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ORIGINAL PAPER

A novel endo-glucanase from the thermophilic bacterium *Geobacillus* sp. 70PC53 with high activity and stability over a broad range of temperatures

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Abstract A thermophilic *Geobacillus* bacterium secreting high activity of endo-glucanase (EC 3.2.1.4) was isolated from rice straw compost supplemented with pig manure. A full-length gene of 1,104 bp, *celA*, encoding this glycosyl hydrolase family 5 endo-glucanase of 368 amino acids was isolated. No related gene from *Geobacillus* has been reported previously. The recombinant CelA expressed in *Escherichia coli* had an optimal activity at 65°C and pH 5.0, and it exhibited tenfold greater specific activity than the commercially available *Trichoderma reesei* endo-glucanase. CelA displayed activity over a broad temperature range from 45 to 75°C and was a thermostable enzyme with 90% activity retained after heating at 65°C for 6 h. Interestingly, CelA activity could be enhanced by 100% in the presence of 2 mM $MnSO_4$. CelA had high specific activity over β-D-glucan from barley and Lichenan, making it a potentially useful enzyme in biofuel and food industries.

Keywords *Geobacillus* · Thermophilic cellulases · Endo-glucanase · Glycosyl hydrolase family 5 · Rice straw compost

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Introduction

Cellulose is the major component of plant biomass, which originally comes from solar energy through photosynthesis and is the most abundant renewable energy feedstock on the planet. It is a greenhouse gas neutral source of sugars and can be converted to ethanol or other chemicals via microbial fermentation (Sticklen 2006). Cellulose hydrolysis could be one of the most important biotechnologies in the 21st century. Therefore, there has been great interest in understanding the biochemical properties and functions of cellulolytic enzymes from fungi, yeasts and bacteria (Zhang and Lynd 2004; Zhang et al. 2006). Based on their structural properties, components of the cellulase system can be classified into three major types: endo-glucanases (EC 3.2.1.4), exo-glucanases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Cellulases are used in the textile industry for cotton softening, in the production of detergents for color care and cleaning, in the food industry for mashing, and in the pulp and paper industries for de-inking, drainage improvement and fiber modification. Several cellulases reported from *Trichodrema* sp. and *Aspergillus* sp. were active only at moderate temperatures (Lynd et al. 2002; Marques et al. 2003; Naika et al. 2007; Sul et al.

2004). Thermophilic cellulases have advantages in many industrial applications because higher processing temperatures can be employed for offering accelerated reaction rates, increased solubility of reactants and reduced contamination. Therefore, a greater understanding of thermoactive cellulases could potentially lead to new and useful applications in industry.

Extreme thermophilic microorganisms, which grow optimally between 60 and 80°C, are widely distributed among genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Thermotoga* and *Aquifex* (Antranikian et al. 2005). *Geobacillus* spp., which are thermophilic and aerobic spore-forming bacteria with growth optima at temperatures between 45 and 70°C, have been classified as the fifth group of genera *Bacillus*, but now considered as a new genus (Nazina et al. 2001). These bacteria are extremophilic microorganisms, have adapted to survive at high temperatures, and secrete various enzymes under suitable conditions. *Bacillus* and *Geobacillus* have high potential for applications in biotechnological processes as sources of various thermostable enzymes including proteases (Chen et al. 2004), amylases, lipases (Abdel-Fattah and Gaballa 2008; Leow et al. 2007), xylanases (Liu et al. 2008; Sharma et al. 2007), cellulases (Tai et al. 2004) and long-chain alkane degradation proteins (Wang et al. 2006). Genome sequences of mesophilic *Bacillus*, e.g. *B. subtilis*, *B. halodurans*, *Oceanobacillus iheyensis*, *B. anthracis* and *B. cereus*, were completed in 2003 (Takami et al. 2004a, b). Regarding the thermophilic *Bacillus*, *Geobacillus*, only genome sequences of *G. kaustophilus* HTA426 (Takami et al. 2004a, b) and *G. thermodenitrificans* NG80-2 (Feng et al. 2007) were recently completed.

In this study, we report on the identification of a novel thermophilic *Geobacillus* isolated from rice straw compost supplemented with pig manure. This bacterium was found to secrete a highly active and thermostable endo-glucanase. The gene encoding this endo-glucanase has been cloned and characterized. This enzyme was over-expressed in *E. coli* and its biochemical properties were characterized.

