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BIOENERGY/BIOFUELS/BIOCHEMICALS

# Efficient production and evaluation of lignocellulolytic enzymes using a constitutive protein expression system in *Penicillium oxalicum*

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**Abstract** Native lignocellulolytic enzyme systems secreted by filamentous fungi can be further optimized by protein engineering or supplementation of exogenous enzyme components. We developed a protein production and evaluation system in cellulase-producing fungus *Penicillium oxalicum*. First, by deleting the major amylase gene *amy15A*, a strain Δ15A producing few extracellular proteins on starch was constructed. Then, three lignocellulolytic enzymes (BGL4, Xyn10B, and Cel12A) with originally low expression levels were successfully expressed with selected constitutive promoters in strain Δ15A. BGL4 and Cel12A overexpression resulted in increased specific filter paper activity (FPA), while the overexpression of Xyn10B improved volumetric FPA but not specific FPA. By switching the culture medium, this platform is convenient to produce originally low-expressed lignocellulolytic enzymes in relatively high purities on starch and to evaluate the effect of their supplementation on the performance of a complex cellulase system on cellulose.

**Keywords** Homologous expression · Strong promoter · Cellulase · Lignocellulolytic enzyme · *Penicillium oxalicum*

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## Introduction

Lignocellulose can be degraded to simple sugars by lignocellulolytic enzyme systems which are composed of classes of cellulases and hemicellulases. These enzymes have been used in industries for many years and are considered to play a critical role in the production of new generation biofuels [1]. The production cost of lignocellulolytic enzymes has become lower because many enzyme high-producing fungal strains were obtained through mutagenesis and genetic engineering.

Native enzyme systems secreted by many fungi can be optimized for more efficient lignocellulose hydrolysis in industry. Strategies for these optimizations include enzyme engineering, overexpression of some low-abundance components, and supplementation of foreign proteins, which need mature protein expression systems to produce and evaluate the performance of single enzymes [12]. Classical heterologous protein expression systems, such as *Escherichia coli, Pichia pastoris,* and *Saccharomyces cerevisiae*, can be used for the expression of cellulases. However, the application of these systems is limited for fungus-derived cellulases, because low-activity or inactive enzymes were often obtained resulting from hyperglycosylation or incorrect protein folding [2, 5, 20]. The widely used expression system

for fungal proteins, *Aspergillus*, is also not suitable for the active expression of some cellulases due to improper glycosylation [9]. Thus, it is better to directly express cellulases in industrial cellulase-producing strains to evaluate their "on-site" performances. On the other hand, a clean protein background is preferred for the purification of cellulases, because multi-step purifications are time consuming and low yielding. Although affinity purification can be used to purify proteins from complex backgrounds, the tags added to proteins may affect the properties of some cellulases [10].

*Penicillium oxalicum* is a fast-growing fungus whose mutants have been used for industrial cellulase production in China. A genetic manipulation system has been established in *P. oxalicum*, and this fungus was found to have relatively high homologous recombination frequencies (can be as high as 33–83 % in wild-type strain 114-2) in targeted gene manipulations [11]. In this study, a dual-purpose protein expression system was constructed in *P. oxalicum* 114-2. The gene *amy15A* encoding the main extracellular protein on starch was deleted to give a clean background for the expression of target proteins. Strong constitutive promoters were used for protein expression with either starch (to get nearly purified protein) or cellulose (to adjust the cellulase system composition) as the carbon source.

## Materials and methods

### Strains and culture conditions

*P. oxalicum* 114-2 (CGMCC 5302) was used for gene deletions [13, 21]. *P. oxalicum* peni-1 (CGMCC 9126) was isolated from cow feces and wheat straw compost.

Liquid glucose minimal medium (GMM) was used for germination and hyphal growth (GMM, per liter: glucose 20 g, $KH_2PO_4$ 3 g, $(NH_4)_2SO_4$ 2 g, $MgSO_4{\cdot}7H_2O$ 0.56 g, $CaCl_2$ 0.56 g, $FeSO_4{\cdot}7H_2O$ 7.5 mg, $MnSO_4{\cdot}H_2O$ 2.5 mg, $ZnSO_4{\cdot}7H_2O$ 3.6 mg, $CoCl_2{\cdot}6H_2O$ 3.7 mg, $CuSO_4$ 3.2 mg) [19]. For phenotypic analyses of wild-type strain 114-2 and its mutants on agar plates, Vogel's minimal medium (VMM) was used with supplementation of glucose or starch (both in concentration of 10.0 g $L^{-1}$) [24], and 1 μL of conidia of each strain was spotted on agar plates. For the determination of hydrolase activities of strain 114-2 and its mutants, strains were first grown in 100 mL of liquid GMM for 24 h. Then, their mycelia were, respectively, collected by vacuum filtration, and 0.5 g of wet mycelia was resuspended in 100 mL liquid VMM supplemented with 10 g $L^{-1}$ starch or 5.0 g $L^{-1}$ microcrystalline cellulose plus 5.0 g $L^{-1}$ wheat bran (CW medium). All the cultivations were performed at 30 °C with rotary shaking at 200 rpm.

For fermentation in bioreactors, conidia of a final concentration of $10^7$/mL were inoculated into a 7.5-L fermentor (BioFlo 310, New Brunswick Scientific Company, USA) containing 5 L of VMM medium with 20 g $L^{-1}$ starch as sole carbon source. The incubation temperature was 30 °C, and an agitation speed of 400 rpm and an air flow rate of 2.1 L $L^{-1}$ $min^{-1}$ were adjusted to keep the dissolved oxygen above 15 %.

For β-glucosidase production by strains derived from peni-1, conidia of a final concentration of $10^7$/mL were, respectively, inoculated into the medium containing 5.0 g $L^{-1}$ wheat bran, 5.0 g $L^{-1}$ crystalline cellulose, and Mandels' solution (per liter: $KH_2PO_4$ 3 g, $NaNO_3$ 2.6 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2$ 0.5 g, peptone 1.0 g, $FeSO_4{\cdot}7H_2O$ 7.5 mg, $MnSO_4{\cdot}H_2O$ 2.5 mg, $ZnSO_4{\cdot}7H_2O$ 3.6 mg, $CoCl_2{\cdot}6H_2O$ 3.7 mg) [15]. For higher β-glucosidase production, the following medium (per liter: grass powder 30.0 g, microcrystalline cellulose 6.0 g, $(NH_4)_2SO_4$ 4.0 g, $KH_2PO_4$ 1.0 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.5 g, peptone 1.0 g, $FeSO_4{\cdot}7H_2O$ 7.5 mg, $MnSO_4{\cdot}H_2O$ 2.5 mg, $ZnSO_4{\cdot}7H_2O$ 3.6 mg, $CoCl_2{\cdot}6H_2O$ 3.7 mg) was used.

