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Screening and Identification of New Isolate: Thermostable *Escherichia coli* with Novel Thermoalkalotolerant Cellulases

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Abstract A cellulase-producing bacterium strain was isolated from soil that produced novel thermoalkalotolerant cellulases after growth on CMC-Na agar screening plate at 37°C. It was identified as *Escherichia coli* using the method of 16S rRNA and intergenic spacer gene analysis combined with morphological, physiological, and biochemical tests. Three major components of the cellulases [carboxymethyl cellulase (CMCase), filter paper cellulase, and β-glucosidase] were produced with maximal activities (0.23, 0.08, and 0.15 U/ml) and maximum specific activities 4.13, 0.56, and 0.50 U/mg protein after 72, 96, and 120 h growth, respectively. Maximum CMCase activity was measured at 50°C and pH 6.0, respectively, and it also retained more than 60% of its maximal activity for at least 20 min at 50–70°C and 10 min at 80°C, respectively, and retained approximately 50% of its maximal activity after incubating at 90°C for 10 min. The enzyme could be applied in bioconversion of lignocellulosic agricultural wastes.

Keywords Screening · Identification · Characteristics · *Escherichia coli* · Cellulase

Introduction

Cellulose is the major component of plant cell wall and is the most abundant renewable resource, and is composed of repeating cellobiose units linked by β-1, 4-glucosidic bonds and forms a crystalline structure. In recent years, the industrial revolution generated an increasing need for energy that was fueled mainly by fossil fuels. With the progress of industrialization, petroleum was in great demand. As a consequence, serious environmental problems have arisen. Recently, products utilizing biomass as an alternative resource have been developed for many markets. One of these, cellulolytic biomass, is known as a carbon-neutral material because it does not increase the amount of carbon dioxide in the air.

Cellulases catalyze the hydrolysis of cellulose which are mainly three types: endoglucanases (EC. 3.2.1.4), cellobiohydrolases (EC. 3.2.1.91), and β-glucosidases (EC.3.2.1.21) [1]. With the recent development of biotechnology, there has been vast interest to use cellulose digestive microorganisms to convert cellulosic biomass to glucose that can be used in different applications such as production of fuel ethanol, use in animal feed, use in waste water treatment, and use in brewing industry.

However, these technologies have hardly been realized in practice because of their high running cost and low yields of this enzyme. Therefore, a decrease in cellulase production cost, an improvement in cellulase performance, and an increase in sugar yields are all vital to decrease processing costs in biorefineries [2].

One strategy is to produce cellulases that are stable over a range of environmental conditions, such as the thermoalkalotolerant cellulases. Most commercial cellulases are produced from fungi, especially *Trichoderma* and *Aspergillus* species, and most of these have limited ranges of temperature and pH. There were few reports about the thermoalkalotolerant isolate strains of *Escherichia coli*, and this study was performed to isolate new *E. coli* strain producing improved thermoalkalotolerant cellulase activities.

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Materials and Methods

Bacterial Strains and Media

Microbes were isolated from soil in Huajiachi campus, Zhejiang University, China, using CMC-Na agar plate, which contained 0.5 g of carboxymethyl cellulose sodium (CMC-Na, Sigma, Saint Louis, USA) as a sole carbon and energy source, 1.5 g of agar and 100 ml of mineral salt solution ($(NH_4)_2SO_4$, 20% (m/v); $MgSO_4$, 0.05% (m/v); K_2HPO_4, 0.1%(m/v); NaCl, 0.05% (m/v), nature pH), and plates were incubated at 37°C for about 36 h. The growing and well-separated individual colonies were isolated and purified. One isolate was tentatively identified as *E. coli* using morphological, physiological, and biochemical assays, and sequence analysis of 16S rRNA and intergenic spacer (ITS) gene.

Identification of the Bacterial Strain

To detect its thermostability, the isolate strain was treated in 80°C water bath for 10 min, and then cultured in LB at 37°C and 225 rpm overnight.

