Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 2011, 23(4) 649–655
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Characterization of the effective cellulose degrading strain CTL-6

Yucai Lü[1,2], Xiaofen Wang[2], Ning Li[1], Xiaojuan Wang[2], Masaharu Ishii[3], Yasuo Igarashi[3], Zongjun Cui[2,*]

1. Alan G. MacDiarmid Research Institute of Renewable Energy, China Three Gorges University, Yichang 443002, China. E-mail: Lv_yucai@hotmail.com
2. College of Agronomy and Biotechnology/Center of Biomass Engineering, China Agricultural University, Beijing 100193, China
3. Graduate School of Agricultural and Life Sciences, The University of Tokyo, I-I-I Yayoi, Bunkyo-Ku, Tokyo 113-8657, Japan

Received 14 May 2010; revised 09 August 2010; accepted 26 October 2010

Abstract

An efficient cellulose degrading bacteria exists in the thermophilic wheat straw-degrading community, WDC2. However, this strain cannot be isolated and cultured using conventional separation techniques under strict anaerobic conditions. We successfully isolated a strain of effective cellulose degrading bacteria CTL-6 using a wash, heat shock, and solid-liquid alternating process. Analysis of its properties revealed that, although the community containing the strain CTL-6 grew under aerobic conditions, the purified strain CTL-6 only grew under anaerobic culture conditions. The strain CTL-6 had a striking capability of degrading cellulose (80.9% weight loss after 9 days of culture). The highest efficiency value of the endocellulase (CMCase activity) was 0.404 μmol/(min·mL), cellulose degradation efficiency by CTL-6 was remarkably high at 50–65°C with the highest degradation efficiency observed at 60°C. The 16S rRNA gene sequence analysis indicated that the closest relative to strain CTL-6 belonged to the genus *Clostridium thermocellum*. Strain CTL-6 was capable of utilizing cellulose, cellobiose, and glucose. Strain CTL-6 also grew with Sorbitol as the sole carbon source, whereas *C. thermocellum* is unable to do so.

Key words: cellulose-degrading bacterium; cellulose degradation capability; anaerobic bacterium; *Clostridium*

DOI: 10.1016/S1001-0742(10)60460-3

Citation: Lü Y C, Wang X F, Li N, Wang X J, Ishii M, Igarashi Y et al., 2011. Characterization of the effective cellulose degrading strain CTL-6. Journal of Environmental Sciences, 23(4): 649–655

Introduction

Conversion and utilization of wheat straw resources by microbes is a major concern for environmental conservation and energy development. Approximately 0.6–0.7 billion tons of agricultural straw is produced in China annually (Editorial Committee of China Agriculture Yearbook, 2005), among which wheat straw accounts for 21%. At present, these rich straw resources are not effectively utilized. Waste straw is stacked, allowed to decay, or is eliminated by centralized combustion. These treatments result in increased environmental pollution, which consequently affects people's quality of life and health.

To reduce the environmental burden that straw combustion causes every year, straw resources are rapidly decomposed, and further converted to available bio-energy products, such as ethanol or biogas (Demain et al., 2005). These measures can effectively clean up the environment and provide inexpensive biomass energy.

Various attempts have been made to develop effective cellulose degradation systems to effectively decompose wheat straw in the environment (Bayer et al., 2006). In our laboratory, a cellulose-degrading bacterial community (designated as WDC2), capable of effectively degrading wheat straw, was successfully enriched from cow manure and wheat straw composting materials. Up to 60% of waste straw was degraded by the community. Clone analysis revealed that community WDC2 was composed of a variety of uncultivated microbes comprised of cellulolytic and non-cellulolytic bacteria (Lü et al., 2009). The ability of community WDC2 to efficiently degrade wheat straw and convert it into various organic acids may have broad applications in biomass resources conversion.

To investigate the micro-biological mechanisms of efficient wheat straw degradation by community WDC2 and to characterize the degradation characteristics of uncultivated cellulolytic bacteria of community WDC2, it is essential to isolate and investigate the properties of the cellulolytic bacteria in the mixed community. However, cellulolytic bacteria are extremely difficult to isolate from the community WDC2, because after long-term domestication and screening, the community WDC2 has obtained stable cellulose degradation capability and community structure, which is composed of many unknown and partly uncultivated anaerobes and facultative aerobes. Since

* Corresponding author. E-mail: acuizj@cau.edu.cn

non-cellulolytic bacteria in the community have developed similar growth characteristics as the cellulolytic bacteria, likely due to some kind of symbiotic relationship within the community, it is difficult to isolate and culture individual strains using conventional anaerobic separation techniques (Lü et al., 2009).

Cellulose degradation strains in WDC2 cannot be isolated using normal isolation methods, even under strictly anaerobic conditions. In this article, an anaerobic cellulose-degrading bacterium (strain CTL-6) was successfully isolated from the community WDC2 by means of adsorption-precipitation of cellulose powder, heat shock, solid-liquid alternating culture protocols and other methods as described in detail below.

