

# Rational Engineering A Xylanase Hyper-Producing System in *Trichoderma Reesei* for Efficient Biomass Degradation

Su Yan

Jiangnan University

Yan Xu

Jiangnan University

Xiao-Wei Yu (✉ [yuxw@jiangnan.edu.cn](mailto:yuxw@jiangnan.edu.cn))

Jiangnan University

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## Research

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# Abstract

**Background:** The degradation of lignocellulose needs the synergetic work of cellulase and xylanase. Filamentous fungi *Trichoderma reesei* has been widely used as a workhorse for cellulase and xylanase fermentation with great producing ability. Previous work for biomass saccharification mainly aims to improve the component and production of cellulase, however the low xylanase activity in *T. reesei* could not meet the needs of a high saccharification efficiency.

**Results:** In this study, for the first time, a xylanase hyper-producing system in *T. reesei* was established by tailoring two transcription factors, XYR1 and ACE1, and homologous over-expression of the major xylanase XYN $\alpha$ . The expression of key xylanolytic enzymes was significantly upregulated with an increase of 6.78- and 1.98-fold in the xylanase activity and pNPXase ( $\beta$ -xylosidase) activity compared to that of the parent, Rut-C30, respectively. Besides, 2310-3085 U/mL of xylanase activities were achieved using soluble lactose or glucose as sole carbon source, which was more efficient and economical than the traditional method of xylan induction. Treated with the crude xylanase cocktails as accessory enzymes, an increase of 39.7% in reducing sugar yield (31.3 mg/mL) in saccharification of alkali pretreated corn stover (5% w/v) was achieved compared to that of the parent.

**Conclusions:** An efficient and economical xylanase hyper-producing platform was developed in *T. reesei* Rut-C30. The novel platform with outstanding ability for crude xylanase cocktails production would greatly fit in biomass degradation and give a new perspective of further engineering in *T. reesei* for industrial purposes.

## Background

Renewable resource lignocellulose consists of cellulose (40%-60%) and hemicellulose (20–40%) [1], which shows significant importance for bioresource conversion and sustainable development. The biodegradation of lignocellulose for biofuels production needs the enzymatical hydrolysis that was mainly functioned by cellulase. However, hemicellulose (mostly xylan) could form a sheath on each cellulose microfibril in lignocellulose [2, 3] which makes a barrier for further hydrolysis [4]. Thus, the degradation of lignocellulose needs the synergetic work of cellulase and xylanase. Xylan is mainly composed of  $\alpha$ -xylose, which is linked with  $\beta$ -1,4-glycosidic bonds, and other  $\alpha$ -galactose and L-arabinose substituent groups, making a complex hetero-polymeric structure [1]. The biodegradation of xylan requires a complex enzyme cocktail that is commonly named xylanase [5]. Xylanase is a group of xylanolytic enzymes consisting of endo-xylanase,  $\beta$ -xylosidase,  $\alpha$ -L-arabinofuranosidase, acetyl xylan esterase and ferulic acid esterase, which decompose xylan into simple monosaccharide and xylo-oligosaccharides [1, 6], showing great potential in feed, biorefinery and pulp paper industry. Generally, the recombinant production of xylanase was achieved by overexpression of endo-xylanases, which shows great specificity to the backbone of xylan. While the insufficient cleave of xylobiose into xylose and the unthorough hydrolysis of the sub-chain group also hinder the thorough degradation of xylan [1], resulting

in low fermentable sugar for downstream biofuels production. Thus, xylanase cocktail exhibits more potential for efficient biomass degradation, compared with addition of single kind of xylanase.

Filamentous fungi *Trichoderma reesei* was widely used as a workhorse for cellulase and xylanase fermentation with great producing ability, and 80.6 g/L extracellular protein, mainly composed of cellulase and xylanase, was achieved in *T. reesei* through rational engineering [7]. It could secrete different kinds of xylanases under the induction of mono/polysaccharide. Moreover, the genomic and transcriptional [8, 9] data from *T. reesei* showed the expression of other xylanolytic enzymes that belonged to different families and exhibited different cleave specificity to xylan [10–13]. The main xylanases secreted under induction condition are XYN $\alpha$  and XYN $\beta$  which belong to GH11 family and account for 90% of the secreted xylanase. Among them, XYN $\beta$  showed higher catalytic activity and stability [14, 15], giving better performance in industrial applications. Traditionally, the production of xylanases in *T. reesei* was achieved using the xylan-based substrate as carbon source. However, the high cost of xylan and its insolubility make unsatisfied and non-economical xylanase production, hindering further industrial fermentation.

The induction and expression of xylanolytic genes in *T. reesei* were co-regulated through multiple transcription factors (TFs), indicating great potential for improving xylanase output with genetic engineering of transcription factors. The widely investigated TF was the negative regulator CRE1, which controlled carbon catabolite repression (CCR) and abolished the induction of cellulase and xylanase with the existence of easy-utilized carbon sources such as glucose [16]. In *T. reesei* Rut-C30, CRE1 was truncated and the xylanase production is partially derepressed. Moreover, the Gal4-like Zn(II)<sub>2</sub>Cys<sub>6</sub> type transcription regulator XYR1 served as a global TF that tightly control the induction of cellulase and xylanase through specific binding to a GGC(A/T)<sub>3</sub> motif in the promoter [17]. The constitutive expression of XYR1 could not only increase the transcription of several xylanase genes [18], but also release the xylanase production from CCR, indicating great potential for xylanase production on other economical carbon sources such as lactose and glucose. ACE1 serves as a negative factor for cellulase and xylanase production, deletion of *ace1* leads to an increase in cellulase and xylanase production [19]. ACE1 could also bind to the AGGCA motif in *cbh1* promoter, and participate in the regulation of *xyn1* with XYR1 [20]. Besides, XPP1 [21] and SxIR [22] were also shown as the negative TFs for a few xylanases (XYN1, XYN2, XYN5, BXL2), deletion of which would cause an increase in xylanase production.

