Biotechnology for Biofuels

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Predominance of *Trichoderma* and *Penicillium* in cellulolytic aerobic filamentous fungi from subtropical and tropical forests in China, and their use in finding highly efficient β-glucosidase

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Abstract

Background: Cellulose is the most abundant biomass on earth. The major players in cellulose degradation in nature are cellulases produced by microorganisms. Aerobic filamentous fungi are the main sources of commercial cellulase. *Trichoderma reesei* has been explored extensively for cellulase production; however, its major limitations are its low β-glucosidase activity and inefficiency in biomass degradation. The aim of this work was to isolate new fungal strains from subtropical and tropical forests in China, which produce high levels of cellulase in order to facilitate development of improved commercial cellulases.

Results: We isolated 305 fungal strains from 330 samples collected from subtropical and tropical virgin forests in China. Of these, 31 strains were found to have Avicelase activity of more than 0.2 U/ml in liquid batch cultivation. Molecular analyses of the 31 strains based on internal transcribed spacer sequences revealed that 18 were *Trichoderma* and 13 were *Penicillium* species. The best-performing isolate was *Trichoderma koningiopsis* FCD3-1, which had similar Avicelase activity to *T. reesei* Rut-C30. Most interestingly, strain FCD3-1 exhibited extracellular β-glucosidase activity of 1.18 U/ml, which was approximately 17 times higher than that of Rut-C30. One β-glucosidase secreted by FCD3-1 was purified, and its gene was cloned and identified. The β-glucosidase belonged to glycosyl hydrolase (GH) family 3, sharing the highest identity of 94% with a GH family 3 protein from *Trichoderma atroviride* IMI 206040, and was designated TkBgl3A. The optimal pH and temperature of TkBgl3A were 4.5 and 65°C, respectively. The enzyme retained over 90% activity for 360 hours at pH 4.0 and 30°C, which are the usual conditions used for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. The enzyme showed significantly higher specific activity toward natural substrate cellobiose (141.4 U/mg) than toward artificial substrate p-nitrophenyl-beta-D-glucopyranoside (108.0 U/mg).

Conclusions: Strains of *Trichoderma* and *Penicillium* were the predominant cellulolytic fungi in subtropical and tropical forests in China. *T. koningiopsis* FCD3-1 was the most efficient producer of cellulase, and also produced a high level of β-glucosidase. The high specific activity toward cellobiose and stability under SSF conditions of the purified β-glucosidase from FCD3-1 indicates its potential application in SSF of cellulose to bioethanol.

Keywords: *Trichoderma*, *Penicillium*, Cellulase, β-glucosidase

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Background

Cellulose, a type of homogeneous polysaccharide that exists as units of cellobiose connected by β-1,4-glycosidic bonds, is the most abundant renewable biomass in nature, with 7.2×10^{10} tons synthesized by photosynthesis every year [1]. Cellulose can be hydrolyzed by cellulase into D-glucose, which can be further transformed into renewable fuel and various valuable chemicals [2]. These characteristics have stimulated studies on the degradation and utilization of cellulose. However, natural cellulose is difficult to be degraded to glucose owing to its structure and the fact that it is often found in combination with lignin and hemicellulose [3]. Cellulose degradation and utilization are part of the global carbon cycle, with the major degradation power of cellulose in nature derived from cellulases produced by microorganisms [4-7].

Hydrolysis of cellulose to glucose requires three groups of enzymes: endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Endo-1,4-β-glucanase randomly cuts cellulose chains, yielding cellulose ends, while cellobiohydrolase attacks the cellulose chains from the reducing or non-reducing ends, yielding cellobiose, and β-glucosidase hydrolyzes cellobiose and cello-oligosaccharides to form glucose [8].

The mechanisms of microorganisms producing cellulases are very diverse. Among these microorganisms are the aerobic filamentous fungi, which produce an extracellular non-composite cellulase system [2,4]. They are the main sources of commercial cellulase, with *Trichoderma reesei* being the most widely used [9].

In the 1970s, Mandels and Sternberge assessed the cellulase production of over 14,000 collected strains, and found that *T. reesei* produced the highest cellulase activity. Various *T. reesei* strains with higher yields of cellulase were then developed by mutagenesis. Of these, *T. reesei* Rut-C30 was one of the most characteristic strains with effective cellulase yield, and has been selected as the reference strain for many studies [10].

However, the hyperproducing mutant strains of *T. reesei* produce cellulase with all essential components, but lack the optimum amount of β-glucosidase for efficient cellulose hydrolysis, and the β-glucosidase it produces is also glucose-sensitive [11]. As β-glucosidase catalyzes the final step of cellulose hydrolysis thus releasing glucose from the inhibitory cellobiose, it plays an important role in the biological conversion of cellulose to glucose [12].

Based on sequence similarities, β-glucosidases are mostly placed in either family 1 or family 3 of GH [13]. Product inhibition and thermal inactivation of β-glucosidases constitute two major barriers to commercial development of enzymatic hydrolysis of cellulose [14]. Therefore, the production costs of cellulosic ethanol would be significantly reduced by improving the glucose tolerance and thermostablity of β-glucosidases [15]. In addition, β-glucosidase activities are generally measured using artificial substrates such as *p*-nitrophenyl-β-D-glycopyranoside (pNPG) or 4-methylumbelliferyl-β-D-glucuronide. When using these artificial substrates, most enzymes show higher catalytic activity toward artificial substrates than toward cellobiose [16-20]. However, UeBgl3A from *Ustilago esculenta* and a recombinant β-glucosidase from *Caldicellulosiruptor saccharolyticus* are exceptions, with higher catalytic activity toward cellobiose than toward artificial substrate [21,22].

Guangxi and Yunnan Provinces are in southwest China, with the Tropic of Cancer passing through them. There is abundant sunlight, bountiful rainfall, and large areas of subtropical and tropical forests. These forests are well preserved because of low levels of human activity, and soils are mainly weakly acidic. These conditions provide abundant biomass and microbial resources. Different climatic types and natural vegetation characteristics have led to the evolution of diverse cellulolytic organisms capable of degrading cellulose [23].

In this work, novel fungal strains producing high cellulase activity were isolated, screened, and identified from soil samples systematically collected from virgin forests in subtropical and tropical climates in Guangxi and Yunnan Provinces in China, with a predominance of *Trichoderma* and *Penicillium*. *Trichoderma koningiopsis* FCD3-1 was the most efficient producer of cellulases, and also had extracellular β-glucosidase activity at levels approximately 17 times higher than that of *T. reesei* Rut-C30. One β-glucosidase purified from FCD3-1 was stable under the usual conditions of simultaneous saccharification and fermentation (SSF), and showed high activity toward the natural substrate cellobiose, indicating its potential application for saccharification of cellulosic biomass for bioethanol production.

Results

Isolation, screening, and identification of fungal strains with higher cellulase activity from forests in subtropical and tropical regions of China

In total, 330 soil and rotten wood samples were collected from sampling sites in virgin forests in 8 natural reserves in Guangxi and Yunnan Provinces, China (Table 1). From these 330 samples, 305 fungal strains were isolated, using agar medium plates containing Whatman No. 1 filter paper as the sole carbon source. Of the 305 fungal strains, 231 could be cultured by shake-flask cultivation, using liquid medium containing microcrystalline cellulose (Avicel PH101) as the sole carbon source. Of these 231 fungal strains (Table 2), 51 produced Avicelase activity of greater than 0.05 U/ml 3 days after cultivation under these conditions (Table 2).