Moreover, we elucidated characteristics such as the isolation procedures of CTL-6, cellulose degradation capability of this strain, the optimum degradation temperature and CMCase activity. We also determined the phylogenetic classification of this strain.

1 Materials and methods

1.1 Media and culture conditions

The CTL-6 was cultivated in DSM122 medium under anaerobic conditions. The medium contained the following components dissolved in 1 L water (pH 7.0): $(NH_4)_2SO_4$ 1.30 g, $MgCl_2{\cdot}6H_2O$ 2.6 g, KH_2PO_4 1.43 g, K_2HPO_4 5.52 g, $CaCl_2{\cdot}2H_2O$ 0.13 g, Na-β-glycerophophate$\cdot 6H_2O$ 6.00 g, yeast extract powder (Aoboxing, Beijing, China) 4.50 g, glutathione 0.25 g, $FeSO_4{\cdot}7H_2O$ 1.10 mg, resazurin 1.00 mg, cellulose (cellulose powder or cellulose paper) 5.00 g. The DSM122-agar medium was the same as the DSM122 medium except that 15 g agar (Yault, Japan) was added.

Culture methods and conditions: strict anaerobic procedures (Zavarzina et al., 2000; Kato et al., 2004) were used for culturing and for physiological and substrate-utilization tests unless stated otherwise. All anaerobic media containing reducing agents were pre-reduced by cooling after sterilization (121°C, 20 min), inside an anaerobic chamber with anaerobic gas mixture (H_2/N_2, 10/90, *V*/*V*). Only fully reduced media, as indicated by the colorless state of resazurin in the media, were used for culture. Bacteria (0.2 mL) were inoculated into 4 mL of DSM122 medium in a 20-mL anaerobic culture tube under static conditions at 60°C. DSM122-agar medium was maintained in a AnaeroPack® Rectangular Jar (Mitsubishi Gas Chemical Company, Tokyo, Japan) with oxygen absorber.

1.2 Total protein measurement

Total protein content was measured according to previously described methods (Han et al., 2003).

1.3 Gravimetrical analysis method of residual cellulose

Media containing strain CTL-6 and cellulose was filtered, and the residual cellulose was dried to a constant weight at 60°C. Cellulose degradation by strain CTL-6 was determined as the difference between cellulose present at the beginning and end of the culture period. The treatment was repeated three times, and a blank control using cellobiose as the carbon source was prepared to eliminate the effect of medium and cell mass (Tailliez et al., 1989).

1.4 CMCase activity

Cellulose degradation activity was expressed as cellulase (CMCase) activity. CMCase activity was measured in a 1-mL mixture. Firstly, 200 μL of culture supernatant (culture solution was centrifuged at 8000 r/min for 20 min) and 200 μL of 1% (*W*/*V*) solution of carboxymethyl cellulose (CMC) were mixed and cultured for 30 min at 60°C. 600 μL of DNS agents (NaOH 1%, Rochelle salt 20%, phenol 2%, sodium sulfite 0.005%, 3,5-dinitrosalicylic acid 1%) was added to the mixture and incubated for 15 min at 100°C. After that the 1 mL mixtures were cooled on ice for 5 min, centrifuged and absorption determined at 600 nm. At the same time, a standard curve was made using 5 mmol/L glucose; a blank control was prepared for each sample. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 mmol of reducing sugars per minute under the above conditions (Faiez et al., 2008; Singh et al., 2001).

1.5 DNA extraction and PCR amplification

Bacterial cells used for DNA extraction were cultured in DSM122 medium for 3 days. Culture solutions were centrifuged at 8000 r/min for 5 min (4°C), the supernatant was then decanted carefully and the cell pellets were resuspended in 500 μL of extraction buffer in 1.5 mL tubes (100 mmol/L Tris-HCl (pH 9.0), 40 mmol/L EDTA (pH 8.0)). After a brief vortex, the mixture was stored at –20°C. Genomic DNA extraction was performed using benzyl chloride method (Zhu et al., 1993; Wang et al., 2006). The concentration of DNA was determined using a DNA/RNA/protein analyzer (Biospecmini Shimadzu, Japan). The extracted DNA was diluted to the appropriate concentration and used as the template in PCR amplification. The PCR amplification was performed using GeneAmp PCR System (Model 9700, Applied Biosystems, USA). The primers used were 357F-GC, 5′-CCTACGGGAGGCAGCAG-3′ (*Escherichia coli* positions, 341–357), which was attached to a GC clamp (5′-CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCACGGGGGG-3′) at the 5′-terminus, and 517R, 5′-ATTACCGCGGCTGCTGG-3′ (*E. coli* positions, 517–534) (Muyzer et al., 1993). Initial DNA denaturation was performed at 94°C for 10 min, followed by 22 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 45 sec, and elongating at 72°C for 1 min 30 sec, followed by a final elongation step at 72°C for 5 min. The products were examined by electrophoresis on 2% agarose gel.

