Production of a high-efficiency cellulase complex via β-glucosidase engineering in *Penicillium oxalicum*

Guangshan Yao¹†, Ruimei Wu¹†, Qinbiao Kan¹†, Liwei Gao¹, Meng Liu¹, Piao Yang¹, Jian Du¹, Zhonghai Li³* and Yinbo Qu¹,²*†

**Abstract**

**Background:** *Trichoderma reesei* is a widely used model cellulolytic fungus, supplying a highly effective cellulase production system. Recently, the biofuel industry discovered filamentous fungi from the *Penicillium* genus as a promising alternative to *T. reesei*.

**Results:** In our study, we present a systematic over-expression analysis of nine β-glucosidase encoding genes in the wild-type strain 114-2 of *Penicillium oxalicum*. We found that the over-expression of BGL1, BGL4, or BGL5 significantly enhanced both β-glucosidase activity and hydrolysis efficiency of the enzyme system on filter paper. We utilised two strategies to over-express β-glucosidase in the strain RE-10 that—although over-producing cellulase, does so at the cost of the cellulase mixture deficiency. The constitutive promoter of gene pde_02864 encoding 40S ribosomal protein S8 was used to over-express three β-glucosidases: BGL1, BGL4, and BGL5. We found that all mutants show significantly enhanced levels of β-glucosidase at transcriptional, protein, and activity levels. Furthermore, the inducible promoter from bgl2 was used to conditionally over-express the β-glucosidases BGL1 and BGL4. Surprisingly, this induced expression strategy enables significantly improved expression efficiency. The BGL1 over-expressing mutant I1-13 particularly improved the β-glucosidase activity at a factor of 65-folds, resulting in levels of up to 150 U/ml. All our BGL over-expression mutants displayed significant enhancement of cellulolytic ability on both microcrystalline cellulose and filter paper. In addition, they substantially reduced the enzyme loads in the saccharification of a natural lignocellulose material delignified corn cob residue (DCCR). The mutant I4-32 with over-expression of BGL4 achieved the highest glucose yield in the saccharification of DCCR at only 25 % enzyme load compared to the parental strain RE-10.

**Conclusions:** In summary, genetically engineering *P. oxalicum* to significantly improve β-glucosidase activity is a potent strategy to substantially boost the hydrolytic efficiency of the cellulase cocktail, which will ultimately lead to a considerable reduction of cost for biomass-based biofuel.

**Keywords:** *Penicillium oxalicum*, Biofuel, β-glucosidase, Genetic engineering
Background

The conversion of plant-based biomass into biofuel and chemicals is a promising course to absorb the upcoming shortage of fossil fuels without any serious environmental problems. Cellulose is the major building block of plant biomass [1]. Fungal cellulas transform plant polysaccharides into fermentable sugars, which in turn determine the cost of plant-based biofuel. Throughout the enzyme industry, the champion for the production of cellulase cocktail is Trichoderma reesei [2–4]. However, an increasing number of publications demonstrate better hydrolysing lignocellulose performance of the cellulase enzyme mixture obtained from Penicillium strains. Thus, Penicillium strains are considered a potent alternative to the T. reesei cellulase cocktail [5].

Three Penicillium oxalicum strains in particular, secrete a more balanced lignocellulose-degrading enzyme system and display higher β-glucosidase activity compared to T. reesei. These are: P. oxalicum 114-2 [6], P. oxalicum GZ-2 [7], and P. oxalicum 16 [8]. P. oxalicum 114-2 has been studied in our laboratory for more than 30 years. Multiple cycles of mutagenesis and screening led to the carbon catabolite repression-resistant mutant JU-A10-T with a volume productivity of 160 IU/Lh. For the last 20 years, this mutant produces cellulase enzyme preparations at industrial scale in China [9]. Recently, its genome has been sequenced, providing valuable information to mine novel components that play key roles in plant cell wall deconstruction [6]. Furthermore, the signal transduction, inducer transportation, and transcription regulation mechanism of the cellulase gene expression of P. oxalicum has been partially uncovered. The sequenced genome, high-efficient gene knock-out techniques, and system biology (RNA-seq and proteomics) are the methods that have led to this advance. Gene disruption analysis allowed the identification of the three major cellodextrin transporters CdtC, CdtD, and CdtG that play crucial roles in cellulase induction [10]. Hu et al. [11] revealed that the G protein-cAMP signaling pathway downregulates the expression of cellulosytic genes. Furthermore, a single-gene deletion library has been established for 470 transcription factors and 15 novel and 4 major transcriptional regulators. Their roles in the cellulase expression regulatory network have been identified and characterized [12]. The Reconstruction of Expression Regulatory Network (REXRN) technology has been developed as a new strategy to engineer fungi that enhance cellulase and protein production. One mutant in particular (RE-10 from REXRN) displayed a drastic increase in cellulase and hemicellulase production, and produced even higher values compared to the industrial strain JU-A10-T [13]. However, the β-glucosidase of RE-10 has not yet been improved to the same level as other inducible cellulas. This makes further improvement of its β-glucosidase activity necessary.