Materials and methods

Isolation and identification of endo-glucanase-producing microorganisms

Rice straw composts included pig manure, pigbone powder, rice hull and rice straw at a ratio of 3:1:12:6 (wt%) were established at temperatures ranging 55–70°C at the Asian Vegetable Research and Development Center (AVRDC), Tainan, Taiwan. Two grams of rice straw composts and 1 g of rice hull were mixed in 100 ml of minimal requirement (MR) medium (Mandels and Reese 1957), which contained 1.0 g carboxyl methyl cellulose (CMC) and then incubated at 65°C overnight (16 h). The MR medium contained 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.34 g $CaCl_2{\cdot}2H_2O$, 0.30 g $MgSO_4{\cdot}7H_2O$, 5 mg $FeSO_4{\cdot}7H_2O$, 1.6 mg $MnSO_4{\cdot}H_2O$, 1.4 mg $ZnSO_4{\cdot}7H_2O$ and 2.0 mg $CoCl_2{\cdot}6H_2O$ per liter. Broth (100 µl) was spatulated to MR plates containing 1% CMC and incubated at the same temperature for 24 h. Bacterial colonies grown on MR-CMC plates were isolated, re-inoculated on a new MR-CMC plate and the plate stained with a Congo red solution (0.2%) and destained with 1 N NaCl solution. Bacteria that generated clear zones on the plate were chosen for further characterization. Identity of the isolated bacterial strain was analyzed by the 16S rDNA sequencing method using universal primers 27f: 5′-AGAGTTTGATCCTGGCT CAG-3′ and 1497r: 5′-AAGTCGTAACAAGGTAACC-3′ (Kim et al. 2000). A bacterial strain that generated large-size clear zone on the stained CMC plate, and with the highest secreted endo-glucanase activity in MR medium, was isolated and designated as strain 70PC53.

Construction and screening of a genomic library for isolation of endo-glucanase genes

Genomic DNA was purified from the *Geobacillus* sp. 70PC53, partially digested with *Eco*RI and resolved on the 0.8% agarose gel. DNA fragments ranging from 3 to 10 kb were recovered from the gel. The vector pBluescriptII SK(+) (Stratagene, USA) was digested with *Eco*RI. The recovered bacterial DNA fragments and the truncated vector were ligated and introduced into *E. coli* DH5α cells to generate a genomic library. The Luria–Bertani (LB) agar plate with 1.0% (w/v) CMC and 100 µg/ml ampicillin was prepared for screening of bacterial colonies that secreted cellulases following the procedure described above. Plasmid DNAs of positive clones were isolated, digested with *Eco*RI and *Hin*dIII and inserted between T7 and T3 promoters in the pBluescript vector which catalyzed the removal of 5′ phosphate groups from DNA with alkaline phosphatase (CIP treatment) to prevent the self-ligation. DNA fragments derived from *Geobacillus* sp. 70PC53 were analyzed by DNA sequencing.

Expression of recombinant endo-glucanase in *E. coli*

The genomic DNA (1,104 bp) encoding a full-length cellulase gene (*celA*) was obtained by screening the *Geobacillus* sp. 70PC53 genomic library. The entire *celA* open reading frame was PCR amplified from the pBluescript vector using forward primer, 5′-GGGAACATATGGTGA AAAAAGCT TTTCTGCCCGTG-3′ (*Nde*I site underlined) and reverse primer, 5′-CGCCCCTCGAGCTCTTTGAACAAACGTT TCCCT-3′ (*Xho*I site underlined). The PCR product was

inserted into the T7 promoter and terminator in the pET-20b(+) vector and introduced into *E. coli* strain Rosetta C41. The bacterium harboring recombinant CelA was cultured in 100 ml of LB medium supplemented with 100 μg/ml ampicillin on a rotary shaker (150 rpm) at 37°C. Production of the recombinant CelA was induced with 1 mM of isopropyl-β-thiogalactopyranoside (IPTG) at $OD_{600\ nm}$ of 0.4 for 6 h.

Purification of recombinant CelA

Bacterium culture with recombinant CelA was collected by centrifugation at 10,000*g* for 15 min at 4°C and washed with deionized water twice. Cells were resuspended in a sodium phosphate buffer (pH 7.4) and disrupted by sonicator. The cell debris was removed by centrifugation at 15,000*g* for 20 min at 4°C and the supernatant was collected. The supernatant was applied to a His-Trap affinity chromatography column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Fractions containing recombinant CelA were eluted with 200 mM imidazole and 300 mM NaCl made up in sodium phosphate buffer (pH 7.4).

Cellulase activity assay

Enzyme activity toward CMC was measured according to the method of Miller (Miller 1959). The reaction mixture was composed of 0.5 ml of 1.0% CMC in 50 mM sodium phosphate buffer, pH 7.0, and 0.5 ml of enzyme solution. After incubation at 65°C for 15 min, the concentration of reducing sugars was determined using the dinitrosalicylic acid (DNS) method. One unit of enzyme activity corresponds to 1 μmol glucose per minute in the reaction. Protein concentrations were determined by the Bradford method (Bradford 1976) using a Bio-Rad Protein Assay Kit with the bovine serum albumin as the standard.

CMC zymogram analysis of CelA

Cellulase samples were denatured by heating for 5 min at 100°C in 1% (w/v) SDS and 2% (w/v) DTT, and applied to a 10% SDS-PAGE gel containing 0.2% (w/v) CMC polymerized within the gel matrix. Following electrophoresis, the gel was washed three times (each 30 min) in 1% Triton X-100 and 10 mM pH 8.0 Tris–HCl buffer, and then soaked in the buffer overnight to allow proteins to renature. Afterwards, the gel was incubated at 65°C in 50 mM pH 5.0 sodium acetate buffer for 30 min, stained with 0.2% (w/v) Congo red solution for 20 min, and destained with 1 M NaCl. Endo-glucanase activity was visible as clear bands against a red background.

