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Thermostable Bacterial Endoglucanases Mined from Swiss-Prot Database

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Abstract As one critical enzyme in deconstructing complicated cellulose matrix, endoglucanase (EG) is needed to exhibit high activity and thermostability under severe industrial conditions. Driven by this purpose, EGtf1 (Q08166) and EGtf2 (Q7X2N2), with relatively high specific activities, were selected out of 43 putative EG genes from SWISS-PROT database. These distinguished EGs were successfully overexpressed in *Escherichia coli* and purified by one-step affinity chromatography. The maximal activity was shown at approximate pH 5.0 and 50 °C. It is worth noting that EGtf1 and EGtf2 displayed outstanding thermostability with a half-life of up to 1,386 h at 50 °C, which is almost 100-fold higher than other reported EGs. Furthermore, the presence of various metal ions (1 mM) or organic solvents (50%, *v*/*v*) did not cause significant effect on the activities of EGtf1 and EGtf2 and even showed 2.1- and 2.7-fold enhancement in the case of dodecanol. All these features, especially the excellent thermostability of EGtf1 and EGtf2, enable them to become a good candidate for further protein engineering to realize the ultimate practical application in biomass industry.

Keywords Endoglucanase (EG) · Carboxymethyl cellulose (CMC) · Thermostability · Enzymatic properties · Data mining · Swiss-Prot

Introduction

The increasing concerns about energy security, sustainability, and global climate change drive to extensive investigations on alternatives to fossil fuels for transportation. One of

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these alternatives, biofuels, like ethanol and butanol, can be derived by converting lignocellulosic biomass to fermentable sugars. Cellulose, the primary constituent of biomass, is usually composed of a linear homopolymer of glucose units linked by β-1,4-glycosidic linkages. In the process of degrading cellulose to fermentable sugars [1, 2], cellulases are regarded as one of the key elements. The most widely accepted mechanism for enzymatic cellulose hydrolysis involves synergistic action of three major enzymes: endoglucanase (EG, EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.9), and β-glucosidase (EC 3.2.1.21) [3]. Among those enzymes, EG is considered to play an important role in the synergy by cutting long-chain cellulose randomly to produce cello-oligosaccharides of various lengths.

Currently, the enzymatic hydrolysis of cellulose is still suffering from several severe shortcomings, such as slow rate, low efficiency, and poor enzyme stability, especially at a high temperature or in an acidic environment. The conventional methods of biocatalysts screening are generally effective but time-consuming and inefficient. Search in genome and protein databases for cellulases with high activity and/or stability is a promising approach to solving the problem. Application of bioinformatics techniques is a better way to increasing the possibility of discovering useful biocatalysts, like mining target genes from micro-organisms with known wealth of data that have accrued from available online databases. In this work, we mined the database of SWISS-PROT for candidate genes encoding EGs.

Although bacteria are important sources of diverse cellulases, their importance was underestimated to a great extent. In fact, majority of thermostable enzymes are often derived from bacteria. A search in the SWISS-PROT resulted in a large number of bacterial cellulases, of which over 85% were not investigated. In this study, EGs with better thermostability were screened by the method of so-called genome data mining. As a result, 15 EGs with outstanding carboxymethyl cellulose (CMC) hydrolytic activities were identified, and two enzymes (EGtf1 and EGtf2) showed not only higher activities at 50 °C but also excellent stabilities.

In addition to evaluating the activity and stability of these newly expressed EGs, their adsorption and desorption behaviors on cellulose were also examined. It is known that the lignolytic enzymes are always adsorbed onto the cellulose surface before hydrolysis [4], and adsorption of cellulase on an insoluble substrate is a critical step in the degradation of lignocellulose [5]. So far, the adsorption behavior of some commercially available enzyme preparations and the purified monocomponents of *Trichoderma reesei* EG on lignocellulosic substrates have been reported as a function of various physical parameters, such as time, temperature, pH, and ionic strength [6]. In this work, adsorption behavior of two thermostable EGs on Avicel was investigated in order to get an insight into the catalytic performance of enzymatic saccharification of cellulose.