1.6 Denaturing gradient gel electrophoresis (DGGE) analysis of PCR product

Denaturing gradient gel electrophoresis analysis of PCR products was performed with the DCode™ system (Bio-Rad Laboratories, Hercules, CA) (Muyzer et al., 1993; Haruta et al., 2002). Briefly, samples were loaded onto 1

mm thick, 6%–12% (*W*/*V*) polyacrylamide gradient gels in 0.5× tris-acetate-EDTA (TAE) electrophoresis buffer (20 mmol/L Tris-HCl pH 8.3, 10 mmol/L acetic acid, 0.5 mmol/L EDTA), with 20%–60% denaturant gradient (where 100% is defined as 7 mol/L urea with 40% formamide). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 61°C for 5 hr. After electrophoresis, the gels were stained with SYBR® Green I (Molecular Probes, Eugene, Oregon, USA) and photographed (Pedro et al., 2001).

1.7 Terminal restriction fragment length polymorphism analysis (T-RFLP)

The 16S rRNA gene sequence of strain CTL-6 and community WDC2 were determined by direct sequencing of the purified PCR-amplified 16S rRNA gene fragment. The PCR protocol was as described above (Section 1.5). The primers used were 27F-6FAM, 5′-AGAGTTTGATCCTGGCTCAG-3′ (*E. coli* positions: 8–27); 907R, 5′-CCCCGTCAATTCCTTTGAGTTT-3′ (*E. coli* positions: 907–929) (Hori et al., 2006). The PCR products were purified on a Wizard® SV Gel and PCR Clean-Up System (Promega, Tokyo, Japan), according to the manufacturer's instructions.

The amplified DNA fragments (3 μL) were digested with a 2.5 U restriction enzyme in a total volume of 10 μL for 16 hr at 37°C for *Msp*I (New England Biolabs Inc., Tokyo, Japan). This restriction enzyme was selected because it produces several short T-RFs (< 300 bases) from most bacteria. The restriction digest products (2 μL) were mixed with 10 μL deionized formamide and 0.5 μL GeneScan-500 ROX standard (Applied Biosystems, Tokyo, Japan). The samples were denatured at 95°C for 2 min, followed by rapid chilling on ice. The fluorescently labeled T-RFs were separated by size on an ABI 3130xl genetic analyzer (Applied Biosystems), with electrophoresis at 60°C and 15 kV, for 30 min with the POP-7 polymer. The electropherograms were analyzed with GeneMapper version 3.7 software (Applied Biosystems), and the fragment sizes were estimated using the local southern method. The T-RFs fluorescence units with peak heights < 30 were excluded from the analysis (Takeshita et al., 2007; Ueno et al., 2006).

1.8 Phylogenetic analysis

The 16S rRNA gene sequence of strain CTL-6 was determined by direct sequencing of the purified PCR-amplified 16S rRNA gene fragment. The 16S rRNA gene sequence of strain CTL-6 was amplified using the PCR protocol described in section 1.5. The PCR was performed with universal bacterial primers complementary to conserved regions of the 5′ and 3′ ends of the 16S rRNA gene, 27F, 5′-AGAGTTTGATCCTGGCTCAG-3′ (*E. coli* positions: 8–27) and 1512R, 5′-ACGGCTACCTTGTTACGACT-3′ (*E. coli* positions: 1512–1493) (Devereux and Willis, 1995).

The 16S rRNA gene was sequenced directly using the ABI PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI PRISM® 3130xl Genetic Analyzer (Applied Biosysytems). The 16S rRNA gene sequences of strain CTL-6 were compared to sequences from GenBank. Sequence similarity searches were performed in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) using the blast database. The sequence information was then imported into DNASTAR and MEGA 4.0 software for assembly and alignment. The tree was constructed by neighbor-joining analysis of a distance matrix obtained from multiple-sequence alignment. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points.

1.9 Nucleotide sequence accession numbers of strain CTL-6

The 16S rRNA gene sequence of strain CTL-6 was deposited with GenBank under accession number FJ599513.

2 Results and discussion

2.1 Isolation of cellulose degrading strain CTL-6

The community WDC2 was cultured in DSM122 medium using cellulose powder as the sole carbon source. The color of the cellulose powder was used as an indication of cellulose degradation (Coughlan and Mayer, 1992) (the color of cellulose powder before cellulose degradation is white and it will turn yellow during the degradation process). After the color turned yellow, the residual cellulose powder was washed with anaerobic buffer (PBS with 1%, *W*/*V*, L-cysteine hydrochloride, pH 6.5) inside an anaerobic chamber and then transferred into fresh DSM122 medium. The medium was maintained under anaerobic conditions at 60°C. The process was repeated three times and community W was obtained.

Community W was further cultured in DSM122 medium (cellulose powder as the sole carbon source). After the color of cellulose powder had turned yellow, Community W was heat-shocked for 5 min at 105°C, and 0.2 mL culture solution was inoculated into 4 mL of fresh DSM122 medium (cellulose powder as the sole carbon source) and cultured under anaerobic conditions at 60°C. This process was repeated three times and a thermophilic enrichment culture F was obtained.

