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Fast Identification of Thermostable Beta-Glucosidase Mutants on Cellobiose by a Novel Combinatorial Selection/Screening Approach

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ABSTRACT: Engineering costly cellulases on natural cellulosic substrates is of importance for emerging biomass-based biorefineries. Directed enzyme evolution is becoming a popular tool, but identification of desired mutants from a large mutant library remains challenging sometimes. In this work, we demonstrated a novel combinatorial selection/screening strategy for finding thermostable beta-glucosidase on its natural substrate—cellobiose. First, selection was conducted through complementation of beta-glucosidase for non-cellobiose-utilizing *Escherichia coli* so that only the cells expressing active beta-glucosidase can grow on a M9 synthetic medium with cellobiose as the sole carbon source (selection plate). Second, the clones on the selection plates were duplicated by using nylon membranes. After heat treatment, the nylon membranes were overlaid on M9/cellobiose screening plates so that remaining activities of thermostable beta-glucosidase mutants hydrolyzed cellobiose on the screening plates to glucose. Third, the growth of an indicator *E. coli* strain that can utilize glucose but not cellobiose on the screening plates helped detect the thermostable beta-glucosidase mutants on the selection plates. Several thermostable mutants were identified from a random mutant library of the *Paenibacillus polymyxa* beta-glucosidase. The most thermostable mutant A17S had an 11-fold increase in the half-life of thermoinactivation at 50°C.

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Introduction

The production of second generation biofuels (e.g., cellulosic ethanol and butanol) and third generation biofuels (e.g., hydrogen) from the most abundant renewable lignocellulosic biomass sugars will bring benefits to the economy, environment, and national energy security (Lynd et al., 2002; Wyman, 2007; Zhang et al., 2007). The largest technical and economical obstacle to the carbohydrate economy is cost-effective release of fermentable soluble sugars from lignocellulosic biomass (Lynd et al., 2008; Wyman, 2007). Biomass saccharification usually involves pretreatment followed by enzymatic hydrolysis (Wyman, 2007; Zhang, 2008). Enzymatic hydrolysis of heterogeneous cellulosic materials requires endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and beta-glucosidase (BGL, EC 3.2.1.21) to work together (Lynd et al., 2002; Zhang and Lynd, 2006). Among them, beta-glucosidase is responsible for converting soluble

cello-oligosaccharides (mainly cellobiose) to glucose and eliminating cellobiose inhibition of cellobiohydrolase and endoglucanase (Zhang and Lynd, 2004b; Zhang et al., 2006).

Enzyme properties can be improved by directed evolution, rational design, and their combination (Hibbert et al., 2005; Schmidt-Dannert and Arnold, 1999; Zhang et al., 2006). Directed enzyme evolution is a widely accepted tool for protein engineering because of its independence of knowledge of enzyme structure and the interaction between enzyme and substrate. The greatest challenge is to develop methods that correctly evaluate the performance of mutants of the interested enzyme, often stated as ''you get what you screen for'' (You and Arnold, 1996). Selection is always preferred over screening because it is several orders of magnitude more efficient (Griffithsa et al., 2004; Olsen et al., 2000; Otten and Quax, 2005; Zhang et al., 2006). However, selection requires smart design that a phenotypic function of the gene product confers selective advantages to the parental microorganism (Zhang et al., 2006). If a selection approach is not available, screening has to be used for finding the desired mutants from the library. Screening can be either (1) facilitated by distinguishing the desired mutants from the rest based on their distinct phenotypes, such as colored products released or halos formed, or (2) random, which involves picking mutants one by one for their performance evaluation (Taylor et al., 2001; Zhang et al., 2006).