The morphological, physiological, and biochemical characterizations of the cellulase-producing isolate were determined by electron microscope and other identification tests. For further identification, 16S rRNA and ITS gene were polymerase chain reaction (PCR) amplified from the genomic DNA of the strains. Isolation of genomic DNA was carried out by using method of lysozyme and protease k. The 16S rRNA and ITS gene primers used in this study are listed in Table 1. The following primer pairs were used for amplifications from the genomic DNA with their specificities listed parenthetically: 16S rRNA pF–16S rRNA pR (universal) and ITS pF–ITS pR (*E. coli* and related genera).

The process of PCR was both under the following conditions: 94°C, 2 min; 35 cycles of 94°C, 1 min; 55°C 1 min; and 72°C, 90 s; 1 cycle of 72°C, 10 min; and then 4°C forever. The 16S rRNA and ITS gene products with the expected size (about 1500 bp) were purified using a DNA Gel Extraction Kit (Axygen, Silicon Valley, USA) and cloned into pMD18-T vector (Takara, Kyoto, Japan) followed by sequencing. Sequence analysis was performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). Bacterial identification was based on 16S rRNA and ITS gene sequence similarity, regarding the results of morphological, physiological, and biochemical tests. The sequences were aligned using ClustalX program, and Neighbor-Joining phylogenetic tree was generated based on the almost complete sequenced 16S rRNA and closely related sequences using Phylip software package version 3.68. The Seqboot, Dnadist, Neighbor, and Consense programs contained in Phylip software package were used for the Neighbor-Joining analysis, and the bootstrap values were set for 1,000 replicates.

Production of Enzyme

Fermentation medium of LB broth with 1% CMC-Na was inoculated with 1 ml overnight-cultured isolate strain, and cultured on a rotary shaker with an agitation rate of 225 rpm at 37°C. Different fermentation treatments were carried out using the shake-flask method. The enzyme activities were assayed at different times (24–168 h). The cultures were centrifuged at 4000 rpm for 10 min and the supernatant was used as crude enzyme in the determination of cellulase activities and protein concentrations.

Enzyme Assays

The carboxymethyl cellulase (CMCase) and filter paper cellulase (FPase) activities were determined using the IUPAC standard procedure [3]. The β-glucosidase activity was assessed using salicin as substrate by Chahal's method [4]. The reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid method [5]. One unit (U) of the activity was defined as the amount of enzyme releasing 1 μmol reducing sugar per 10 min at 50°C using glucose as a standard. Protein content was determined using the Modified Bradford Protein Assay Kit.

Table 1 Oligodeoxynucleotide primers used for PCR amplifications of bacterial genomic DNA (16S rRNA, ITS, or 23S rRNA) from isolate strain sample

Deoxyoligonucleotide name	Deoxyoligonucleotide sequences (5′ to 3′)	Location on 16S rRNA[a]	Specificity
16S rRNA pF	AGAGTTTGATCCTGGCTCAG	8–27	Universal
16S rRNA pF	AAGGAGGTGATCCAGCC	1525–1541	Universal
ITS pF	CTTGACATCCACGGAAGTTT	990–1009	*E. coli* and related genera
ITS pR	GTTCTTTTTCACTCCCCTCG	490–509	*E. coli* and related genera

[a] *E. coli* numbering system. ITS pF anneals to the 16S rRNA, whereas ITS pR anneals to the 23S rRNA. ITS pF–ITS pR amplifies the 3′ region of the 16S rRNA gene (nucleotide numbers 1010–1542), the ITS, and the 5′ region of the 23S rRNA gene (nucleotide numbers 1–489)

Characteristic Assays of Enzyme

The culture time profile of the isolate strain cultured in fermentation medium of LB containing 1% CMC-Na was determined by measuring the CMCase, FPase, and β-glucosidase activities at the environmental condition of 50°C, pH 5.0, and different fermentation times (24–168 h) with CMC-Na, filter paper, and salicin as the substrates, respectively.