Community F was then inoculated into DSM122-agar medium (cellobiose as the sole carbon source) under anaerobic conditions at 60°C. After the colony had grown, the isolated colony was inoculated into fresh DSM122 medium (cellulose powder as the sole carbon source) and cultured under anaerobic conditions at 60°C. Procedures of solid-liquid alternating process were repeated three times and strain CTL-6 capable of efficiently degrade cellulose was obtained.

2.2 Analysis of the cellulose degradation process performed by strain CTL-6

The cellulose degradation process performed by strain CTL-6 and the cells comprising strain CTL-6 were

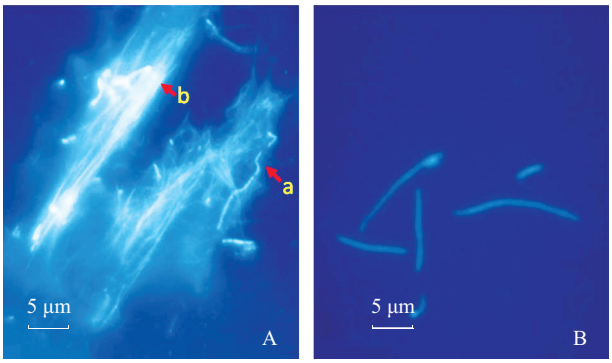

Fig. 1 Phase-contrast micrograph of cells of strain CTL-6. (A) profile of cellulose degradation by strain CTL-6, including the cell of strain CTL-6 (a), and cellulose filaments (b); (B) profile of cells of strain CTL-6.

observed under a phase-contrast microscope (Axioplan II imaging; Carl Zeiss) (Fig. 1). The cellulose powder appeared dispersed and had a filamentous structure. Strain CTL-6 looked embedded in the cellulose filament and swam freely in the culture medium (Fig. 1A). Strain CTL-6 had rod-shaped vegetative cells (about 5–10 μm long), with the majority of the cells appearing as single cells and a few cells forming small clusters and oval spores (Fig. 1B).

2.3 Relationship between strain CTL-6 and community WDC2

The DNA from enriched cultures of strain CTL-6, community W and community WDC2 was extracted and analyzed. Figure 2 shows the DGGE patterns of the V3 region of 16S rRNA gene PCR products. The DGGE profiles of both community WDC2 and W showed a strong band that corresponds to strain CTL-6. This result indicates that strain CTL-6 was a dominant bacterium in community WDC2. Community W showed four bands, indicating that it was mixed community composed of a variety of bacteria. Only one band corresponding to that of community WDC2 remained in strain CTL-6 through several cycles of restricted culture. These data also showed

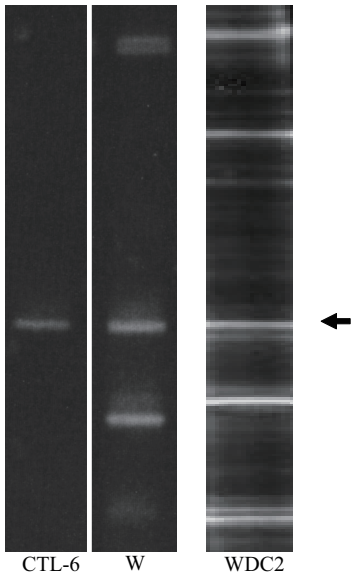

Fig. 2 Denaturing gradient gel electrophoresis profiles of strain CTL-6, community W, WDC2.

that the washing protocol utilized alone was not sufficient for the purification of cellulose degrading bacteria and that multiple methods must be used.

The PCR was performed on the 16S rRNA gene amplified from the DNA extracted from strain CTL-6 and community WDC2 after five days in culture using fluorescently labeled primers. The T-RFLP profiles resulting from the *Msp*I digestions of the DNA sample are shown in Fig. 3. The T-RFLP analyses indicated that a characteristic peak corresponding to strain CTL-6 was also present in the T-RFLP profile of community WDC2, which consisted of a total of seven peaks. The functions of bacterial species represented by the other peaks in community WDC2 are not clear, but strain CTL-6 had efficient cellulose degradation capability, and may be the primary functional strain of community WDC2 capable of degrading cellulose.

Results (Figs. 1, 2, and 3) indicated that strain CTL-6 existed in community WDC-2 and strain CTL-6 as pure culture was obtained through enrichment isolation and cultivation.

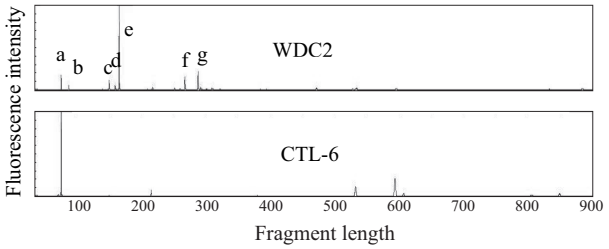

Fig. 3 Terminal restriction fragment length polymorphism analysis (T-RFLP) maps of strain CTL-6 and community WDC2.