The recombinant expression of xylanase in *T. reesei* has already been performed using different promoters including *Pxyn2*, *Pegl1*, *Pcbh2*, *Pcbh1* and *Ppdc*, and achieved different results [23–25]. Li *et al.* made the highest xylanase activity of 9266 U/mL through homologous expression of native XYNII under the strong pyruvate decarboxylase (*pdcc*) promoter with a high concentration of glucose (7%) [26]. However, the strategy to recombinant expression of XYNII in *T. reesei* QM9414 (a carbon catabolite repressed strain) only increased the endo-xylanase activity, further application in xylan degradation and other application needs the addition of other associated xylanolytic enzymes. Therefore, it is requisite to build a xylanase hyper-producing system that could produce different kinds of xylanases for efficient xylan and biomass degradation, and fit in other applications.

In this study, we developed a strategy that combined not only regulation of two transcription factors, XYR1 and ACE1, to obtain higher transcription of xylanolytic genes, but also homologous expression of the major xylanase XYNII under *cbh1* promoter in *T. reesei* for improved production of a xylanase cocktail. The xylanase hyper-producing strain could mainly produce endo-xylanase, associated with  $\beta$ -xylosidases and other xylanolytic enzymes for efficient xylan degradation and saccharification of biomass. Besides, the recombinant *T. reesei* could also achieve higher xylanase production in other economical carbon source such as lactose or glucose medium, suggesting a more economical-friendly and flexible strategy for xylanase production. Our work presented here would give new perspective for efficient biomass degradation and meet the increased demand for biofuel production.

## Results And Discussion

### Overexpression of XYNII under *cbh1* promoter results in elevated xylanase production

The strong inducing *cbh1* promoter has been widely used for recombinant protein expression in *T. reesei* [27, 28]. In this study, the major xylanase XYNII was expressed under *cbh1* promoter. The expression cassette was inserted into the chromosome randomly or site-directed to the *cbh1* locus (Fig. 1a) for the construction of the strains C30/*xyn2* and C30/*xyn2* $\Delta$ *cbh1* (Additional file 1: Table S1). The results showed that the strain C30/*xyn2* achieved 1734 U/mL xylanase activity after 5 days' induction, which was about 3.4-fold higher than that of the parent strain Rut-C30 under 2% Avicel induction. The highest xylanase activity of 2330 U/mL (Fig. 1c) was also achieved in C30/*xyn2* $\Delta$ *cbh1*, which was about 1.3-fold higher than that of C30/*xyn2*. The higher xylanase activity in C30/*xyn2* $\Delta$ *cbh1* was mainly contributed by the higher transcription rate in the *cbh1* locus [29]. Besides, the analysis of FPase activity in C30/*xyn2* $\Delta$ *cbh1* also showed a dramatic decrease caused by the deletion of the main cellulase CBHI (Fig. 1b, 1d), which would release the room for the recombinant protein expression [30]. For further study of the xylanase producing ability under repressing condition, the recombinant strains were cultured on 3% glucose. As shown in Fig. 1e, although the recombinant strains displayed a 16.3-fold increase in xylanase activity compared to that in Rut-C30, the xylanase outputs in all three strains were significantly repressed in the glucose medium, which was about 5% of that using Avicel as inducing carbon source. The results suggested that the recombinant *xyn2* expression cassette was also under the regulation of CCR through binding of CRE1 to the 'SYGGR' motif in *cbh1* promoter [16].

### Effect of transcription factors regulation on xylanase production

The global transcription factor XYR1 participated in the regulation of cellulase and xylanase production. Overexpression of XYR1 showed a significant increase in cellulase/xylanase production [31] combined with derepressed cellulase/xylanase production on repressing medium. In contrast, the deletion of *xyr1* would lead to despaired cellulase/xylanase production [32] and slower growth in xylose containing medium. In our study, transcription factor XYR1 was constitutively expressed under *gpd* promoter for moderate expression. Simultaneously, the negative TF ACE1 which was reported for competition with XYR1 [20] and negative regulation in the transcription of cellulase/xylanase [19] was also deleted through

insertion of *xyr1* expression cassette into *ace1* locus (Fig. 2a). The recombinant with correct insertion to *ace1* locus was confirmed by anchor PCR and named OExyr1 $\Delta$ ace1. We also constructed a recombinant strain OExyr1 that the *xyr1* expression cassette was inserted to the *ace1* locus by single-crossover homologous recombination, leaving an intact *ace1* in the chromosome (Fig. 2a) (Additional file 1: Table S1). The transformants both showed similar growth trend compared to the parent strain on the PDA plate. In this study, the copy number of *xyr1* expression cassette was verified to be single copy (data not shown), and the discrepancy of insertion sites was also excluded because the cassette was specifically inserted into the *ace1* locus.