Materials and Method

Strains and Vectors

Mycobacterium tuberculosis AS1.562, *Pseudomonas stutzeri* AS1.1803, *Bacillus licheniformis* AS1.813, and *Bacillus pumilus* AS1.1165 were purchased from the China General Microbiological Culture Collection Center. *Acetobacter xylinus* DSM6513, *Erwinia tasmaniensis* DSM17950, *Gluconacetobacter diazotrophicus* DSM5601, *Paenibacillus provencensis* DSM22280, *Pectobacterium carotovorum* subsp DSM30168, *Thermomono-*

spora fusca DSM43792, and *Dickeya dadantii* DSM18020 were purchased from the German Resource Center for Biological Material.

Escherichia coli DH5α (Genotype: F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ (*lacZYA-argF*) U169, hsdR17 (r_K^- m_K^+), λ–), and *E. coli* BL21 (DE3) (Genotype: F^- ompT gal dcm lon hsdS_B (r_B^- m_B^-) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])) were used, respectively, as the cloning and expression hosts. Ampicillin (100 μg/mL) was used for the selection of recombinant *E. coli* containing correct plasmids. The plasmid pET43.1a (+) for the heterogeneous expression studies was obtained from Novagen (Shanghai, China).

Cloning and Plasmid Construction

The genomic DNAs of all the above bacteria were extracted and purified using the TIANamp Bacteria DNA Kit (Tiangen, China). DNA fragments containing the different full-length EG-encoding genes were amplified by polymerase chain reaction (PCR) using primers with overlap 15 bases from either side of the site of linearization on the vector by the CloneEZ Kit (GenScript Corp.). PCRs were performed in a 30-μL reaction volume, containing 15 μL of 2× Tag Plus PCR MasterMix, 1 μL of each primer, 1 μL of genomic DNA, and 12 μL of ddH_2O. The thermal cycling conditions were as follows: 95 °C for 3 min for initial denaturation; 32 cycles of 94 °C, 1 min for denaturation; 55–65 °C (different genes in the different degree), 30 s for annealing; 72 °C, and 1 min for extension. The amplified DNAs were directly cloned into the *Sma*I and *Hind*III-linearized vector pET43.1a (+) and then introduced into *E. coli* DH5α and *E. coli* BL21 (DE3) cells. More details can be found in the supplementary materials (Table S3). All the primers were synthesized by and the restriction enzymes were ordered from Sangon Co. (Shanghai, China).

Expression and Purification of EGs

The genes encoding bacterial EGs were inserted into pET43.1a (+) and were expressed in transformed *E. coli* BL21 (DE3) cells. Cultures were grown at 37 °C in Luria Bertani medium (Tryptone 10 g/L, Yeast extract 5 g/L, and NaCl 10 g/L) containing 100 μg/mL ampicillin. When the optical density at 600 nm (OD_{600}) of the cultures reached 0.6–0.8, isopropy β-D-thiogalactoside was added to a final concentration of 0.5 mM. The gene expression was induced at 25 °C for a period of 12 h. Harvested by centrifugation (7,500×*g*, 6 min, 4 °C), the cells were washed twice with 0.85% physiological saline and resuspended in a binding buffer (20 mM phosphate buffer, 500 mM NaCl, 10 mM imidazole, pH 7.4). Cell suspensions were disrupted by sonication (JY92-II, Scientz Biotechnology Co.), and the cell lysate was centrifuged at 7,500×*g* for 20 min. The supernatant was loaded onto a Ni-NTA column (His trap Ni-NTA FF crude column, 5 ml, GE Healthcare Co.), equilibrated with the binding buffer after filtrating by a syringe filter (0.22 μm). The column was washed with the binding buffer, and the retained proteins were eluted with an increasing gradient from 10 to 500 mM imidazole in the binding buffer at a flow rate of 5 mL/min. The purity of fractions was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The fractions containing the desired protein were pooled and dialyzed against phosphate buffer for desalting. SDS–PAGE was performed on 15% polyacrylamide gel with Tris–glycine buffer system. Protein bands were visualized by staining the gel with Coomassie Brilliant Blue.

Assay of Enzyme Activity

The assay of endoglucanase EG activity was performed as follows: an aliquot (100 μL) of enzyme solution was added to 100 μL solution of 0.5% (*w*/*v*) CMC (Acros Organics; average molecular weight, 90,000; degree of carboxymethyl substitution, 0.7) in 50 mM citrate buffer (pH 5.0). The reaction mixture was incubated at 50 °C for 1 h. The reaction was stopped by the addition of 3 mL 3,5-dinitrosalicylic acid solution, boiled for 5 min, and then cooled in water for color stabilization. The optical absorbance was measured at 540 nm, and the amounts of liberated reducing sugars (glucose equivalents) were estimated against a standard glucose curve. One unit of enzyme activity is defined as the amount of enzyme that librates 1 μmol glucose per hour under the assay conditions.