2.4 Cellulose degradation ability of Strain CTL-6

Strain CTL-6 was cultured in DSM122 medium under anaerobic conditions using cellulose paper (60 mg) as the sole carbon source (Fig. 4). During 9 days cultivation residual cellulose paper was gravimetrically determined using the method described in Section 1.3. After 1 day of culture, the amount of residual cellulose paper in the medium was 58.98 mg; indicating that little degradation occurred. After 6 days of culture, however, substantial cellulose paper degradation was observed as the paper softened and broke while turning yellow in color and reaching a residual weight of 19.7 mg. At the end of culture (9 days), the residual cellulose paper weight was 11.49 mg, and total degradation ratio reached 80.9%. Noticeably, the highest degradation ratio of the cellulose paper was observed between the first and sixth day of culture, with the degradation ratio for this period accounting for 65.5% of total paper degraded.

The utilization of the substrate was confirmed by monitoring pH drop in the culture solution (Kato et al., 2004). The initial pH of the culture solution was 6.83. The pH decreased quickly during culture (pH 6.8, day 1; pH 5.7, day 6). At the end of the culture period (9 days) the pH was 5.63. The pH value dropped rapidly between the first and sixth day of culture, indicating that strain CTL-6 had a remarkable cellulose degradation efficiency during this

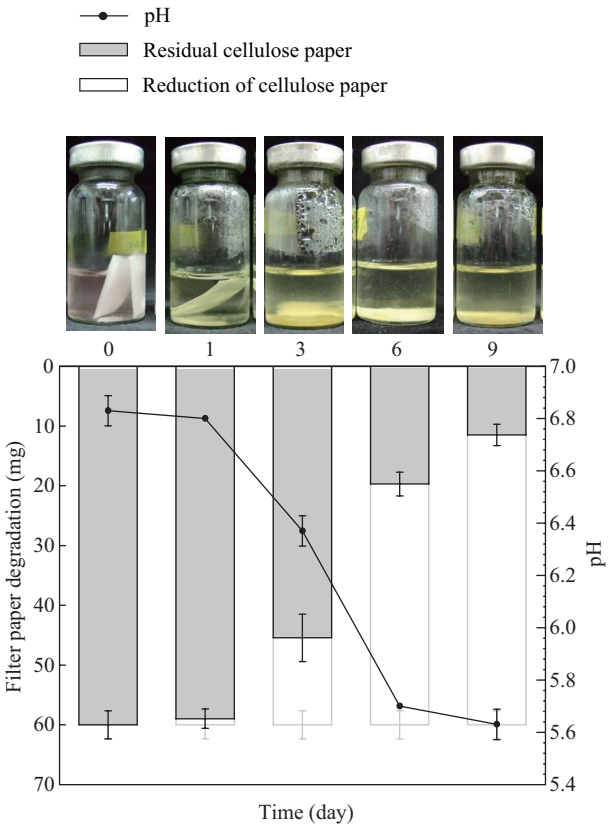

Fig. 4 Cellulose paper degradation by strain CTL-6 and pH value of culture solution. Sixty milligram of cellulose paper was added as the sole carbon source according to observations in anaerobic culture bottles, and this initial weight of cellulose paper was reduced appreciably at different days of culture.

period.

In nature, cellulosic materials can be degraded by many bacteria. More than 50 species have been isolated (www.wzw.tum.de/mbiotec/cellmo.htm). However, various strains possess different cellulose degradation capabilities. Research by Shiratori et al. (2006) has reported that the degradation ratio of photocopy paper after 3 days of digestion with *Clostridium* sp. EBR45, *C. thermocellum* JCM9323 and *Clostridium straminisolvens* were 79%, 74% and 68%, respectively. Previous studies have reported that the degradation ratio of cellulose paper by community MC1 was up to 95%, even though community MC1 was comprised of cellulolytic bacteria and non-cellulolytic bacteria (Cui et al., 2004; Haruta et al., 2002). Degradation efficiency of a cellulose degrading microbial community based on good microbial ecological relationship is usually more active than that of a single isolate. In this study, however, the cellulose paper degradation ratio by strain CTL-6 was 80.9% after nine days of culture, suggesting that strain CTL-6 as a single isolate had outstandingly high cellulose paper degradation capability.

2.5 CMCase activity and growth of strain CTL-6

Endocellulase efficiency (CMCase activity) was 0.115 μmol/(min·mL) at the beginning of culture (Fig. 5) but increased during culture (0.396 μmol/(min·mL) after 3 days and 0.404 μmol/(min·mL) after 6 days), before decreasing to 0.363 μmol/(min·mL) at the end of the culture period (9