Beta-glucosidase is the rate-limiting enzyme because it is responsible for the final step of lignocellulose hydrolysis and converts cellobiose as well as short cellodextrins into glucose [14]. Fungal β-glucosidases are classified into glycosyl hydrolases families 3 and 1 (GH3 and GH1) [15, 16]. Different proteins exhibit various characteristics in specific activity, substrate specificity, stability and others in the same family of β-glucosidase [17]. The genome of P. oxalicum includes 11 β-glucosidase encoding genes, of which only β-glucosidase 1 (BGL1) and β-glucosidase 2 (BGL2) have been identified [18, 19].

To solve the shortage of β-glucosidase in T. reesei enzyme cocktails, a time-consuming and laborious process is used to supplement heterologous β-glucosidases from Aspergillus into commercial cellulase preparations [14]. We systemically screened the β-glucosidases via over-expression analysis and found three β-glucosidases from P. oxalicum to have a promising enzymatic performance. The three BGLs were over-expressed either constitutively, or inductively by means of two different types of promoters. We obtained a number of high-yield β-glucosidase producers and their β-glucosidase yields were elevated from twofold to 65-fold. Microcrystalline cellulose, filter paper and corn cob residue were used as substrates to assess hydrolysis efficiency of the enzyme complex.

Results and discussion

Systematic over-expression analysis of nine β-glucosidases in P. oxalicum 114-2

The β-glucosidases of P. oxalicum are encoded in a total of 11 genes. Among these, four β-glucosidases belong to the glucoside hydrolase family 1 (GH1) with the other seven BGLs belonging to GH3. Using multiple algorithms, we found the signal peptides for five β-glucosidases (Table 1), implying a high likelihood for these enzymes to secret extracellularly. However, of these, only BGL1 had been detected in a previous secretome analysis [6]. In order to systematically examine all β-glucosidases in P. oxalicum, we performed a systematic over-expression analysis, using a constitutive promoter from gene pde_02864 to drive BGL expression. We obtained 9 out of 11 BGLs over-expression transformants, while over-expression mutants for the remaining two genes were not available after several rounds of genetic transformation. The promoter of gene pde_02864 that decodes the 40S ribosomal protein S8, has previously been identified as a strong and constitutively promoter [12]. We then evaluated the pNPase activities and filter paper hydrolysis rate of all the obtained β-glucosidase over-expression mutants (obtaining at least two transformants for each gene). Both the BGL(X)
Table 1 Systematic screening the major β-glucosidases

| BG(X) | Gene ID       | Signal peptide | GH family | Fold change (transcript level) | Fold change (pNPGase activity) | Fold change (filter paper hydrolysis rate) |
|-------|---------------|----------------|-----------|-------------------------------|-------------------------------|-------------------------------------------|
| BGL1  | PDE_02736     | Yes            | GH3       | 3.93                          | 2.023                         | 1.37                                      |
| BGL2  | PDE_00579     | No             | GH1       | 4.48                          | 1.537                         | 0.68                                      |
| BGL3  | PDE_01277     | Yes            | GH1       | 40.4                          | 0.650                         | 0.92                                      |
| BGL4  | PDE_01565     | Yes            | GH3       | 492.1                         | 9.335                         | 1.20                                      |
| BGL5  | PDE_02905     | Yes            | GH3       | 132.0                         | 6.439                         | 1.10                                      |
| BGL6  | PDE_09019     | Yes            | GH3       | 17.8                          | 1.797                         | 0.89                                      |
| BGL7  | PDE_02108     | No             | GH3       | 79.6                          | 2.427                         | 0.73                                      |
| BGL8  | PDE_04859     | No             | GH1       | 68.0                          | 0.868                         | 0.89                                      |
| BGL9  | PDE_03485     | No             | GH3       | 13.2                          | 0.868                         | 0.90                                      |

Yes with signal peptides, No without signal peptides

over-expression mutants and RE-10 were cultured in cellulose medium for enzyme production. Our results (Table 1) show that the over-expression of seven BGLs significantly increased pNPG activity. Among these, over-expression of BGL1, BGL4, BGL5, and BGL7 resulted in more than twice β-glucosidase activities of that of WT. In parallel, the crude enzyme from BGL1, BGL4, and BGL5 over-expression mutants significantly improved the hydrolysis rate of filter paper (see Table 1). However, over-expression of both bgl3 and bgl8 slightly reduced the extracellular β-glucosidase (less than twofold) and FPase activities. BGL8 is an intracellular β-glucosidase and thus hydrolyze intracellular cellobiose into glucose, which enhancing the repression and alleviating the induction of cellulosytic system. BGL3 had been expressed and purified in Pichia pastoris. However, the BGL3 did not showed activities against both pNPG and salicin in vitro (data not shown), and their roles (BGL3 and BGL8) in regulation of the expression of cellulase and β-glucosidase required further investigation. Therefore, BGL1, BGL4, and BGL5 are considered as the most efficient β-glucosidases and hence, we targeted them in the following strain engineering for a cellulase high-producer.