Biochemical characterization of CelA

Optimal temperature and thermostability

Endo-glucanase activity was analyzed with 1.0% CMC in 50 mM sodium acetate buffer (pH 5.0), incubated at 45, 55, 60, 65, 70 or 75°C. Thermostability was first investigated by incubating the enzyme at 45, 55, 65, 70 or 75°C for 6 h. The residual enzyme activity was determined with 1.0% CMC in 50 mM sodium acetate buffer pH 5.0, at 65°C.

Optimal pH and pH stability

Enzyme was analyzed with 1.0% CMC in 50 mM buffer with variable pH values, i.e. pH 4–5 in sodium acetate buffer and pH 6–9 in sodium phosphate buffer, at 65°C. The pH stability was analyzed by incubating the enzyme in 50 mM buffers with variable pH values for 16 h, and then measuring the residual enzyme activity in 1.0% CMC at pH 5.0, which was made up in 50 mM sodium acetate buffer, at 65°C.

Effect of chemical reagents and metal ions

Various chemicals, such as $CaCl_2$, $CoCl_2$, $CuCl_2$, $CuSO_4$, EDTA, $MgCl_2$, $MnSO_4$, NaN_3, $ZnSO_4$, DTT, and 2-mercaptoethanol, were included in the 1.0% CMC substrate at specified concentrations of 2, 5 and 10 mM. The amount of reducing sugars released from CMC after 15 min of incubation at 65°C pH 5.0 was measured.

Substrates specificity

The enzyme was analyzed with 1.0% of different substrates, including Avicel (Fluka), acid swollen Avicel, CMC (Sigma), cellulose fiber (Sigma), β-D-glucan (barley, Sigma), filter paper (Whatman No.1), Lichenan (Sigma), Xylan-birchwood (Sigma) and Xylan oat spelts (Sigma), at the optimal temperature and pH.

Cellulolytic activity comparisons between *Trichoderma reesei* and recombinant CelA

Cellulases from *Trichoderma reesei* (ATCC 26921), 4-nitrophenyl β-D-glucopyranoside (pNPG), 4-nitrophenyl β-D-cellobioside (pNPC) and 4-nitrophenyl β-D-cellotrioside (pNPT) were purchased from Sigma. Enzyme activities were analyzed at a concentration of 1.0 mM with different substrates at their optimal temperatures, i.e. *T. reesei* at 37°C and CelA at 65°C. The activity was calculated by the calibration curve of 4-nitrophenol, with 1 U defined as 1 μmol 4-nitrophenol equivalent amount at

specific condition. For all substrates, assays were carried out with the same analytical conditions.

Nucleotide sequence accession number

The sequences of 16S rDNA gene and amino acid of CelA of *Geobacillus* sp. 70PC53 have been deposited in the GenBank database under accession numbers EU860293 and EU860294, respectively.

Results

Strain 70PC53 is a unique *Geobacillus* sp.

Over 100 *Geobacillus* sp. strains were isolated from the rice straw compost. Seven of them displayed a strong ability to hydrolyze CMC (Fig. 1a–d). Strain 70PC53 showed the highest cellulolytic activity when cultivated in the MR-CMC medium. The 16S rDNA sequence analysis indicated that strain 70PC53 was a *Geobacillus* sp., a thermophilic bacterium with an optimal growth temperature at 65°C. The 16S rDNA of many *Geobacillus* showed high G+C content (Nazina et al. 2001), e.g. 58.5% for *G. thermodenitrificans*, 59.7% for *G. stearothermophilus*, 60.0% for *G. thermocatenulatus* and 59.1% for *G. kaustophilus*. Strain 70PC53 had a total G+C content of 59.7% in its 16S rDNA, consequently, it was very difficult to distinguish strain 70PC53 from other *Geobacillus* sp. only based on the phylogenetic tree analysis of 18 closely related species (Fig. 2). The nearly completed 16S rDNA sequence (i.e. 1,519 bp) of strain 70PC53 showed 99.8% sequence similarity to *G. thermocatenulatus* (GenBank accession no. AY550104). However, this bacterium was only known to be able to degrade nylon and adsorb cadmium previously (Hetzer et al. 2006; Tomita et al. 2003), and to date no report has been published for its cellulolytic activity. Comparison of strain 70PC53 with other common *Geobacillus* strains revealed several unique features, such as the optimal growth temperature at ~65°C and the production of cellulolytic enzymes, making this new isolate a distinct member within the genus *Geobacillus*.

The *Geobacillus* CelA is a novel endo-glucanase

By screening 6,000 colonies of the genomic library of *Geobacillus* sp. 70PC53, one positive clone expressing cellulolytic activity with an insert DNA fragment of 19 kb (Fig. 1e) was obtained. The entire DNA insert was not completely sequenced due to its high G/C content. The 19-kb DNA fragment was then digested with *Hin*dIII, and a smaller 6-kb DNA fragment was cloned. *E. coli* containing the 6-kb DNA fragment had an endo-glucanase activity as visualized by a large clear zone against the Congo red stain