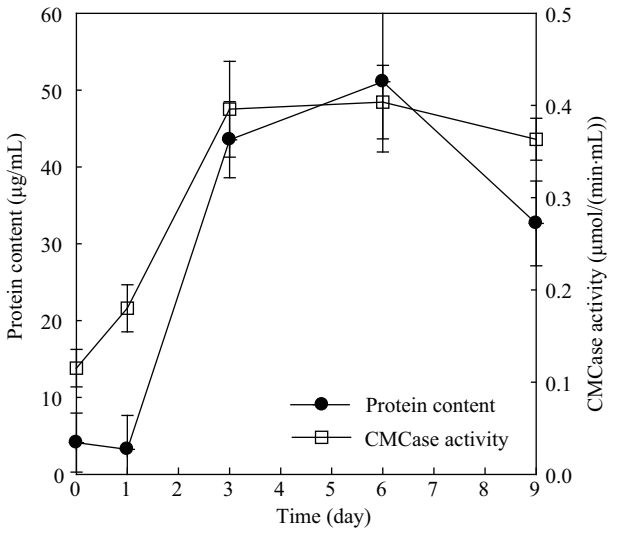

Fig. 5 Cellulose degradation efficiency (CMCase activity) and total amount of protein of strain CTL-6.

days). Reynolds et al. (1985) reported extremely high CMCase activity for *C. thermocellum* (0.928 μmol/(min·mL)). They isolated thermophilic degrading-cellulose anaerobes (TP8, KP8, TP11), whose CMCase activities were only 0.324, 0.214, and 0.264 μmol/(min·mL), respectively. In our study, the highest value of CMCase activity (0.404 μmol/(min·mL)) was lower than that of *C. thermocellum*, this result revealed significant differences between strain CTL-6 and *C. thermocellum*.

The biomass of strain CTL-6 was calculated from total protein (TP) in the culture solution (Fig. 5). The initial TP content of strain CTL-6, immediately after inoculation in culture solution, was 4.13 μg/mL. The most rapid biomass growth of strain CTL-6 was observed during day 1–3 of culture, with TP content increasing from 3.25 to 43.53 μg/mL. The highest value of TP content (51.09 μg/mL) was detected after 6 days of culture, before decreasing to 32.66 μg/mL at the end of culture (9 days). The plots of strain CTL-6 CMCase activity and TP followed a similar trend and consequently were accompanied by biomass growth, CMCase activity of the culture solution increased. The cellulose degradation activity of CTL-6 was reflected in the biomass. Figure 5 shows that CTL-6 possessed the highest activity of cellulose degradation between the first and sixth days of culture. This is because carbon needed for bacterial grow and metabolism was supplied by the degradation of cellulose.

2.6 Effect of temperature on cellulose paper degradation of strain CTL-6

Figure 6 shows the temperature profile for the degradation. The degradation ratio after nine days cultivation was calculated. The ability of strain CTL-6 to degrade cellulose paper changed significantly at various temperatures ranging from 45°C to 70°C. The optimum temperature for its growth and cellulose degradation was observed at 60°C, with the highest degradation ratio of 79.5%. The degradation capability was significantly reduced by either a drop or increase in the temperature of the culture solution, with the degradation ratios being 16.8% and

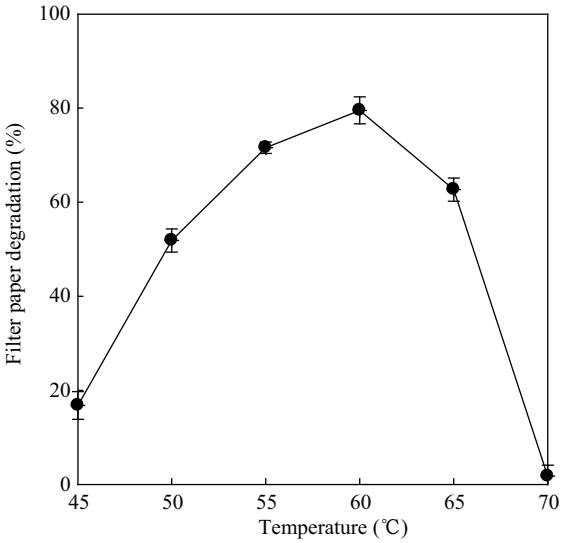

Fig. 6 Changes in cellulose paper degrading capability of strain CTL-6 at different temperatures. The highest degradation ratio of 79.5% was observed at 60°C.

1.75% at 45°C and 70°C, respectively.

2.7 16S rRNA gene phylogenetic analysis of strain CTL-6

The 16S rRNA gene sequence obtained for strain CTL-6 was compared with known sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) using the blast database. A phylogenetic tree was constructed using the neighbor-joining method (Fig. 7). The results of the sequence-similarity calculations indicated that strain CTL-6 belonged to cluster III (Collins et al., 1994) of the genus *Clostridium*. The closest relative of strain CTL-6 was *C. thermocellum* ATCC 27405 (99.9% similarity) (1462/1463). Most of these clusters were reported as being anaerobic, cellulolytic bacteria (Ng et al., 1977; Yang et al., 1990).

2.8 Utilization of different carbon source substrate

Strain CTL-6 was cultured in DSM122 medium supplemented with various substrates as the carbon source (Table 1) at 0.5%. The results indicate that strain CTL-6 can utilize cellulose, cellobiose, glucose, and sorbitol as the sole carbon source. No growth of strain CTL-6 was observed with glycerol, mannose, sucrose, and raffinose.