The optimum pH and temperature (Table 3) of the cellulases produced by the 51 fungal strains were measured against Avicel. Following this, the Avicelase activity of

Table 1 Samples collected from forests of national natural reserves in subtropical and tropical regions of China

Sample location	Date of sampling	Latitude and longitude of one sampling point	Altitude, m	Temperature, °C	Samples, n
Shankou Mangrove National Natural Reserve	Sep 10, 2007	N21°34′31.1″ E109°41′06.1″	0	30 to 34	31
Xishuangbanna National Natural Reserve	Aug 23, 2008	N21°36′06.3″ E101°35′09.1″	300 to 500	28 to 34	56
Golden Camellia National Natural Reserve	Oct 15, 2007	N21°44′52.1″ E108°06′50.7″	50 to 200	24 to 28	62
Shiwandashan National Natural Reserve	July 20, 2007	N21°51′56.6″ E107°50′37.2″	300 to 600	26 to 30	26
Longgang National Natural Reserve	July 18, 2007	N22°28′18.1″ E106°57′26.4″	200 to 300	28 to 32	19
Dawangling National Natural Reserve	July 13, 2007	N23°49′23.4″ E106°28′01.5″	200 to 400	26 to 30	37
Huaping National Natural Reserve	Oct 30, 2008	N25°35′59.9″ E109°54′23.7″	800 to 1000	24 to 28	37
Baimaxueshan National Natural Reserve	Aug 27, 2008	N28°14′23.7″ E99°18′02.6″	3600 to 3900	20 to 26	62

cellulases produced by the 51 strains at peak enzyme production was measured under conditions of the respective optimum pH and temperature of the crude enzyme. Of the 51 strains, 31 produced Avicelase activity of greater than 0.2 U/ml (Tables 2 and 3), compared with 0.39 U/ml for *T. reesei* Rut-C30 under the same cultivation conditions. Two fungal strains, FCD3-1 and BM48-3, had Avicelase activity of 0.37 and 0.36 U/ml, respectively, which did not differ statistically from that of Rut-C30. Strains HP35-3 and HP29-3 also produced relatively high Avicelase activity at 0.34 and 0.33 U/ml, respectively.

The 31 strains were further identified by sequence analysis of internal transcribed spacer (ITS) and observation of morphological characteristics. Thus, 18 strains were identified as belonging to genus *Trichoderma* and 13 to *Penicillium* (Table 3).

Notably, 9 of the 31 strains were isolated from Huaping National Nature Reserve in Guangxi, which was the sample location that gave the highest number of strains with Avicelase activity of greater than 0.2 U/ml, and 5 of these 9 strains were identified as *Trichoderma harzianum* (Table 3). Of the 31 strains, 8 were isolated from Xishuangbanna National Nature Reserve in Yunnan, which was the sample location with the second highest number of strains producing Avicelase activity greater than 0.2 U/ml, and 3 of these 8 strains were identified as *T. harzianum* (Table 3).

Enzymatic characterization of cellulases from fungal strains producing higher Avicelase activity

The cellulase produced by the four highest-producing strains, FCD3-1, BM48-3, HP35-3, and HP29-3, were further measured against substrates of filter paper, carboxylmethylcellulose sodium salt (CMC-Na), and pNPG, in addition to Avicel. Of the four strains tested, *T. koningiopsis* strain FCD3-1 had the most efficient enzyme activity toward Avicel, filter paper, and CMC-Na (Figure 1). Interestingly, strain FCD3-1 produced a high level of β-glucosidase activity at 1.18 U/ml, which was approximately 17 times higher than that of Rut-C30 (Figure 1).

The ITS of strain FCD3-1 was amplified by PCR and sequenced (GenBank accession number KJ619600). Using

Table 2 Isolation and screening of fungal strains producing Avicelase activity from forests of national natural reserves in subtropical and tropical regions of China

Sample location	Samples, n	Strains isolated, n			
		Culture method		Avicelase activity	
		Filter paper	Avicel	> 0.05 U/ml[a]	> 0.2 U/ml[b]
Shankou Mangrove National Natural Reserve	31	20	5	2	2
Xishuangbanna National Natural Reserve	56	63	63	15	8
Golden Camellia National Natural Reserve	62	64	51	8	3
Shiwandashan National Natural Reserve	26	25	15	3	3
Longgang National Natural Reserve	19	14	14	1	1
Dawangling Natural Reserve	37	20	10	1	0
Huaping National Natural Reserve	37	53	53	16	9
Baimaxueshan National Natural Reserve	62	46	20	5	5
Total	330	305	231	51	31

[a]Indicates that Avicelase activity was measured under conditions of pH 5.0 and 50°C 5 days after cultivation of fungal strains in liquid culture.
[b]Indicates that Avicelase activity was measured under optimum pH and temperature of the crude enzyme produced by each fungal strain in liquid culture at peak enzyme production.

Table 3 The hydrolytic performance of the culture supernatants from the 31 fungal strains against Avicel