Under 3% Avicel induction, the FPase and xylanase activities in OExyr1 were achieved at 7.17 U/mL and 1095 U/mL respectively, which was 56.2% and 62.4% higher than that of Rut-C30 (Fig. 2b, 2c). Moreover, for analyzing other enzymes that participated in xylan degradation,  $\beta$ -xylosidase (pNPXase) activity in OExyr1 was also analyzed and showed a 1.27-fold increase compared to that of the parent Rut-C30, which demonstrated the positive effect of XYR1 overexpression. However, for strain OExyr1 $\Delta$ ace1 which was absent in the negative TF ACE1, the xylanase activity of 1015 U/mL was achieved, which was a little lower than that in OExyr1. The analysis of FPase and pNPXase activities also showed similar trends (Fig. 2b, 2c, 2d). ACE1 was identified as a repressor for cellulase production [19], and its deletion can cause increased transcription on cellulase and xylanase related genes. Furthermore, ACE1 could specifically bind to the AGGCA sequence of the *cbh1* promoter [33]. Overexpression of recombinant endoglucanase EGI under *cbh1* promoter with *ace1* disruption made a 132.7% increase compared with the parent strain [34]. Moreover, higher xylanase and cellulase activities were achieved by the combination of *xyr1* overexpression and *ace1* suppression through RNAi compared with sole *xyr1* overexpression [18], which was opposite to our result. However, researches also demonstrated that ACE1 could compete with XYR1 in the binding of the GGCTAA motif, and both regulators might form a protein complex through regulating transcription of *xyn1* [20]. Thus, we speculated that in this study the constitutive expression of *xyr1* might disturb the competition between XYR1 and ACE1, and further deletion of *ace1* could hinder the interaction between XYR1 and ACE1 which would lead to decreased improvement. Our results give a new perspective that ACE1 might also be involved in the functional regulation of XYR1, but the mechanism still needs to be further investigated.

Moreover, to test whether the expression of xylanase in repression medium showed similar trends, OExyr1 and OExyr1 $\Delta$ ace1 were cultured in 5% glucose medium. The results demonstrated that cellulase and xylanase production was also released by constitutive overexpression of *xyr1*, while OExyr1 $\Delta$ ace1 still showed less output for cellulase and xylanase compared with OExyr1 (Fig 2e, 2f), which suggested the different role of the repressor ACE1 participated in transcriptional regulation.

### **Transcription level of xylanolytic genes in OExyr1 and OExyr1 $\Delta$ ace1**

To give a deep understanding of the attribution to elevated xylanase activity by *xyr1* overexpression and *ace1* disruption, the transcription level of the xylanolytic genes was investigated, including *xyn1* (Trire2:74223), *xyn2* (Trire2:123818) that encode for two major endo-xylanases, *xyn3* (Trire2:120229) that

encodes for a GH10 endo-xylanase, *xyn4* (Trire2:111849) encoding for GH30 xylanase harbored both exo- and endo-xylanase activity, *bxl1* (Trire2:121127) encoding for  $\beta$ -xylosidase, *axe1* (Trire2:73632) and *abf1* (Trire2:123283) that encode for acetyl xylan esterase and  $\alpha$ -L-arabinofuranosidase respectively which degrade side chain of xylan.

The results demonstrated that the transcription level of *xyr1* in OExyr1 and OExyr1 $\Delta$ ace1 was upregulated by 1.13- and 1.74-fold at 24 h, respectively, due to the overexpression of *xyr1*. XYR1 was indispensable for the induction of cellulase and xylanase, and could specially bind to the GGC(A/T)<sub>3</sub> motif in the promoter [35], which then activate the transcription of downstream genes. Several GGC(A/T)<sub>3</sub> motif has been found in the promoter of xylanolytic genes (Fig. 3a), and the transcription level of xylanolytic genes was also upregulated in OExyr1, especially for *xyn1*, which was increased by 3.84-fold compared to that in Rut-C30 at 48 h. *xyn1* was under the positive regulation of XYR1 by direct binding to an inverse repeat of GGCTAA [20], and ten GGC(A/T)<sub>3</sub> sites could be found in its promoter sequences (Fig. 3a), consistent with the dramatical increase in *xyn1* transcription. Besides, the transcription level of *xyn3*, *xyn4*, *bxl1*, *axe1* was upregulated at least 1.28- to 1.81-fold in OExyr1 at 48 h, suggesting a wide regulation of XYR1 and increased transcription in xylanolytic genes. Moreover, the transcription of *xyn2* which encodes for the major endo-xylanase was only increased by 0.56-fold at 48 h, probably due to the less XYR1 binding sites in its promoter. Mach-Aigner *et al.* also reported a less pronounced increase in the transcription level of *xyn2* compared to *xyn1* [36], which was in agreement with our data. Besides, the transcription of *abf1* was slightly increased in OExyr1, and only four XYR1 binding sites were present in its promoter, suggesting less regulation by XYR1 compared to other xylanolytic genes. For strain OExyr1 $\Delta$ ace1 that lacks intact *ace1*, the upregulated transcription level of *xyn1*, *bxl1* and *axe1* were detected both at 24 h and 48 h, which was consistent with the enzyme activity data (Fig. 2c, 2d). While the transcription level of *xyn2*, *xyn3*, *xyn4* and *abf1* was unchanged or downregulated compared to Rut-C30, probably because of loss of ACE1 function and the lower transcription level of *xyr1* in OExyr1 $\Delta$ ace1 at 48 h. ACE1 was generally recognized as a repressor for cellulase and xylanase, and could antagonize *xyr1* transcription [36] through direct binding to the promoter of *xyr1* [37]. Thus, after the deletion of *ace1*, the transcription level of *xyr1* at 24 h in OExyr1 $\Delta$ ace1 was 0.61-fold higher than that of OExyr1. However, the higher transcription of *xyr1* did not result in the upregulation of other xylanolytic genes in OExyr1 $\Delta$ ace1. It was assumed that ACE1 did not only serve as a repressor, and it might assist the functional regulation of XYR1 by forming a complex with XYR1 [20].