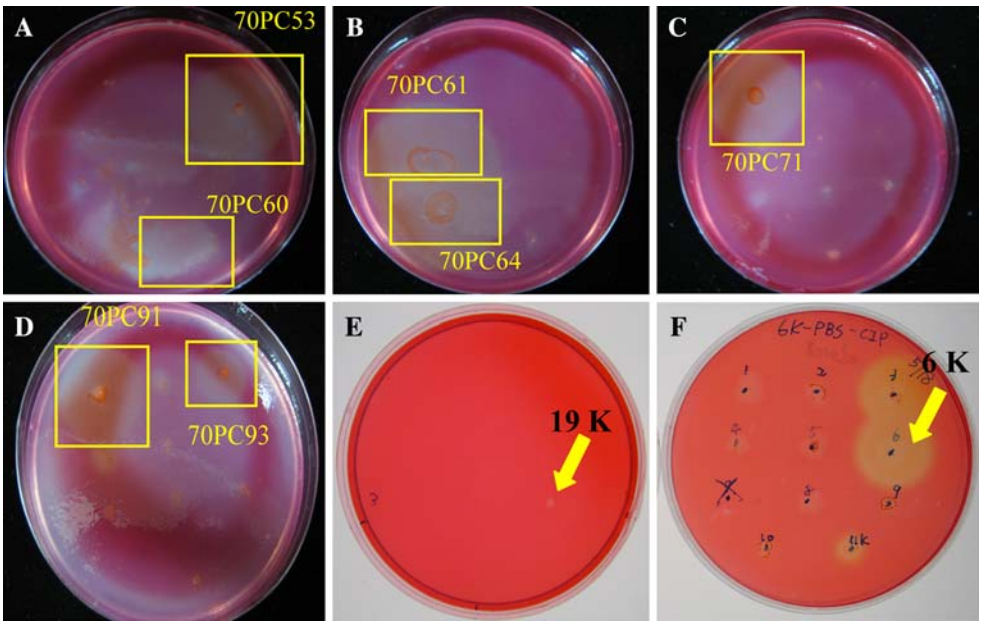

Fig. 1 Identification of bacterial strains capable of hydrolyzing CMC and cloning of endo-glucanase genes. All bacterial strains from rice straw compost were cultured in CMC-agar plates at 65°C overnight, stained with 0.2% Congo red and destained with 1 N NaCl solution. Endo-glucanase activity was visible as clear bands against a red background. **a** Strains 70PC53 and 70PC60, **b** strains 70PC61 and 70PC64, **c** strain 70PC71, **d** strains 70PC91 and 70PC93, **e** *E. coli* contained a 19-kb DNA fragment derived from the genomic library of *Geobacillus* sp. 70PC53 in pBluescriptII SK(+), and **f** *E. coli* contained a 6-kb DNA fragment derived from the 19-kb DNA fragment indicated in (**e**) in pBluescriptII SK(+) vector

Fig. 2 Phylogenetic tree analyzed by 16S rDNA sequences with the neighbor-joining methods constructed with the MEGA 4.0 software. Strain 70PC53 is most closely related to *Geobacillus* sp. GenBank accession numbers for all species are given in *parenthesis*. The *numbers* at the branch nodes are bootstrap values based on 1,000 re-samplings. *Scale bar* indicates nucleotide divergence as 0.2%

(Fig. 1f). The 1,176-kb open reading frame encodes a putative endo-glucanase of 392 amino acids, designated as CelA, with a putative signal peptide of 24 amino acids and three conserved domains (Fig. 3a). Amino acid sequence comparison against protein databases indicated that this novel endo-glucanase shared only 53.1% similarity with enzymes belonging to the glycosyl hydrolase (GH) family 5.

The amino acid sequence of CelA endo-glucanase was further compared with other members in the GH family 5 by a phylogenetic analysis. The CelA endo-glucanase from *Geobacillus* sp. 70PC53 was more closely related to *Pectobacterium*, *Paenibacilus* and *Bacillus* than to *Clostridium* (Fig. 3b). The highest sequence identity of CelA was 45.1% with that of the CelN from *Pectobacterium atrosepticum* (accession no. Q59394) followed by 43.6% with that of CelV from *Panibacillus polymyxa* (accession no. ABV08875), 41.9% with that of Cel from *Bacillus* sp. NBL420 (accession no. AAK73277) and 40.6% with that of CelA from *Clostridium saccharobutylicum* (accession no. P15704). These sequence identities with respect to CelA were all relatively low (<50%), which confirmed that *Geobacillus* sp. 70PC53 CelA represented a unique branch from other GH members.

CelA endo-glucanase is stable over a broad range of temperatures and pH values

The yield of recombinant CelA expressed in *E. coli* was 85 ± 1.1 mg per liter at final concentration. The enzymatic activity of purified recombinant CelA was further confirmed by a CMC-zymographic analysis (Fig. 4). The