### Construction of targeting cassettes and transformation of two *P. oxalicum* strains

Genomic DNA was isolated from the mycelia of *P. oxalicum* 114-2 and *P. oxalicum* peni-1 using the method previously described [4]. All targeting cassettes were constructed using the double-joint PCR technique [26].

For gene deletions, the 5′ and 3′ flanking regions of *amy13A* and *amy15A* were amplified from the genomic DNA of 114-2 with primer pairs 13A-F/13A-R-ptrA, 13A-F-ptrA/13A-R, and 15A-F/15A-R-hph, 15A-F-hph/15A-R, respectively. The templates of *amy13A* and *amy15A* deletion cassettes were obtained by fusing the 5′ and 3′ flanking sequences of *amy13A* and *amy15A* to the pyrithiamine resistance gene *ptrA* and the hygromycin B resistance gene *hph* cassette which were amplified with primer pairs PtraF/PtraR and hphF/hphR, respectively. The 5063 and 5793 bp deletion cassettes were then obtained by nest-PCR primer pairs 13A-NF/13A-NR and 15A-NF/15A-NR.

For glycoside hydrolases overexpression cassettes, *cel12A*, *xyn10B*, *bgl1,* and *bgl4* were amplified with the primer pairs Ubi-EG12-F/EG12-R, Ubi-XYN-F/XYN10-R, Beta-F/Beta-R, and B4-F/B4-R from genomic DNA of strain 114-2, respectively. We used upstream nucleotides with lengths of 879, 965, and 1097 bp as the promoter regions of *aciA*, *ubiD*, and *pgmC*, respectively. The three promoters P*aci,* P*ubi,* and P*pgm* were amplified with primer pairs aci-F/aci-R, ubi-F/ubi-R, and pgm-F/pgm-R, respectively. The template of overexpression cassettes were obtained by fusing those promoters and *ptrA* to the glycoside hydrolase coding regions and their terminator regions (Supplementary material Fig. S1). Finally, the overexpression cassettes were amplified and transformed

**Table 1** Strains used in this study

| Strains | Genotype | Parent strain |
|---|---|---|
| 114-2 | Wild type | |
| Δ15A | Δ*amy15A*::*hph* | 114-2 |
| Δ13A | Δ*amy13A*::*ptrA* | 114-2 |
| Δ1315A | Δ*amy13A*::*ptrA*;Δ*amy15A*::*hph* | Δ13A |
| Δ15A-BG | Δ*amy15A*::*hph*; *pgmC(p)*::*bgl4*::*ptrA* | Δ15A |
| Δ15A-Xyn10B | Δ*amy15A*::*hph*; *ubiD(p)*::*xyn10B*::*ptrA* | Δ15A |
| Δ15A-Cel12A | Δ*amy15A*::*hph*; *ubiD(p)*::*cel12A*::*ptrA* | Δ15A |
| peni-1 | Wild type | |
| P1, P2, P3, P4 | *pgmC(p)*::*bgl1*::*ptrA* | peni-1 |
| A1, A2, A3 | *aciA(p)*::*bgl1*::*ptrA* | peni-1 |
| U1, U2, U3 | *ubiD(p)*::*bgl1*::*ptrA* | peni-1 |

to strain Δ15A or peni-1. Primer pairs P-YZF/13A-R and H-YZF/15A-R were used to test whether those two deletion cassettes exchanged the coding regions of *amy13A* and *amy15A,* respectively. 13A-TF/13A-TR and 15A-TF/15A-TR were applied to further detect the existence of *amy13A* and *amy15A* by amplified their partial coding regions. The detailed information of primers used in this study is described in Table S1 (Supplementary material Table S1).

Transformation of *P. oxalicum* was performed according to the method previously described [6, 11]. Strains 114-2 and peni-1 were used as the parent strains for *amy13A* or *amy15A* single deletion and glycoside hydrolases overexpression, respectively (Supplementary material Fig. S1). Δ13A was used to construct the double-deletion strain Δ1315A, while considering the limited number of resistance selection marker, Δ15A was used to construct the overexpression strains of BGL4, Xyn10B, and Cel12A. All fungal strains used in this study are summarized in Table 1.

### Enzyme assays, protein determination, and SDS-PAGE analysis

Total cellulase activity (FPA) was analyzed with Whatman No. 1 filter paper as the substrate. Amylase, β-glucosidase, endoglucanase, and xylanase activities were assayed according to the methods previously described [22]. The amounts of released reducing sugars were determined using the dinitrosalicylic acid method, as described by Miller [17]. One enzyme unit (U) was defined as the amount of enzyme needed for releasing 1 μmol of glucose equivalent per minute. Protein concentration in the culture supernatant was determined by using a Bradford reagent kit (Sangon Biotech, China). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed in polyacrylamide gel slabs and gels were stained with Coomassie Brilliant Blue R-250 (Sangon Biotech, China).

### Lignocellulose saccharification

Enzymatic saccharification was performed on delignified corncob residue (DCCR) [3] or 20 % NaOH-pretreated corn stover (PCS) prepared in our lab. The DCCR powder of 80–100 mesh was used in the experiments. The reaction mixture in a total volume of 20 mL, which contained 2 % DCCR or PCS (w/v) and 18 mL crude enzyme in pH 4.8 acetate buffer, was incubated for designated period at 50 °C with shaking at 150 rpm. The crude enzyme was prepared as follows: 0.5 g pre-grown mycelia was resuspended in 100 mL of liquid VMM containing 0.5 % cellulose plus 0.5 % wheat bran (w/v) as the sole carbon source and cultured for 24 h at 30 °C and 200 rpm. The supernatants of relevant cultures were collected for saccharification experiments.

### RNA isolation, cDNA synthesis, and quantitative RT-PCR

For real-time PCR, total RNA was extracted using Trizol reagent (TaKaRa, Japan) according to the manufacturer's instructions. Reverse transcription was performed using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan) according to the manufacturer's protocols. Quantitative PCR was performed using SYBR *Premix Ex Taq*™ (Perfect Real Time) (TaKaRa, Japan) on Roche 480 LightCycler (Roche, Mannheim, Germany) with LC480 software as previously described [25]. Each sample with a total reaction volume of 20 μL was performed with two biological replicates and three experiment replicates. The actin gene was used as the internal standard. All the primers are listed in Table S1 (Supplementary material Table S1).