### **Combined strategy of transcriptional regulation and homologous expression of XYNII**

Transcriptional data showed an upregulation of xylanolytic genes in OExyr1 and OExyr1 $\Delta$ ace1, however, the transcription of *xyn2* was not significantly increased compared to other genes. It was reported that recombinant expression using *cbh1* promoter could be influenced by the elements lying on the promoter and inner transcription factors [38, 39]. For further enhancement of the xylanase output in *T. reesei*, a strategy that combined transcriptional regulation with homologous expression was conducted by overexpression of the major xylanase XYNII in OExyr1 and OExyr1 $\Delta$ ace1 recombinant strain. Similarly, the expression cassette was inserted randomly or site-directed to the *cbh1* locus, resulting in 4 recombinant

strains which were defined as OExyr1/xyn2, OExyr1/xyn2 $\Delta$ cbh1, OExyr1 $\Delta$ ace1/xyn2 and OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1, respectively (Additional file 1: Table S1).

Induced by 3% Avicel for four days, the highest xylanase activity of 5256 U/mL was achieved by OExyr1/xyn2 $\Delta$ cbh1, which was 6.79- and 1.93-fold higher compared to that of the parent strain Rut-C30 and C30/xyn2 $\Delta$ cbh1, respectively (Fig 4a). Moreover, the transcription level of *xyn2* in OExyr1/xyn2 $\Delta$ cbh1 and OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1 was higher than that in C30/xyn2 $\Delta$ cbh1 (Fig. 5a), contributed by the improved transcriptional strength of the *cbh1* promoter. For OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1, disrupted in *ace1*, the xylanase activity was slightly lower than that in OExyr1/xyn2 $\Delta$ cbh1, which was consistent with the decreased activity in OExyr1 $\Delta$ ace1 compared with OExyr1 (Fig. 2). Meanwhile, the deletion of *ace1* in OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1 did not cause a further increase in the transcription level of *xyn2* (Fig. 5a) that was in agreement with the decreased enzyme activities in Fig. 4a. The  $\beta$ -xylosidase (pNPXase) activities of different recombinants were also analyzed, and the highest pNPXase activity of 9.25 U/mL at day 4 was achieved in OExyr1/xyn2 $\Delta$ cbh1, which was about 1.98-fold higher than that of Rut-C30 (Fig. 4d). The results showed that all the strains with *xyr1* overexpression cassette give higher pNPXase, especially in the strains that *cbh1* was disrupted (Fig. 4d). It indicated that the recombinant strains harbored intact CBHI would suffer more serious endoplasmic reticulum stress, which further trigger a feedback mechanism called repression under secretion stress (RESS) that was exclusively present in filamentous fungi [40, 41], and the lower xylanase activity in C30/xyn2, OExyr1/xyn2 and OExyr1 $\Delta$ ace1/xyn2 might also be attributed to the negative effect of RESS. Moreover, the transcription analysis in OExyr1/xyn2 $\Delta$ cbh1 and OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1 also demonstrated the upregulation of other xylanolytic genes (Fig. 5b), which led to significantly improved xylanase activity and showed great potential for efficient xylan degradation.

### **Improved xylanase activity was achieved using lactose or glucose as the sole carbon source**

Lactose and glucose were easily utilized, soluble and economical carbon sources for industrial fermentation. In *T. reesei*, lactose has been shown to moderately induce production of cellulase and xylanase while glucose has been widely reported as a repressor since CCR. Therefore, the xylanase activities of the recombinant strains constructed above were also assessed using lactose and glucose as the sole carbon source respectively.

When lactose was used as sole carbon source, the highest xylanase activity reached 3085 U/mL in OExyr1/xyn2 $\Delta$ cbh1 after 4 days' fermentation, which was 0.38-, 2.67- and 28.38-fold higher than that in OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1, C30/xyn2 $\Delta$ cbh1 and Rut-C30, respectively (Fig. 4b). Besides, the xylanase activity of OExyr1/xyn2 $\Delta$ cbh1 on lactose at day 4 was higher than that C30/xyn2 $\Delta$ cbh1 produced on Avicel (Fig. 4a, 4b), showing great potential for large-scale fermentation on lactose. The pNPXase activity of 1.41 U/mL achieved in OExyr1/xyn2 $\Delta$ cbh1 was 0.36-, 2.71, 1.66-fold higher than that in OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1, C30/xyn2 $\Delta$ cbh1 and Rut-C30, respectively (Fig. 4e), which was also consistent with the increased transcription level of *bx11* (Fig. 5c). The transcriptional analysis of other xylanolytic

genes also showed an increase in OExyr1/xyn2 $\Delta$ cbh1 and OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1 on lactose (Fig. 5c), that together give an enhanced ability for xylan degradation.

When glucose was used as the sole carbon source, all the transformants with *xyr1* overexpression showed elevated xylanase output, suggesting the derepression of the transcription of xylanolytic genes. The highest xylanase and pNPXase activity of 2310 U/mL and 0.61 U/mL were achieved in OExyr1/xyn2 $\Delta$ cbh1 using 5% glucose as carbon source (Fig. 4c, 4f), respectively, which was less than that on lactose. The master repressor CRE1 would be activated and mediate CCR with the presence of high nutritional value carbon sources such as glucose, and it could repress the induction of cellulase and xylanase through binding to the 'SYGGRG' motif of promoters [16], which would cause less xylanase production compared to that on lactose medium. Moreover, CRE1 mediated XYR1 repression would further abolish induction on glucose, which influenced xylanase production. In our study, the recombinant XYR1 was constitutively expressed under the *gpd* promoter that was not repressed by CRE1, and activated XYR1 could further trigger xylanases expression and alleviate negative regulation by CRE1 on xylanolytic genes. Transcriptional data of OExyr1/xyn2 $\Delta$ cbh1 and OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1 on glucose also showed an increasing trend compared to Rut-C30 except for *abf1* (Fig. 5d), which was nearly 3- to 4-fold downregulated. Promoter sequence analysis revealed that fewer XYR1 binding sites (Fig. 3a) and several CRE1 recognition sites were presented (data not shown), showing a complicated regulation network for *abf1* on glucose.