The pH profile of enzyme was evaluated by measuring the CMCase activity at the optimum fermentation time with CMC-Na as the substrate at 50°C and different pH. The following buffers were used: 0.1 M citrate buffer (pH 3.0–6.0), 0.2 M phosphate buffer (pH 7.0–8.0), 0.2 M Glycine/NaOH buffer (pH 9.0–10.0).

The reaction temperature profile of the enzyme was evaluated by measuring the CMCase activity at the optimum fermentation time and pH, at the different reaction temperatures (40–90°C) with CMC-Na as the substrate.

The thermotolerance of CMCase was determined at the optimum fermentation time, pH, and reaction temperature with CMC-Na as the substrate, after incubating the enzymes at different temperatures (50–90°C) for different times (10–60 min). Then, the enzymes were assayed for the remaining activity.

Cloning and Sequence Analysis of Cellulase Gene from Isolate TCP-1

According to the published sequence of *E. coli* cellulase gene in the GenBank (Accession No. CP000970), two DNA oligonucleotide primers (up: 5′-TTGAAGATGAAT GTGTTGCG-3′ and down: 5′-TTAGTGTGAATTTGCGC ATTC-3′) were designed and synthesized to amplify the entire cellulase gene CelTCP-1 from Chromosomal DNA of the isolated strain *E. coli* TCP-1. The condition of PCR was as follows: 94°C, 2 min; 35 cycles of 94°C, 1 min; 60°C 1 min; and 72°C, 90 s; 1 cycle of 72°C, 10 min; and then 4°C forever. The product (size about 1100 bp) was purified using a DNA Gel Extraction Kit (Axygen) and cloned into pMD18-T vector (Takara) followed by sequencing. The deduced amino acid sequence was predicted by an open reading frame (ORF) finder online in NCBI (http://www.ncbi.nih.gov) and was drawn by DNAMAN program. Sequence alignment was performed using the ClustalX software package version 1.81 and alignment picture was generated by GeneDoc software package version 2.7.0.

Statistical Analysis

Analysis of variance (ANOVA) was done with Statistica software package (version 5.0). Before analysis, the assumptions of ANOVA were tested, which are data normality, variance homogeneity, and factor additivity. Tukey honest significant difference (HSD) test and Dunnett *t*-test (2-tailed) were used for multiple comparisons. All experiments were performed in triplicate.

Results

Isolation of Cellulase-Producing Bacteria

To search for characteristic microbe which can produce cellulase, we collected some soil samples near tree roots. By screening the size of opaque circular around the colonies on CMC-Na selective plates after Congo red staining, we isolated a colony, which demonstrated high cellulase activity (Fig. 1).

Identification of the Isolate Strain

After treating the isolate strain in 80°C water bath for 10 min, it could be survived and still grew in LB at 37°C and 225 rpm. It confirmed that this isolate strain has good thermostability.

To identify the isolate strain, the morphological, physiological, and biochemical tests were then carried out and the results showed that this cellulase-producing bacterium belonged to gram-negative Bacillus. Finally, according to the widely used method of 16S rRNA and ITS gene analysis, we cloned and sequenced the 16S rRNA and ITS gene of this thermotolerant cellulase-producing isolate strain, named as TCP-1 (16S rRNA accession no. FJ823386, ITS accession no. FJ823387). The almost complete sequence of 16S rRNA gene showed 99% homology with *E. coli* and *Shigella* strains (GenBank: AB251925, CU928162,

Fig. 1 Screening for cellulase-producing bacteria using CMC-Na plate followed by Congo red staining. After colony purification and culturing for 3D, the clear haloes appeared around the cellulase-producing bacterium colonies

