. Other OTUs made up a minor portion of the clone library; however, they may still play an important role in the cellulose degradation process. For example, it was reported that *Caldicoprobacter oshimai* was a xylanolytic, extremely thermophilic bacterium (Yokoyama et al., 2010). The OTU which showed the closest similarity with *C. oshimai* might also be a xylanolytic bacterium, which would play an important role in lignocellulose degradation. Another example was one OTU which represented 4% of the clone library, and one strain from the OTU which had been isolated as pure culture. This strain was named ASX2 and shared 90.4% 16S rRNA gene sequence identity with *Desulfotomaculum halophilum* SEBR 3139. ASX2 was able to hydrolyze CMC, as determined by formation of a clear zone on a

![Fig. 2. Rarefaction curve for the 16S rRNA gene clone library with a confidence threshold of 95%](image-url)
Fig. 3. Phylogenetic tree of the 16S rRNA gene and their closest related strains from the GenBank. Phylogenetic relationships were inferred by the NJ method. Bootstrap values were calculated based on 1000 replicates.
Congo Red agar plate (data not shown). Beta-glucosidase was also found in strain ASX2. It is noteworthy that most of the clones represented by the clone library shared 16S rRNA similarities lower than 90%, and all of them shared 16S rRNA similarities below 94%, which meant that they were novel at least at species level. One example was the isolation of strain ASX2 mentioned above. Another example was the isolated pure culture which shared 93% 16S rRNA sequence identity with Bacillus thermolactis. The general low 16S rRNA similarity might be attributable to the fact that the coastal marine environment was thought to be hypothermal, so thermophilic bacteria were ignored. In our experiment, the selection pressure put on by thermophilic and anaerobic conditions and the limited carbon source eliminated bacterial species which were commonly found by traditional isolation methods under low temperature and aerobic conditions.

The BLAST results showed that the known strains most closely related to the sequenced clones were all from a terrigenous environment, for example Planifilum yunnanense isolated from a hot spring, Sporosalibacterium faouarense isolated from oil-contaminated soil, D. halophilum isolated from an oilfield brine and C. oshimai isolated from sheep feces (Tardy-Jacquenod et al., 1998; Zhang et al., 2007; Yokoyama et al., 2010; Rezgui et al., 2011). A few of them such as D. halophilum and S. faouarense were reported as moderately halophilic bacteria (Tardy-Jacquenod et al., 1998; Rezgui et al., 2011). As we know, the marine environment was of high salinity and there is a possibility that these bacteria from land had adapted to the marine saline conditions and at last settled down in the ocean. However, the isolation environment and the low 16S rRNA similarity might indicate the opposite possibility, which was that the evolution positions of our clones pre-dated the isolated terrigenous strains. Briefly, the halophilic and thermophilic properties of these bacteria are unexpected and provide an interesting area for future studies.

The phylogenetic tree of these clones and their closest related strains from the GenBank were constructed through the NJ method (Fig. 3). As shown in Fig. 3, all of the clones formed separate branches from the known species. The 16S rRNA gene sequences were mainly from the order Clostridiales, with a proportion of 76% (38/50) by RDP Naive Bayesian rRNA Classifier. The clones of the top cluster of the tree were mainly classified to the genus Acetivibrio and were closely related to C. thermocellum and C. straminisolvens (Fig. 3). It is known that C. thermocellum is a cellulosome-producing bacterium. It will be important to determine whether the strains in the community isolated can produce a cellulosome. To our knowledge, cellulosome-producing bacteria have never been found in any marine environment.

A theory explaining how the thermophiles accumulated in the cold ocean concludes that the thermophiles are produced by seabed fluid flow from warm subsurface petroleum reservoir and ocean crust ecosystems (Hubert et al., 2009). These authors also found that all these thermophilic bacteria are spore-forming Firmicutes species.

**Diversity of GHF48 genes**

The diversity of cellulases of GHF48 was explored as a functional gene indicative of truly cellulolytic bacteria (Izquierdo et al., 2010). GHF48 gene is known for its ability to enhance cellulose solubilization in synergistic interactions with family 9 glycosyl hydrolases and mostly single copies in the genomes of cellulolytic microbes (Irwin et al., 2000; Berger et al., 2007). The cloned
GHF48 sequences were blasted against the NCBI database. The results showed that these sequences shared the closest similarities to the uncultured bacterial clone from the thermophilic biocompost enrichments, *Clostridium lentocellum* and *C. straminisolvens* (Izquierdo et al., 2010). The diversity of GHF48 was low, which is in accordance with our result that most of the 16S rRNA of the cellulolytic bacteria were the most closely related to *C. thermocellum*.

The phylogenetic tree of these sequences and their closest related strains from the GenBank were constructed (Fig. 4). The GHF48 clones were classified to two general branches (Fig. 4). All GHF48 sequences belonged to *Clostridia*. The upper branch contained clones G2, G7 and G19 (with a total proportion of 72%). They were most similar to the uncultured bacterium clone CO6-G1 and CO6-G35 GHF48 gene, and *C. straminisolvens* strain CSK1 GHF48 gene, respectively, with only 70% amino acid sequence similarity to *C. straminisolvens* strain CSK1 GHF48 gene, and with 71% amino acid sequence similarity to the GHF48 protein. The lower branch contained clones G6, G11 and G22, accounting for 28% of the clone library, with 71% amino acid sequence similarity to the GHF48 identified in *Herpetosiphon aurantiacus*.

**Acknowledgements**

This work was supported by grants from the National Basic Research Program of China (No. 2011CB707404) and National Key Technology R&D Research Program (2011BAD22B02-01).

**References**


Weimer PJ & Zeikus JG (1977) Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and