Beta-glucosidase activities can be measured based on (1) glucose released from a natural substrate—cellobiose (Zhang et al., 2006), and (2) fluorescence or colored products released from analogs of cellobiose, for example, *p*-nitrophenyl-beta-D-1,4-glucopyranoside (*p*NPG) (Strobel and Russell, 1987), 4-methylumbelliferyl-beta-D-glucopyranoside (Setlow et al., 2004), naphthyl-beta-D-glucopyranoside, and 6-bromo-2-naphthyl-beta-D-glucopyranoside (Polacheck et al., 1987; Zhang et al., 2006). Chromogenic substrate-based assays featuring high sensitivity and easy operation have been widely used to facilitate screening of beta-glucosidase mutants. So far, nearly all beta-glucosidase engineering via directed evolution is based on a chromogenic substrate *p*NPG (Arrizubieta and Polaina, 2000; Gonzalez-Blasco et al., 2000; Lebbink et al., 2000). However, there is usually no clear relationship between the activities of beta-glucosidase on the analog substrates and natural substrate cellobiose (González-Candelas et al., 1989; Kaur et al., 2007; McCarthy et al., 2004). A similar situation occurs with endoglucanase, whose activity on an analog substrate carbomethylcellulose (CMC) has no correlation with its activity on the natural substrate cellulose (Himmel et al., 1996; Wolfgang and Wilson, 1999; Zhang et al., 2000, 2006; Zhou et al., 2004). To find beta-glucosidase mutants with desired performance on the natural substrate cellobiose, Eveleigh and his co-workers screened a mutant library of *Thermotoga neapolitana* 1,4-beta-D-glucan glucohydrolase by using 96-well microplates (McCarthy et al., 2004). Such random screening is time-consuming, labor-intensive, and costly, therefore not suitable for a large library.

Since large amounts of cellulases are consumed for cellulosic ethanol production (e.g., about 100–200 g of cellulase per gallon of cellulosic ethanol; Zhang et al., 2006), any improvement in cellulase performance (e.g., thermostability and catalytic efficiency; Zhang et al., 2006) and/or cellulase recycling (Tan et al., 1986; Tu et al., 2007) will greatly reduce ethanol production costs. As one of the key enzymes in cellulose hydrolysis, beta-glucosidase that has no cellulose-binding module cannot be adsorbed by cellulose (Zhu et al., 2009) or even most pretreated biomass (Lynd et al., 2002; Zhang and Lynd, 2004b). Hence, it can be recycled easily as long as it remains active. Therefore, improving thermostability of beta-glucosidase, along with cellulase recycling would significantly reduce production costs of cellulosic ethanol. Although thermostable beta-glucosidases can be cloned from thermophiles or extremophiles (Lebbink et al., 2000; Pouwels et al., 2000), it is still important to enhance thermostability of mesophilic enzymes through directed evolution and study the relationship among amino acid sequence, structure, and thermostability. For example, van der Oost and his coworkers have investigated to improve the catalytic performance of the hyperthermophilic *Pyrococcus furiosus* beta-glucosidase at a decreased temperature by directed evolution (Lebbink et al., 2000).

In this study, we described a novel high-efficiency selection and screening strategy for identifying thermostable beta-glucosidase mutants using cellobiose as substrate. A well-studied beta-glucosidase from *Paenibacillus polymyxa* (González-Candelas et al., 1989; Painbeni et al., 1992) was chosen as the model enzyme to evaluate our new approach. Its crystalline structure has been resolved (Sanz-Aparicio et al., 1998b) and its thermostability has been improved by directed evolution (Gonzalez-Blasco et al., 2000; Lopez-Camacho et al., 1996). In this work, several new mutants were generated and identified, one of which was found to be more thermostable than the previous ones.

Materials and Methods

Chemicals and Media

All chemicals were reagent grade, purchased from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Luria-Bertani (LB) medium and M9 minimal medium were prepared as described elsewhere (Sambrook and Russel, 2001). The M9/cellobiose media was prepared by mixing the filtration-sterilized cellobiose solution and the autoclaved M9 minimal medium at a final cellobiose concentration of 0.4% (w/v) as the sole carbon source. *P. polymyxa* (ATCC 842) was grown on the ATCC potato medium and its genomic DNA was purified as described elsewhere by using commercial DNA kits. Plasmid pOCB containing the *P. polymyxa bglA* gene was constructed as described previously (Hong et al., 2008).