The hydrolytic activity of β-glycosidase was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-xylopyranoside, or *p*-nitrophenyl β-D-galactopyranoside.

Characterization of EGs

The optimum pH was estimated by activity assay at different pH (2.0–10.0), with citrate buffer for the pH ranging from 2.0 to 6.0, phosphate buffer for the pH ranging from 6.0 to 7.0, and glycine-NaOH buffer for pH ranging from 7.0 to 10.0. The temperature optimum was examined in citrate buffer (50 mM, pH 5.0) in the temperature range of 30–80 °C.

Thermal stability was tested by incubating the enzymes in the citrate buffer (50 mM, pH 5.0) at 40, 50, and 60 °C for the enzyme EGbs, and at 50, 60, and 70 °C for enzymes EGtf1 and EGtf2. The residual activity was assayed as described above. All the experiments were performed in triplicate.

Influences of various metal ions and additives were investigated on enzymes EGtf1 and EGtf2. By preincubating the enzyme with different compounds (the final concentration was 1 mM) in citrate buffer (50 mM, pH 5.0) for 20 min at room temperature (25 °C), the residual activity was estimated.

To determine the effect of organic solvents on the endoglucanse activity, EGtf1 and EGtf2 were preincubated with different solvents (50%, *v*/*v*) for 20 min at 30 °C and 900 rpm on a thermostatic mixer (Thermostatic mixing device MHR23, HLC). The residual activity was then measured and was expressed as a percentage to the activity in the absence of the solvents.

Kinetic parameters (K_m and V_{max}) of EGs were measured in terms of Michaelis–Menten kinetics using the Lineweaver–Burk plots. The substrate (CMC) concentrations were in the range from 1.25 to 13.75 mg/mL.

The hydrolytic activities of EGtf1 and EGtf2 on alkylglycosides was assayed in a system of 20 mL mixture, containing various alkylglycosides at a final concentration of 20 mg/mL and a proper amount of enzyme in citrate buffer (50 mM, pH 5.0). The reaction was carried out at 50 °C and 150 rpm for 72 h. Samples were withdrawn to determine the glucose concentration by HPLC (Aminex HPX-87H Cloumn, 300×7.8 mm, mobile phase 0.005 M H_2SO_4) and to calculate the yield.

Adsorption Studies

Various amounts of the purified EGs in 50 mM citrate buffer (pH 5.0) were mixed with a fixed amount of Avicel cellulose (Avicel pH 101, from Sigma) to give a final volume of 0.5 ml. The enzyme concentration varied between 0.1 and 1.0 mg (protein)/mL. The final

cellulose concentration was 20 mg/mL. The mixture was incubated for 1 h at 4 °C in 50 mM citrate buffer (pH 5.0). After centrifugation for 10 min at 2,000×*g*, the protein concentration in the supernatant was analyzed by Nanodrop 2000c Spectrophotometer (Thermo Scientific).

Reversibility of the EG adsorption was studied by examining the enzyme desorption at suitable enzyme protein concentration, which was 50% saturation of enzyme protein adsorbed on cellulose (0.4 mg protein/mL in this work). After being adsorption for 1 h under the above condition, the suspension was centrifuged to separate cellulose and adsorbed enzyme. The pellet was resuspended in 50 mM fresh citrate buffer (pH 5.0) and further incubated for 8 h at 4 °C. Then, the mixture was centrifuged again to measure the free protein in the supernatant.

Result and Discussion

Cloning, Expression, and Purification of Recombinant EGs

As many as 43 putative full-length genes encoding EGs were chosen from the SWISS-PROT. All these EG genes were contained in the genome of various bacteria. Approximately half (22) of them were successfully expressed in the form of soluble proteins with the N-terminal His-tags. Among the 22 proteins, 15 showed significant activity towards the CMC substrate (Table S1). Eight out of the 15 enzymes have not been investigated yet, and two showed higher specific activity even after freeze-drying. These two enzymes, EGtf1 and EGtf2, were purified to nearly electrophoretic homogeneity by immobilized metal affinity chromatography (Fig. 1). The specific activities of EGtf1 and EGtf2 after purification were 3.44 and 3.23 U/mg protein, respectively, indicating about 2.5-fold purification compared to the crude extract.