Meanwhile, a recombinant strain C30/pdcxyn2 harboring a constitutively expressed *xyn2* cassette under *pdc* promoter in *T. reesei* Rut-C30 was constructed according to Li *et al.* [26]. The recombinant strain C30/pdcxyn2 exhibited about 1398 U/mL xylanase activity using 5% glucose as sole carbon source after 5 days' fermentation. While Li *et al.* reported a rapid increase of xylanase up to 9266 U/mL after 7 days' fermentation [26]. The obvious differences between these two experiments might be attributed to the different cultivation methods, insert locus and copy numbers, which has positive effects for the recombinant xylanase expression. In our study, the xylanase activity produced by OExyr1/xyn2 $\Delta$ cbh1 and OExyr1 $\Delta$ ace1/xyn2 $\Delta$ cbh1 was 0.65- and 0.33-fold higher than that in C30/pdcxyn2 on 5% glucose. And the highest xylanase activity of 5256 U/mL was achieved by OExyr1/xyn2 $\Delta$ cbh1 on Avicel inducing medium, which was approximately 2.76-fold higher than that of C30/pdcxyn2, associated with increased expression of other xylanolytic enzymes for thorough xylan degradation, indicating a potential ability for xylanase production in OExyr1/xyn2 $\Delta$ cbh1. Moreover, the slightly lower xylanase activity on soluble carbon source could be conquered through fed-batch fermentation due to the rapid consumption of glucose and lactose compared to Avicel, together with a rapid drop of pH below 4 (data not shown), that was not suitable for xylanase fermentation [42]. Controlling the concentration of carbon source and fermentation conditions in a bioreactor might further increase the yield of xylanase, showing the great potential for industry application.

**Better performance in saccharification of alkali pretreated corn stover with enzyme cocktails produced by recombinant strains**

The degradation of plant biomass needs the co-work of both cellulase and xylanase. Hu *et al.* have reported a synergistic interaction between xylanase and cellulase that significantly improved cellulose accessibility [4], showing great potential for biomass degradation with higher efficiency and yield. In our study, the saccharification of APCS using enzyme cocktails produced by different transformants were also tested, and equal FPU (4 FPU/g APCS) was loaded to the reaction (Additional file 1: Table S4). The results showed that 24.5 mg/mL reducing sugar was released with the enzyme supernatant produced by C30/xyn2 (Fig. 6), which was 9.4% higher compared to that in Rut-C30 (23.1 mg/mL) after 72 h saccharification. A similar result was also obtained with OExyr1/xyn2 and OExyr1 that the reducing sugar released by OExyr1/xyn2 was 8.2% higher than that of OExyr1 (Additional file 1: Fig. S1). Due to the absence of major cellulase CBHI in the xylanase hyper-producing strain OExyr1/xyn2 $\Delta$ cbh1, an enzyme mixture (Additional file 1: Table S4) composed of the supernatant of Rut-C30 and OExyr1/xyn2 $\Delta$ cbh1 with equal FPU was also conducted for APCS degradation. The highest reducing sugar of 31.3 mg/mL was achieved after 72 h with the ratio of 6:4 (Additional file 1: Table S4), which was 39.7% higher than that in Rut-C30. Ohgren *et al.* reported that the glucose and xylose released by adding xylanase were higher than that without xylanase addition [43], which was attributed to the increased accessibility caused by hydrolysis of xylan that crosslinked within nature lignocellulose [4]. Besides, our result showed great potential for saccharification using low FPU load with high reducing sugar yield. It also indicated that the improved output not only in endo-xylanase but also in  $\beta$ -xylosidase and other xylanolytic enzymes resulted in efficient and thorough degradation of biomass, which has great potential to be utilized in downstream biorefinery industry.

## Conclusion

A xylanase hyper-producing platform in *T. reesei* was established by constitutive expression of XYR1 and homologous expression of the native XYNII under the strong *cbh1* promoter. OExyr1/xyn2 $\Delta$ cbh1 exhibited improved xylanase producing ability using several carbon sources, 5256 U/mL xylanase activity and 9.26 U/mL pNPXase activity were achieved under Avicel induction, together with increased expression of several xylanolytic enzymes for efficient and complete xylan degradation. Unexpectedly, deletion of ACE1 on the strain OExyr1/xyn2 $\Delta$ cbh1 did not give any positive outputs, which might be the reason that it also disturbed the function of the complex formed between ACE1 and XYR1. Meanwhile, a 39.7% increase in APCS saccharification was also attained using xylanase cocktail produced by OExyr1/xyn2 $\Delta$ cbh1 as accessory enzymes. The novel platform with outstanding ability for crude xylanase cocktails production would greatly fit in biomass degradation and give a new perspective of further engineering in *T. reesei* for industrial purposes.

## Methods

### Strains and culture conditions

*Escherichia coli* TOP10, used as a cloning host for plasmid construction, was cultured at 37°C in Luria–Bertani (LB) medium. *Agrobacterium tumefaciens* AGL1 was used as a media for the transformation of *T.*

*reesei* and was cultured in LB medium or inducing medium (IM) at 28 °C according to purposes. *T. reesei* Rut-C30 (CICC 13052) was a parent strain that was kindly provided by Doctor Chengcheng Li [28]. *T. reesei*  $\Delta$ ura3 was a uracil-deficient phenotype of Rut-C30 which was constructed previously in our lab by homologous recombination at native *ura3* locus. All the *T. reesei* were cultured on potato dextrose agar (PDA) plate at 28 °C for conidiation. 10mM uracil was added when uracil-deficient *T. reesei*  $\Delta$ ura3 was cultured. For enzyme induction, 0.5 mL conidia suspension ( $10^7$ /mL) was inoculated into 20 mL Sabouraud Dextrose Broth (SDB) and incubated for 40 h at 28 °C with 200 rpm. After the accumulation of mycelia, the culture mixture was transferred into a 250 mL flask containing 50 mL of inducing medium with the inoculum rate of 10% (v/v). The flask was routinely cultured at 28 °C with constant shake of 200 rpm for 5-6 days. The Avicel inducing medium was prepared as described previously [22], and wheat bran was added for sufficient source. The glucose or lactose medium was similar to the Avicel inducing medium that Avicel and wheat bran were replaced by lactose and glucose respectively.