Similar to previous cellodextrin transporter studies [10], gene knockout analysis is unhelpful for identification of the essential genes that are responsible for β-glucosidase activity, due to high functional redundancy [20]. We propose that the single-gene over-expression analysis we establish in this study is effective to discriminate the important β-glucosidases, and other high-redundant genes (or gene families) in filamentous fungi.

Sequence and phylogenetic analysis of three major β-glucosidases from P. oxalicum and other cellulytic fungi

BGL1 was the most conserved extracellular β-glucosidase, and its homolog could be found in many cellulytic fungi, such as P. brasilianum, T. reesei, Neurospora crassa, Aspergillus nidulans, A. fumigatus, A. niger and Talaromyces cellulolyticus, and plant pathogens with a capacity of degrading plant cell wall, including Botrytis cinerea, Magnaporthe oryzae (Fig. 1), implying its essential role in lignocellulose deconstruction in the natural system. Orthologs of BGL5 can be found in most of the above cellulytic fungi, with the exception of T. reesei and N. crassa (Fig. 1). Interestingly, BGL4 could only be identified in P. oxalicum and the Fusarium genus and forms a clade separate from BGL1 and BGL5 (Fig. 1). This suggests an evolutionary development from gene expansion. However, most of the β-glucosidases from the Fusarium genus remain unidentified.

All three efficient β-glucosidases are classified as members of the GH3 family (Table 1), and have three functional domains: the N-terminal domain, the C-terminal domain, and a fibronectin-like domain with unknown function (see Additional file 1: Figure S1). According to a previous study, the former two domains are likely forming a catalytic pocket [21].

Over-expression of β-glucosidases in RE-10 using a constitutive promoter

The cellulase over-producer RE-10 has previously been obtained by redesigning the regulatory pathway of cellulase gene expression in P. oxalicum. As a result, most enzyme proteins and their corresponding activities, were significantly enhanced, with the exception of BGL1 and pNPGase activity remaining unaffected [13]. Therefore, improvement of the β-glucosidase activity in RE-10 was the main goal of our study. Via systematic screening, we identified BGL1, BGL4, and BGL5 as the most promising β-glucosidases for industrial scale Penicillium cellulase production.

The three β-glucosidases encoding genes pde_02736, pde_01565 and pde_02905, were driven by the promoter...
of \textit{pde}_02864 and expressed in the background of RE-10. Following single spore isolation, three BGL1, two BGL4, and two BGL5 over-expression mutants were selected for further analysis. All the putative over-expression mutants showed increased pNPGase activity. As shown in Fig. 2, all the BGL over-expression mutants also had a significant increase ($p \leq 0.01$) in β-glucosidase activity. Three BGL1 over-expression mutants increased pNPGase activity over 10, 12, fivefold, respectively, compared to the parental strain RE-10. Remarkably, the pNPGase of one of them (C1-57) reached up to 30 IU/ml, which is almost the highest value reported throughout all \textit{Penicillium} strains with the ability for lignocellulose degradation. This possibly correlates with the fact that BGL1 has a lower Km than the BGLs from other fungi [18]. In addition, both BGL4 and BGL5 over-expression mutants displayed twofold higher β-glucosidase activity over RE-10 at 120 and 144 h. Cellobiose is proposed as the natural substrate of β-glucosidase and is the product of CBH and EG during cellulose hydrolysis. To further illuminate their enzymatic performance of the BGL(X) over-expression mutants, we examined their cellobiose hydrolysis activity, and found that all the BGL(X) over-expression mutants improved glucose release from cellobiose by more than tenfold, which is consistent with the above pNPGase activity analysis (see Additional file 2: Figure S2).

