Curr Microbiol (2009) 59:393 399
DOI 10.1007/s00284-009-9450-7

Escherichia coli v

. . .
.

Received: 19 Ma 2009 / Accepted: 18 June 2009 / Published online: 9 Jul 2009

A cellulase-producing bacterium strain as isolated from soil that produced novel thermoalkalotolerant cellulases after gro th on CMC-Na agar screening plate at 37 C. It as identi ed as *Escherichia coli* using the method of 16S rRNA and intergenic spacer gene anal sis combined ith morphological, ph siological, and biochemical tests. Three major components of the cellulases [carbo meth l cellulase (CMCase), lter paper cellulase, and β-glucosidase] ere produced ith ma imal activities (0.23, 0.08, and 0.15 U/ml) and ma imum speci c activities 4.13, 0.56, and 0.50 U/mg protein after 72, 96, and 120 h gro th, respectivel . Ma imum CMCase activit as measured at 50 C and pH 6.0, respectivel , and it also retained more than 60% of its ma imal activit for at least 20 min at 50 70 C and 10 min at 80 C, respectivel , and retained appro imatel 50% of its ma imal activit after incubating at 90 C for 10 min. The en me could be applied in bioconversion of lignocellulosic agricultural astes.

Screening · Identi cation · Characteristics · *Escherichia coli* · Cellulase

Cellulose is the major component of plant cell all and is the most abundant rene able resource, and is composed of repeating cellobiose units linked b β-1, 4-glucosidic bonds and forms a cr stalline structure. In recent ears, the industrial revolution generated an increasing need for energ that as fueled mainl b fossil fuels. With the progress of industriali ation, petroleum as in great demand. As a consequence, serious environmental problems have arisen. Recentl , products utili ing biomass as an alternative resource have been developed for man markets. One of these, cellulol tic biomass, is kno n as a carbon-neutral material because it does not increase the amount of carbon dio ide in the air.

Cellulases catal e the h drol sis of cellulose hich are mainl three t pes: endoglucanases (EC. 3.2.1.4), cellobioh drolases (EC. 3.2.1.91), and β-glucosidases (EC.3.2.1.21) [1]. With the recent development of biotechnolog , 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 aste ater treatment, and use in bre ing industr .

Ho ever, these technologies have hardl been reali ed in practice because of their high running cost and lo ields of this en me. Therefore, a decrease in cellulase production cost, an improvement in cellulase performance, and an increase in sugar ields are all vital to decrease processing costs in biore neries [2].

One strateg 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, especiall *Trichoderma* and *Aspergillus* species, and most of these have limited ranges of temperature and pH. There ere fe reports about the thermoalkalotolerant isolate strains of *Escherichia coli*, and this stud as performed to isolate ne *E. coli* strain producing improved thermoalkalotolerant cellulase activities.

X. Li · R. Bhaskar · H. Yang · D. Wang · Y. Miao (✉)
Ke Laborator of Animal Epidemic Etiolog & Immunological Prevention of Ministr of Agriculture, College of Animal Sciences, Zhejiang Universit , 310029 Hang hou, People's Republic of China
e-mail: miao g@ ju.edu.cn

Bacterial Strains and Media

Microbes ere isolated from soil in Huajiachi campus, Zhejiang Universit , China, using CMC-Na agar plate, hich contained 0.5 g of carbo meth l cellulose sodium (CMC-Na, Sigma, Saint Louis, USA) as a sole carbon and energ 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 ere incubated at 37 C for about 36 h. The gro ing and ell-separated individual colonies ere isolated and puri ed. One isolate as tentativel identi ed as *E. coli* using morphological, ph siological, and biochemical assa s, and sequence anal sis of 16S rRNA and intergenic spacer (ITS) gene.

Identi cation of the Bacterial Strain

To detect its thermostabilit , the isolate strain as treated in 80 C ater bath for 10 min, and then cultured in LB at 37 C and 225 rpm overnight.