Strains	Source/reference	Closest match of ITS in GenBank		Species identification	ITS GenBank accession number	Optimal pH of Avicelase	Optimal temperature of Avicelase,°C	Avicelase activity, U/ml[a]
		Closest strain (accession number)	Maximum identity					
Rut-C30	[24]	*Trichoderma reesei* strain ATCC 56765 (Rut-C30) (X93938.1)	100%	*T. reesei*	–	4.0 to 5.0	55	0.39 ± 0.004
FCD 3-1	Golden Camellia NNR	*Trichoderma koningiopsis* strain T-1 (KC884758.1)	100%	*T. koningiopsis*	KJ619600	4.0 to 5.0	55	0.37 ± 0.007
BM 48-3	Baimaxueshan NNR	*Hypocrea cremea* strain GJS 91-125 (AY737760.1)	99.2%	*H. cremea*	KJ619591	4.0 to 4.5	50	0.36 ± 0.006
HP 35-3	Huaping NNR	*Trichoderma atroviride* voucher TriAtv_JSB791 (KC569351.1)	100%	*T. atroviride*	KJ619607	4.0 to 5.0	55	0.34 ± 0.006
HP 29-3	Huaping NNR	*Trichoderma harzianum* RP1-4 (JX493008.1)	100%	*T. harzianum*	KJ619604	5.0	55	0.33 ± 0.012
HP37-2	Huaping NNR	*Trichoderma harzianum* voucher TriH_JSB301 (KC569359.1)	100%	*T. harzianum*	KJ619608	–	–	0.33 ± 0.030
BM16-3	Baimaxueshan NNR	*Penicillium oxalicum* strain a1s2_d38 (KC344971.1)	100%	*P. oxalicum*	KJ619590	4.5 to 5.0	50	0.32 ± 0.014
ML5-2a	Xishuangbanna NNR	*Trichoderma* sp. FKI-6626 (AB733349.1)	97%	*Trichoderma* sp.	KJ619623	4.5	55	0.32 ± 0.003
ML29-3	Xishuangbanna NNR	*Trichoderma* sp. SQR037 (GQ497168.1)	100%	*Trichoderma* sp.	KJ619624	5.0	55	0.31 ± 0.009
HP33-1	Huaping NNR	*Trichoderma tawa* strain LIPIMC0569 (KC847183.1)	100%	*T. tawa*	KJ619605	–	–	0.30 ± 0.011
SK9-1	Shankou mangrove NNR	*Penicillium* sp. 3 BRO-2013 (KF367495.1)	100%	*Penicillium* sp.	KJ619620	4.5	45	0.30 ± 0.004
HP21-3	Huaping NNR	*Trichoderma harzianum* voucher TriH_JSB301 (KC569359.1)	100%	*T. harzianum*	KJ619603	–	–	0.30 ± 0.004
HP37-4	Huaping NNR	*Trichoderma piluliferum* isolate wxm149 (HM061325.1)	100%	*T. piluliferum*	KJ619609	4.5	55	0.29 ± 0.011
HP7-2	Huaping NNR	*Trichoderma harzianum* Ir.112C (AY154949.1)	100%	*T. harzianum*	KJ619602	–	–	0.29 ± 0.007
ML14-1	Xishuangbanna NNR	*Trichoderma harzianum* strain LIPIMC0572 (KC847182.1)	100%	*T. harzianum*	KJ619614	4.0	55	0.29 ± 0.005
ML12-1	Xishuangbanna NNR	*Trichoderma harzianum* voucher TriH JSB301 (KC569359.1)	99.7%	*T.harzianum*	KJ619613	4.0 to 5.0	55	0.29 ± 0.003
BM12-1	Baimaxueshan NNR	*Penicillium* sp. 0312 F1 (EU926977.1)	100%	*Penicillium* sp.	KJ619589	5.0	50	0.28 ± 0.014
HP35-1	Huaping NNR	*Trichoderma harzianum* strain IPBCC07_547 (KC8471910.1)	100%	*T. harzianum*	KJ619606	4.0 to 5.0	55	0.27 ± 0.012
ML16-1	Xishuangbanna NNR	*Trichoderma harzianum* voucher TriH_JSB301 (KC569359.1)	100%	*T. harzianum*	KJ619615	5.0	55	0.26 ± 0.009
ML4-2	Xishuangbanna NNR	*Trichoderma hamatum* strain T-17 (FR87274.1)	100%	*T. hamatum*	KJ619610	4.5	55	0.26 ± 0.006
S17-1	Shiwandashan NNR	*Penicillium aculeatum* isolate A11 (EU781668.1)	100%	*P. aculeatum*	KJ619618	5.0	60	0.25 ± 0.003
SK22-2	Shankou mangrove NNR	*Penicillium* sp. 3 BRO-2013 (KF367495.1)	100%	*Penicillium* sp.	KJ619621	3.5 to 5.0	40 to 45	0.24 ± 0.021
ML5-2	Xishuangbanna NNR	*Penicillium* sp. 3 BRO-2013 (KF367495.1)	100%	*Penicillium* sp.	KJ619611	4.5 to 5.0	55 to 60	0.24 ± 0.016
S17-2	Shiwandashan NNR	*Penicillium aculeatum* strain LP67 (HQ392497.1)	100%	*P. aculeatum*	KJ619619	4.0 to 5.0	55 to 60	0.24 ± 0.008
SY20-5	Golden Camellia NNR	*Penicillium oxalicum* isolate C1-5 (KF986426.1)	100%	*P. oxalicum*	KJ619622	4.5 to 5.0	50 to 55	0.24 ± 0.004
NG9	Longgang NNR	*Penicillium* sp. 3 BRO-2013 (KF367495.1)	100%	*Penicillium* sp.	KJ619616	4.0 to 4.5	50	0.23 ± 0.009
FC3-3	Golden Camellia NNR	*Penicillium* sp. ZH7-E1 (FJ037747.1)	100%	*Penicillium* sp.	KJ619599	4.0 to 5.0	55	0.22 ± 0.016
S15-3	Shiwandashan NNR	*Penicillium* sp. 3 BRO-2013 (KF367495.1)	100%	*Penicillium* sp.	KJ619617	5.0	55	0.21 ± 0.022
HP7-1	Huaping NNR	*Penicillium* sp. 3 BRO-2013 (KF367495.1)	100%	*Penicillium* sp.	KJ619601	5.0	55	0.21 ± 0.015

Table 3 The hydrolytic performance of the culture supernatants from the 31 fungal strains against Avicel *(Continued)*

BM58-1	Baimaxueshan NNR	*Trichoderma viride* strain NEFU26 (KF944470.1)	100%	*T. viride*	KJ619592	–	–	0.21 ± 0.008
ML11-1	Xishuangbanna NNR	*Penicillium oxalicum* strain HL6 (KF746061.1)	100%	*P. oxalicum*	KJ619612	5.0	55	0.21 ± 0.004
BM58-4	Baimaxueshan NNR	*Trichoderma viride* isolate OTU220 (GU934567.1)	100%	*T. viride*	KJ619593	–	–	0.20 ± 0.003

ITS, internal transcribed spacer; NNR, National Natural Reserve.
[a]Indicates that Avicelase activity was measured under optimum pH and temperature of the crude enzyme produced by the strain in liquid culture at peak enzyme production. Avicelase activity produced by strains whose optimum pH and temperature for the crude enzyme were not tested was measured under conditions of pH 4.5 and 55°C 5 days after cultivation of fungal strains in liquid culture. Data are means ± standard deviation from three replicates. The experiments were repeated three times and similar results were obtained.

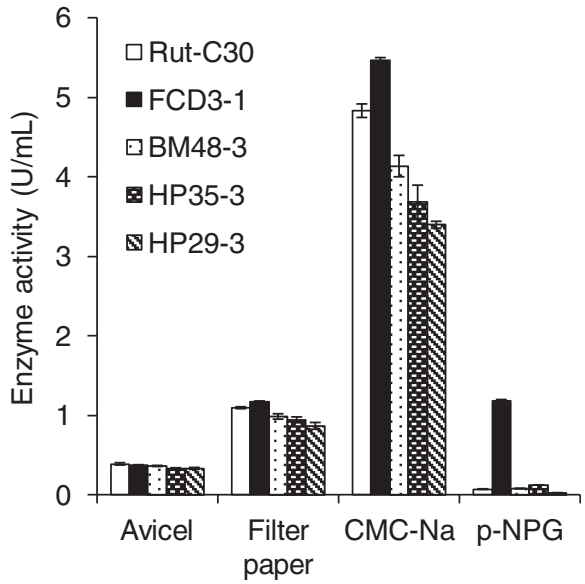

Figure 1 Hydrolytic performance of secretomes of the four fungal strains with highest cellulase activity. The four fungal strains were cultivated in liquid medium in flasks at 30°C and 180 rpm for 4 to 7 days to reach their peak of enzyme production. pNPG was used to evaluate β-glucosidase activity. The reactions were performed at the optimum pH and temperature of the culture supernatant against Avicel. Data are means ± standard deviation from three replicates. The results presented are from a representative experiment; similar results were obtained in the other two independent experiments.

the BLAST algorithm, the ITS sequence of FCD3-1 was compared with sequences in the GenBank database, and the best BLAST match was found to be the ITS of *T. koningiopsis* strain T-1 (GenBank accession number KC884758) at 100% identity. The morphological identification of FCD3-1 (see Additional file 1) and the molecular identification of the strain were consistent. Therefore, fungal strain FCD3-1 was identified as *T. koningiopsis.*

Glucose was the sole hydrolytic product of Avicel hydrolyzed by the crude cellulase produced by strain FCD3-1 (Figure 2). In comparison, glucose and cellobiose were the major hydrolytic products of Avicel hydrolyzed by the crude cellulases produced by the fungal strains BM48-3, HP35-3, and HP29-3, and the reference strain Rut-C30 (Figure 2; see Additional file 2).

Purification and characterization of one β-glucosidase secreted by *T. koningiopsis* FCD3-1

To determine the β-glucosidase responsible for the high level of β-glucosidase activity by *T. koningiopsis* FCD3-1, the extracellular proteins secreted by this strain were subjected to protein purification. One β-glucosidase secreted by FCD3-1 was purified by using ammonium sulfate precipitation, followed by hydrophobic interaction chromatography and ion exchange chromatography. The homogeneity of the purified enzyme was confirmed by non-denaturing polyacrylamide gel electrophoresis (PAGE) analysis, which showed a single protein band after staining with Coomassie blue (Figure 3A), coincident with a β-glucosidase activity band detected using esculin as the substrate (Figure 3A). A single protein band was also detected after electrophoresis under denaturing conditions (SDS-PAGE), with molecular mass of about 100 kDa (Figure 3A).