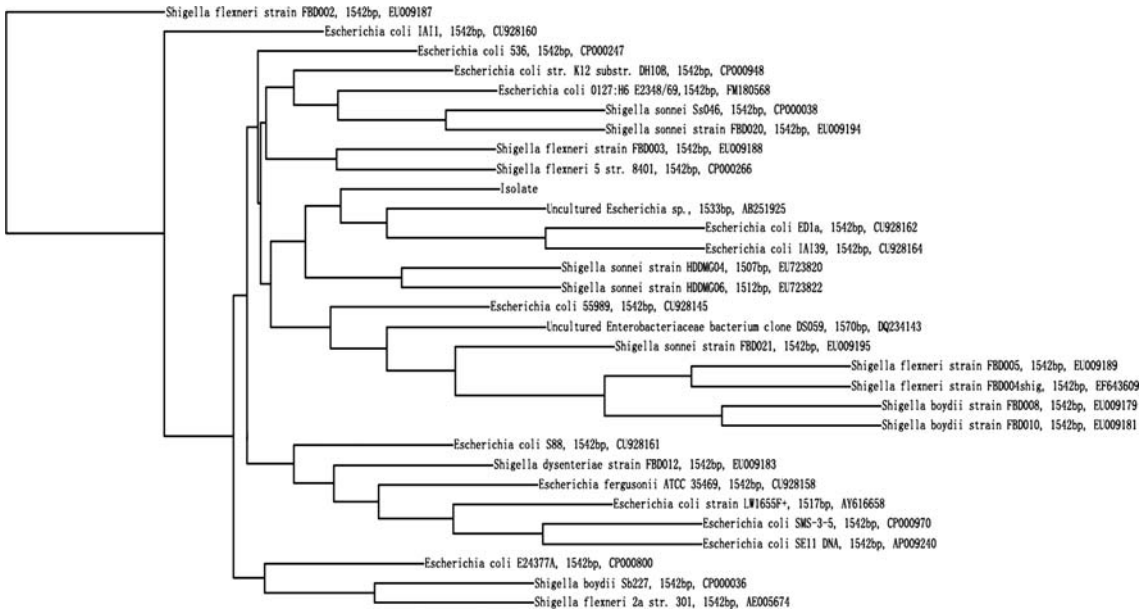

Fig. 2 Phylogenetic tree based on the almost complete 16S rRNA gene sequences showing relationships between isolate strain TCP-1 and representatives of *E. coli* and *Shigella* strains from the database using Neighbor-Joining method. The bootstrap values were generated from 1,000 replicates. The sequences were aligned using ClustalX program and the phylogenetic tree was booted by Phylip software package version 3.68. The number of nucleotides analyzed and the corresponding GenBank number are at the right of the sequence

EU723820, and so on), and the phylogenetic tree based on their 16S rRNA gene sequences was shown in Fig. 2.

Sequence analysis of one ITS gene between 16S rRNA and 23S rRNA showed that top 13 sequences closely related to ITS with 99% homology were all from *E. coli* (GenBank: AP009048, U00096, CU928145, and so on). Thus, it confirmed with regards to above additional tests that this thermotolerant cellulase-producing isolate strain TCP-1 was *E. coli*.

Characteristics of Cellulase Excreted by Isolate Strain

The major cellulase component activities were characterized by assays for CMCase, FPase, and β-glucosidase [2]. The ANOVA showed that there were significant differences of the cellulase activities between different culture time, different pH, and different temperatures, respectively (ANOVA, $P < 0.01$).

Optimum Culture Time

The cellulase enzymes excreted by isolate TCP-1 which were cultured in LB with 1% CMC-Na fermentation medium exhibited significant maximum CMCase, FPase, and β-glucosidase activities (Tukey HSD test, $P < 0.01$) at 72, 96, and 120 h of culture time, respectively (Fig. 3a), that is, CMCase was 0.23, FPase was 0.08, and β-glucosidase was 0.15 U/ml, with specific activities of 4.13, 0.56, and 0.50 U/mg protein, respectively.

This new isolate produced all the required main components of the cellulase enzyme complex, namely CMCase, FPase, and β-glucosidase, which work synergistically on the hydrolysis of cellulose to glucose. The decrease in cellulase production after 120 h incubation time may be due to catabolite repression by glucose.