The morphological, ph siological, and biochemical characteri ations of the cellulase-producing isolate ere determined b electron microscope and other identi cation tests. For further identi cation, 16S rRNA and ITS gene ere pol merase chain reaction (PCR) ampli ed from the genomic DNA of the strains. Isolation of genomic DNA as carried out b using method of l so me and protease k. The 16S rRNA and ITS gene primers used in this stud are listed in Table 1. The follo ing primer pairs ere used for ampli cations from the genomic DNA ith their speci cities listed parentheticall : 16S rRNA pF 16S rRNA pR (universal) and ITS pF ITS pR (*E. coli* and related genera).

The process of PCR as both under the follo ing conditions: 94 C, 2 min; 35 c cles of 94 C, 1 min; 55 C 1 min; and 72 C, 90 s; 1 c cle of 72 C, 10 min; and then 4 C forever. The 16S rRNA and ITS gene products ith the e pected si e (about 1500 bp) ere puri ed using a DNA Gel E traction Kit (A gen, Silicon Valle , USA) and cloned into pMD18-T vector (Takara, K oto, Japan) follo ed b sequencing. Sequence anal sis as performed using the BLAST algorithm (http:// .ncbi.nlm.nih.gov).

Characteristic Assa s of En me

The culture time pro le of the isolate strain cultured in fermentation medium of LB containing 1% CMC-Na as determined b 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) ith CMC-Na, lter paper, and salicin as the substrates, respectivel .

The pH pro le of en me as evaluated b measuring the CMCase activit at the optimum fermentation time ith CMC-Na as the substrate at 50 C and different pH. The follo ing buffers ere used: 0.1 M citrate buffer (pH 3.0 6.0), 0.2 M phosphate buffer (pH 7.0 8.0), 0.2 M Gl cine/NaOH buffer (pH 9.0 10.0).

The reaction temperature pro le of the en me as evaluated b measuring the CMCase activit at the optimum fermentation time and pH, at the different reaction temperatures (40 90 C) ith CMC-Na as the substrate.

The thermotolerance of CMCase as determined at the optimum fermentation time, pH, and reaction temperature ith CMC-Na as the substrate, after incubating the en mes at different temperatures (50 90 C) for different times (10 60 min). Then, the en mes ere assa ed for the remaining activit .

Cloning and Sequence Anal sis of Cellulase Gene from Isolate TCP-1

According to the published sequence of *E. coli* cellulase gene in the GenBank (Accession No. CP000970), t o DNA oligonucleotide primers (up: 5′-TTGAAGATGAAT GTGTTGCG-3′ and do n: 5′-TTAGTGTGAATTTGCGC ATTC-3′) ere designed and s nthesi ed to amplif the entire cellulase gene CelTCP-1 from Chromosomal DNA of the isolated strain *E. coli* TCP-1. The condition of PCR as as follo s: 94 C, 2 min; 35 c cles of 94 C, 1 min; 60 C 1 min; and 72 C, 90 s; 1 c cle of 72 C, 10 min; and then 4 C forever. The product (si e about 1100 bp) as puri ed using a DNA Gel E traction Kit (A gen) and cloned into pMD18-T vector (Takara) follo ed b sequencing. The deduced amino acid sequence as predicted b an open reading frame (ORF) nder online in NCBI (http:// .ncbi.nih.gov) and as dra n b DNAMAN program. Sequence alignment as performed using the ClustalX soft are package version 1.81 and alignment picture as generated b GeneDoc soft are package version 2.7.0.

Statistical Anal sis

Anal sis of variance (ANOVA) as done ith Statistica soft are package (version 5.0). Before anal sis, the assumptions of ANOVA ere tested, hich are data normalit , variance homogeneit , and factor additivit . Tuke honest signi cant difference (HSD) test and Dunnett *t*-test (2-tailed) ere used for multiple comparisons. All e periments ere performed in triplicate.