Table 1 Comparison of different substrates utilization by *C. thermocellum* and strain CTL-6

Substrate utilization	*C. thermocellum*	Strain CTL-6
Cellulose	+	+
Cellobiose	+	+
Glucose	+	+
Glycerol	–	–
Mannose	–	–
Sorbitol	–	+
Sucrose	–	–
Raffinose		–

+: utilized; –: not utilized.

The closest relative of strain CTL-6 was *C. thermocellum* (high similarity) by comparing the obtained 16S rRNA gene sequence with known sequences from GenBank. It has been reported, however, that *C. thermocellum* cannot grow with sorbitol as the sole carbon source, a characteristic indicating that strain CTL-6 was different from *C. thermocellum.* Strain CTL-6 has considerable value in the application of natural cellulose degradation because of its efficient degradation capability of cellulose paper.

3 Conclusions

Strain CTL-6 is a strict anaerobic, thermophilic, and cellulolytic bacterium, which was isolated from a cellulose-degrading bacterial community WDC2 using a cellulose absorbing-wash, heat shock, and solid-liquid alternating process. The degradation rate of the cellulose paper of strain CTL-6 reached 80.9% (weight). The successful isolation of strain CTL-6 provides an opportunity for further research on degrading-straw cellulose mechanisms and symbiotic relationships between cellulolytic bacteria and non-cellulolytic bacteria in community WDC2. These results have the potential to significantly aid the conversion of biomass resources from accumulating straw in nature.

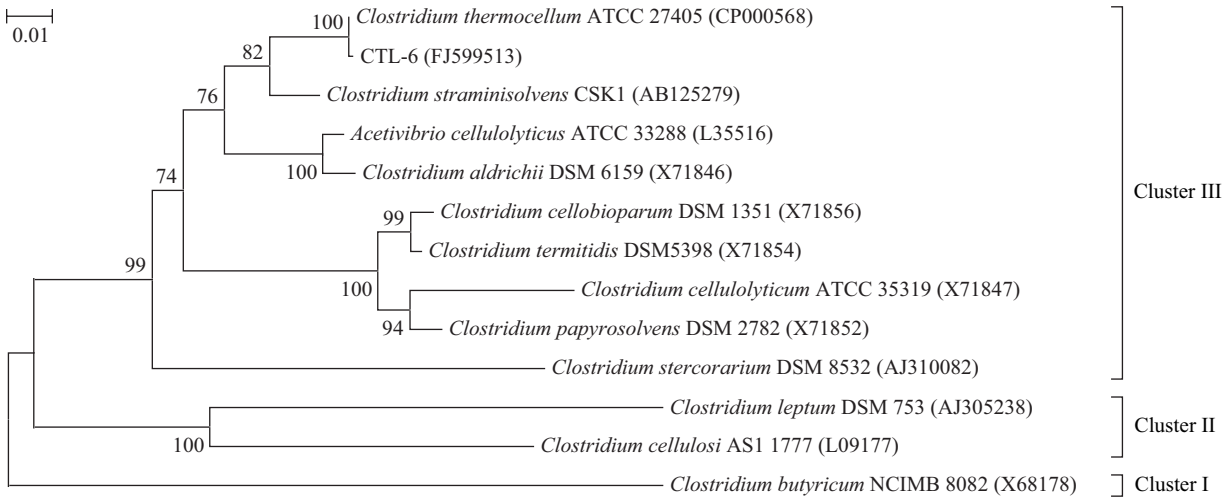

Fig. 7 Phylogenetic dendrogram indicating the relative position of strain CTL-6 as inferred by the neighbor-joining method of complete 16S rRNA gene sequences. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree. Accession numbers are shown in parentheses. The bar indicates 1% sequence divergence.

Acknowledgments

This work was supported by the Key Projects in the National Science & Technology Pillar Program during the Eleventh Five-Year Plan Period (No. 2006BAD10B05-02, 2006BAD07A01) and the National Public Benefit Research Sector (No. 200803033).

References

Bayer E A, Shoham Y, Lamed R, 2006. Cellulose-decomposing bacteria and their enzyme systems. *The Prokaryotes*, 2: 578–617.

Coughlan M P, Mayer F, 1992. The cellulose-decomposing bacteria and their enzyme systems. In: The Prokaryotes I (2nd ed.) (Balows A, Trüper H G, Dworkin M, Harder W, Schleifer K H, eds.). Springer, Berlin. 460–516.

Collins M D, Lawson P A, Willems A, Cordoba J J, Fernandez-Garayzabal J, Garcia P et al., 1994. The phylogeny of the genus *Clostridium*: Proposal of five new genera and eleven new species combinations. *International Journal of Systematic Bacteriology*, 44: 812–826.

Cui Z, Piao Z, Wang W, Su B, Yu H, 2004. Conditions of cellulase production in the presence of microbial consortia MC1 capable of degrading cellulose with high efficiency. *Journal of Agro-environmental Science*, 23(2): 296–299.

Demain A L, Newcomb M, Wu J H D, 2005. Cellulase, clostridia, and ethanol. *Microbiology and Molecular Biology Reviews*, 69(1): 124–154.