Bacterial Strains and Plasmids

Escherichia coli JM109 strain was used for cloning and mutant library construction and the Stratagene *E. coli* BL21-CodonPlus(DE3)-RIL strain (La Jolla, CA) was used for BglA expression. The *bglA* gene encoding the family I beta-glucosidase from *P. polymyxa* was amplified using primers bglA-f-SalI (5′-GGAAGCGTCGACCATGACTATTTTTT-CAATTTCCGC, *Sal*I restriction site underlined) and bglA-r-stop (5′-GCAGCCGGATCCTTAGCGTCTAGTCTCC, *Bam*HI restriction site underlined) from plasmid pOCB. The DNA fragments were digested by *Sal*I and *Bam*HI and ligated with the digested plasmid pUC19 (New England Labs, Ipswich, MA) to give plasmid pUC19-bglA. For protein characterization, the wild-type and mutant *bglA* genes in the plasmid pUC19-bglA were amplified using the primers bglA-f-SacI (5′-GGACTGGAGCTCGAATGACTA-TTTTTCAATTTCC, *Sac*I site underlined) and bglA.Pp-r21c (5′-AACATGCTCGAGTTTGTCGTCATCATCGCGTCTA-GTCTCCAACC, *Xho*I site underlined). The DNA fragments were digested by *Sac*I and *Xho*I and ligated with the expression vector pET21c (Novagen, Madison, WI).

Construction of a Random Mutant Library

A randomly mutated *bglA* library was generated by error-prone PCR (Leung et al., 1989). The PCR mixture contained 0.2 ng/μL plasmid pUC19-bglA as the template, 0.2 mM dATP, 0.2 mM dGTP, 1 mM dCTP, 1 mM dTTP, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, and 0.4 μM each of the primers bglA-f-SalI and bglA-r-stop. The PCR reaction was carried out with the New England Labs Taq DNA polymerase under the following conditions: 95°C denaturation for 2 min, 30 cycles of 94°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min, followed by 72°C extension for 10 min by using a Stratagene RoboCycler96 Temperature Cycler. The amplified PCR products were then digested by *Sal*I and *Bam*HI, ligated with plasmid pUC19, and transformed into *E. coli* JM109.

Identification of BglA Mutants With Improved Thermostability

Identification of desired beta-glucosidase mutants was performed in a two-step process that involves the selection for mutants with adequate cellobiase activity and the subsequent screening for improved thermostability (Fig. 1). The majority of the transformed JM109 cells were grown on solid agar plates (selection plates) containing 1.5% (w/v) agar, M9 minimal medium with 0.4% cellobiose as the sole carbon source, 100 μg/mL ampicillin, and 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG); a small aliquot of the transformed cells were grown on a LB plate containing 100 μg/mL ampicillin as the reference plate. The plates were incubated at 30°C until the clones could be visualized easily. Colonies on the selection plates were then duplicated with nylon membranes. After incubation at 60°C for 10 min (heat treatment) that killed all *E. coli* cells, lyzed them, and released intracellular beta-glucosidase, the membranes containing thermophilic beta-glucosidases were overlaid on the soft agar screening plates that contained M9 minimal medium with 1% cellobiose as the sole carbon source, 100 μg/mL ampicillin, and 0.5% agar mixed with an indicator strain *E. coli* JM109/pUC19 that can utilize glucose but not cellobiose. After overnight incubation at 37°C, the growth of the indicator strain on the screening plates helped identify the clones expressing thermostable BglA mutants on the selection plates.

Enzyme Purification and Characterization

Wild-type BglA and three thermostable mutants N52D, N437K, and A17S were produced from the expression vector pET21c in *E. coli* strain BL21-CodonPlus(DE3)-RIL growing on LB medium supplemented with 50 μg/mL ampicillin and 50 μg/mL chloramphenicol at 37°C. When A_{600} reached 0.6–0.7, IPTG was added at a final concentration of 50 μM and then the growth temperature was decreased to 16°C. After overnight culture, the cells were harvested by centrifugation and resuspended in a 50 mM sodium phosphate and 0.3 M NaCl buffer (pH 8.0). The cell suspension was sonicated and after centrifugation, the supernatant was loaded onto the column packed with Bio-Rad Profinity IMAC Resins precharged with Ni^{2+} (Hercules, CA). The bound proteins were eluted with 50 mM imidazole that was then removed using GE Healthcare PD-10 desalting columns (Piscataway, NJ). Protein mass concentration was determined by the Bradford method using the Bio-Rad protein assay dye reagent with bovine serum albumin as the standard protein. The purity of the purified protein was checked by SDS–PAGE followed by staining with Coomassie Blue.