Isolation of Cellulase-Producing Bacteria

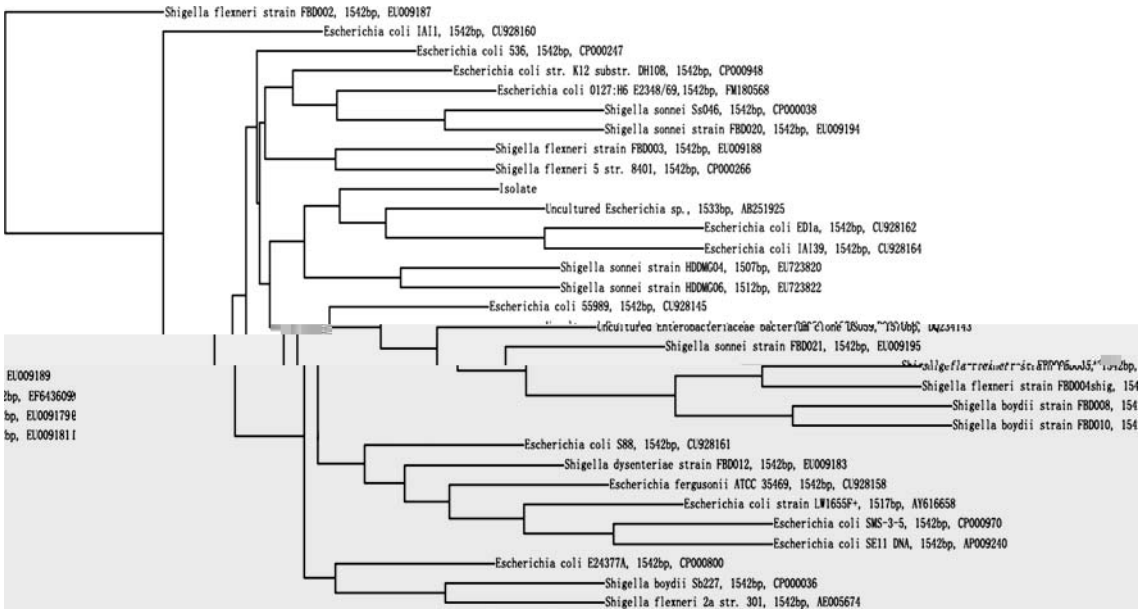
To search for characteristic microbe hich can produce cellulase, e collected some soil samples near tree roots. B screening the si e of opaque circular around the colonies on CMC-Na selective plates after Congo red staining, e isolated a colon , hich demonstrated high cellulase activit (Fig. 1).

Identi cation of the Isolate Strain

After treating the isolate strain in 80 C ater bath for 10 min, it could be survived and still gre in LB at 37 C and 225 rpm. It con rmed that this isolate strain has good thermostabilit .

To identif the isolate strain, the morphological, ph siological, and biochemical tests ere then carried out and the results sho ed that this cellulase-producing bacterium belonged to gram-negative Bacillus. Finall , according to the idel used method of 16S rRNA and ITS gene anal sis, e 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 sho ed 99% homolog ith *E. coli* and *Shigella* strains (GenBank: AB251925, CU928162,

Screening for cellulase-producing bacteria using CMC-Na plate follo ed b Congo red staining. After colon puri cation and culturing for 3D, the clear haloes appeared around the cellulase-producing bacterium colonies



Ph logenetic tree based on the almost complete 16S rRNA gene sequences sho ing relationships bet een isolate strain TCP-1 and representatives of *E. coli* and *Shigella* strains from the database using Neighbor-Joining method. The bootstrap values ere generated from 1,000 replicates. The sequences ere aligned using ClustalX program and the ph logenetic tree as booted b Ph lip soft are package version 3.68. The number of nucleotides anal- ed and the corresponding GenBank number are at the right of the sequence

EU723820, and so on), and the ph logenetic tree based on their 16S rRNA gene sequences as sho n in Fig. 2.

Sequence anal sis of one ITS gene bet een 16S rRNA and 23S rRNA sho ed that top 13 sequences closel related to ITS ith 99% homolog ere all from *E. coli* (GenBank: AP009048, U00096, CU928145, and so on). Thus, it con rmed ith regards to above additional tests that this thermotolerant cellulase-producing isolate strain TCP-1 as *E. coli*.

Characteristics of Cellulase E creted b Isolate Strain

The major cellulase component activities ere character- -i ed b assa s for CMCase, FPase, and β-glucosidase [2]. The ANOVA sho ed that there ere signi cant differences of the cellulase activities bet een different culture time, different pH, and different temperatures, respectivel (ANOVA, $P < 0.01$).