### Accession numbers

The GenBank accession numbers of the studied proteins are as follows: Amy13A, EPS26265; Amy15A, EPS34453; BGL1, ADB82653; BGL4, EPS26627; Cel12A, EPS31484; Xyn10B, ADM34973; AciA, EPS26144; PgmC, EPS26399; UbiD, EPS29015; GpdA, EPS34987.

## Results

### Deletion of amylase gene *amy15A* resulted in a cleaner extracellular protein background

Two major amylases, belonging to the glycoside hydrolase (GH) families 15 (Amy15A, gene ID PDE_9417) and 13 (Amy13A, PDE_1201), respectively, were estimated to occupy 29 and 11 % of total secreted proteins produced by 114-2 on cellulose-wheat bran medium [13]. The mutants of 114-2 lacking the coding sequence of *amy15A*

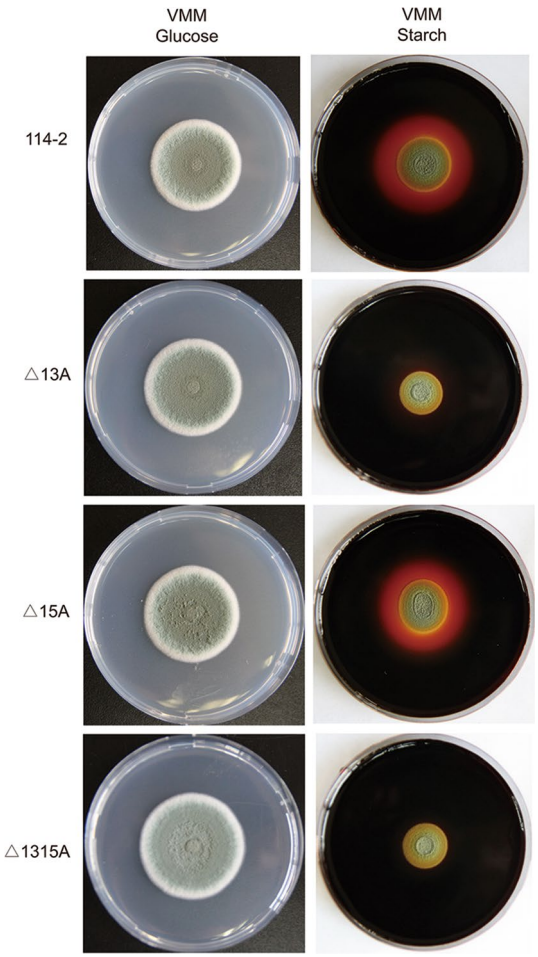


**Fig. 1** Growth of 114-2 and mutants on different carbon sources. The plates were incubated at 30 °C for 5 days. Starch-containing plates were dyed with iodine

or *amy13A* (named Δ15A and Δ13A, respectively) were constructed by targeted gene replacement followed by PCR identification (Supplementary material Fig. S2). A Δ1315A strain that lacks both *amy15A* and *amy13A* genes was also constructed. The colonies of these three mutants on glucose showed no differences when compared to that of wild-type 114-2. The hydrolysis halo of Δ15A on starch agar was smaller compared with that of 114-2, while deletion of *amy13A* or both genes led to almost complete disappearance of hydrolysis halo (Fig. 1). In liquid glucose or starch medium, where cellulases were not induced, deletion of the two major amylase genes or only *amy15A* gave few extracellular proteins (Fig. 2). These provided a clean background for the expression of specific proteins.

When the single- and double-deletion mutants were grown on cellulose, significant decreases in amylase activity and extracellular protein content were observed (Fig. 3a, b). On the other hand, cellulase activity measurement and SDS-PAGE analysis demonstrated that cellulase production was not affected in the mutants (Fig. 3c, d).

## Selection of three potential constitutive promoters

Three potentially strong promoters, P*ubiD*, P*pgmC,* and P*aciA*, were selected for further evaluation according to the analysis of highly expressed genes in transcriptomic study of strain 114-2 (unpublished data). Genes *ubiD*, *pgmC,* and *aciA* were predicted to encode polyubiquitin (PDE_03961), phosphoglycerate mutase family protein (PDE_01335), and dehydrogenase (PDE_01080), respectively. The expression levels of these three genes at different time points on different carbon sources were further analyzed by real-time

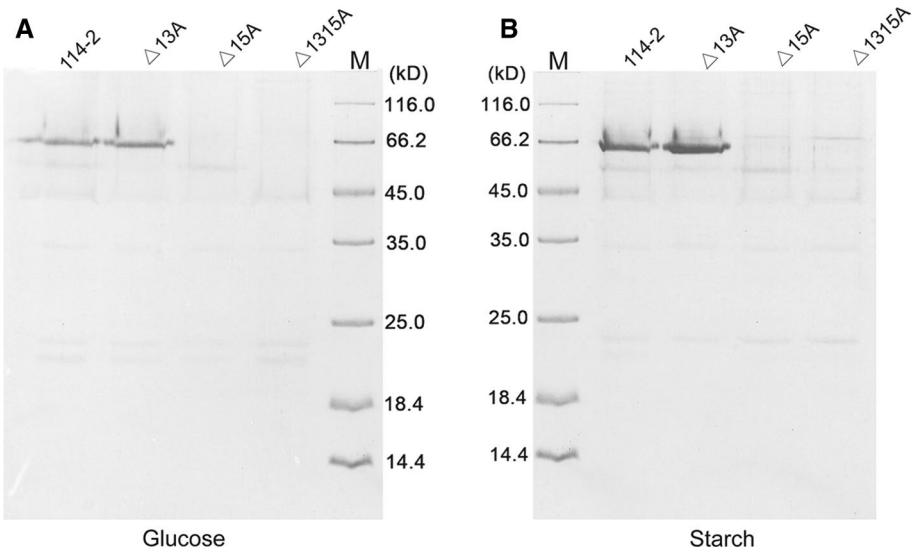


**Fig. 2** Extracellular proteins of 114-2 and mutants on glucose or starch analyzed by SDS-PAGE. 32 μL culture supernatant of each strain was loaded after cultivating in 1 % (w/v) glucose medium (**a**) or 1 % starch medium (**b**) for 48 h, respectively. *Lane M* was the molecular weight marker