### Plasmid construction and fungal transformation

All the recombinant plasmid was constructed based on pCAMBIA1301G, which was derived from pCAMBIA1301 by replacing the promoter of hygromycin resistant gene from *CaMV 35S* to a shorten glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter of *Aspergillus nidulans* for constant transcription of the resistant gene in *T. reesei*.

For overexpressing major xylanase gene *xyn2* (Trire2:123818) of *T. reesei* under *cbh1* promoter, 700-bp *xyn2* coding sequence including signal peptide sequence was amplified from cDNA of Rut-C30 with primers *xyn2F/R* (Additional file 1: Table S2). The 1.3-kb promoter and 1.1-kb terminal sequence of *cbh1* were amplified from genomic DNA, and the resulting three sequences were ligated through overlap extension PCR using the primers *Pcbh1F/Tcbh1R*. The *xyn2* expression cassette was ligated to the *Kpn*I and *Sna*B sites of linearized pCAMBIA1301G by T4 ligase (NEB, England), resulting in pCbhxyn2 (Fig. 1a). pPdcxyn2 was constructed by changing the *cbh1* promoter with *pdc* promoter. To insert the expression cassette into *cbh1* locus, about 1.3-kb upstream of the *cbh1* promoter was cloned into pCbhxyn2, resulting in pCbhxyn2 $\Delta$ cbh1 (Fig. 1a).

For overexpression of *xyr1* (Trire2:122208) and deletion of *ace1* (Trire2:75418) in *T. reesei*, the *xyr1* coding sequence was amplified from genomic DNA of Rut-C30 with primers listed in Additional file 1: Table S2, and inserted into the *hyg* locus of pCAMBIA1301G resulting in a 3.8-kb *xyr1* overexpression cassette. Then the 1.3-kb upstream and downstream of *ace1* was amplified from the genomic DNA of Rut-C30, combined with the *xyr1* overexpression cassette and *ura3* cassette, cloned to the backbone of pCAMBIA1301G using MultiF SeamLess Assembly Mix (ABclonal, Wuhan, China), resulting in pOExyr1 $\Delta$ ace1. All the vectors constructed above were verified by PCR and sequencing.

The transformation of *T. reesei* was conducted by *Agrobacterium tumefaciens* mediated transformation (ATMT) as described previously [44]. The selection of transformants was using MM plate [45] for uracil-deficient  $\Delta$ ura3, and PDA containing 20 mg/L hygromycin for others. The putative transformants and

correct insert of the cassette were verified by PCR, single spore was isolated through successively streaking. The recombinant strains mentioned in this paper were listed in Additional file 1:Table S1.

## Biochemical analysis

Specifically, 1 mL mixture of shake flask fermentation was sampled every 24 h, the sample was centrifuged at 4 °C 12000 rpm for 10 min to remove hypha pellet, and the supernatant was transferred to another tube and filtered for further analysis. Cellulase and xylanase activity was determined by DNS method [14, 46] with a few modifications. In brief, 6.6 mg filter paper (Whatman NO.1) was added into 180 µL 50 mM sodium citrate buffer with pH of 4.8, then 20 µL enzyme solution diluted to appropriate concentration was added to the mixture and incubated at 50 °C for 60 min. The reaction was stopped by the addition of 300 µL alkaline 3,5-dinitrosalicylic (DNS) and boiled for 5 min, followed by immediate ice incubation. Then the mixture was diluted for 4-fold and the absorbance at 540 nm was detected for correction of FPase activities. For xylanase activity determination, 180 µL 1% oat spelt xylan (TCL, Japan) in 50 mM sodium citrate buffer with pH of 5.3 was mixed with 20 µL diluted enzyme and incubated for 5 min, the following step was similar with cellulase activity analysis. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol reducing sugar per minute. For β-xylosidase activity assay, 90 µL 4 mM pNPX in 50 mM sodium acetate (pH 5.0) was mixed with 20 µL appropriately diluted enzyme and incubated for 10 min at 50 °C. The reaction was stopped by the addition of 2% sodium carbonate, and then absorbance at 410 nm was detected for β-xylosidase (pNPXase) activity. SDS-PAGE analysis was performed on 9% Tris–HCl polyacrylamide gels using 3.75 µL supernatant of different *T. reesei* strains. All experiments were performed in three biological replicates.

## RNA extraction and RT-qPCR analysis

Culture mixtures were sampled at different time, mycelia were collected by centrifuged at 4 °C 12000 rpm for 10 min and washed twice with distilled water, then the pellet was rapidly frozen in liquid nitrogen and stored at -80 °C for further analysis. RNA extraction was conducted using RNAiso plus (Takara, Japan) according to the manual's instruction. The quality and quantity of extracted RNA were assessed by the absorbance at 260 nm on NanoDrop 8000 (Thermo Scientific, U.S.A.). Reverse transcription was performed using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan) with total RNA extracted from different transformants cultured in inducing medium. Quantitative real-time PCR (RT-qPCR) was conducted in 20 µL per tube with 2\* SG Fast qPCR Master Mix (High Rox) (Sangon Biotech, Shanghai, China) using primers listed in Additional file 1: Table S3, and the transcription level of *sar1* was used as inner standard. The PCR protocol run in the ABI StepOne instrument Plus (ABI, Germany) with software Version 2.3 (ABI, Germany) consisted of 3 min of initial denaturation at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. A melting curve was performed after each run to check the PCR product specificity. The data was calculated using  $(2^{-\Delta\Delta CT})$  for relative quantification and was represented with  $\log_2^{(\text{target strain/Rut-C30})}$ . All PCRs were carried out in triplicate on a plate.