The 100-kDa band on SDS-PAGE was excised, subjected to trypsin digestion, and analyzed by liquid chromatography–tandem mass spectrometry (LC/MS/MS). Mascot (A software from Matrix Science, London, UK) search showed that eight sequences in a GH family 3 protein (GenBank accession number EHK21862) from *Trichoderma virens* Gv29-8 matched (Table 4). The gene encoding the β-glucosidase was amplified from the genomic DNA of strain FCD3-1 and sequenced. This gene, with four introns, encoded a polypeptide with 866 amino acids, which contained the eight matched peptide sequences in the GH family 3 protein from *T. virens* Gv29-8, confirming that the cloned gene encoded the purified β-glucosidase.

The encoded polypeptide by the cloned gene shared its highest identity (94%) with a GH3 protein (GenBank accession number EHK46786) from *Trichoderma atroviride* IMI 206040. Simple Modular Architecture Research Tool (SMART) analysis showed that the polypeptide composed of 4 domains (aa residues of 1 to 21 belonged to the signal peptide, aa 88 to 313 belonged to the GH3 N-terminal domain, aa 413 to 652 belonged to the GH3 C-terminal domain, and aa 788 to 856 was described as a fibronectin type III-like domain), indicating that the polypeptide was a GH3 β-glucosidase. Thus, the enzyme was named TkBgl3A.

Characteristics of β-glucosidase TkBgl3A from *T. koningiopsis* FCD3-1

The purified β-glucosidase TkBgl3A was most active toward pNPG at pH 4.5 (Figure 3B), and this enzyme was stable in the pH range of 3.0 to 9.5 (Figure 3B). The β-glucosidase activity was optimal at 65°C (Figure 3C); and after 1 hour of incubation, TkBgl3A retained more than 50% activity at 60°C (Figure 3C). The enzyme retained more than 90% activity for 360 hours at pH 4.0 and 30°C, which are the usual conditions for SSF of cellulose to produce ethanol (Figure 3D). The Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) for hydrolysis of pNPG were 1.21 mM and 314 μmol/min/mg, respectively. The catalytic constant (K_{cat}) was 523/s. The enzyme was competitively inhibited by glucose, with an inhibition constant (Ki) value of 3.29 mM.

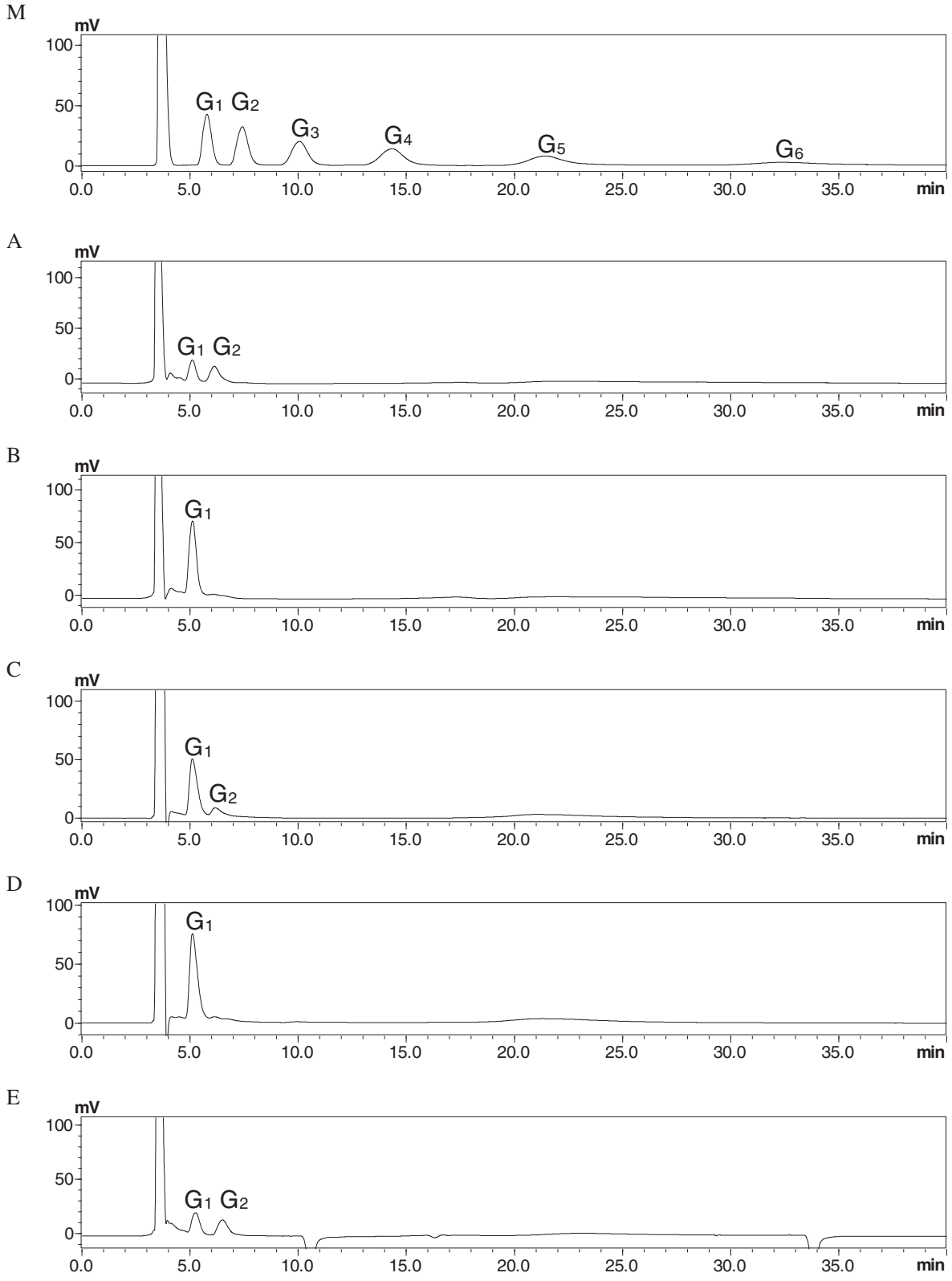

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 Analysis of hydrolytic products of Avicel by secretomes of four fungal strains by high-performance liquid chromatograpy. (A) *Trichoderma reesei* Rut-C30, (B) *Trichoderma koningiopsis* FCD3-1, (C) *Hypocrea cremea* BM48-3, (D) *Trichoderma atroviride* HP35-3, and (E) *Trichoderma harzianum* HP29-3. M: sugar standards including glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentose (G5), and cellohexose (G6). Enzyme load: 10 FPU/g Avicel; substrate concentration: 2% Avicel. Hydrolysis was performed at pH 5.0 and 45°C for 32 hours. The results from samples incubated for 0, 2, 4, 8, 16, and 32 hours, respectively, are provided in Additional file 2. FPU, filter paper units.

Specific activity of TkBgl3A toward cellobiose was 141.4 U/mg, which was significantly higher than that toward artificial substrate pNPG (108 U/mg) (Table 5). It is noteworthy that high-performance liquid chromatography (HPLC) analysis showed that the purified enzyme TkBgl3A could also hydrolyze cello-oligosaccharides (cellotriose, cellotetraose, cellopentaose, cellohexaose) and mainly produced glucose with high efficiency (see Additional file 3).

Metal ions had no significant effect on TkBgl3A when using pNPG as substrate, whereas the surfactant SDS significantly inhibited the activity of TkBgl3A. The reaction mix containing 0.25% SDS retained 13.5% of enzyme activity relative to the untreated sample (see Additional file 4).