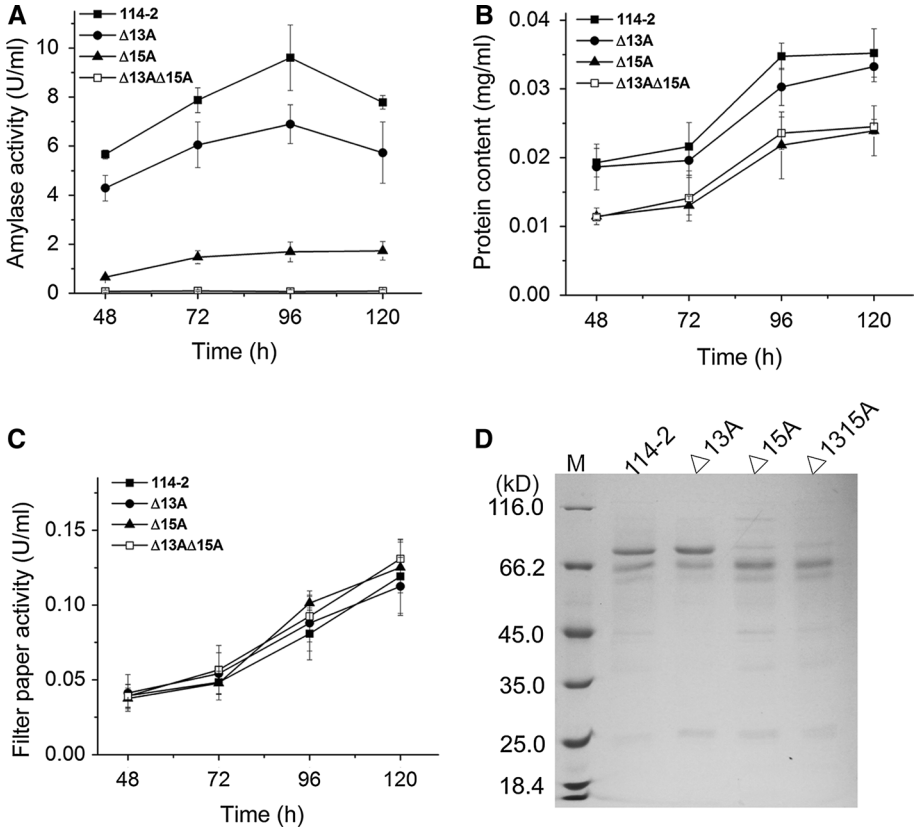


**Fig. 3** Enzyme activities and composition of extracellular proteins of 114-2 and mutants on cellulose. Amylase activity (**a**), total protein concentration (**b**), and filter paper activity (**c**) in 1 % (w/v) cellulose medium were shown. *Error bars* indicated standard deviations of three independent cultivations. **d** SDS-PAGE analysis of extracellular proteins of different strains after Coomassie blue staining. 32 μL culture supernatant of each strain was loaded after cultivating in 1 % cellulose medium for 72 h. *Lane M* was the molecular weight marker

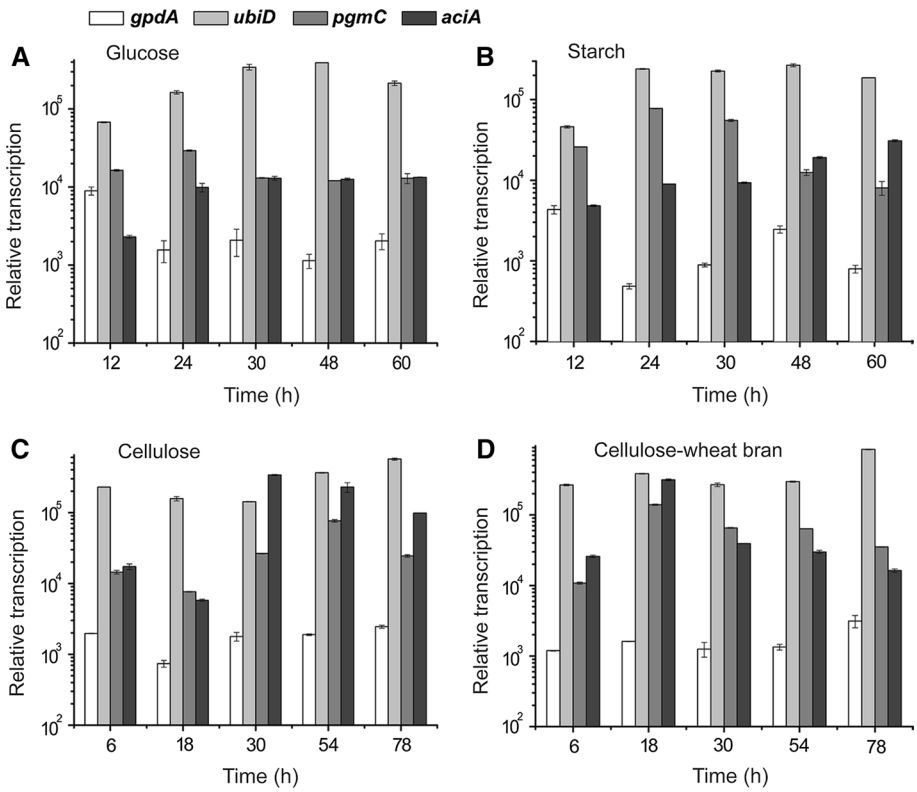


**Fig. 4** Comparison of the expression levels of four genes on different carbon sources at different time points. **a**–**d** Indicated the relative expression levels of the genes in the medium with 1 % (w/v) glucose, 1 % starch, 1 % cellulose, and 0.5 % cellulose plus 0.5 % wheat bran as sole carbon source, respectively. Conidia of strains at a final concentration of $10^7$ $mL^{-1}$ were inoculated into 1 % glucose or 1 % starch minimal medium and grown for indicated time periods, while mycelia were transferred to 1 % cellulose or 0.5 % cellulose plus 0.5 % wheat bran medium after growth on glucose liquid medium for 18 h. Relative transcription values represent the transcript copy numbers of each gene per $10^5$ copies of actin gene transcript. *Error bars* indicated standard deviations of three independent measurements