Biochemical Properties of the Two Thermostable Endoglucanases

Effect of pH on the enzyme activity was examined in the range of pH 2.0 to 10.0. The maximum activities of enzymes EGtf1 and EGtf2 were observed around pH 4.0 (Figure S1). The optimum temperature was examined by measuring the enzyme activities at 30–80 °C, and the maximum activities of enzymes EGtf1 and EGtf2 were observed at 50 and 60 °C, respectively (Fig. 2).

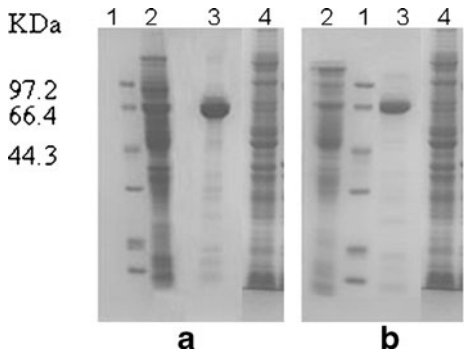

Fig. 1 SDS–PAGE analysis of the purified EGs. *Lane 1* protein markers; *Lane 2* crude extract; *Lane 3* purified enzyme; *Lane 4* uninduced cell lysate. Protein bands were visualized by Coomassie Brilliant Blue. **a** EGtf1; **b** EGtf2

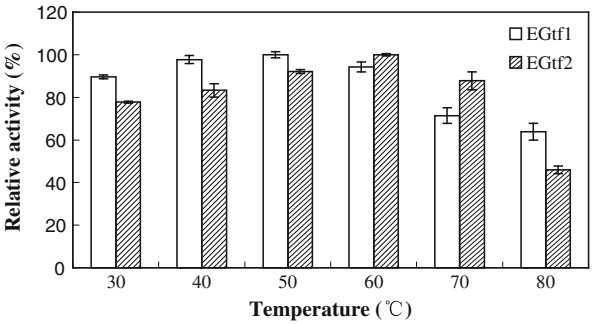

Fig. 2 Effect of temperature on EGs activity. The optimum temperature of EGs was estimated at various temperatures (30–80 °C) using the standard assay procedure. Relative activity was expressed as a percentage of maximum activity under the experimental conditions

A number of EGs share similar temperature optima, e.g., *YEG*1 and *YEG*2 from *Reticulitermes sperattuts* [7], NtEG from *Nasutitermes takasagoensis* [8], cellulases IIA, IIB, and IIC from *Trichoderma viride* [9, 10], and EGI from *T. viride* HK75 [11]. However, the enzymes EGtf1 and EGtf2 have lower pH optima than the general optimum of pH 5 or 6. Industrial processes often require acidophilic and thermostable enzymes to guarantee the degradation of fiber particles. Therefore, these enzymes were expected to be very useful and were therefore further studied.

Preliminary experiments on the thermostability (data not shown) indicated that the activities of EGtf1 and EGtf2 were very stable at high temperatures. Therefore, EGtf1 and EGtf2 were preincubated in citrate buffer (50 mM, pH 5.0) at temperatures of 50, 60 or 70 °C for varied periods of time and the residual activity was then assayed as described in Materials and Method (Fig. 3). As shown in Table 1, the half-lives of enzymes EGtf1 and EGtf2 at 50 °C are approximately 100-fold longer than the others.

Compared with the other EGs, the stabilities of EGtf1 and EGtf2 were quite outstanding. This performance is an important criterion for selecting enzymes of potential in industrial applications. Besides, the activities of EGtf1 and EGtf2 during the first 3 h of incubation at 50 and 60 °C were even increased. The reason might be that a higher temperature makes the recombinant enzymes refold into three-dimensional structures more similar to the native and active conformation [1].