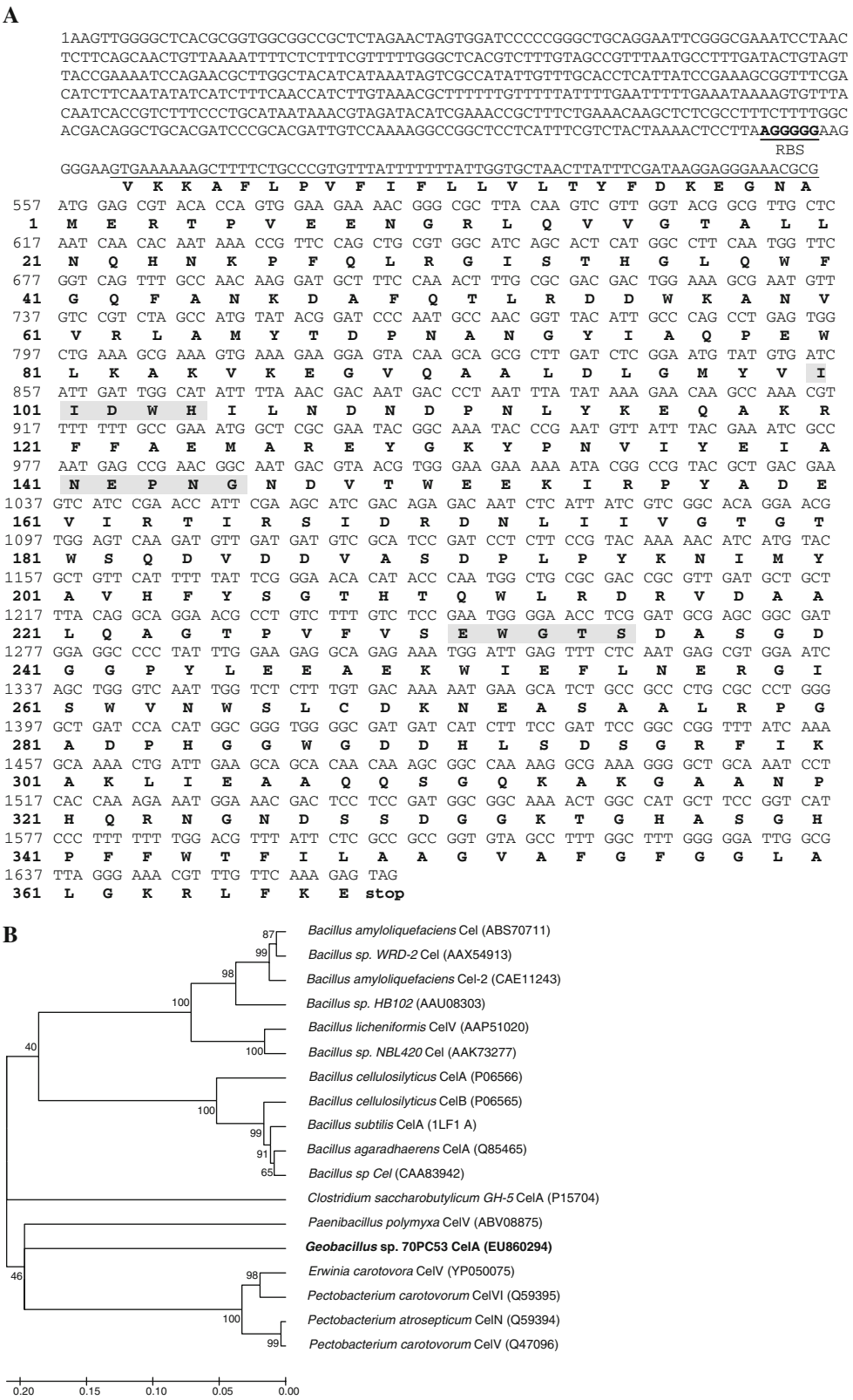

Fig. 3 **a** Nucleotide sequence of the *Geobacillus* sp. 70PC53 *celA*. The deduced amino acid sequence of the open reading frame is shown below the nucleotide sequence. The putative Shine–Dalgarno-type ribosomal binding site (RBS) is shown in *bold* and the three conserved domains of GH5 are shown as *shaded boxes*. Deduced amino acids are numbered from the initiator methionine, with the upstream 24 amino acids (*underlined*) as signal peptide predicted by the SignalP 3.0 Server. **b** Amino acid sequences of CelA and endo-glucanases in the GH family 5 were compared by a phylogenetic analysis. GenBank accession numbers for all enzymes are given in *parenthesis*

major protein CelA showing highest hydrolytic activity had a molecular weight of 43 kDa.

Biochemical properties of the purified recombinant CelA were further characterized. CelA endo-glucanase was active over broad ranges of temperature (45–75°C) (Fig. 5a). Over 80% of CelA endo-glucanase activity was maintained for 4 h at 75°C, indicating that it was a thermophilic enzyme (Fig. 5b). The optimal activity of CelA