**Inducible over-expression β-glucosidases in RE-10**

Intracellular β-glucosidase BGL2 in \textit{P. oxalicum} has previously been identified as a negative regulator for cellulase gene expression [19]. Interestingly, its expression pattern is similar to other inducible cellulase genes, such as \textit{cel7A-2}, \textit{cel5B} and \textit{cel7B}. As a result, the \textit{bgl2} promoter was designed to over-express secretory β-glucosidases. Multiple rounds of transformation and screening led to identification of three mutants for BGL1 over-expression (I1-2, I1-3, and I1-4), and two mutants for BGL4...
over-expression (I4-32 and I4-33). Despite considerable effort, no sur-resistant transformants for BGL5 could be obtained. Notably, all inducible mutants (I1-12, I1-13, I1-14, I4-32, and I4-33) displayed drastic increases in β-glucosidase activity towards both pNPG and cellobiose, compared to their respective constitutive over-expression mutants. One of them in particular (I1-13) has a pNPGase activity of 150 U/ml (Fig. 2) and a cellobiose activity of 36 U/ml (see Additional file 2: Figure S2). These activities are more than 65- and 93-fold higher than those of RE-10. In T. reesei, the strong cbh1 promoter was previously used to drive bggl expression, but the recombinant strain produced a pNPGase activity of only about 30 U/ml [22], which is significantly lower compared to that found in our study.

Genetic engineering of the regulatory machinery of cellulolytic genes is a promising strategy to create protein over-producers. Cellobiohydrolase, endo-glucanase, and other proteins that play significant roles in cellulose degradation, were all synchronously improved in our previous work, except for β-glucosidase [13]. Another pathway-specific regulatory mechanism for the expression of β-glucosidase is present in cellulolytic fungi and the β-glucosidase specific regulator BglR, which was found in T. reesei further strengthened this proposal [23]. As a consequence, additional improvement of β-glucosidase levels in the engineered cellulase over-producers is crucial for producing efficient as well as more balanced enzyme mixtures.

A recent study reports a strong cellulose induction of the gene bgl2 and corresponding repression by glucose-mediated carbon catabolite repression (CCR) [12]. The expression pattern of this gene is highly synchronous with cellulase gene induction. Furthermore, the cellulase gene transcription activator ClrB activates its level, while the repressor CreA represses its level. Interestingly, both the bgl2 [20, 24] and its regulators CreA/Crel [25, 26] are highly conserved in the cellulolytic fungi N. crassa and T. reesei. Our data further highlights the bgl2 promoter to be a highly efficient and inducible promoter in P. oxalidum and likely also in other cellulolytic fungi, such as N. crassa and T. reesei. It will be a particularly valuable tool for strain genetic engineering, to conditionally super-express other essential enzymes or proteins.

The sugars produced during the fermentation process were examined by high-performance liquid chromatography (HPLC). As expected, cellobiose was only detected in RE-10, but not in the other β-glucosidase over-expression mutants (see Additional file 3: Figure S3). In fact, the cellobiohydrolase and endo-glucanase activities were slightly enhanced in the β-glucosidase mutants (data not shown). The expression level of CBH and EG encoding genes were further analyzed (Additional file 4: Figure S4). The result showed that the expression levels of major CBH encoding gene cbh1 in C1-9, and major EG gene eg1 in C1-9, I1-12 and C4-1 were up-regulated significantly. On the other hand, previous assumptions pointed towards cellobiose to be the inhibitor of both cellobiohydrolase and endo-glucanase [27]. As a result, it was suggested that over-expression of β-glucosidase might indirectly increase both cellobiohydrolase and endo-glucanase activities by removing the product feedback inhibition caused by cellobiose.

**Improvement of microcrystalline cellulose degradation via over-expression of β-glucosidases in RE-10**

We assayed fungal hyphae extension rates of all mutants and used higher concentration cellulose in the plate medium to evaluate cellulolytic ability. Hyphae extension speeds and colony diameters of most of the BGL mutants resemble that of RE-10 (Fig. 3), which indicates that over-expression of BGLs did not affect fungal growth. However, the mutants C1-36 and C5-2 decreased fungal growth.

![Fig. 2](image-url) Comparative analysis of pNPGase activity between BGL(X) over-expression mutants and the parental strain RE-10. The culture supernatants of 12 BGL(X) over-expression mutants and the parental strain RE-10 were sampled at 120 and 144 h. β-Glucosidase activity was measured with the pNPG as the substrate. Data are the means of three biological replicates and error bars show the standard deviation. Asterisks indicates the significantly difference between the mutant and the RE-10 (*p ≤ 0.05; **p ≤ 0.01)
rate. A possible reason for this could lie in the genomic sites of expression cassette insertion, so we removed these two strains from further analysis. The cellulolytic zones of 8 over-expression strains increased significantly compared to RE-10, including C1-9, C1-36, C1-57, C5-1, I1-12, C4-1, I1-14, I4-32 and I4-33 (Fig. 3). This correlates with our results for the β-glucosidase activity assay. Our data demonstrates that over-expression of β-glucosidases significantly improves the fungal cellulolytic ability.