Beta-glucosidase activity was measured by monitoring the formation of the reaction product using cellobiose as the substrate. All the assays were performed in a 50 mM phosphate buffer (pH 7.0) at 37°C. The reactions were stopped by boiling for 5 min, and the product glucose was determined using the Pointe Scientific Liquid Glucose (Hexokinase) Reagent Set (Canton, MI) (Zhang and Lynd, 2004a). Kinetic parameters (K_m and k_{cat}) of beta-glucosidase were determined using 2–50 mM cellobiose through direct fitting of the hyperbolic Michaelis–Menten curves by using the Origin software (Microcal Software, Northampton, MA). For determination of the half-lives of the enzymes, the purified samples were diluted in a prewarmed buffer to 10 μg/mL and incubated at 50°C for different lengths of times. After the preincubation, the samples were chilled on ice and the residual enzymatic activity was assayed at 37°C, as described above.

Structural Analysis

The structural model of the different mutants was built by using the graphic program RasWin, a variant of RasMol—a

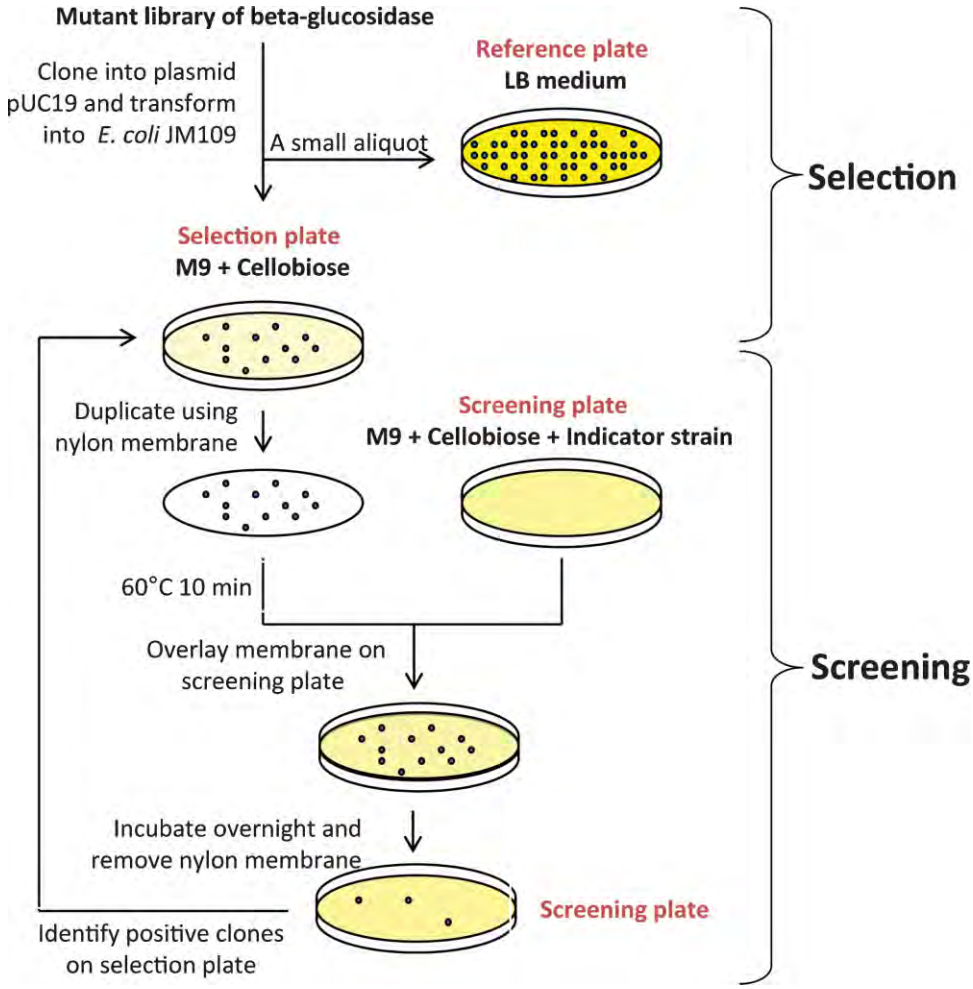

Figure 1. Scheme of the selection/screening approach for fast identification of thermostable beta-glucosidase mutants active on cellobiose. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

public domain program for examining protein structure, based on the reported crystal structure of the *P. polymyxa* beta-glucosidase A complex with gluconate ligand with Protein Data Base (PDB) code 1BGG (Sanz-Aparicio et al., 1998b).

Results

Combinatorial Selection/Screening Approach