Devereux R, Willis S G, 1995. Amplification of ribosomal RNA sequences. In: Molecular Microbial Ecology Manual (Akkermans A D L, van Elsas J D, de Bruijn F J, eds.). Kluwer, Dordrecht. 3.3.1. 1–11.

Editorial Committee of China Agriculture Yearbook, 2005. China Agriculture Yearbook. China Agriculture Press, Beijing. 177.

Faiez A, William A A, Murray M, 2008. New isolate of *Streptomyces* sp. with novel thermoalkalotolerant cellulases. *Biotechnology Letters*, 30(1): 123–126.

Han S O, Yukawa I, Inui A, Doi R H, 2003. Regulation of expression of cellulosomal cellulase and hemicellulase genes in *Clostridium cellulovorans*. *Journal of Bacteriology*, 185(20): 6067–6075.

Haruta S, Cui Z, Huang Z, Li M, Ishii M, Igarashi Y, 2002. Construction of a stable microbial community with high cellulose-degradation ability. *Applied Microbiology and Biotechnology*, 59(4): 529–534.

Hori T, Haruta S, Ueno Y, Ishii M, Igarashi Y, 2006. Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. *Applied and Environmental Microbiology*, 72(2): 1623–1630.

Kato S, Haruta S, Cui Z, Ishii M, Igarashi Y, 2004. Effective cellulose degradation by a mixed-culture system composed of a cellulolytic *Clostridium* and aerobic non-cellulolytic bacteria. *FEMS Microbiology Ecology*, 51(1): 133–142.

Lü Y, Zhu W, Cui Z, Wang X, Ling C, Cheng X, 2009. Characterization and community diversity of cellulose-degrading microflora WDC2 during degrading wheat straw. *Journal of China Agricultural University*, 14(5): 40–46.

Muyzer G, De Waal E C, Uitterlinden A G, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, 59(3): 695–700.

Ng T K, Weimer P J, Zeikus J G, 1977. Cellulolytic and physiological properties of *Clostridium thermocellum*. *Archives of Microbiology,* 114: 1–7.

Pedro M S, Haruta S, Hazaka M, Shimada R, Yoshida C, Hiura K et al., 2001. Denaturing gradient gel electrophoresis analyses of microbial community from fieldscale composter. *Journal of Bioscience and Bioengineering*, 91(2): 159–165.

Reynolds P H S, Sissons C H, Daniel R M, Morgan H W, 1985. Comparison of cellulolytic activities in *Clostridium thermocellum* and three thermophilic, cellulolytic anaerobes. *Applied and Environmental Microbiology*, 51(1):12–17.

Shiratori H, Ikeno H, Ayame S, Kataoka N, Miya A, Hosono K et al., 2006. Isolation and characterization of a new *Clostridium* sp. that performs effective cellulosic waste digestion in a thermophilic methanogenic bioreactor. *Applied and Environmental Microbiology*, 72(5): 3702–3709.

Singh J, Batra N, Sobti R C, 2001. A highly thermostable, alkaline CMCase produced by a newly isolated *Bacillus* sp. VG1. *World Journal of Microbiology and Biotechnology*, 17(8): 761–765.

Takeshita T, Nakano Y, Yamashita Y, 2007. Improved accuracy in terminal restriction fragment length polymorphism phylogenetic analysis using a novel internal size standard definition. *Oral Microbiology and Immunology*, 22(6): 419–428.

Tailliez P, Girard H, Millet J, Beguin P, 1989. Enhanced cellulose fermentation by an asporogenous and ethanol-tolerant mutant of *Clostridium thermocellum*. *Applied and Environmental Microbiology*, 55(1): 207–211.

Ueno Y, Sasaki D, Fukui H, Haruta S, Ishii M, Igarashi Y, 2006. Changes in bacterial community during fermentative hydrogen and acid production from organic waste by thermophilic anaerobic microflora. *Journal of Applied Microbiology*, 101(2): 331–343.

Wang X, Haruta S, Wang P, Ishii M, Igarashi Y, Cui Z, 2006. Diversity of a stable enrichment culture which is useful for silage inoculant and its succession in alfalfa silage. *FEMS Microbiology Ecology*, 57(1): 106–115.

Yang J C, Chynoweth D P, Williams D S, Li A, 1990. *Clostridium aldrichii* sp. nov., a cellulolytic mesophile inhabiting a wood-fermenting anaerobic digester. *International Journal of Systematic Bacteriology*, 40: 268–272.

Zavarzina D G, Zhilina T N, Tourova T P, Kuznetsov B B, Kostrikina N A, Bonch-Osmolovskaya E A, 2000. *Thermanaerovibrio velox* sp. nov., a new anaerobic, thermophilic, organotrophic bacterium that reduces elemental sulfur, and emended description of the genus *Thermanaerovibrio*. *International Journal of Systematic and Evolutionary Microbiology*, 50: 1287–1295.

Zhu H, Qu F, Zhu L H, 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. *Nucleic Acids Research*, 21(22): 5279–5280.