Up-regulation of BGL(X) in the mutants at both transcription and protein levels

Although all the above mutants have been verified by PCR using specific primers (data not show), the transcript abundance of β-glucosidase genes has not been examined. Fluorescence quantitative polymerase chain reaction (qPCR) was performed to investigate the transcript abundance of BGL(X). The results are shown in Fig. 4. Both the C1-9 and I1-12 strains substantially up-regulated their bgl1 expression levels in both glucose and cellulose conditions. Markedly, after cellulose induction for 4 h, the transcriptional level of bgl1 in C1-9 increases about 1000-fold, and in I1-12 up-regulates 10,000-fold. The expression level of bgl5 in C5-1 was also greatly up-regulated with values of up to 5000-fold in comparison to RE-10. The expression level of bgl4 in C4-1 and I4-32 was also significantly elevated. Our data confirmed that all of the β-glucosidase mutants we used, improved the corresponding β-glucosidase at the transcription level. Given that the expression levels of all three bgl genes were comparable in their over-expression strains, we assumed that higher specific activity and substrate affinity of BGL1 contributes to higher β-glucosidase activity in the bgl1-over-expressing strains C1-9, C1-36, C1-57, I1-13, and I1-14. Furthermore, the SDS-PAGE and MS analysis confirmed that the BGL bands were significantly improved compared to those of RE-10 in the over-expression mutants (Fig. 5). The BGL1 amount of I1-12 in particular, is more abundant compared to that of CBHI (PDE_07945), which is the dominant protein in the cellulolytic secretome [6, 28, 29].

The regulation model of the three β-glucosidases was analyzed based on three kinds of different promoter-driven expression patterns. Three different promoters were from gene bgl(x), pde_02864 and bgl2, respectively. The expression pattern of BGL1 and BGL4 was similar: low-level expression under glucose and high-level expression when induced by cellulose (Fig. 4 and Additional file 5: Figure S5), whose regulation model resemble CBH and EG. However, the bgl5 displayed constitutive expression and independent of carbon source (Fig. 4c).

Over-expressing β-glucosidases enhanced the filter paper hydrolysis

For the process of lignocellulose hydrolysis, β-glucosidases affect the last step and are also the important rate-limiting enzymes. To further test the cellulolytic ability of the above β-glucosidase over-expression strains, filter paper was used as substrate. The result is shown in Fig. 6. All β-glucosidase mutants exhibit a significant increase in glucose release compared to RE-10. This suggests that high β-glucosidase activity has a strong impact on filter paper hydrolysis (Fig. 3). Most of β-glucosidase over-expression mutants increase the FPase activity over twofold compared to RE-10. This result further confirmed the significant role of β-glucosidase as rate-limiting enzyme in
the lignocellulose-glucose conversion. The constitutive β-glucosidase over-expression strain had a slight higher activity compared to those of corresponding inducible mutants. Especially, C1-9 displayed the highest FPase activity among all with up to 8.0 U/ml (Fig. 6).

Cellulase enzyme complex with β-glucosidase over-expression showed higher hydrolysis efficiency against delignified corn cob residue (DCCR)

In nature, neither cellulose, nor filter paper is the staple lignocellulose materials. In order to clarify the hydrolysis ability of the cellulase mixture of all BGL(X) mutants on natural cellulosic materials, the enzyme mixture from their supernatants were used to hydrolyze DCCR. The released glucose for DCCR gradually increased with time from 24 to 72 h. With equal FPU loading, all strains produced similar amounts of glucose from hydrolyzing DCCR, with approximately 40 g/l during 72 h. However, we only required 27 % of enzyme loadings for C4-1, 36 % for C5-1, 31 % for I1-12, and 38 % for I4-32 to obtain the same hydrolysis rates compared to RE-10. These results signify that these β-glucosidase over-expression mutants noticeably reduce total crude enzyme loading, and therefore reduce enzyme cost. The amount of released glucose per mg protein is illustrated in Fig. 7. The data clearly shows that all the BGL(X) mutants produce more than twice or three times the glucose (from 158 to 212 g glucose/mg crude enzyme complex) at the equivalent protein basis compared to the parental strain RE-10 (65 g glucose/mg crude enzyme complex) (Fig. 7). Although the released glucose of C1-9 was slightly less than that of RE-10 at 72 h, only 22 % protein load was supplemented compared to RE-10. We assume that the mild decrease of hydrolysis rate in C1-9 to be due to a lack of other proteins that play a role in the DCCR hydrolysis. Noteworthy, the strain I4-32 displayed significantly higher glucose yield compared to that of RE-10 at lower protein loading. Engineering the β-glucosidase as described in this study contributes to manufacture high-efficiency cellulase complex.