For directed enzyme evolution, efficient identification of desired mutants from a large number of mutants, most of which are negative or neutral, is still challenging. Selection is always preferred due to its several orders of magnitude higher efficiency relative to screening. Figure 1 presents the new combinatorial selection/screening scheme for efficient identification of thermostable beta-glucosidase on a natural substrate—cellobiose. Selection design is based on the hypothesis that introduction of a heterologous beta-glucosidase that can hydrolyze cellobiose to glucose should enable non-cellobiose-utilizing *E. coli* to grow on cellobiose as the sole carbon source. Wild-type *E. coli* cannot utilize cellobiose as carbon source to support its growth but has a cellobiose transport system (Hall et al., 1986). On the LB medium, both *E. coli* JM109/pUC19 (a negative control) and *E. coli* JM109/pUC19-bglA that expresses beta-glucosidase grew well (Fig. 2A). On the contrary, on the M9 minimal medium supplemented by cellobiose as the sole carbon source, only *E. coli* JM109/pUC19-bglA grew (Fig. 2B). The experimental data clearly validated the selection hypothesis. The selection/screening power can be estimated from the ratio of the number of colonies on the M9/cellobiose selection plate, where the only the transformants expressing active beta-glucosidase can grow, to that on the LB reference plate, where all transformants can grow.

In order to avoid using labor-intensive screening (e.g., microplate) or costly instruments (e.g., fluorescence-activated

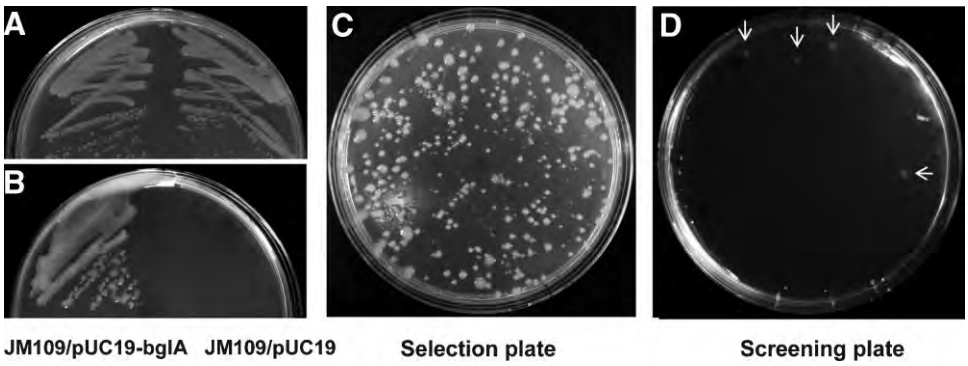

Figure 2. Growth of *E. coli* JM109 strains on the LB and M9/cellobiose plates. *E. coli* strains JM109/pUC19-bglA (*Paenibacillus polymyxa* beta-glucosidase A) and the control JM109/pUC19 were streaked on the plates containing (**A**) LB medium or (**B**) M9 minimal medium with 0.4% cellobiose. **C**: Selection plate with the colonies expressing active beta-glucosidase. **D**: Screening plate showing growth of the indicator strain (JM109/pUC19) indicated by the arrows.