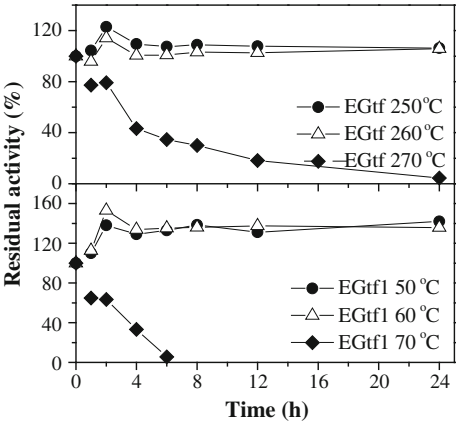

Fig. 3 Thermostability of the EGs. The residual activity of EGs was assayed after incubating the enzymes in the citrate buffer (50 mM, pH 5.0) at 50 °C (*black circle*), 60 °C (*white up-pointing triangle*), or 70 °C (*black diamond*) for 24 h

Table 1 The half-lives of the EGs

EG resources	$t_{1/2}$ (h)[a]			References
This work				
EGtf1	1386 (50 °C)	256 (60 °C)	1.7 (70 °C)	
EGtf2	1386 (50 °C)	198 (60 °C)	5.1 (70 °C)	
Other references				
Clostridium thermocellum	1 (86 °C)			[20]
Penicillium funiculosum ATCC 11797	23 (50 °C)	3 (60 °C)		[21]
Clostridium phytofermentans	0.25 (60 °C)			Liu et al. [2]
Salinivibrio sp. strain NTU-05	10 (50 °C)	6 (60 °C)	3 (70 °C)	[22]
Trichoderma reesei QM9414	1 (70 °C)			[23]
Chaetomium thermophile	1 (70 °C)[b]			[24]

[a] The half life period was represented by $t_{1/2}$

[b] 30% residual relative activity

Further Study on Enzymes EGtf1 and EGtf2

Influence of Metal Ions and Organic Solvents

By incubating the enzymes with different metal ions at a final concentration of 1 mM, it was found that metal ions had no significant effect on the two enzymes activity (Table S2). It is worth noting that both the enzymes displayed a slightly higher activity upon the addition of Co^{2+}. On the other hand, the presence of EDTA considerably diminished the activity. Inhibition of enzymes in presence of EDTA and the positive action of Co^{2+} suggest that the enzyme activities probably require divalent metal ions such as Co^{2+}, which can be chelated with EDTA [12]. The inhibition of enzymes by metal ions may also suggest the presence of at least one sulfhydryl group such as cysteine in the active site, whose oxidation by cations destabilizes the folded conformation of enzymes [13].

Residual activities of EGs were assayed after preincubation with different solvents (50%, *v*/*v*) for 20 min. In the presence of methanol, ethanol, isopropanol, acetone, DMSO, or propanol, the activities of EGs decrease slightly, while DMF and THF inhibit the EGs activity obviously. On the contrary, the presence of hydrophobic hexanol, octanol, and dodecanol enhanced the enzymes activity dramatically (Fig. 4). Since the solubility of neither CMC nor cellulobiose was significantly enhanced in hexanol, octanol, or dodecanol, the reason for this phenomenon is unclear at the present stage.

The kinetic parameters, K_m and V_{max}, of the EGs were determined from the data of EGs activities at various concentrations of CMC (Table 2). The K_m of a reported recombinant cellulase (Cel5D) towards CMC was 28.4 gL^{-1} [14], much higher than that of the enzymes in this work. Therefore, the two enzymes reported herein have higher affinity to the substrate CMC.

Substrate Specificity

The substrate specificity of EGtf1 and EGtf2 is partially summarized in Table 2. CMC is amorphous cellulose that has more exposed attacking sites to the EGs, while Avicel is crystalline cellulose which is hard to attack. Therefore, both the EGs showed the higher

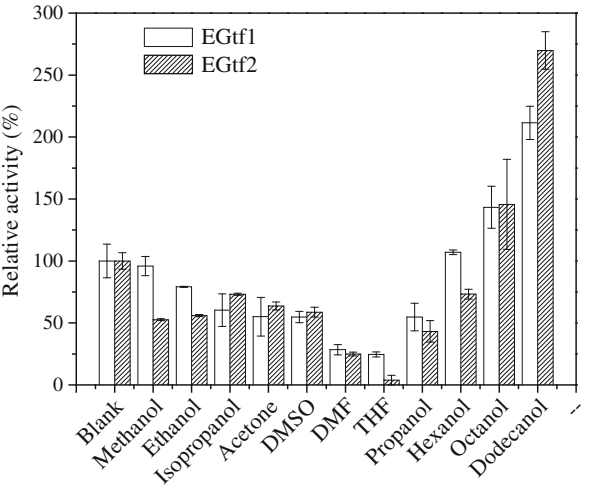

Fig. 4 Effect of organic solvents on EGs activity. Enzymes were preincubated with different solvents (50%, *v*/*v*) for 20 min at 30 °C and 900 rpm. Relative activity was expressed as a percentage of the activity in the absence of any test compound

activity toward CMC, while lower activities were observed on Avicel. In addition, these two enzymes displayed glycosidase activity to a certain degree, which was higher on pNP-galactopyranoside than those on pNP-glucopyranoside and pNP-xylopyranoside.