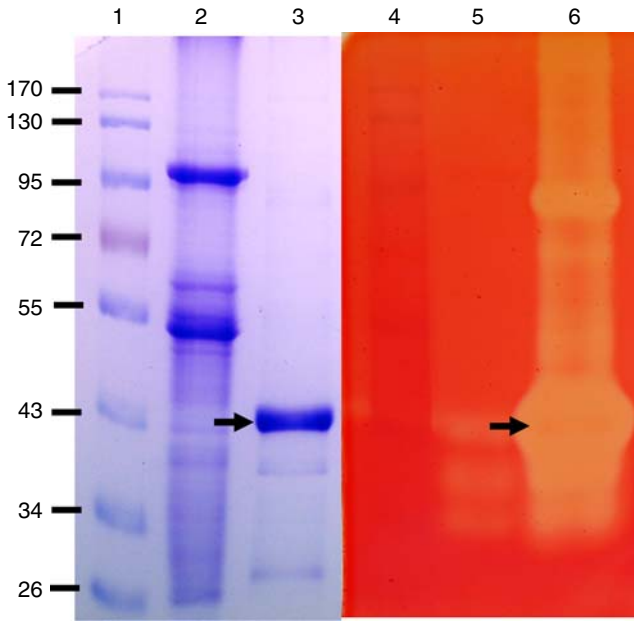

Fig. 4 The *Geobacillus* sp. 70PC53 CelA is a highly active endo-glucanase. Crude proteins (5 μg) from strain 70PC53 and recombinant CelA expressed in *E. coli* (2 μg) were extracted and subjected to SDS-PAGE and Coomassie blue R-250 staining (*lanes* 2, 3), or subjected to a CMC-zymographic analysis and Congo red staining, for detection of endo-glucanase activity (*lanes* 5, 6). *Lanes* 1 and 4: protein markers in kDa; *lanes* 2 and 5: crude proteins from strain 70PC53; *lanes* 3 and 6: recombinant CelA after purification

was obtained at 65°C in the pH 5.0 sodium acetate buffer (Fig. 5c). This was in agreement with the biochemical property of *Geobacillus thermoleovorans* T4 reported by Tai et al. (2004). The endo-glucanase activity declined by only 20% after incubation for 16 h at pH ranging from 5.0 to 9.0 (Fig. 5d). These results suggest that CelA is a novel enzyme not only being thermostable but also stable over a broad range of pH values.

Although enzymatic catalysis is usually influenced by pH, as their active sites are composed of ionizable groups, proper ionic form is also needed to maintain proper protein conformations for substrate binding and catalytic activity. CelA exhibited a high degree of tolerance in basic and acidic solutions, with over 70% of activity remained in a broad range of pH (4–9) (Fig. 5d). CelA was strongly inhibited by Cu^{2+} and Zn^{2+}, depending on the concentration tested (Fig. 6). However, other divalent cations ($Mn^{2+} > Co^{2+} > Ca^{2+}$) stimulated the endo-glucanase activity, and especially 2 mM $MnSO_4$ increased the enzyme activity by as much as 100%. The metal ions, Na^{+}, Mg^{2+} and EDTA did not have any effect on the enzyme activity. Increasing concentration of reducing reagents, DTT and 2-ME, dramatically enhanced the endo-glucanase activity.

CelA has a distinct substrate specificity and higher enzymatic activity than *T. reesei* cellulases

CelA could more efficiently hydrolyze amorphous substrates (including acid swollen Avicel, CMC and β-D-glucan) than crystalline substrates (including Avicel, cellulose fiber and filter paper) as shown in Table 1. CelA had little xylanase activity but could hydrolyze both β-D-glucan (1,267 U/mg) and Lichenan (945 U/mg), which had not been reported in *Geobacillus* sp. previously.

Cellulases from *T. reesei*, including endo-, exo- and β-glucosidase, were well known for their high activities (Fujita et al. 2002; Teeri et al. 1998). The composition of enzyme complexes from *T. reesei* had been reported to be 20% endo-glucanase, 70% exo-glucanase and 10% β-glucosidase and xylanases (Markov et al. 2005). Comparison between *T. reesei* and CelA endo-glucanases indicated that the latter exhibited a tenfold greater specific activity toward CMC, pNPT and pNPC (all were soluble substrates) (Table 2). This is probably due to the fact that CelA was a highly active endo-glucanase, and the cellulase activity in *T. reesei* was mainly exo-glucanase and some minor β-glucosidase.

Discussion

Rice straw composts supplemented with pig manure contain an active and diverse microbial community that includes bacteria and fungi producing cellulolytic enzymes. To screen for thermophilic bacteria, we collected samples from the hyper-thermostable compost at temperatures over 70°C where *Geobacillus* is known to grow at such high temperatures and can become the dominant species in the community. *Geobacillus* sp. 70PC53 was isolated from the compost at AVRDC, Tainan, Taiwan, as an endo-glucanases-producing bacterium. Many *Bacillus* sp. are known to produce endo-glucanases (Li et al. 2008; Liu et al. 2004) but there is only one report describing endo-glucanase from *Geobacillus* (Tai et al. 2004). It is interesting to note that annotations of complete genome sequence did not predict any cellulase homologous in *Geobacillus kaustophilus* and *Geobacillus thermodenitrificans*. Therefore, *cel*A described in the current work appears to be a unique cellulase gene belonging to a particular group of cellulolytic *Geobacillus* bacteria that has not yet been reported. Our ability of expressing high level of recombinant CelA cellulase in *E. coli* further facilitates production and improvements of this unique enzyme.