cell sorter or automated robot), we designed a new high-efficiency screening method as follows. The colonies on the selection plates were duplicated by using nylon membranes, and heat treatment was applied to the membranes to deactivate most of the beta-glucosidase mutants as well as the wild-type. After the heat treatment, only the thermostable beta-glucosidase mutants can hydrolyze cellobiose to glucose on the screening plates. Differentiation of glucose and cellobiose on solid agar plates was relatively challenging because both of them are colorless, soluble reducing sugars. To solve this problem, we proposed for the first time to use an indicator microorganism to detect glucose released from cellobiose. The heat-treated membranes were put on the second M9/cellobiose synthetic medium plates (screening plates), the soft agar mixed with the indicator microorganism (*E. coli* JM109/pUC19) that can grow on glucose but not cellobiose. Based on the position of the indicator microorganism growing on the screening plates, the *E. coli* clones containing thermostable beta-glucosidase mutants were located on the selection plates.

Identification of Thermostable Beta-Glucosidase Mutants

The *P. polymyxa* BglA was chosen for testing of the selection/screen method described above. The *bglA* mutant library was generated by error-prone PCR at an estimated average mutation rate of 0.3% (1–2 mutations per *bglA* gene). The mutated DNA fragments were ligated into plasmid pUC19 and transformed into *E. coli* JM109 strain. The majority of *E. coli* transformants were spread on the selection plates and a small aliquot on the reference plate (LB plate). The selection to screen efficiency can be adjusted from very low to very high by altering the mutation rate of error-prone PCR. In this study, only about 3% of the transformants had adequate beta-glucosidase activity for growth on cellobiose. Due to different activities of beta-glucosidase mutants on cellobiose, the sizes of clones on the screening plates varied (Fig. 2C).

The cells duplicated on the nylon membranes were incubated at 60°C for 10 min. Such heat treatment killed *E. coli* cells, lyzed them, and deactivated thermolabile beta-glucosidases, including the wild-type BglA (data not shown). Such conditions deactivated about 99% of the beta-glucosidases, that is, of the 200–300 clones on each membrane, only up to four remained active after the heat treatment and they enabled to support growth of the indicator microorganism on the screening plates (Fig. 2D). The heat treatment conditions (e.g., temperature and time) can be adjusted to obtain different thermo-inactivation effects. In this study, one thermostable mutant was identified from about 3,300 random mutants.

Approximately 30 putative thermostable clones were observed from a mutant library of about 100,000 clones, of which 20 clones of at least an average size (with a diameter of about 1 mm) on the selection plates were chosen for DNA sequencing. Several mutants with single amino acid substitutions were identified, including N437K (AAT → AAG), E96K (GAG → AAG), N52D (AAT → GAT), and A17S (GCC → TCC). The mutants N437K and E96K had been previously reported (Gonzalez-Blasco et al., 2000). The mutant N52D previously constructed by rational design was reported to have no appreciable improvement in thermostability compared to the wild-type BglA (Gonzalez-Blasco et al., 2000). The mutant A17S was identified first in this work.

Basic Characteristics of Wild-Type and Mutants

The wild-type BglA and the thermostable mutants N437K, N52D, and A17S were selected for further analyses because the mutant E96K has been characterized well in the literature

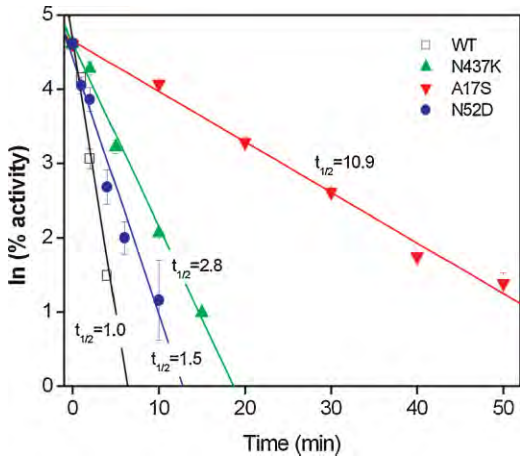

Figure 3. Thermostability analysis of wild-type BglA and mutants on cellobiose. Purified enzymes were diluted in prewarmed assay buffer to 10 μg/mL and incubated at 50°C. Half-lives ($t_{1/2}$) in terms of min at 50°C are indicated. Standard deviations were calculated from triplicate experiments. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