Discussion

We carried out large-scale isolation and screening of secretomes with Avicelase activity from fungal strains isolated from tropical and subtropical forests in China. Kaisa *et al.* isolated approximately 600 mesophilic lignocellulolytic fungi from soil samples and decaying plant materials, and found that the majority of these strains were *Trichoderma* spp., with two *Penicillium* strains also found to be

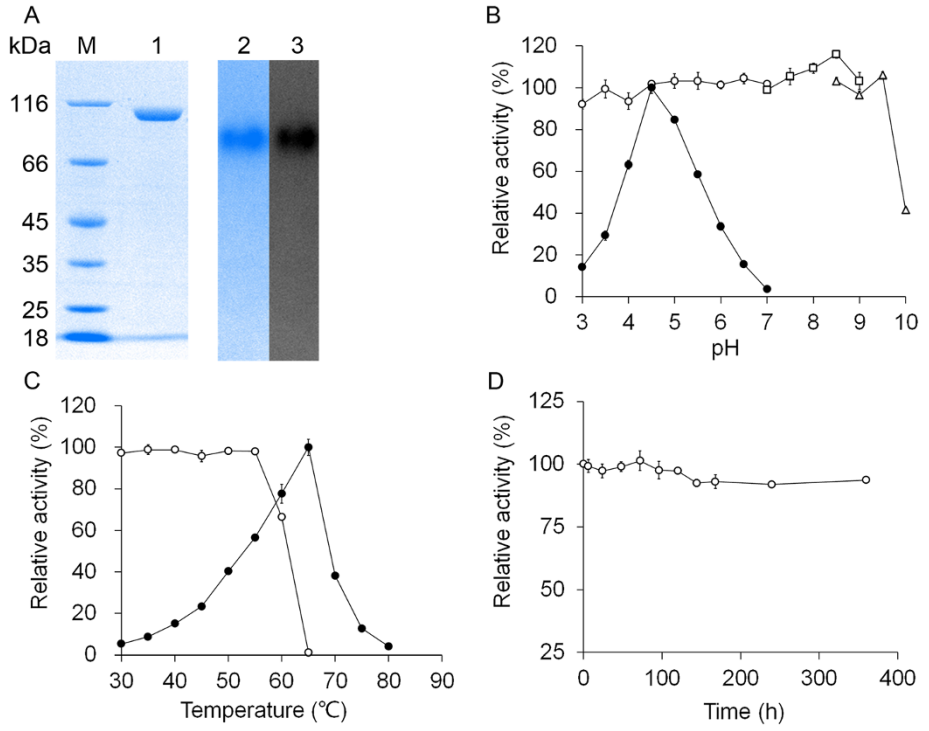

Figure 3 Identification of purified β-glucosidase from *Trichoderma koningiopsis* FCD3-1. (A) (Left) SDS-PAGE analyses of the β-glucosidase. Lane M, protein molecular marker; lane 1, the purified β-glucosidase. (Right) Native PAGE analyses of the β-glucosidase. Lane 2, the gel stained with Coomassie blue; lane 3, the gel soaked in a 50°C solution containing 0.1% esculin and 0.25% ammonium iron (III) citrate (pH 4.5) for 30 minutes. **(B)** Effect of pH on TkBgl3A activity and stability toward substrate pNPG. For effect of pH on TkBgl3A activity (filled circles), enzyme activity was measured in 0.1 M citrate–phosphate buffer (CPB) pH 3.0 to 7.0 for 10 minutes at 50°C. For effect of pH on TkBgl3A stability, the enzyme was incubated in CPB, pH 3.0 to 7.0 (open circles), or 0.1 M Tris–HCl buffer, pH 7.0 to 9.0 (open squares), or 0.1 M glycine–NaOH buffer, pH 8.5 to 10.0 (open triangles) at 4°C for 24 hours. The residual activity was measured under optimal conditions. All values are expressed as percentages of the activity of untreated sample. **(C)** Effect of temperature on TkBgl3A activity and stability toward substrate pNPG. To assess the effect of temperature on TkBgl3A activity (filled circles), enzyme activity was measured at pH 4.5 in 0.1 M CPB at the indicated temperature for 10 minutes. To assess the effect of temperature on TkBgl3A stability (open circles), the enzyme was incubated in 0.1 M CPB (pH 4.5) at various temperatures for 1 hour; the residual enzyme activity was measured under optimal conditions. All values are expressed as percentages of the activity of untreated sample. **(D)** Stability of TkBgl3A under SSF conditions (30°C and pH 4.0); 100 μl of purified enzyme was added to 4.9 ml of 0.1 M CPB (pH 4.0) , followed by incubating the enzyme mixture at 30°C for varying times before checking the remaining enzyme activity.

Table 4 The matched peptide sequences to the mass spectra of the peptides released from TkBgl3A

Peptide sequence	Species	Accession no.	Annotation
LGFPGLCNQDSPLGVR	*Trichoderma virens* Gv29-8	EHK21862	GH family 3 protein
GKGVDTQLGPVAGPIGR	*T. virens* Gv29-8	EHK21862	GH family 3 protein
HFIGNEQEHYR	*T. virens* Gv29-8	EHK21862	GH family 3 protein
LDDMVVR	*T. virens* Gv29-8	EHK21862	GH family 3 protein
SIAVIGNDAHDNPAGPNACSDR	*T. virens* Gv29-8	EHK21862	GH family 3 protein
VAPAGGAPGGNPGLYDVLYTVTAQIENTGK	*T. virens* Gv29-8	EHK21862	GH family 3 protein
GFDDIEILPGR	*T. virens* Gv29-8	EHK21862	GH family 3 protein
TVYVGSSSR	*T. virens* Gv29-8	EHK21862	GH family 3 protein

good cellulase producers [25]. The predominance of *Trichoderma* and *Penicillium* strains in hydrolyzing cellulose in Guangxi and Yunnan Provinces indicated that fungal strains from these two genera may play important roles in cellulose degradation and therefore in carbon recycling in tropical and subtropical forest soils in China.

Several experimental studies have concluded that effect of the physical properties of cellulose on cellulase binding and substrate accessibility by the enzyme is responsible for the rate of hydrolysis of lignocellulose by cellulase. Physical properties of cellulose include crystallinity, degree of polymerization, and accessible surface area [26-28]. Crystalline cellulose is the main form of cellulose in most plant cell walls, and crystallinity is a key factor affecting hydrolysis of cellulose because the glycosidic bonds in crystalline regions are difficult to be hydrolyzed compared with those in amorphous regions [27,28]. Avicel is one form of crystalline cellulose with a high degree of crystallinity, and it is very difficult to be hydrolyzed [29].

Desrchers *et al*. reported a fungal strain that can produce an enzyme complex with high carboxymethycellulase and cellobiase activity but low Avicelase activity (0.02 U/ml) [30]. Whereas the Avicelase activity produced by an actinomycete strain isolated by Li *et al*. was only 0.008 U/ml [31], Douglas *et al*. isolated a fungal strain with higher Avicelase activity (0.12 to 0.17 U/ml) [32]. In the present study, 17% of the strains isolated using Whatman No. 1 filter paper as the sole carbon source had significant Avicelase activity (greater than 0.05 U/ml), and 10% of the strains had Avicelase activity greater than 0.2 U/ml, which is a high proportion of strains producing high levels of Avicelase activity.

Table 5 Substrate specificity of the purified β-glucosidase produced by *T. koningiopsis* FCD3-1

Substrate (concentration 1 mM)	Specific activity, U/mg[a]	Relative activity, %[a]
pNPG	108.0 ± 1.3	100 ± 1.2
Cellobiose	141.4 ± 3.9	124.22 ± 2.76
Cellotriose	99.9 ± 0.7	85.70 ± 0.7
Cellotetraose	91.9 ± 4.6	78.86 ± 5.0
Cellopentaose	84.4 ± 0.8	72.41 ± 0.9
Cellohexaose	88.6 ± 1.5	76.02 ± 1.7

[a]Data are means ± standard deviation from three replicates. The experiments were repeated three times, and similar results were obtained.