Optimum Culture Time

The cellulase en me s e creted b isolate TCP-1 hich ere cultured in LB ith 1% CMC-Na fermentation medium e hibited signi cant ma imum CMCase, FPase, and β-glucosidase activities (Tuke HSD test, $P < 0.01$) at 72, 96, and 120 h of culture time, respectivel (Fig. 3a), that is, CMCase as 0.23, FPase as 0.08, and β-glucosidase as 0.15 U/ml, ith speci c activities of 4.13, 0.56, and 0.50 U/mg protein, respectivel .

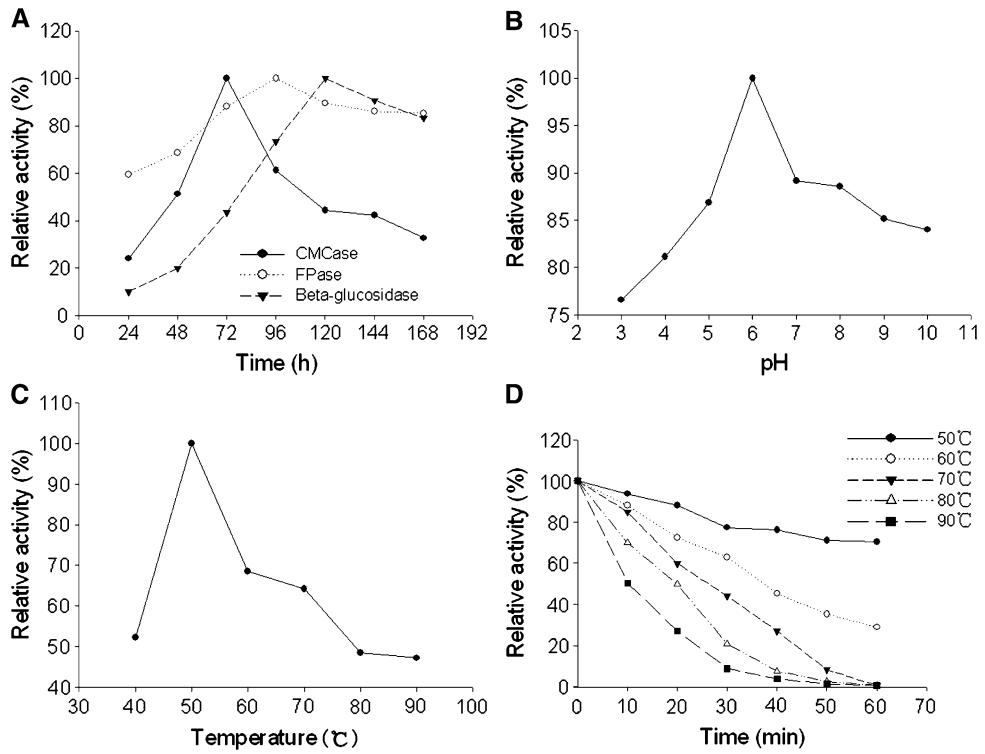
This ne isolate produced all the required main components of the cellulase en me comple , namel CMCase, FPase, and β-glucosidase, hich ork s nergisticall on the h drol sis of cellulose to glucose. The decrease in cellulase production after 120 h incubation time ma be due to catabolite repression b glucose.

Optimum pH

The effect of pH on CMCase activit as determined under the standard conditions for the CMCase assa (50 C for 30 min) and optimum culture time (72 h) (Fig. 3b). The CMCase activit attained signi cant highest (Tuke HSD test, $P < 0.01$) at the environmental condition of pH 6.0, hich as 0.17 and 2.01 U/mg protein. It as stable at pH range of 5.0 to 10.0, still retained nearl 85% of its ma - imum activit at pH 10.0, suggesting that this isolate's CMCase as alkali resistant. This abilit to retain high activit at elevated pH is a potentiall useful propert in processes emplo ing alkaline deligni cation.