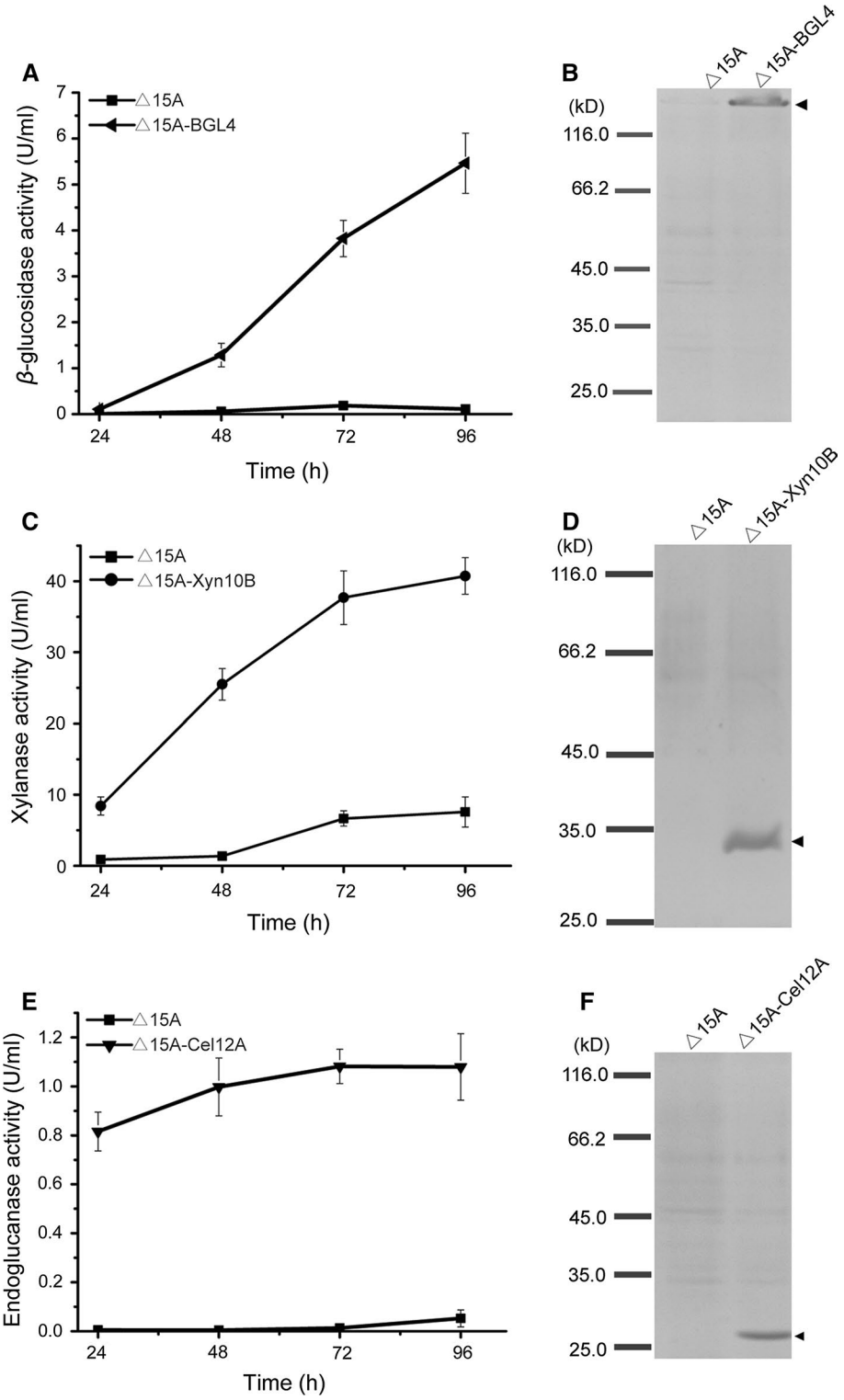


**Fig. 5** Overexpression of β-glucosidase BGL4, xylanase Xyn10B, and Cel12A in Δ15A with starch as carbon source. β-Glucosidase activity (**a**), xylanase activity (**c**), and endoglucanase activity (**e**) of strains in the medium with 1 % (w/v) starch. **b**, **d**, and **f** Extracellular proteins of indicated strains on starch for 72 h were analyzed by SDS-PAGE. 32 μL culture supernatant of each strain was loaded. *Triangular arrows* showed the corresponding overexpressed enzyme

quantitative PCR. The expression levels of glyceraldehyde 3-phosphate dehydrogenase gene *gpdA,* whose promoter is often used for constitutive gene expression in eukaryotes [8], were also analyzed. Generally, *pgmC* showed the highest while *gpdA* showed the lowest expression levels under different conditions. When the expression levels were compared within genes, *ubiD*, *pgmC,* and *gpdA* showed constitutive expression over the conditions, while *aciA* had higher expression levels on cellulose or cellulose-wheat bran than those on glucose or starch (Fig. 4). These results suggested