T. koningiopsis FCD3-1 was the best-performing isolate, producing a similar level of Avicelase activity to *T. reesei* Rut-C30. Interestingly, strain FCD3-1 produced about 17 times as much β-glucosidase as *T. reesei* Rut-C30. Although *T. reesei* has been extensively studied and reported to produce a highly active extracellular cellulase system, most of its strains produce β-glucosidase at low levels compared with other cellulolytic fungi such as *Aspergillus* species, because most of its β-glucosidase is bound to its cell wall. Exogenous supplementation of β-glucosidase in *T. reesei* cellulase preparations has been used as an alternative strategy to increase the rate and extent of cellulose hydrolysis [33]. Nevertheless, wild-type *Trichoderma* strains producing significant quantities of β-glucosidase have been reported. Kovács *et al*. screened more than 150 wild-type *Trichoderma* strains isolated from 30 countries based on the overall cellulase production (filter paper activity). *Trichoderma atroviride* TUB F-1505 was the best wild-type extracellular cellulase producer, producing significant quantities of β-glucosidase, but it had 10 to 25% less extracellular cellulase activity than *T. reesei* Rut-C30. TUB F-1505 was isolated from a tropical rain forest soil sample collected near Manaus, close to the Rio Negro, Brazil [34]. Cellulase production by six species of *Trichoderma* cultivated on medicinal plant processings was comparatively evaluated, and *Trichoderma citrinoviride* MTCC No. 2418 was found to be the most efficient producer of cellulases and also produced a high level of β-glucosidase. *T. citrinoviride* produced five times more β-glucosidase than produced by *T. reesei* MTCC No. 164 [35]. However, purification of β-glucosidase and cloning of the gene encoding the enzyme from *T. atroviride* and *T. citrinoviride* were not reported.

In the present study, the purified β-glucosidase TkBgl3A was most active at pH 4.5. Most fungal β-glucosidases have an optimal pH range of 3.5 to 6.5 [36]. The optimal

pH for the β-glucosidase was similar to the commercial β-glucosidase Novozyme S188 [37]. TkBgl3A was stable at pH 3.0 to 9.5, and its activity did not vary substantially within this range; its pH stability was notably better than that of previously reported β-glucosidases [21]. TkBgl3A had an optimum temperature of 65°C; in general, the optimum temperatures for fungal β-glucosidases are in the range of 50 to 70°C [21]. In addition, TkBgl3A displayed good thermostability, similar to Bgl3A from *P. funiculosum* NCL1 [38].

Most microbial β-glucosidases have glucose Ki ranging from as low as 0.5 mM to 100 mM or more [14]. Although glucose acted as a competitive inhibitor of pNPG hydrolysis by TkBgl3A, with a Ki of 3.29 mM, the glucose inhibition of β-glucosidase could be overcome by an SSF process in which glucose is rapidly converted to ethanol by yeast [39]. The purified TkBgl3A was stable under SSF conditions, which facilitates the use of the enzyme in SSF.

TkBgl3A exhibited a similar affinity toward the synthetic substrate pNPG (1.21 mM) as that of the commercially available β-glucosidase Novozyme S188 (1.03 mM). However, the specific activity of TkBgl3A for cellobiose was approximately four times higher than that of Novozyme S188 [16]. The higher activity of TkBgl3A toward cellobiose and cello-oligosaccharides again supports the potential application of the enzyme in the final step of cellulose saccharification [40,41].

Conclusions

Large-scale isolation and screening of secretomes with Avicelase activity for fungal strains from tropical and subtropical forests in China led to the isolation and identification of 31 fungal strains producing Avicelase activity of greater than 0.2 U/ml in liquid cultivation. All 31 fungal strains were identified as *Trichoderma* or *Penicillium* species, indicating the predominance of these genera in cellulose degradation in subtropical and tropical forests in China. *T. koningiopsis* FCD3-1 was the most efficient producer of cellulases and also had a high level of extracellular β-glucosidase. The purified β-glucosidase TkBgl3A from FCD3-1 showed high specific activity toward natural substrate cellobiose and good stability under SSF conditions, indicating its potential application in SSF of cellulose to produce bioethanol.

Methods

Collection of soil and rotten wood samples

Soil and rotten wood in virgin forests were collected in 2007 and 2008 (July to October) from eight natural reserves in both tropical and subtropical areas in different biotopes in China. The sampling route was set at intervals of 100 meters, with one sample collected per interval. Surface soil at sampling sites was removed, and the soil at a depth of 50 to 200 mm was collected, with about 50 g per sample.

Cultivation and isolation of fungal strains

The suspensions of soil samples were serially diluted. Three dilutions of each sample were chosen, and 0.1 ml of each sample was spread onto the surface of Medium P agar plates containing Whatman No. 1 filter paper as the sole carbon source. The composition of Medium P was as follows: 2 g/l $NaNO_3$, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l NaCl, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 1.0 g/l KH_2PO_4, 0.4 g/l yeast, extract, 10 g/l Whatman No. 1 filter paper, and 15.0 g/l agar, at pH 5.0. After incubation at 28°C for 3 to 5 days, the single colonies of fungi were picked using an inoculating hook and inoculated onto a new Medium P agar plate.

Shake-flask cultivation of fungal strains

The medium used for the shake-flask cultivation was named Medium A, and contained microcrystalline cellulose powder (Avicel PH101, Sigma, St. Louis, MO, USA) as the sole carbon source. The composition of Medium A was as follows: 10 g/l Avicel (Sigma, USA), 2 g/l KH_2PO_4, 1.4 g/l $(NH_4)_2SO_4$, 0.3 g/l $MgSO_4 \cdot 7H_2O$, 0.3 g/l $CaCl_2$, 1 g/l $CaCO_3$, 1 g/l peptone (Difco, USA), 1 g/l Tween-80, 0.3 g/l urea, 10 mg/l $FeSO_4 \cdot 7H_2O$, 3.2 mg/l $MnSO_4$, 6.9 mg/l $ZnSO_4 \cdot 7H_2O$, and 0.4 mg/l $CoCl_2 \cdot 6H_2O$. All materials were supplied by Sinopharm Chemical Reagent Co., Ltd., Shanghai, China unless otherwise stated. The pH was adjusted to 5.0 before sterilization.

Shake-flask cultivation was carried out in 500 ml cotton-plugged Erlenmeyer flasks containing 100 ml of Medium A. After autoclaving at 121°C for 20 minutes, the flasks were inoculated by removing the mycelia from a single colony of fungi on a Medium P agar plate. Flask cultivation was performed at 28°C on a rotary shaker at 200 rpm. After 3 days of cultivation, samples were removed and centrifuged at 4°C and 9,000 × g (Centrifuge Model:himac CF 16RX, Rotor type: T15A29, Hitachi Koki Co., Ltd., Tokyo, Japan) for 10 minutes, and the clear supernatants were removed and used for enzyme assays.

Enzyme assay

Avicelase activity produced by isolated fungal strains was determined using the 3,5-dinitrosalicylic acid (DNS) method [42]. The reaction system was prepared by mixing 50 μl of crude enzyme (appropriately diluted) with 450 μl of 1.1% (w/v) Avicel. The buffer used for resuspending the substrate was 0.1 M sodium citrate buffer at appropriate pH. The mixtures were incubated at 50°C for 1 hour, and then the reactions were stopped by adding 1 ml of DNS reagent. All mixtures were heated in boiling water for 5 minutes for color development. Subsequently, 200 μl of each sample was transferred to a 96-well microplate, and

absorbance was measured at 540 nm. 1 U of Avicelase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars equivalent to glucose per minute during the reaction.