Optimum Reaction Temperature

The ma imum CMCase activit as attained at 50 C (Tuke HSD test, $P < 0.01$) (Fig. 3c). This en me as also ver active at relativel higher temperatures, maintained 64, 48, and 47% activit at 70, 80 and 90 C, respectivel , indicating that the en me can be considered to be thermotolerant.



Characteristics of cellulases e creted b isolate strain TCP-1 cultured in LB ith 1% CMC-Na fermentation medium. Optimum culture time of isolate on the activit of CMCase, FPase, and *β*-glucosidase. Culture time pro le as determined b incubating the e me at 50 C in 0.1 M citrate buffer (pH 5.0) for 30 min ith CMC-Na, lter paper, and salicin as the substrate, respectivel , at var ing times (24 168 h). Optimum pH on the activit of CMCase. pH pro le as determined in the same a as ( ) at the optimum culture time and var ing pHs (pH 3.0 10.0). Optimum reaction temperature on the activit of CMCase. Reaction temperature pro le as determined in the same a as ( ) at the optimum culture time, optimum pH, and var ing reaction temperatures (40 90 C). Thermotolerance of CMCase. The thermal stabilit of CMCase as carried out b incubating at different temperatures for 10 60 min (as indicated in Fig. 3d) before the remaining activit as assa ed in the same a as ( ) at the optimum culture time, optimum pH, and optimum reaction temperature

Thermotolerance

The CMCase thermostabilit as assessed b incubating the e me at different temperatures (50 90 C) for different times (10 60 min). The e me as found to retain more than 60% of its ma imal activit for at least 20 min at 50 70 C and 10 min at 80 C, respectivel . It retained appro imatel 50% of its ma imal activit after incubating at 90 C for 10 min (Fig. 3d), suggesting that this isolate's CMCase as heat resistant.

The thermostabilit of this e me ma lend itself to various industrial applications such as in the food, sugar, and fuel ethanol industries, here higher temperature process conditions are used. Other applications ma be in paper, aste treatment, and agricultural industries that process cellulose-derived materials [6].

Cloning and Sequence Anal sis of Cellulase Gene from Isolate TCP-1

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

The CelTCP-1 gene as generall 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% identit ith the reported cellulase (GenBank: ZP_03067850 and YP_001745806, respectivel), endo-1,4-D-glucanase (GenBank: NP_417988 and YP_001465001, respectivel), and gl cos 1 h drolase,

4 Alignment of CelTCP-1 sequence ith similar cellulase amino acid sequences from *E. coli*. The deduced CelTCP-1 as aligned using ClustalX soft are ith 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*, hereas similar residues are sho n in *gray*

famil 8 (GenBank: ZP_03002204) of *E. coli*. Whereas the predicted amino acid sequence sho ed lo homolog (<50%) ith the cellulases that came from other species, such as *Ralstonia metallidurans*, *Pseudomonas putida*, and *Burkholderia graminis* (GenBank: YP_584399, YP_001669423, and ZP_02883242, respectivel). This further con rmed that e cloned an *E. coli* cellulase gene.

It is convenient to isolate some cellulase-producing microbial strains using CMC-Na screening plate. The isolated TCP-1 in this research sho ed cellulase activities. The ratio of the h drol sis halo diameter to the colon diameter (H/C) stained b Congo red as >1.5. The modern genetic identi cation methods using PCR and sequence anal sis combined ith the traditional identi - cation methods of morphological, ph siological, and biochemical tests have allo ed us to rapidl and effectivel identif the isolate strain. The molecular ph logenetic tree based on sequence anal sis of 16S rRNA gene sho ed that the isolate TCP-1 as closel related to *E. coli* and *Shigella* strains. It is kno n that some species of bacteria have highl variable ITS sequences in the rRNA operon that allo for strain differentiation [7]. In an attempt to uncover greater sequence heterogeneit , e ampli ed and cloned one ITS region bet een the 16S and 23S genes from the isolate TCP-1. The sequences of *E. coli* ere dominantl close to the TCP-1 ITS sequence. Although the ITS had been kno n to be highl conserved bet een different isolates in some species, such as *Tropheryma whippelii* [8] and *Mycobacterium leprae* [9], e still speculated that the isolate strain TCP-1 as a ne strain of *E. coli* based on the additional evidences of morphological, ph siological, and biochemical tests.