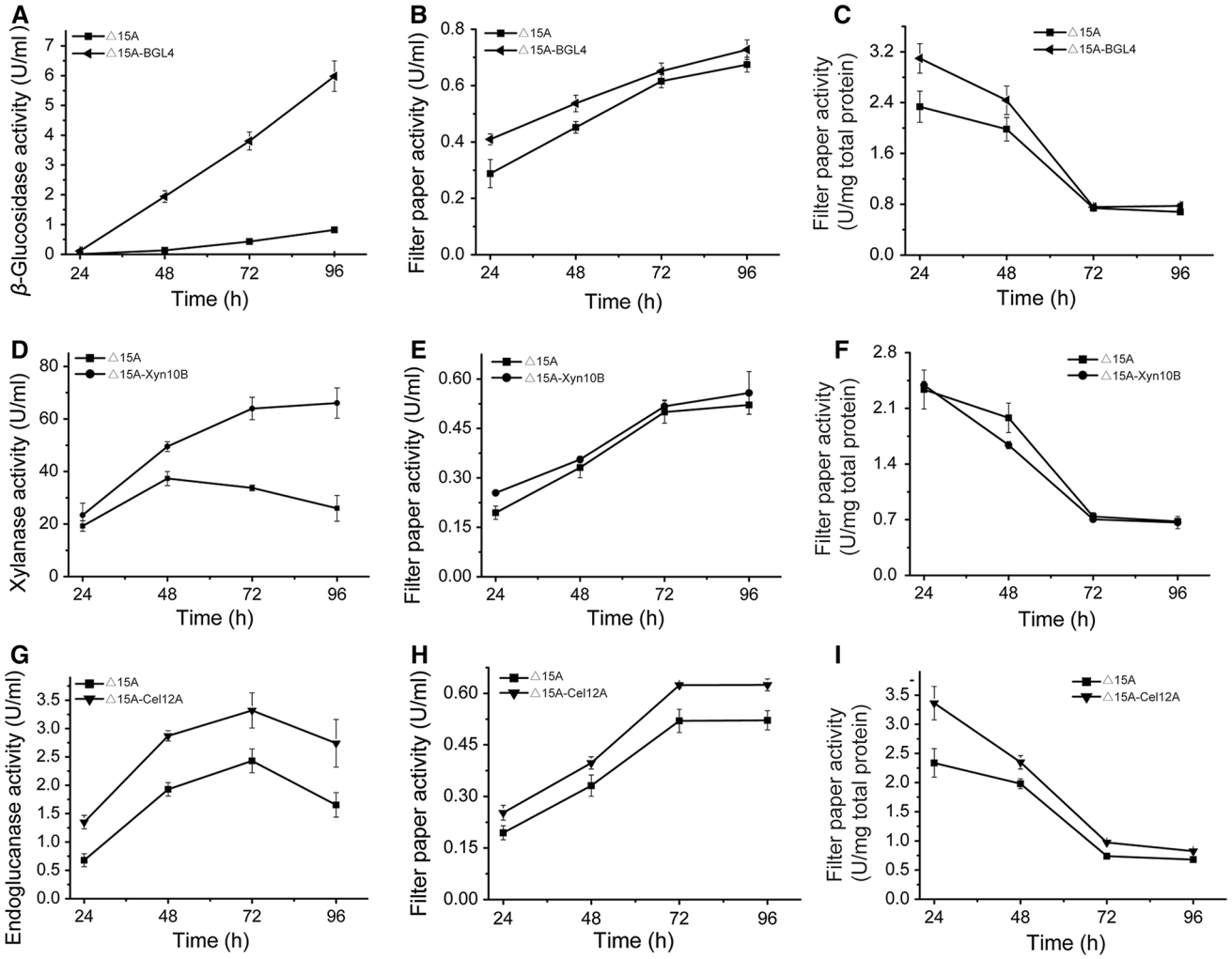


**Fig. 6** Overexpression of BGL4, Xyn10B, and Cel12A in Δ15A with cellulose-wheat bran as carbon source. β-Glucosidase activity (**a**), xylanase activity (**d**), and endoglucanase activity (**g**) of strains in the medium with 0.5 % (w/v) cellulose plus 0.5 % wheat bran were shown. **b**, **c** Volumetric and specific filter paper activities of β-glucosidase overexpression strain. **e**, **f** Volumetric and specific filter paper activities of xylanase overexpression strain. **h**, **i** Volumetric and specific filter paper activities of endoglucanase overexpression strain

that the selected three promoters, especially P*ubiD* and P*pgmC*, can be used for constitutive and high-level expression of target genes in *P. oxalicum.*

## Efficient production of lowly expressed lignocellulolytic enzymes in strain Δ15A

With the mutant Δ15A producing a low extracellular protein background and those selected strong promoters, we tried to overexpress several enzymes with originally low expression levels in *P. oxalicum*. Three proteins potentially involved in lignocellulose degradation, including BGL4 (PDE_01565), Xyn10B (PDE_02418), and Cel12A (PDE_06439), were chosen for overexpression. BGL4 was predicted to be a β-glucosidase hydrolyzing cellobiose to glucose. Xyn10B is a hypothetical endo-β-1,4-xylanase, and Cel12A is a hypothetical endo-β-1,4-glucanase. While all the three proteins were predicted to have signal peptides, they were not experimentally found (BGL4 and Xyn10B), or was detected at a very low abundance (Cel12A, 0.13 % of total protein) in the secretome of 114-2 [13].

The *bgl4* and *xyn10B, cel12A* genes, fused to the promoters P*pgmC* and P*ubiD,* respectively (Supplementary material Fig. S1), were transformed to strain Δ15A. Those overexpressed transformants were verified by PCR and one of them was selected for the following analysis. The *bgl4* transformant Δ15A-BGL4 reached maximum β-glucosidase activities of 5.2 U mL at 96 h on starch, almost 50 fold higher than that of the parent strain (Fig. 5a). The Xyn10B overexpression strain produced as high as 40.7 U $mL^{-1}$ of xylanase at 96 h on starch, which was also much higher than that of the parent strain

(Fig. 5c). The *cel12A* transformant showed obvious endoglucanse activity when compared to strain Δ15A during the whole cultivation period (Fig. 5e). The extracellular proteins after 72 h cultivation were then analyzed by SDS-PAGE. While there were few proteins in the secretome of the parent strain Δ15A, one protein (glycosylated BGL4, Xyn10B, and Cel12A, respectively) was the major component in the secretomes of Δ15A-BGL4, Δ15A-Xyn10B, and Δ15A-Cel12A (Fig. 5b, d, f).

### The effects of enzyme overexpression on the performance of the lignocellulolytic enzyme system

As we used constitutive promoters to overexpress enzymes, the composition of secretome produced in cellulose-wheat bran medium (cellulase-inducing condition) is assumed to be changed in the overexpression strains. As expected, the β-glucosidase activity of Δ15A-BGL4, xylanase activity of Δ15A-Xyn10B and endoglucanase activity of Δ15A-Cel12A in cellulose-wheat bran media were also significantly higher than those of the parent strain, respectively (Fig. 6a, d, g). The three recombinant cellulases systems which contain the overexpression enzyme exhibited different cellulose degradation abilities. The overall cellulase activity (measured as filter paper activity, FPA) of Δ15A-BGL4 was higher than that of the parent strain Δ15A at early periods of cultivation (Fig. 6b for volumetric activity, c for specific activity). Unlike BGL4, the overexpression of Xyn10B led to slightly higher volumetric but similar specific FPA compared with those of the parent strain (Fig. 6e, f). Notably, overexpression of endoglucanase Cel12A contributed to improved cellulase activity during the whole cultivation period (Fig. 6h, i). SDS-PAGE analysis of the extracellular proteins of those three strains after 24 h cultivation confirmed the overexpression of corresponding enzymes in this strain (Fig. 7). Consistent with these results, the recombinant cellulase systems showed increased sugar release from complex lignocellulosic materials (delignification corncob residue and pretreated corn stover) to different extents (Fig. 8).