(Gonzalez-Blasco et al., 2000; Lopez-Camacho et al., 1996) and its structure has been resolved (Sanz-Aparicio et al., 1998a). Figure 3 shows the activities of the wild-type and three mutants after being incubated at 50°C for different lengths of time. The mutants A17S and N437K had half-lives ($t_{1/2}$) of 10.9 min and 2.8 min, representing about 11- and 3-fold improvement over the wild-type ($t_{1/2} = 1$ min), respectively. The mutant N52D had only a slight improvement in the half-life compared to the wild-type. Kinetic parameters of the wild-type and the three mutants are presented in Table I. The newly identified mutant A17S had a slightly lower K_m of 7.0 ± 0.3 mM and a higher k_{cat} of 14.6 ± 0.3 s^{-1} than the wild-type ($k_{cat}/K_m = 1.0 \pm 0.1 \times 10^3$ $M^{-1}\,s^{-1}$), resulting in a doubled catalytic efficiency (k_{cat}/K_m) of $2.1 \pm 0.1 \times 10^3$ $M^{-1}\,s^{-1}$ at 37°C. The mutant N52D had a 57% increase in catalytic efficiency and N437K showed no improvement (Table I).

Discussion

Engineering cellulases by directed evolution will play an important role for improving cellulase performance (Zhang et al., 2006) because (i) the relationships between substrates and enzymes are very complicated (Zhang and Lynd, 2004b, 2006), (ii) advances through rational design are limited so far (Wolfgang and Wilson, 1999; Zhang et al., 2000, 2006; Zhou et al., 2004), and (iii) directed evolution is independent of knowledge of substrate and enzyme. Lack of efficient selection or high-throughput screening is limiting advances of cellulase engineering.

Here we proposed a novel selection/screening method for identification of thermostable beta-glucosidase mutants by using cellobiose as substrate. Selection was based on beta-glucosidase complementation to non-cellobiose-utilizing *E. coli*, and its selection/screening power depended on DNA mutagenesis rates. In addition to selection, high-efficiency screening was conducted on soft agar plates with help of an indicator microorganism whose growth represented glucose availability from cellobiose; the number of mutants isolated was associated with heat treatment conditions and the percentage of the desired mutants in library and their characteristics. In addition, the use of nylon membrane for plate duplication and heat treatment is better than the use of two-layer agar plate because agar melts at high temperatures.

So far, nearly all cellulase engineering via directed evolution is based on cellulose analogs. Several beta-glucosidases have been improved by directed evolution previously (Arrizubieta and Polaina, 2000; Gonzalez-Blasco et al., 2000; Lebbink et al., 2000), but the beneficial mutants were identified based on facilitated screening by using a chromogenic substrate—*p*NPG, an analog of cellobiose. This could be a problem because cellulase activities on analog substrates are not associated with their activities on natural substrates most of the times (Zhang et al., 2006). For example, several endoglucanases have been improved by directed evolution based on CMC, but no evidence for their enhanced performance on cellulose was observed (Catcheside et al., 2003; Kim et al., 2000; Murashima et al., 2002; Wang et al., 2005). To our knowledge, only one directed evolution study of cellulase is based on the natural substrate—cellobiose, conducted by Eveleigh and his coworkers based on 96-well microplate-based blind screening (McCarthy et al., 2004).