It is ver important to kno that ampli cation of microbial 16S rRNA and ITS gene b PCR is ver sensitive to contamination b microbial DNA in laborator reagents and solutions [10, 11]. Therefore, e anal- ed negative controls b t o methods. First, a control as prepared ithout the addition of sample (i.e., isolate TCP-1) and orked up in the same manner as the true sample. Second, a control as performed during PCR that lacked input genomic DNA. No ampli cation as observed in these negative controls.

To our kno ledge, this is the rst reported thermostable *E. coli* ith novel thermoalkalotolerant cellulases. The characteristics of cellulase produced b the isolate TCP-1, hich ere cultured in LB ith 1% CMC-Na fermentation medium, sho ed high thermal and pH stabilit and included all the major h drol tic activities of cellulase comple es ith the abilit to liberate glucose from soluble cellulose CMC-Na, but not from insoluble cr stalline cellulose Avicel pH-101 (Sigma) (data not sho n). The reason for undecomposing insoluble cr stalline cellulose ma be related to the particularit of its cellulase genes. But the en me's thermoalkalotolerant propert as still attractive for potential biore neries in hich either native lignocellulosic feedstock (crop stra s, ood pulps, etc.) are pretreated in some a s to be transformed to soluble cellulose, or cloning and altering TCP-1's cellulase gene in the desire to endo some bacteria ith the abilit to decompose insoluble cr stalline cellulose. The application

of the ne isolate could reduce the need for the neutral-i ation of the pretreated feedstock.

To adopt to the high temperature, organisms ma change the thermal stabilit of speci c proteins, such as endogenous e- me. The increase in the thermal stabilit can be achieved b change of amino acids or b addition of suitable stabili ing effectors [12]. Here, e screened and identi ed a ne *E. coli* TCP-1 from soil, a bacterium strain that could produce thermoalkalotolerant cellulases. The e- me retained >60% of its ma imal activit for at least 20 min at 50 70 C and 10 min at 80 C, respectivel , and retained appro imatel 50% of its ma imal activit after incubating at 90 C for 10 min. There have been some thermostable cellulases reported. For e ample, the cellulase of *Caldocellum saccharolyticum* could retain high e- me activit at 70 C [13] and that of *Bacillus* sp. KSM-S237 could retained 30% of the original activit after boiling at 100 C for 10 min [14]. The optimal temperature of the cellulase using CMC-Na as substrate in this stud as 50 C, this as similar to *Poronia punctata* [15] and *Bacillus* sp. KSM-S237 [14].

To further character- e this thermoalkalotolerant e- me, the gene encoding CelTCP-1 as cloned and sequence assa as performed. Compared ith the reported ve amino acid sequences of cellulases and the related e- mes in *E. coli*, there as a similarit in function and structure, but CelTCP-1 differed at 24 amino acid residues (three in NP_417988; four in YP_0017458; t o 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 t o strong h drophobic substituents, hereas most amino acids contained onl one. Previous research sho ed that h drophobic interactions ere one of the factors that in uenced the thermal stabilit of an e- me [16]. Thus, e speculated that the strong h drophobic amino acids (I and V) probabl increase the tolerance to the high temperature of CelTCP-1.

In conclusion, e isolated and identi ed a ne *E. coli* hich could produce cellulase ith high-level activities, and anal- ed the thermal and alkaline stabilities of the e- me. The cellulase encoding gene CelTCP-1 as cloned, sequenced, and aligned. Thus, e character- ed a cellulase in *E. coli* and provided a potential thermoalkalotolerant e- me for application in the industr .

This stud as supported b a ke project of Zhejiang Government No. 2008C24011 and b the Hi-Tech Research and Development Program of China (Nos. 2008AA10Z132 and 2006AA10A119).

1. Thongekkae J, Ikeda H, Mesaki K, Lefuji H (2008) An acidic and thermostable carbo meth l cellulase from the east *Cryptococcus* sp. S-2: puri cation character- ation and improvement of its recombinant e- me production b high cell-densit fermentation of *Pichia pastoris*. Protein E pr Purif 60(2):140 146