### The processes of β-glucosidase and xylanase production in fermenters

To effectively monitor the processes of protein production on starch, we cultivated two strains Δ15A-BGL4 and Δ15A-Xyn10B in 7.5-L fermentors with a working volume of 5 L. 2 % starch was used as the sole carbon source, and no additional control of pH and dissolved oxygen was performed during the fermentation period of 120 h. The measured biomass (mycelial dry weight) and pH indicated that both strains underwent a fast growth phase with starch utilization, a second growth phase (probably consuming some organic acids or endogenous nutrients), and finally a mycelial lysis phase (Fig. 9). The maximum production of extracellular proteins and enzyme activities occurred at the mycelial lysis phase, with 7.48 U mL of β-glucosidase (Fig. 9a) and 99.42 U mL of xylanase (Fig. 9b) activities detected, respectively. Moreover, starch gave final enzyme yield of 374 U $gds^{-1}$ (gram of dry fermented substrate) for BGL4 and 4971 U/gds for Xyn10B. Different from xylanase production by Δ15A-Xyn10B, the production of β-glucosidase by Δ15A-BG L4 was significantly faster in the later fermentation stage than that in the early stage (Fig. 9a). The probable reason of this is β-glucosidase can be "trapped" by the cell wall and is released during mycelia lysis [16, 18].

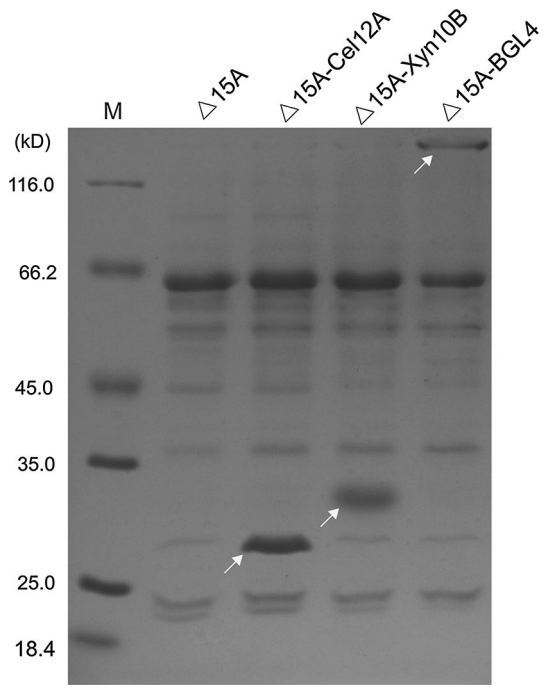


**Fig. 7** SDS-PAGE analysis of extracellular proteins of indicated strains on cellulose-wheat bran. Extracellular proteins of strains Δ15A-BGL4, Δ15A-Xyn10B, and Δ15A-Cel12A on cellulose-wheat bran for 24 h analyzed by SDS-PAGE. 32 μL culture supernatant of each strain was loaded. *Arrows* indicated the corresponding overexpressed enzymes. *Lane M* showed the molecular weight marker

### Using the strong promoters to improve β-glucosidase production in *P. oxalicum* strain peni-1

To further evaluate the efficiencies of the three selected promoters from 114-2, we used them to express β-glucosidase BGL1 from 114-2 [3] in another strain peni-1. Strain peni-1 was also identified as *P. oxalicum* based on phylogenetic analysis of internal transcribed space sequences (Supplementary material Fig. S3), and has a higher original production level of β-glucosidase than 114-2. Considering

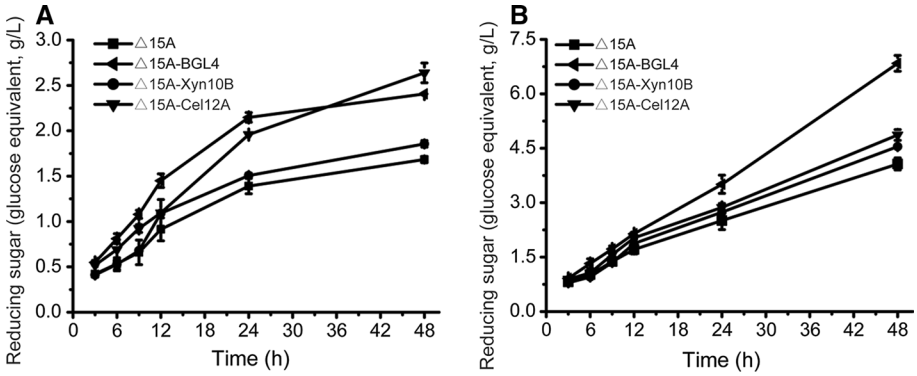


**Fig. 8** Saccharification efficiencies of recombinant cellulase systems on complex lignocellulosic materials. Sugars from the saccharification of DCCR (**a**) and PCS (**b**) by enzymes from Δ15A or overexpression strains were measured. *Error bars* indicated standard deviations of three saccharification reactions

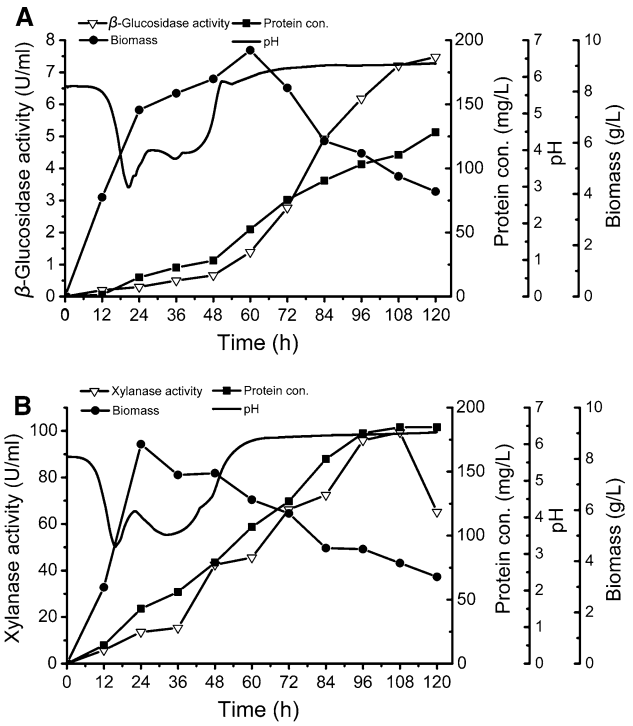


**Fig. 9** Enzyme productions of BGL4 and Xyn10B in 7.5-L fermentors. Δ15A-BGL4 (**a**) and Δ15A-Xyn10B (**b**) were cultured in a 7.5-L fermentor at 30 °C for 120 h by using starch as the carbon source. Cultures were sampled every 12 h

the integration position or copy number of overexpression cassettes may affect the expression level of *bgl1*, several transformants were evaluated for each promoter. Most of the overexpression strains produced higher β-glucosidase activities than the parent strain peni-1, with variations in production levels between different transformants using the same promoter (Fig. 10a, b). When the selected high-producing transformants P2, A1, and U2 (using promoters P*pgmC*, P*aciA,* and P*ubiD*, respectively) were cultivated in a high carbon source medium (containing 0.6 % cellulose and 3.0 % grass powder), higher β-glucosidase activities (14.5, 13.1 and 15.7 U $mL^{-1}$, respectively) were produced after 5 days' cultivation (Fig. 10c). This results together with the former analysis of transcription levels on different carbon sources revealed the high efficiency and extensive adaptivity of screened promoters in *Penicillium.*