Optimum pH

The effect of pH on CMCase activity was determined under the standard conditions for the CMCase assay (50°C for 30 min) and optimum culture time (72 h) (Fig. 3b). The CMCase activity attained significant highest (Tukey HSD test, $P < 0.01$) at the environmental condition of pH 6.0, which was 0.17 and 2.01 U/mg protein. It was stable at pH range of 5.0 to 10.0, still retained nearly 85% of its maximum activity at pH 10.0, suggesting that this isolate's CMCase was alkali resistant. This ability to retain high activity at elevated pH is a potentially useful property in processes employing alkaline delignification.

Optimum Reaction Temperature

The maximum CMCase activity was attained at 50°C (Tukey HSD test, $P < 0.01$) (Fig. 3c). This enzyme was also very active at relatively higher temperatures, maintained 64, 48, and 47% activity at 70, 80 and 90°C, respectively, indicating that the enzyme can be considered to be thermotolerant.

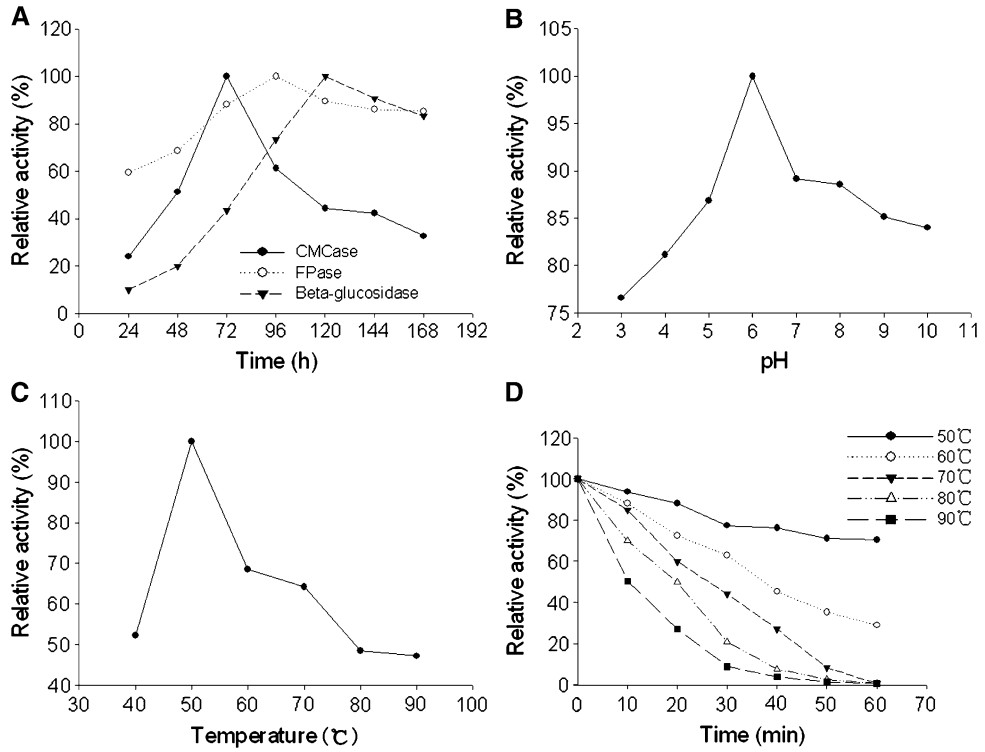

Fig. 3 Characteristics of cellulases excreted by isolate strain TCP-1 cultured in LB with 1% CMC-Na fermentation medium. **a** Optimum culture time of isolate on the activity of CMCase, FPase, and *β*-glucosidase. Culture time profile was determined by incubating the enzyme at 50°C in 0.1 M citrate buffer (pH 5.0) for 30 min with CMC-Na, filter paper, and salicin as the substrate, respectively, at varying times (24–168 h). **b** Optimum pH on the activity of CMCase. pH profile was determined in the same way as (**a**) at the optimum culture time and varying pHs (pH 3.0–10.0). **c** Optimum reaction temperature on the activity of CMCase. Reaction temperature profile was determined in the same way as (**a**) at the optimum culture time, optimum pH, and varying reaction temperatures (40–90°C). **d** Thermotolerance of CMCase. The thermal stability of CMCase was carried out by incubating at different temperatures for 10–60 min (as indicated in Fig. 3d) before the remaining activity was assayed in the same way as (**a**) at the optimum culture time, optimum pH, and optimum reaction temperature

Thermotolerance

The CMCase thermostability was assessed by incubating the enzyme at different temperatures (50–90°C) for different times (10–60 min). The enzyme was found to retain more than 60% of its maximal activity for at least 20 min at 50–70°C and 10 min at 80°C, respectively. It retained approximately 50% of its maximal activity after incubating at 90°C for 10 min (Fig. 3d), suggesting that this isolate's CMCase was heat resistant.