A growing number of publications report the reduction of enzyme loadings in the saccharification process and supplementing β-glucosidase undoubtedly becomes one of the most efficient strategies [22, 30, 31]. In this study, the β-glucosidases were directly improved by strain engineering, which is both time and labor-saving. Lytic polysaccharide monooxygenase (LPMO) [32, 33], expansion and expansion-like proteins [34], and other proteins [35] were reported to play important and synergetic roles in lignocellulose degradation. Their supplementation could significantly improve the lignocellulose-to-glucose conversion rate. With the highly efficient promoters found in this study, we were able to enhance their levels by over-expressing those proteins, and thereby ultimately reduce the enzyme cost.
Conclusions
This study presents over-expression analysis of nine β-glucosidase genes in wild-type *P. oxalicum* and identifies three major players of lignocellulose degradation: BGL1, BGL4 and BGL5. Two different types of promoters were used to over-express the three β-glucosidases in the cellulase over-producer RE-10. Compared to the parental strain, we observed significant boost in beta-glucosidase activity for all recombinant strains obtained. The inducible promoter from gene *bgl2* is particularly more efficient in activating the *bgl1* or *bgl4* gene expression and is proposed to be a potentially valuable tool to conditionally over-express other essential genes in *P. oxalicum* as well as other cellulolytic fungi. The crude cellulase mixture from these β-glucosidase over-producers was significantly more efficient in hydrolyzing microcrystalline cellulose, filter paper, and DCCR compared to that
of RE-10. This substantially reduced the enzyme cost of lignocellulose-based biofuel.

Methods

Strains and culture conditions

*P. oxalicum* 114-2 (CGMCC 5302), the cellulase high-producer RE-10 [13] and all mutants we obtained from these two, were maintained on malt extract agar. The glucose and cellulose medium comprised 1 × Vogel’s salts [51] and 2 % glucose or microcrystalline cellulose as the sole source of carbon. The enzyme-producing medium was composed as follows: corn cob residue (2.0000 %), cellulose (0.6000 %), wheat bran solid (4.6571 %), soybean cake powder plate (1.0000 %), (NH₄)₂SO₄ (0.2000 %), NaNO₃ (0.2789 %), urea (0.1000 %), KH₂PO₄ (0.3000 %), and MgSO₄ (0.0500 %). To induce enzyme production, 10⁵ spores were pre-cultured for 22 h in glucose medium and subsequently transferred into 1 × Vogel’s solution devoid of any carbon source, where it stayed for 2 h. As a final step, the cultures were then transferred to cellulose or enzyme-producing media at 30 °C, and 200 rpm for 144 h. All samples were collected at the respective time points as described in the text.

Bioinformatics analysis

The sequences of BGL1, BGL4, and BGL5 were used as the search queries in the NCBI database (http://www.ncbi.nlm.nih.gov/). All BGL sequences form *P. oxalicum* and their orthologs from other fungi were downloaded from NCBI’s protein database. The software package MEGA 6.0 [36] was used to construct phylogenetic trees with neighbor-joining algorithms and using 500 bootstrap replications. The domain predictions for BGL1, BGL4, and BGL5 were performed using Pfam 28.0 (http://pfam.xfam.org/) and the signal peptide prediction was based on SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP-3.0/).

Generation of the BGL(X)-over-expression mutant

Throughout this study, we used high-fidelity FastPfu DNA polymerase (TransGen Biotech, Beijing, China) for all PCR. PEG-mediated fungal transformation protocols were performed following published protocol [37]. The constitutive promoter from gene *pde_02864* was PCR-amplified with primers DF and DR, using the *P. oxalicum* 114-2 genome as the template. We amplified the gene encoding regions of nine β-glucosidases from *P. oxalicum* 114-2 with 20 bp overlapping fragments including the promoter and the downstream *hph* cassette. The resistance cassette of *hph* was amplified with primers HPHsF and HPHsR, and via the plasmid pSilent-1 [38] template. These gene over-expression cassettes were fused in the order *pde_02864* (p) *bglx* (coding region) *hph* by Double-joint PCR [39] with nest primers DF2 and HPHsR. All the BGL(X) over-expression cassettes were gel-purified and transformed into the protoplast of *P. oxalicum* 114-2.

In order to generate the *bgl(x)* over-expression mutant in RE-10, the mark gene *hph* of the over-expression cassettes of BGL1, BGL4, BGL5 were replaced with a novel resistance gene *sur* [12] using the primer pair SurF + SurR. The inducible expression cassettes for BGL1 and BGL4 were generated by individually amplifying their encoding region with corresponding primers (see Additional file 6: Table S1) and fused with both the *bgl2* promoter and the mark gene *sur*. All the over-expression cassettes were concentrated by gel purification and transformed into *P. oxalicum* RE-10 protoplasts.