The enzyme activities toward filter paper and CMC-Na were determined as described by Ghose [43]. β-glucosidase activity was measured using Berghem's method [44].

To determine the optimal pH of the crude enzyme produced by isolated fungal strains, 50 μl of supernatant from flask cultivation were incubated with 450 μl of Avicel (1.1% w/v) at 50°C with a range of different pH levels (3.0 to 6.0 at intervals of 0.5). To determine the optimal temperature of crude enzyme produced by isolated fungal strains, 50 μl of supernatant from flask cultivation were incubated with 450 μl of Avicel (1.1%, w/v) at the optimal pH and a temperature range of 30 to 65°C with an interval of 5°C. The maximum Avicelase activity obtained at different pH or temperature levels was considered to be 100%.

Hydrolysis products of Avicel by the crude enzymes produced by fungal strains FCD3-1, BM48-3, HP35-3, HP29-3, and Rut-C30 were analyzed by HPLC. After 5 days of shaking cultivation at 28°C in 500 ml cotton-plugged Erlenmeyer flasks containing 100 ml of Medium A, the cultures of fungal strains were centrifuged at 3,000 × g (Centrifuge Model:himac CF 16RX, Rotor type: T5SS31, Hitachi Koki Co., Ltd., Tokyo, Japan) for 10 minutes, and the filter paper activity of each collected supernatant was measured. Each enzyme solution was respectively added to 100 mg of Avicel in a test tube at a dose of 10 U/g Avicel and the total volume of the mixture was adjusted to 5 ml with 0.1 M citrate–phosphate buffer (CPB) at pH 5.0. The hydrolysis was performed in a water bath shaker with shaking at 200 rpm at 45°C. Hydrolysis was stopped after reactions were conducted for 0, 2, 4, 8, 16 and 32 hours by boiling for 10 minutes. After the cooled sample was filtered through a 0.22 μm filter (Nylon 66 Syringe Filter, Jinteng, China), the collected filtrate was analyzed with HPLC (LC-10A VP Plus, Shimadzu Kyoto, Japan) equipped with a refractive index detector (Shimadzu). Sugars were separated using a Hypersil NH_2 column (Dalian Elite Analytical Instruments Co., Ltd., China) at 30°C, with 75% acetonitrile as eluent, at a flow rate of 1.0 ml/min.

Identification of isolated fungal strains

Cell mycelia of fungal isolates were obtained from pure culture in Yeast Mold (YM) broth (10 g/l glucose, 3 g/l yeast extract, 3 g/l malt extract, and 5 g/l peptone). Genomic DNA of the isolates was extracted and used as PCR template. PCR was performed to amplify the ITS (approximately 600 bp, including ITS1, the 5.8 S rRNA gene, and ITS2 region) of fungal ribosomal DNA with primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TC CTCCGCTTATTGATATGC-3′. The 25 μl mixtures were composed of 1 μl of template DNA, 12.5 μl of 2 × *Taq* PCR Master Mix (purchased from Tiangen Biotech, Beijing, China; containing 0.5 U *Taq* DNA polymerase, 500 μM of each dNTP, 20 mM Tris–HCl pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, and bromophenol blue), 1 μl of each primer (10 μM) and 9.5 μl of double-distilled H_2O. The PCR procedure was as follows: primary denaturation for 5 minutes at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, then an additional reaction for 10 minutes at 72°C. The PCR products were separated in 0.8% agarose gels to confirm purity, quantity, and size, and sent to Sangon Biotech (Shanghai) Co., Ltd. (China) for sequencing. The ITS sequences were compared with other ITS sequences in GenBank using the BlastN program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Media and culture conditions for cultivation of *T. koningiopsis* FCD3-1

The media used for cultivation of *T. koningiopsis* FCD3-1 were potato dextrose agar (PDA), Avicel agar medium, or fungal cultivation medium. The Avicel agar medium contained: 3 g Avicel PH-101 (Sigma), 2.0 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.3 g $CaCl_2$, 5 mg $FeSO_4 \cdot 7H_2O$, 1.6 mg $MnSO_4$, 1.7 mg $ZnCl_2$, 2 mg $CoCl_2$, and 20 g agar in 1 litre ultra-pure water. The fungal cultivation medium contained: 4.0 g KH_2PO_4, 2.8 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4 \cdot 7H_2O$, 0.6 g $CaCl_2$, 0.6 g urea, 10 mg $FeSO_4 \cdot 7H_2O$, 3.2 mg $MnSO_4$, 2.8 mg $ZnSO_4$, 2.4 mg $CoCl_2$, 20 g wheat bran, and 30 g Avicel PH-101 in 1 litre of ultra-pure water. Each medium was adjusted to pH 5.0.

To cultivate the *T. koningiopsis* FCD3-1 for high-level production of β-glucosidase, FCD3-1 was firstly inoculated onto PDA plates at 28°C for 72 hours. The hyphae were then incubated in Avicel agar medium at 28°C for 96 hours, and incubated in fungal cultivation medium at 28°C on a rotary shaker at 200 rpm for 1 week to assess extracellular β-glucosidase production.

Purification and characterization of one β-glucosidase secreted by *T. koningiopsis* FCD3-1

The culture of FCD3-1 was centrifuged for 20 minutes at 9,000 × g (Centrifuge Model:himac CF 16RX, Rotor type: T15A29, Hitachi Koki Co., Ltd., Tokyo, Japan) and 4°C after cultivation. The supernatant was carefully transferred to a sanitized container. Purification of β-glucosidase was mainly based on the methods of Parry *et al.* [45]. The supernatant (crude enzyme) was subjected to 40% $(NH_4)_2SO_4$ precipitation. After centrifugation, the supernatant was collected and then subjected to 60% $(NH_4)_2SO_4$ precipitation overnight. The precipitated protein was dissolved in 1.5 M $(NH_4)_2SO_4$ solution and loaded onto a HiPrep 16/10 Phenyl FF (high sub) column (GE Healthcare, Amersham Place, Buckinghamshire, UK). The eluted

β-glucosidase was then loaded on an anion column (5 ml HiTrap™ Q FF, GE Healthcare, UK).

The purified protein sample was separated by SDS-PAGE and the protein band was excised. The band pieces were washed with 100 μl dH_2O for 15 minutes and were further successively washed for 10 minutes with 100 μl CH_3CN and 200 μl 50 mM NH_4HCO_3/CH_3CN (50:50 v/v). After the band pieces were treated with 100 μl of CH_3CN to dehydrate the proteins for 10 minutes, the band pieces were treated with 20 μl of 10 ng/μl modified trypsin in 20 mM NH_4HCO_3 for 30 minutes, then more 20 mM NH_4HCO_3 was added to cover the band pieces. After overnight incubation at 37°C, an equal volume of CH_3CN was added. After incubation at room temperature for 30 minutes and centrifugation, the supernatant was transferred to a new clean Eppendorf tube, and then lyophilized by vacuum centrifugation and stored at −80°C.

Liquid chromatography was performed on a nano Acquity UPLC system (Waters Corporation, Milford, USA) connected to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source (Michrom Bioresources, Auburn, USA). Peptides were resuspended with 25 μl of solvent A (5% acetonitrile and 0.1% formic acid in water), then 20 μl of peptide solution, was loaded onto a Captrap Peptide column (0.5 mm internal diameter × 2.0 mm; Michrom Bioresources) using a 20 μl/min flow rate of solvent A for 5 minutes, and then separated on a Magic C18AQ reverse phase column (100 μm internal diameter × 15 cm; Michrom Bioresources) with a linear gradient, starting from 5% solvent B (90% acetonitrile and 0.1% formic acid in water) to 45% B (that is, from 95% A to 55% A) in 70 minutes. The column flow rate was maintained at 500 nl/min and column temperature at 35°C. An electrospray voltage of 1.4 kV versus the inlet of the mass spectrometer was used.