The hydrolytic activities of EGtf1 and EGtf2 towards alkyl glycosides were further examined, using methyl glycoside, ethyl glycoside, propyl glycoside, butyl glycoside, pentyl glycoside, hexyl glycoside, and heptyl glycoside as the substrates. They exhibited significant activities on all the substrates. Figure 5 indicates that although the conversions were very low, they were clearly improved with the growing length of the alkyl chain.

Adsorption Behavior of EGs on Avicel

The adsorption of enzymes EGtf1 and EGtf2 was examined at 4 °C to minimize substrate change due to enzymatic activity [15]. By incubation of varied amounts of the purified EGs with a fixed amount of cellulose Avicel, the change of free protein concentration [*P*] (milligram per milliliter) was determined, which was used to calculate the amount of protein adsorbed. Rearrange the Langmuir-type adsorption isotherm equation, we get

$$\frac{[P]}{[P_{\mathrm{ads}}]} = \frac{1}{K_{\mathrm{p}}[P_{\mathrm{ads,m}}]} + \frac{1}{[P_{\mathrm{ads,m}}]}[P]$$

Table 2 Activity and kinetics parameters of EGtf1 and EGtf2

Enzyme	Specific activity[a] (U/mg)		K_m[b] (mg·mL^{-1})	V_{max}[b] (mg·mL^{-1}·min^{-1})
	CMC	Avicel		
EGtf1	1.15	0.244	12.8	0.36
EGtf2	1.25	0.205	13.0	0.29

[a] One unit (U) of enzyme activity was defined as the amount of enzyme that librates 1 μmol glucose per hour under the assay conditions

[b] The kinetic parameters were calculated from the data of EGs activities at 50 °C and various concentrations of CMC

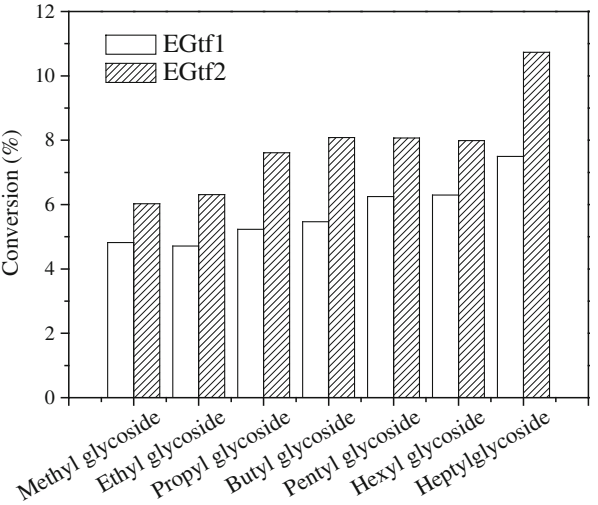

Fig. 5 The conversions of alkyl glycosides by the three recombinant EGs. An aliquot (1 mL) of the crude enzyme solutions was added to 1 mL of 20 mg/mL alkyl glycoside solution in 50 mM citrate buffer (pH 5.0). The reaction mixture was incubated at 50 °C for 1 h

$[P]$	the free protein concentration (mg/mL)
$[P_{ads}]$	the amount of adsorbed protein per gram of cellulose (mg protein/g cellulose)
K_p	the adsorption equilibrium constant (mL/mg)
$[P_{ads,\ m}]$	the maximum amount of adsorbed protein per gram of cellulose (mg protein/g cellulose).

Straight lines were obtained by using the experimental data and the above equation (Fig. 6). Adsorption parameters obtained from these lines are listed in Table 3. R was introduced to indicate the intercept of the straight line; a low R stands for a high maximal protein adsorption. One can see from the data that the degree of interaction between the EGs and Avicel has the dimension of mL/g cellulose [16].