The thermostability of this enzyme may lend itself to various industrial applications such as in the food, sugar, and fuel ethanol industries, where higher temperature process conditions are used. Other applications may be in paper, waste treatment, and agricultural industries that process cellulose-derived materials [6].

Cloning and Sequence Analysis of Cellulase Gene from Isolate TCP-1

The putative cellulase gene (CelTCP-1) was PCR amplified from *E. coli* TCP-1 genomic DNA according to the reported *E. coli* cellulase gene (GenBank accession no. CP000970). In desire to further characterize the cellulase gene and understand the reason for thermoalkalotolerant cellulase produced by this thermostable *E. coli*, we then sequenced the inserted fragment in recombinant plasmid pMD 18-T/CelTCP-1. The sequence result indicated that the cloned sequence contained an ORF which started with a TTG start codon and terminated with a TAA stop codon. The ORF of CelTCP-1 consists of 1,113 nucleotides encoding a protein of 370 amino acids with a predicted molecular weight of about 42 kDa. Further sequence analysis showed that the amino acids sequence consisted of a endo-1,4-D-glucanase domain, which included 366 amino acids from 2nd to 367th amino acid, but the function of residual amino acids was unclear (Fig. 4).

The CelTCP-1 gene was generally similar to that reported for other *E. coli* cellulase genes (GenBank: CP000948, AP009240, CP000800, CU928162, and so on). The amino acid sequence had above 95% identity with the reported cellulase (GenBank: ZP_03067850 and YP_001745806, respectively), endo-1,4-D-glucanase (GenBank: NP_417988 and YP_001465001, respectively), and glycosyl hydrolase,

Fig. 4 Alignment of CelTCP-1 sequence with similar cellulase amino acid sequences from *E. coli*. The deduced CelTCP-1 was aligned using ClustalX software with the other *E. coli* (101-1, GenBank accession no. ZP_03067850; *E. coli* SMS-3-5, GenBank accession no. YP_001745806; *E. coli* str. K-12 substr. MG1655, GenBank accession no. NP_417988; *E. coli* E24377A, GenBank accession no. YP_001465001 and *E. coli* 53638, GenBank accession no. ZP_03002204). Conserved residues are highlighted in *black*, whereas similar residues are shown in *gray*

family 8 (GenBank: ZP_03002204) of *E. coli*. Whereas the predicted amino acid sequence showed low homology (<50%) with the cellulases that came from other species, such as *Ralstonia metallidurans*, *Pseudomonas putida*, and *Burkholderia graminis* (GenBank: YP_584399, YP_001669423, and ZP_02883242, respectively). This further confirmed that we cloned an *E. coli* cellulase gene.

Discussion

It is convenient to isolate some cellulase-producing microbial strains using CMC-Na screening plate. The isolated TCP-1 in this research showed cellulase activities. The ratio of the hydrolysis halo diameter to the colony diameter (H/C) stained by Congo red was >1.5. The modern genetic identification methods using PCR and sequence analysis combined with the traditional identification methods of morphological, physiological, and biochemical tests have allowed us to rapidly and effectively identify the isolate strain. The molecular phylogenetic tree based on sequence analysis of 16S rRNA gene showed that the isolate TCP-1 was closely related to *E. coli* and *Shigella* strains. It is known that some species of bacteria have highly variable ITS sequences in the rRNA operon that allow for strain differentiation [7]. In an attempt to uncover greater sequence heterogeneity, we amplified and cloned one ITS region between the 16S and 23S genes from the isolate TCP-1. The sequences of *E. coli* were dominantly close to the TCP-1 ITS sequence. Although the ITS had been known to be highly conserved between different isolates in some species, such as *Tropheryma whippelii* [8] and *Mycobacterium leprae* [9], we still speculated that the isolate strain TCP-1 was a new strain of *E. coli* based on the additional evidences of morphological, physiological, and biochemical tests.