Beta-glucosidase activity, SDS-PAGE, and HPLC assays

The fermentation broth was collected via centrifugation, and the aliquots of the supernatant were diluted to measure enzyme activity. To examine β-glucosidase activity, pNPG and cellobiose (Sigma, USA) were used as substrates. We conducted the enzyme reaction in acetate buffer (pH 4.8) at 50 °C for a total of 30 min, after which we added 10 % Na₂CO₃ to stop the reaction. pNP release was measured and the absorbance was read at 405 nm. The glucose level was measured with the Biosensor. One enzyme activity unit represents the amount of enzyme required to either produce one µmol glucose, or pNP per minute under the above condition. To measure the FPase activity, the enzyme reaction was conducted in 0.2 mol/L acetate buffer (pH 4.8) at 50 °C for a total of 60 min with 0.05 g Whatman No. 1 paper as the substrate. DNS method was used to quantify the released reducing sugars. A protein concentration assay was performed using a Bradford kit (Sangon Biotech, Shanghai, China). Three biological triplicates were performed throughout all described experiments. Both equal quality and volume of culture supernatants were performed for SDS-PAGE analysis, and the predicted β-glucosidase bands were excised for MALDI-TOF–MS identification. We measured the released sugars within the broth supernatants via LC-10 AD HPLC (Shimadzu, Japan) by a Bio-Rad Aminex HPX-42A carbohydrate column (Bio-Rad, USA).

Fungal growth, microcrystalline cellulose hydrolysis and qRT-PCR analysis

Equal volumes of conidia (10⁴ per ml) of all *bgl(x)* mutants as well as the parental strains RE-10 were spotted on a solid plate using 2 % glucose or 3 % ball-milled cellulose at 30 °C for a total of 6 days, after which, they were photographed. The diameters of all colonies on glucose plates were measured, and this was used to evaluate fungal growth. Two biological triplicates were performed in the analysis. For RNA
extraction, we inoculated the spores into glucose medium and pre-culturing them for 22 h, followed by a starvation period of 2 h and transferral into cellulose medium at 30 °C and 200 rpm for a total of 4 h. The RNAsiso™ reagent and PrimeScript RT Reagent Kit (Takara, Japan) were used to extract RNA and to synthesize cDNA, respectively, following the manufacturer's description. We performed the qPCR on LightCycler equipment as previously described [10]. We used the expression level of actin as the internal control for data normalization.

Saccharification of delignified corncob residue
The delignified corncob residue was provided by LONGLIVE Co., Yucheng, Shandong province, China (http://www.longlive.cn/). Alkaline extraction of the lignin from corncob residues was performed and left the delignified corncob residue. The crude enzymes were collected at 144 h, and we removed the mycelia and residual medium. The saccharification reaction was implemented at 50 °C in a 5 ml Eppendorf tube containing a mixture of 0.1 g DCCR (5 %), 1 % NaN₃ and 10 FPU/g glucan per crude enzyme with added pH 4.8 sodium acetate buffer to increase total volume to 2 ml. The glucose release was measured using the SBA-40C biological sensor (Shanto, China). The experiments were performed in three biological repetitions.

Statistics
We performed one-tail t-Student tests with equal variance using the software Microsoft Office 2010 Excel. All mean values, standard deviations as well as p values were calculated in the quantitative analyses throughout this study.
12. Li Z, Yao G, Wu R, Gao L, Kan Q, Liu M, Yang P, Liu G, Qin Y, Song X, et al. Synergistic and dose-controlled regulation of cellulase gene expression in *Penicillium oxalicum*. PLoS Genet. 2015;11:e1005509.

13. Yao G, Li Z, Gao L, Wu R, Kan Q, Liu G, Qu Y. Redesigning the regulatory pathway to enhance cellulase production in *Penicillium oxalicum*. Biotechnol Biofuels. 2015;8:71.

14. Singhania RR, Patel AK, Sukumaran RK, Larroche C, Pandey A. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol. 2013;127:500–7.

15. Henrissat B. A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J. 1991;280(Pt 2):309–16.

16. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 2014;42:D4940–5.

17. Bohlin C, Olsen SN, Morant MD, Patkar S, Borch K, Westh P. A comparative study of activity and apparent inhibition of fungal beta-glucosidases. Biotechnol Bioeng. 2010;107:943–52.

18. Chen M, Qin Y, Liu Z, Wang F, Qu Y. Isolation and characterization of a new Zn(II)(2)Cys(6)-type transcription factor BglR regulates beta-glucosidase activity in *Trichoderma reesei* and *Penicillium decumbens* glucosidase gene from *Penicillium oxalicum*. Enzyme Microb Technol. 2010;46:444–9.