## Saccharification of alkali pretreated corn stover

Milled corn stover was purchased from the country of Jiangsu (China), then treated with 2% NaOH at 121 °C for 30 min. The mixture was washed with distilled water until neutral pH, and dried at 60 °C for 24 h, then milled again for better utilization. The alkali pretreated corn stover (APCS) was stored at 4 °C for further study. For saccharification experiments, 50±0.5 mg APCS was mixed with culture supernatant after 5 days' induction as 4 FPU/g APCS, 0.25% sodium benzoate was also added to inhibit contamination, and finally making up to 1 mL with 50 mM sodium acetate (pH 5.0) in a 2 mL tube. The reaction was performed at 50 °C 200 rpm for 72 h. Samples were collected every 12 h and reducing sugar was analyzed by DNS method described above. All the experiments were conducted in three biological replicates.

## Abbreviations

TFs: transcription factors; CCR: carbon catabolite repression; SDB: Sabouraud Dextrose Broth; MM: minimal medium; RESS: repression under secretion stress; APCS: alkali pretreated corn stover; ATMT: *Agrobacterium tumefaciens* mediated transformation.

## Declarations

### Author' contributions

SY, YX and XWY designed the study. SY conducted the experiments. SY and XWY analyzed the data and wrote the paper. All authors read and approved the final manuscript.

### Acknowledgments

Not applicable.

### Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional file.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

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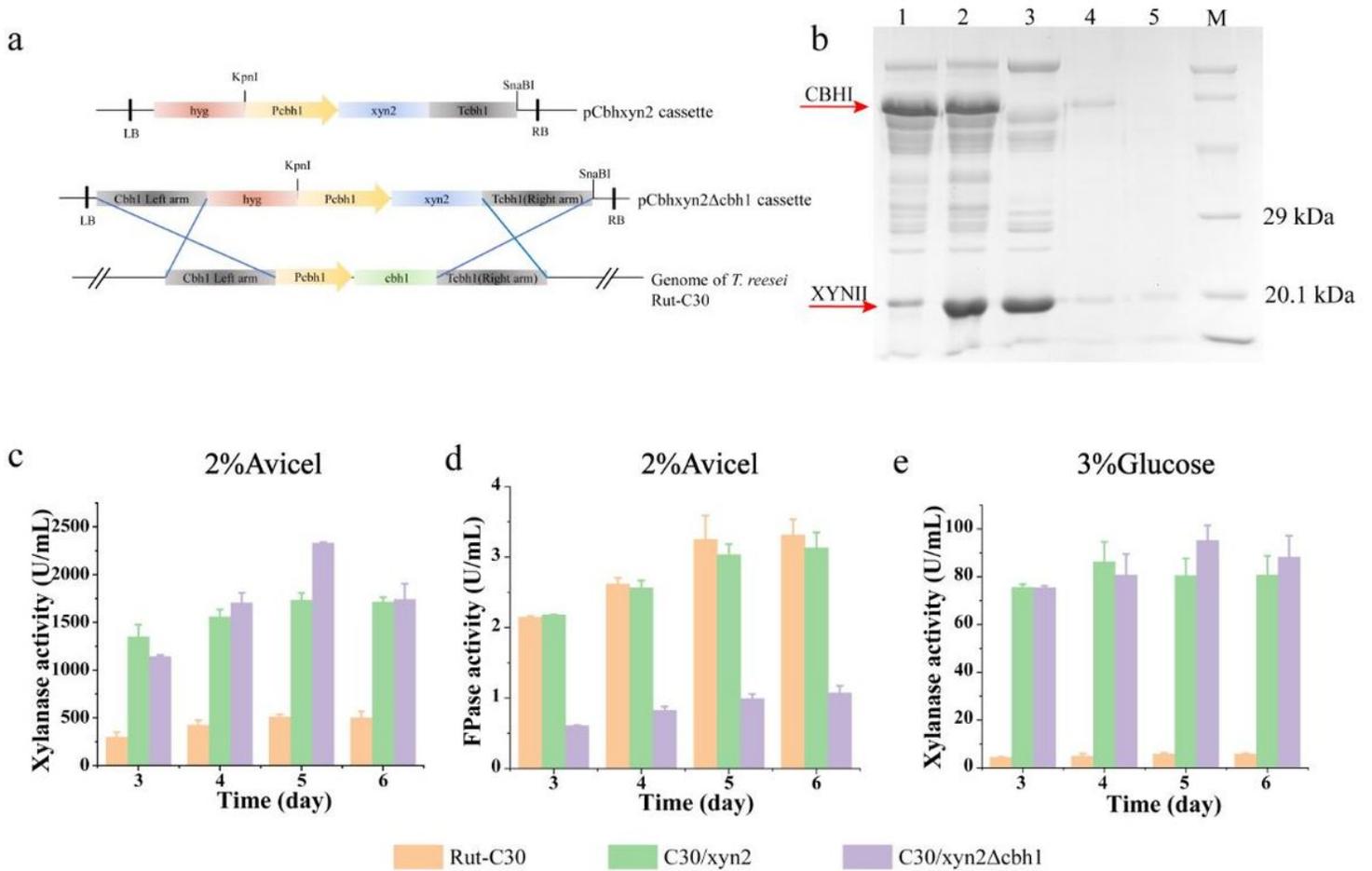
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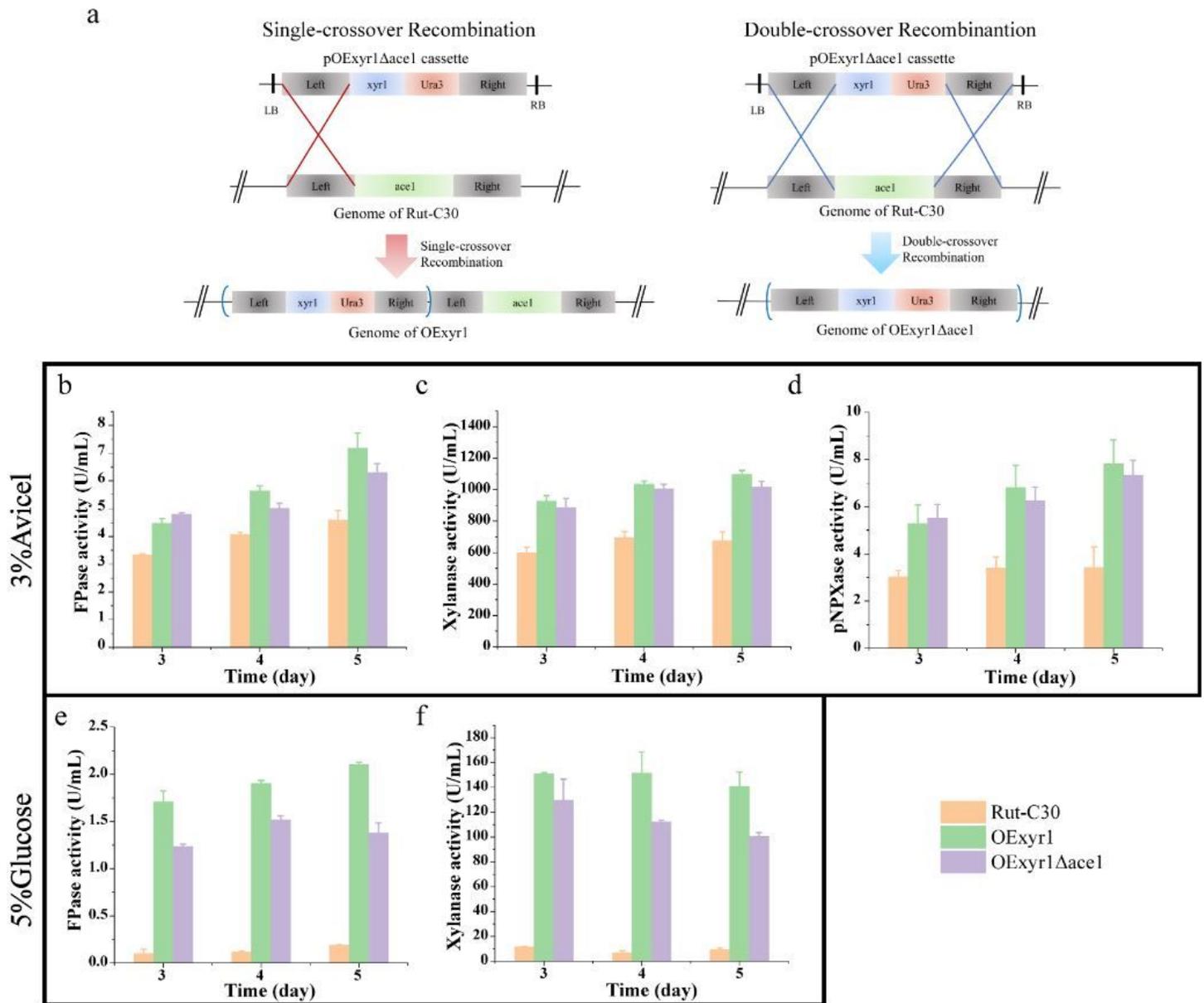
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## Figures