Furthermore, the adsorption equilibrium constant K_p values (mL/mg) of EGs were similar to those reported for the CBD bearing CBHI and CBHII cellobiohydrolases of *T. reesei* (0.9 and 1.9 L/mol, respectively), where the adsorption of EGs on Avicel was also studied at 4 °C [17]. A higher value of the equilibrium constant indicated a higher binding affinity of the enzyme for the specific substrate [13].

The activity of an enzyme is different from the conversion rate of a substrate, since the latter is also dependent on the enzyme adsorption. On the other hand, desorption of the

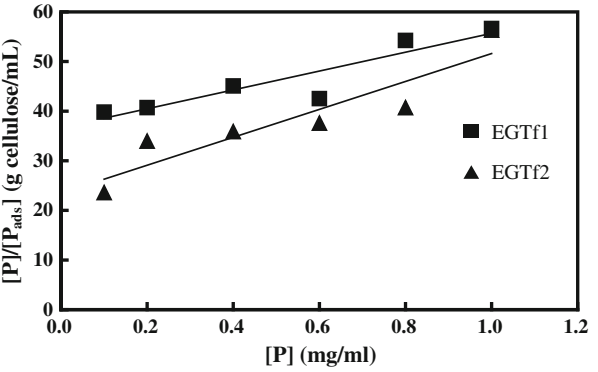

Fig. 6 Langmuir-type adsorption plots of EGs (EGtf1 *black square*; EGtf2 *black up-pointing triangle*)

Table 3 Adsorption parameters of the two recombinant EGs in comparison with other reported cellulases

Enzyme	K_p[a] (mL/mg)	$[P_{ads,\ m}]$[b] (mg protein/g cellulose)	R[c] (ml/g)	Desorption (%)	References
EGtf1	0.514	53	0.027	6.3	This work
EGtf2	1.183	36	0.042	4.4	
Thermobifida fusca E3 (50 °C)	n.a.	26	0.0031	n.a.	[25]
Thermobifida fusca E4 (50 °C)	n.a.	31	8.5E-4	n.a.	
Thermobifida fusca E5 (50 °C)	n.a.	31	0.0048	n.a.	
Trichoderma viride EndoI (30 °C)	n.a.	130	8.8E-4	n.a.	Beldman et al. [16]
Trichoderma viride EndoIII (30 °C)	n.a.	26	0.012	n.a.	
P. brasilianum EG Cel5C	4.0E-5	40	n.a.	n.a.	[26]

n.a. not available

[a] K_p (mL/mg) means the adsorption equilibrium constant

[b] $[P_{ads,\ m}]$ means the amount of adsorbed protein divided by the amount of cellulose (mg protein/g cellulose)

[c] R is the intercept of the straight line obtained using the equation in the section of "Adsorption behavior of EGs on Avicel"

enzyme might be beneficial to its further re-adsorption to the new available sites of substrate. Therefore, enzyme desorption was also investigated by separating the cellulose from the adsorbed enzyme via centrifugation and resuspending in a fresh buffer. The amount of released protein ($[\Delta P_{des}]$, mg protein/g cellulose) was measured and expressed as percentage of the protein amount that had been adsorbed (Table 3). These indicate that adsorption of these EGs on Avicel was almost irreversible. Formation of stable complexes with and irreversible binding of cellulolytic enzymes to cellulose have been reported [18]. Various studies on fungal cellulases have also shown that once adsorbed, the desorption of enzyme from the substrate is not easy and that the binding is, at least, partially irreversible [19].

Conclusions

This study demonstrated a successful example of screening a set of cellulolytic enzymes by combination of applied bioinformatics with gene cloning techniques. The process of identifying useful biocatalysts capable of hydrolyzing the target polysaccharide was very efficient and easy-to-handle. In this work, several bacterial EGs were overexpressed, purified, and characterized. Among them, enzymes EGtf1 and EGtf2 showed outstanding thermostability. They kept active after being incubated at 60 °C for about 200 h, and their half-life periods were even more than 1,000 h when incubated at 50 °C. Presence of dodecanol in the reaction mixture greatly enhanced the hydrolytic activity of these endoglucanases. All these features are attractive and promising for potential application like composing the 'cocktails' for enzymatic saccharization of liginocellulosics.

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