It is very important to know that amplification of microbial 16S rRNA and ITS gene by PCR is very sensitive to contamination by microbial DNA in laboratory reagents and solutions [10, 11]. Therefore, we analyzed negative controls by two methods. First, a control was prepared without the addition of sample (i.e., isolate TCP-1) and worked up in the same manner as the true sample. Second, a control was performed during PCR that lacked input genomic DNA. No amplification was observed in these negative controls.

To our knowledge, this is the first reported thermostable *E. coli* with novel thermoalkalotolerant cellulases. The characteristics of cellulase produced by the isolate TCP-1, which were cultured in LB with 1% CMC-Na fermentation medium, showed high thermal and pH stability and included all the major hydrolytic activities of cellulase complexes with the ability to liberate glucose from soluble cellulose CMC-Na, but not from insoluble crystalline cellulose Avicel pH-101 (Sigma) (data not shown). The reason for undecomposing insoluble crystalline cellulose may be related to the particularity of its cellulase genes. But the enzyme's thermoalkalotolerant property was still attractive for potential biorefineries in which either native lignocellulosic feedstock (crop straws, wood pulps, etc.) are pretreated in some ways to be transformed to soluble cellulose, or cloning and altering TCP-1's cellulase gene in the desire to endow some bacteria with the ability to decompose insoluble crystalline cellulose. The application

of the new isolate could reduce the need for the neutralization of the pretreated feedstock.

To adopt to the high temperature, organisms may change the thermal stability of specific proteins, such as endogenous enzyme. The increase in the thermal stability can be achieved by change of amino acids or by addition of suitable stabilizing effectors [12]. Here, we screened and identified a new *E. coli* TCP-1 from soil, a bacterium strain that could produce thermoalkalotolerant cellulases. The enzyme retained >60% of its maximal activity for at least 20 min at 50–70°C and 10 min at 80°C, respectively, and retained approximately 50% of its maximal activity after incubating at 90°C for 10 min. There have been some thermostable cellulases reported. For example, the cellulase of *Caldocellum saccharolyticum* could retain high enzyme activity at 70°C [13] and that of *Bacillus* sp. KSM-S237 could retained 30% of the original activity after boiling at 100°C for 10 min [14]. The optimal temperature of the cellulase using CMC-Na as substrate in this study was 50°C, this was similar to *Poronia punctata* [15] and *Bacillus* sp. KSM-S237 [14].

To further characterize this thermoalkalotolerant enzyme, the gene encoding CelTCP-1 was cloned and sequence assay was performed. Compared with the reported five amino acid sequences of cellulases and the related enzymes in *E. coli*, there was a similarity in function and structure, but CelTCP-1 differed at 24 amino acid residues (three in NP_417988; four in YP_0017458; two in ZP_0306785; eight in YP_0014650; seven in ZP_0300220). In addition, CelTCP-1 contained 9 Isoleucine (I) and 19 Valine (V) residues in its total of 370 amino acid residues, and both kinds of them contained two strong hydrophobic substituents, whereas most amino acids contained only one. Previous research showed that hydrophobic interactions were one of the factors that influenced the thermal stability of an enzyme [16]. Thus, we speculated that the strong hydrophobic amino acids (I and V) probably increase the tolerance to the high temperature of CelTCP-1.

In conclusion, we isolated and identified a new *E. coli* which could produce cellulase with high-level activities, and analyzed the thermal and alkaline stabilities of the enzyme. The cellulase encoding gene CelTCP-1 was cloned, sequenced, and aligned. Thus, we characterized a cellulase in *E. coli* and provided a potential thermoalkalotolerant enzyme for application in the industry.

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