19. Chen M, Qin Y, Cao Q, Liu G, Li J, Li Z, Zhao J, Qu Y. Promotion of extracellular lignocellulolytic enzymes production by restraining the intracellular beta-glucosidase in *Penicillium decumbens*: Bioresearch Technol. 2013;127:33–40.

20. Znameroski EA, Coradetti ST, Roche CM, Tsai JC, Iavarone AT, Cate JT, Fushinobu S. Crystal structures of glycoside hydrolase family 3 beta-glucosidase in complex with cellodextrins. Proc Natl Acad Sci USA. 2011;109:6012–7.

21. Suzuki K, Sumitani J, Nam YW, Nishimaki T, Tani S, Kawaguchi T, Fushinobu S. Crystal structures of glycoside hydrolase family 3 beta-glucosidase from *Aspergillus awamori*. Biochem J. 2013;452:211–21.

22. Ma L, Zhang J, Zou G, Wang C, Zhou Z. Improvement of cellulase activity in *Trichoderma reesei* by heterologous expression of a beta-glucosidase gene from *Penicillium decumbens*. Enzyme Microb Technol. 2011;49:366–71.

23. Liao X, Wang H, Zhang J. Purification and characterization of a new beta-glucosidase from *Penicillium piceum* and its application in enzymatic degradation of delignified corn stover. Bioresour Technol. 2013;147:658–61.

24. Muller G, Varnai A, Johansen KS, Eijjink VG, Horn SJ. Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. Biotechnol Biofuels. 2015;8:187.

25. Eibinger M, Ganner T, Rosker S, Kracher D, Haltrich D, Ludwig R, Pflank H, Nidetzky B. Cellulose surface degradation by a lytic polysaccharide monooxygenase and its effect on cellulase hydrolytic efficiency. J Biol Chem. 2014;289:35929–38.

26. Liu X, Ma Y, Zhang M. Research advances in expansins and expansion-like proteins involved in lignocellulose degradation. Biotechnol Lett. 2014;36:4275–83.

27. Antonieto AC, dos Santos Castro L, Silva-Rocha R, Persinoti GF, Silva RN. Defining the genome-wide role of CRE1 during carbon catabolite repression in *Trichoderma reesei* using RNA-Seq analysis. Fungal Genet Biol. 2014;73:93–103.

28. Sorensen A, Mubeck M, Lubeck PS, Ahring BK. Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials. Biomolecules. 2013;3:612–31.

29. Jun H, Guan Y, Cao Q, Liu G, Chen Y. Insights into enzyme secretion by filamentous fungi: comparative proteome analysis of *Trichoderma reesei* grown on different carbon sources. J Proteomics. 2013;89:191–201.

30. Phillips CM, laverone AT, Marletta MA. Quantitative proteomic approach for cellulose degradation by *Neurospora crassa*. J Proteome Res. 2011;10:4177–85.

31. Yan X, Zhang X, Zhang Y. A new beta-glucosidase from *Hypocrea jecorina* and its potential of activity and apparent inhibition of fungal beta-glucosidases. Biotechnol Bioeng. 2010;107:366–76.

32. Muller G, Varnai A, Johansen KS, Eijjink VG, Horn SJ. Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. Biotechnol Biofuels. 2015;8:187.

33. Eibinger M, Ganner T, Rosker S, Kracher D, Haltrich D, Ludwig R, Pflank H, Nidetzky B. Cellulose surface degradation by a lytic polysaccharide monooxygenase and its effect on cellulase hydrolytic efficiency. J Biol Chem. 2014;289:35929–38.

34. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2014;30:2725–9.

35. Kim IJ, Lee HJ, Choi IG, Kim KH. Synergistic proteins for the enhanced enzymatic hydrolysis of cellulose by cellulase. Appl Microbiol Biotechnol. 2014;98:8469–80.

36. Shida Y, Ogasawara W, Okada H. Utilization of recombinant *Trichoderma reesei* expressing Aspergillus aculeatus beta-glucosidase I (UN11) for a more economical production of ethanol from lignocellulosic biomass. J Biosci Bioeng. 2015;120:657–65.

37. Li ZH, Du CM, Zhong YH, Wang TH. Development of a highly efficient cellulase for bioethanol production. Bioresour Technol. 2013;127:500–7.

38. Nakayashiki H, Hanada S, Nguyen BQ, Kadotani N, Tosa Y, Mayama S. Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725–9.

39. Li Z, Yao G, Wu R, Gao L, Kan Q, Liu M, Yang P, Liu G, Qin Y, Song X, et al. Synergistic and dose-controlled regulation of cellulase gene expression in *Penicillium oxalicum*. PLoS Genet. 2015;11:e1005509.