A LTQ Orbitrap XL mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra with one microscan (350 to 1600 m/z) was acquired in the Obitrap with a mass resolution of 100,000 at 400 m/z, followed by MS/MS of the eight most intense peptide ions in the LTQ analyzer. The automatic gain control (AGC) was set to 1,000,000 ions, with maximum accumulation times of 500 ms. For MS/MS, we used an isolation window of 2 m/z, and the AGC of LTQ was set to 20 000 ions, with maximum accumulation time of 120 ms. Single charge state was rejected, and dynamic exclusion was used with two microscans using exclusion durations of 10 and 90 seconds. For MS/MS, precursor ions were activated using 35% normalized collision energy at the default activation q of 0.25 and an activation time of 30 ms. The spectra were recorded with Xcalibur (v2.0.7) software.

The mass spectra were searched using the Mascot Daemon software (v2.3.0; Matrix Science, London, UK) based on the Mascot algorithm. We searched the National Center for Biotechnology Information database (downloaded on 27 March 2013, taxonomy is *Trichoderma* with 39.001 entries). To reduce false-positive identification results, a decoy database containing the reverse sequences was appended to the database. The searching parameters were set up as follows: full trypsin cleavage with two missed cleavages was considered; oxidation on methionine and aceytlation of the protein N-terminus were set as variable modifications.; the peptide mass tolerance was 10 ppm; and the fragment ion tolerance was 1.0 Da. Peptides for which Mascot scores exceeded the 99.9% confidence level score (ions score ≥ 40) were accepted as correct matches.

Protein and enzyme activity assay of β-glucosidase secreted by *T. koningiopsis* FCD3-1

Determination of the total protein was performed according to the method of Branford [46]. Standard curves were prepared with bovine serum albumin. For enzyme assay, each reaction was performed at 50°C and pH 5.0 (in triplicate if not otherwise mentioned). The reaction mixture was 116 μl of 0.1 M CPB (pH 5.0), 14 μl of substrate solution (25 mM pNPG) and 10 μl of enzyme solution. After addition of enzyme, the mixture was incubated for 10 minutes at 50°C and stopped with 70 μl of 0.4 M Na_2CO_3. The developed color was read at 410 nm on a microplate reader (BioTek, USA). One unit of β-glucosidase activity was expressed as the amount of enzyme required to release 1 μmol of p-nitrophenol (pNP) per minute.

The optimal pH of β-glucosidase activity was determined by incubating the purified enzyme at 50°C for 10 minutes in CPB with pH values in the range of 3.0 to 7.0 at intervals of 0.5. To determine the optimal temperature, the enzyme was incubated in CPB (0.1 M and pH 4.5) for 10 minutes at different temperatures in the range of 30 to 80°C at intervals of 5°C [47].

The pH stability of purified enzyme was assessed by incubating 500 μl of purified enzyme at 4°C for 24 hours in various buffers adjusted to different pH values, followed by checking the residual activity [17]. To determine the thermostability of β-glucosidase activity, the purified enzyme was incubated at different temperatures (30, 35, 40, 45, 50, 55, 60, and 65°C) in the absence of substrate. After incubation for 1 hour, the residual β-glucosidase activity was determined as described above. Stability of the purified enzyme under SSF conditions (30°C and pH 4.0) was assessed by mixing 100 μl of purified enzyme with CPB pH 4.0 to a volume of 5 ml, followed by incubating the enzyme mixture at 30°C for varying times before checking the remaining activity.

The influence of metal ions on enzyme activity was determined by mixing the enzyme with CPB (pH 4.5) that contained various metal ions and reagents.

The kinetic parameters (V_{max} and K_m) in hydrolysis of pNPG by the purified β-glucosidase were determined under optimal conditions, and the values for K_m and V_{max} were estimated by applying a nonlinear curve fit using GraphPad Prism4 from GraphPad Software (San Diego, CA, USA). Catalytic constants (K_{cat}) and catalytic efficiency ratios (K_{cat}/K_m) were determined from the obtained kinetic parameter values [17].

The activity of the purified β-glucosidase against pNPG and cello-oligosaccharides (degree of polymerization from 2 to 6) was measured in 0.1 M CPB (pH 4.5) at 65°C for 10 minutes. For the hydrolysis of the pNPG, 250 μl reaction mixtures were set up by adding 0.1 μg purified enzyme and pNPG stock solution to final concentration of 1 mM. For hydrolysis of cello-oligosaccharides, 250 μl reaction mixtures were set up by adding 0.28 μg purified enzyme and cello-oligosaccharide to final concentration of 1 mM. The amount of released glucose was determined under the same conditions as described above using a glucose oxidase kit (Maker Co., Ltd., China). One unit of β-glucosidase activity was expressed as the amount of enzyme required to release 1 μmol of pNP or glucose per minute [16]. In addition, the hydrolysates of cello-oligosaccharides produced by the purified β-glucosidase TkBgl3A were analyzed by HPLC using a refractive index detector (RI detector k-2301; Knauer, Germany). Sugars were separated using a Hypersil NH_2 column (Dalian Elite Analytical Instrument Co., Ltd., China) at 25°C, with 70% acetonitrile as eluent, at a flow rate of 1.0 mL/min.

Cloning of *Tkbgl3A* gene

For isolation of genomic DNA from *T. konigiopsis* strain FCD3-1, the fungal strain was cultivated on PDA plates for 3 days. Genomic DNA was isolated from the lyophilized mycelia using the hexadecyl trimethyl ammonium bromide (CTAB) extraction method [48]. To amplify the *TkBgl3A* gene, the forward primer 5′-ATGTTGTCAA ATTCAATCATCGCCGTTG-3′ and reverse primer 5′-CTAAGGAAGAACCTGGCTCAAGGGTAAA-3′ were used, which were designed based on the coding sequence (GenBank accession number ABDF02000059) of GH family 3 protein from *T. virens* GV29-8 using vector NTI 10 software. The extracted genomic DNA from *T. konigiopsis* FCD3-1 was used as template. The PCR amplification was carried out using the following condition: one cycle at 95°C for 4 minutes, followed by 30 cycles at 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1.5 minutes, and then one cycle at 72°C for 10 minutes. The PCR product was cloned into the T-vector, and then transformed into *Escherichia coli* DH5α. Three of the transformants were sequenced by BGI TechSolutions Co., Ltd. (Shenzhen, China). The sequence analyses were carried out using the Lasergene DNASTAR software package, Vector NTI 10 software, NCBI-BLAST, and SMART (http://smart.embl-heidelberg.de).

Nucleotide sequence accession numbers

All the ITS sequences of the 31 fungal strains reported in this paper were deposited in the GenBank database under accession numbers KJ619589 to KJ619593 and KJ619599 to KJ619642. The β-glucosidase gene *TkBgl3A* is under GenBank accession number KJ623247.

Additional files

Additional file 1: Morphological characteristics of *T. koningiopsis* FCD3-1.

Additional file 2: Analysis of hydrolytic products of Avicel by secretomes of four fungal strains by HPLC.

Additional file 3: The hydrolysates of cello-oligosaccharides by the purified β-glucosidase TkBgl3A at different time intervals.

Additional file 4: Effects of metal ions and reagents on enzyme activity of the purified β-glucosidase produced by *T. koningiopsis* FCD3-1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZZ collected soil samples, isolated the fungal strains, assayed the fungal enzyme production, identified the fungal isolates, and was involved in drafting the manuscript. JLL and JYL purified and characterized the β-glucosidase TkBgl3A, cloned the encoding gene, and participated in drafting the manuscript. CJD participated in data analysis. QSM participated in conceiving and designing the study. JXF conceived, designed the study, and critically revised and finally approved the manuscript. All authors read and approved the final manuscript.

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