## Discussion

In this study, we constructed a constitutive protein expression system which can be used for both the production and evaluation of lignocellulolytic enzymes from fungi. The results provided deeper understanding of the polysaccharide-degradation by *P. oxalicum*, and will facilitate the improvement of current lignocellulolytic enzyme systems.

We identified the major amylases for starch utilization by *P. oxalicum*. Secretome analysis suggested that Amy13A and Amy15A accounted for nearly 40 % of total secreted proteins when the wild *P. oxalicum* strain 114-2 was cultivated on cellulose-wheat bran [13]. Our double-gene deletion and the followed amylase assays showed that these two amylases contributed to nearly all the amylase activities (Fig. 3a). As an α-amylase breaking starch in a random manner, Amy13A played an important role in starch degradation as revealed by hydrolysis halo analysis (Fig. 1), even though its content was much lower than the glucoamylase Amy15A. Deletion of *amy15A* remarkably reduced amylase activity, but hardly affected the growth rate of the strain on pure starch (Fig. 1).

Deletion of *amy15A* resulted in few extracellular proteins when the strain was cultivated on glucose or starch. This allowed the construction of the dual-function protein expression system. On starch, the overexpressed enzymes reached relatively high purities without contamination of other cellulases, which made the characterization of these enzymes easily. On cellulose, deletion of *amy15A* had no effect on cell growth and cellulase production. Thus, once an enzyme is expressed in the *amy15A*-deletion strain, it is possible to directly evaluate

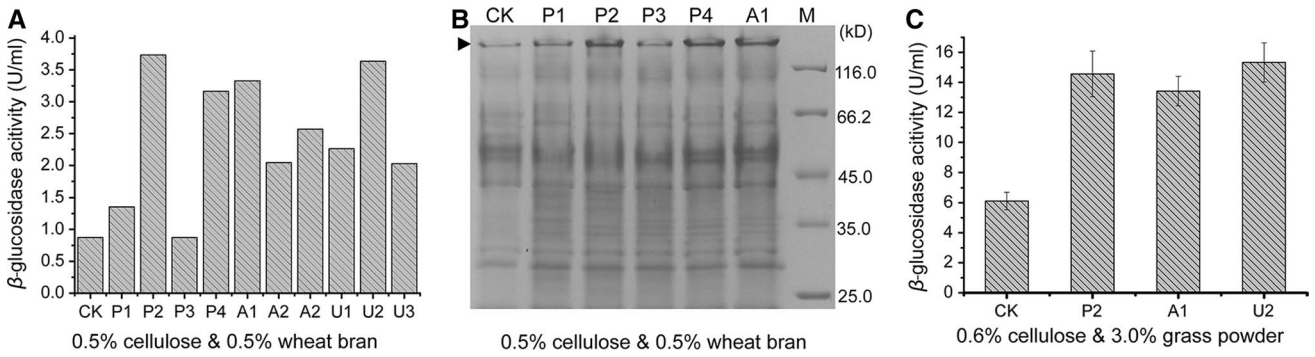


**Fig. 10** Overexpression of β-glucosidase with three strong promoters in peni-1. **a** β-glucosidase activities in low carbon source medium (0.5 % wheat bran plus 0.5 % cellulose). **b** SDS-PAGE of the extracellular proteins of selected transformants after Coomassie blue staining. 32 μL culture supernatant of each strain was loaded after cultivating in cellulose-wheat bran medium for 120 h. *Lane M* showed the molecular weight marker. *CK* represented the parent strain peni-1. *Triangular arrow* indicated the β-glucosidase BGL1. **c** β-glucosidase activities in high carbon source medium (3 % grass powder plus 0.6 % cellulose). *Error bars* indicated standard deviations of three independent cultivations

the performance of the new cellulase system without special protein purification and supplementation steps. This strategy, which constitutively expresses target proteins under both low-background and working conditions, may be applicable to the study and engineering of other complex enzyme systems.

The overexpressions of single enzymes have multiple effects on the production of lignocellulolytic enzyme systems. BGL4 and Cel12A overexpression resulted in increased specific FPA, suggesting the β-glucosidase and endoglucanase activity in the parent strain are not enough for efficient cellulose hydrolysis. As a xylan-degrading enzyme, the overexpression of Xyn10B improved volumetric FPA but not specific FPA. We speculate that Xyn10B overexpression may facilitate biomass formation (which could benefit to volumetric FPA production) due to release of more sugars from xylan-containing wheat bran during the cultivation.

Genes encoding lignocellulolytic enzymes are widely found in the genomes of filamentous fungi [14]. However, only a small set of these genes are highly expressed under the typical inducing conditions [7, 14]. Here, we proved all the three tested lowly expressed enzymes are active enzymes. Although the proteins secreted by strain Δ15A were not very high, they are enough for the characterization of their properties. As this protein expression platform has an appropriate protein glycosylation system and either produces near-pure proteins on starch or recombinant cellulase systems on cellulose, it is possible to use it to mine more lowly expressed fungal enzymes in a high-throughput manner in the future. Furthermore, the identification of three strong constitutive promoters allows the co-overexpression of several enzymes in one strain after a first round of single-enzyme expression and evaluation.

## Conclusion

We reported a convenient protein expression system in *P. oxalicum* based on a low-background host Δ15A and several constitutive promoters. By using different carbon sources to grow strains, this system can be used to obtain single enzymes with high purities, or to evaluate the effect of cellulase system supplementations. More homologous and heterologous proteins are expected to be expressed using this system in the future. Moreover, this system can be further improved for large-scale protein production with the advantage of using low-cost starch as the sole carbon source.

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