We had tried to identify strain 70PC53 by comparisons with *rec*N (Zeigler 2005) and 16S rDNA sequences, all data indicated that this strain was a new species in the

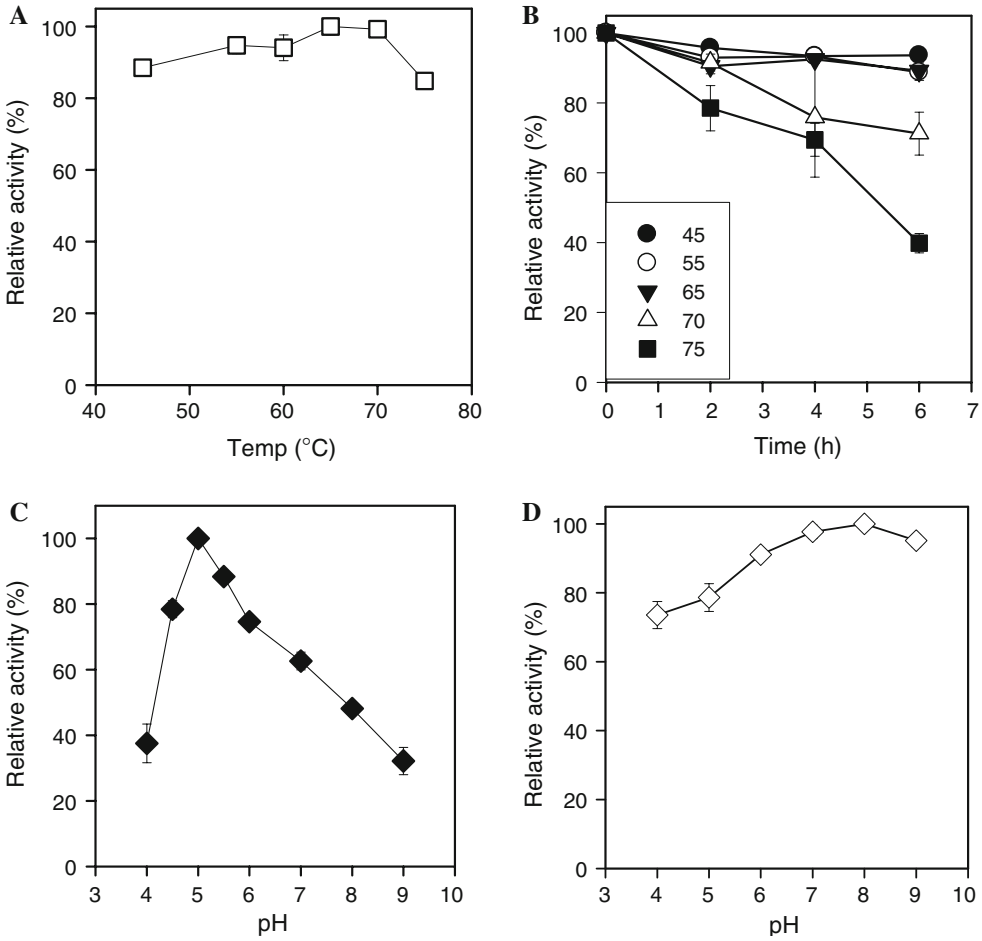

Fig. 5 Effect of temperature and pH on the activity and stability of CelA. **a** Optimal temperature (*open squares*). **b** Thermostability: enzyme was determined by incubating the enzyme at 45°C (*filled circles*), 55°C (*open circles*), 65°C (*filled inverted triangles*), 70°C (*open triangles*) and 75°C (*filled squares*) for 6 h. The residual enzyme activity was determined in pH 5.0 at 65°C. **c** Optimal pH (*filled diamonds*), **d** pH stability (*open diamonds*): enzyme was analyzed first by incubating the enzyme in 50 mM buffers with variable pH for 16 h. The residual enzyme activity was then determined in 50 mM sodium acetate buffer, pH 5.0 at 65°C

Geobacillus genus. The taxonomy of *Geobacillus* is complicated (Nazina et al. 2005) and the purpose of this study was to clone a novel endo-glucanase in a thermophilic environment, thus further identification of strain 70PC53 was not carried out in the current work. To positively identify strain 70PC53, one needs to carry out further elucidations in terms of its fatty acid compositions, DNA–DNA hybridization kinetics, morphology analysis, physiology evaluations, etc.

Gene cloning via screening genomic DNA library has been routinely used in studies of cellulolytic enzyme genes (Cho et al. 2000; Liu et al. 2004). In the present study, *celA* was isolated by screening an expressing library of more than 6,000 clones for specific clones expressing enzymatic activity capable of hydrolyzing carboxy methyl cellulose, a commonly used substrate for endo-glucanase. Interestingly, CelA from *Geobacillus* was not related to endo-glucanase genes in *Bacillus* species, such as *B. amyloliquefaciens*, *B. licheniformis* and *B. substilis* (Fig. 3). It appeared to be a novel endo-glucanase belonging to glycosyl hydrolase family 5 (GH5) with 45.1% amino acid identity to the most