The crystal structure of *P. polymyxa* BglA indicates that it is a tetramer of dimers with fourfold symmetry, and each monomer has the $(\alpha/\beta)_8$ barrel topology with the active center located along the axis of the barrel (Sanz-Aparicio et al., 1998b). Figure 4A shows the positions of the three mutations characterized in this work on the enzyme monomer. Amino acids Asn437 and Asn52 are both located near the surface of the subunit interface, with the former in a helix and the latter in a loop. The N437K mutation enables the formation of a bifurcated salt bridge from its side chain to two residues of the neighboring subunit, which strengthens the inter-subunit interactions and thus enhances thermostability of the enzyme (Gonzalez-Blasco et al., 2000). The mutation N52D may improve enzyme thermostability through another mechanism—decreasing deamidation. At high temperatures, asparagine can be degraded to

Table I. Kinetic parameters of the wild-type BglA and mutants on cellobiose at 37°C.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ $s^{-1}\,M^{-1}$)
WT	12.0 ± 1.0	12.3 ± 0.3	1.0 ± 0.1
N52D	4.8 ± 0.4	7.8 ± 0.2	1.6 ± 0.1
N437K	10.9 ± 1.0	11.1 ± 0.3	1.0 ± 0.1
A17S	7.0 ± 0.3	14.6 ± 0.3	2.1 ± 0.1

Standard deviations were calculated from triplicate experiments.

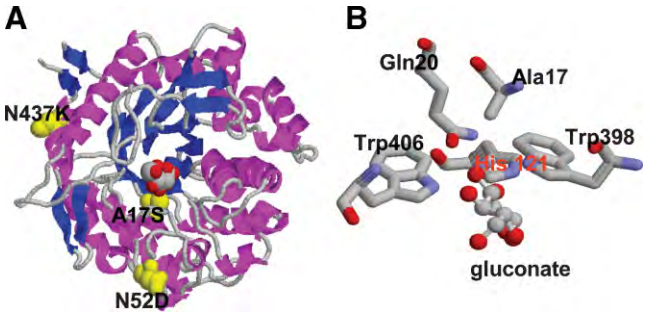

Figure 4. Structural analysis of the thermostable mutations. **A**: Structure of a *P. polymyxa* BglA subunit (Sanz-Aparicio et al., 1998b) with the positions of the thermostable mutations mapped. Alpha-helices are colored in magenta, beta-sheets in blue, and loops in gray. Mutated residues and gluconate ligand are displayed as space-filling spheres in yellow and CPK color scheme (vide infra), respectively. **B**: Ala17 and the surrounding residues at the active center. Amino acids are in sticks, and gluconate ligand is in ball-and-stick representation. All atoms are in CPK color scheme, with carbon atoms in white, oxygen atoms in red, and nitrogen atoms in blue. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

succinamide that can lead to peptide bond hydrolysis (Daniel et al., 1996). The replacement of thermolabile Asn with Asp might avoid the deamidation of the exposed Asn side chain, resulting in an increase in thermostability of BglA. Different from the two surface mutations above, the mutation A17S occurs in a loop near the active center (Fig. 4B). The side chain of this residue is buried among Gln20, His121, Trp398, and Trp406, all of which interact with the gluconate ligand either by hydrogen bonding or by ring stacking (Sanz-Aparicio et al., 1998b). Notably, Trp398 is part of the hydrophobic platform that is highly conserved in this enzyme family and is critical for transition state stabilization (Nerinckx et al., 2003). The substitution of alanine at position 17 with serine results in an increase of residue volume, which is believed to be important for enhanced thermostability of enzymes, possibly by filling internal cavities and reducing the entropy of the unfolded protein backbone (Haney et al., 1999). In addition, the A17S mutation results in a change of the side chain, which faces toward the gluconate ligand, from neutral to polar. This change in the side chain packing of the active center may affect the hydrogen bonds between the sugar hydroxyls of the substrate and the polar side chains of the active center residues (Sanz-Aparicio et al., 1998b), which influences the substrate binding affinity, as reflected by a slight decrease of K_m from 12.0 mM (wild-type) to 7.0 mM (A17S), as shown in Table I.

Conclusion

A combinatorial selection/screening strategy was developed for fast identification of thermostable beta-glucosidases. This new approach based on the natural substrate cellobiose drastically reduced labor as compared to screening and did not require any costly instruments. The most thermostable mutant *P. polymyxa* BglA A17S identified by this method had an 11-fold increase in the half-life of thermoinactivation at 50°C. Now we are working on further improvement of thermostability of beta-glucosidase and other cellulase components via directed evolution.

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