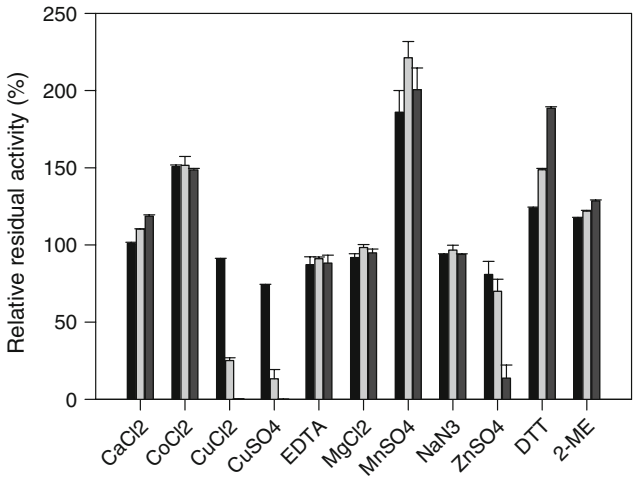

Fig. 6 Effect of various reagents and metal ions on CelA. Concentrations of all chemicals ranged are from 2 mM (*dark*), 5 mM (*bright gray*) to 10 mM (*dark gray*). The residual relative enzyme activities were measured in 1.0% CMC in 50 mM sodium acetate buffer, pH 5.0, at 65°C for 15 min. All measurements were analyzed with triplicates with *error bars* indicated

Table 1 Substrate specificity of *Geobacillus* sp. 70PC53 CelA

Substrate	Specific activity[a] (U/mg)
Avicel	0
Acid swollen Avicel	41.4
CMC	116.4
Cellulose fiber	0
β-D-Glucan (barley)	1,267.3
Filter paper	1.0
Lichenan	945.4
Xylan-birchwood	5.3
Xylan oat spelts	0.1

[a] Activities were measured at 65°C in 50 mM sodium acetate buffer (pH 5.0) using 1.0% of different substrates. 1 U is defined to 1 μmol glucose equivalent amount per minute

Table 2 Comparison of cellulase activity between *Geobacillus* sp. 70PC53 CelA and *Trichoderma reesei* (ATCC 26921) using different substrates

Substrates	*Geobacillus* sp. 70PC53 CelA (U/mg, 65°C)	*Trichoderma reesei* (U/mg, 37°C)
CMC	116.44	19.11
Avicel	0.71	3.84
β-1,3 Glucan (from *Euglena gracilis*)	1.08	0.23
Filter paper	1.06	19.13
pNPT	84.48	7.87
pNPC	166.89	13.61
pNPG	0.35	11.56

1 U is defined to 1 μmol glucose equivalent amount per minute when using CMC, Avicel, β-1,3 glucan and filter paper as substrates while 1 U is defined to 1 μmol of 4-nitrophenol amount per minute when using pNPT, pNPC and pNPG as substrates

closely related endo-glucanase in *Pectobacterium atrosepticum* CelN.

In general, thermophilic microorganisms produce enzymes more thermostable than their counterparts in mesophilic microorganisms. Thermally tolerant cellulases from *Acidothermus cellulolyticus*, in which the disulfide bond is involved in thermal stability, are the best known thermostable endo-glucanase with optimal enzyme activities at ~80°C (Ransom et al. 2007; Sakon et al. 1996; Skopec et al. 2003). CelA described in this work has only one Cys268 in the full-length amino acid sequence, thus it is unlikely to form an intramolecular disulfide linkage. Interestingly, the activity of this enzyme was stimulated by 50–80% when treated with 5–10 mM DTT and to lesser extent by β-mercaptoethanol. In addition, an extra activity band at 95 kDa, which was about twice the size of predicted size of CelA, appeared on SDS gel. This implied that the enzyme could form a dimer that was not fully dissociated on SDS-PAGE. Thermostability of an enzyme is a very important factor for developing its potential industrial usage. We found that endo-glucanase CelA was a thermostable enzyme as it retained almost all of its original activity at 65°C over 6 h and only a 20% decay was observed at 70°C. This enzyme is active over a broad temperature ranges from 37 to 75°C and it is also stable over broad pH values, ranging from pH 5 to 9.

During the past two decades, several cellulase genes have been cloned from *Trichoderma reesei*, including at least five encoding endo-glucanases. In this study, we cloned and analyzed an endo-glucanase gene, *cel*A, from a thermophilic *Geobacillus* and compared it with endo-glucanases from *T. reesei*. The *Geobacillus* endo-glucanase described in this work possess specific activity of 116.4 U/mg with CMC, 84.48 U/mg with 4-nitrophenyl β-D-cellotrioside and 166.89 U/mg with 4-nitrophenyl β-D-cellobioside. From the comparison with commercially available *T. reesei* cellulases, it is apparent that CelA endo-glucanase described in this work is a highly active enzyme, which is rare among bacterial cellulolytic enzymes. Besides, we found a synergistic effect of about 15% when we added CelA to *T. reesei* cellulase system.

Conclusions

In recent years, there has been considerable interest in the utilization of plant materials as a renewable source of fermentable sugars that could be subsequently converted to useful products such as liquid fuels, solvents, chemicals or animal feeds. Many bioconversion processes are particularly attractive for the elimination of residues and wastes produced by agriculture and forestry. As a result of this interest, a wealth of knowledge on cellulolytic enzymes has been accumulated. Judging from the biochemical properties of *Geobacillus* sp. 70PC53 CelA observed in this work, such as high specific activity, activity over a broad range of temperatures, and stability over broad ranges of temperature and pH, we believe that this is a unique enzyme potentially useful in, but not limited to, applications in biomass conversion, detergent enhancement, paper pulping, textile manufacturing and juice clarification.

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