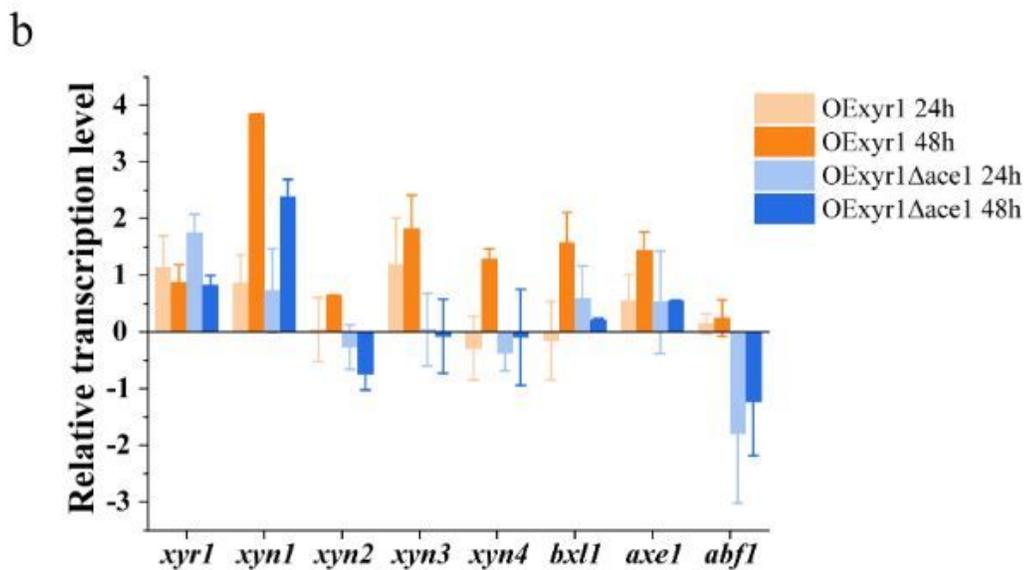
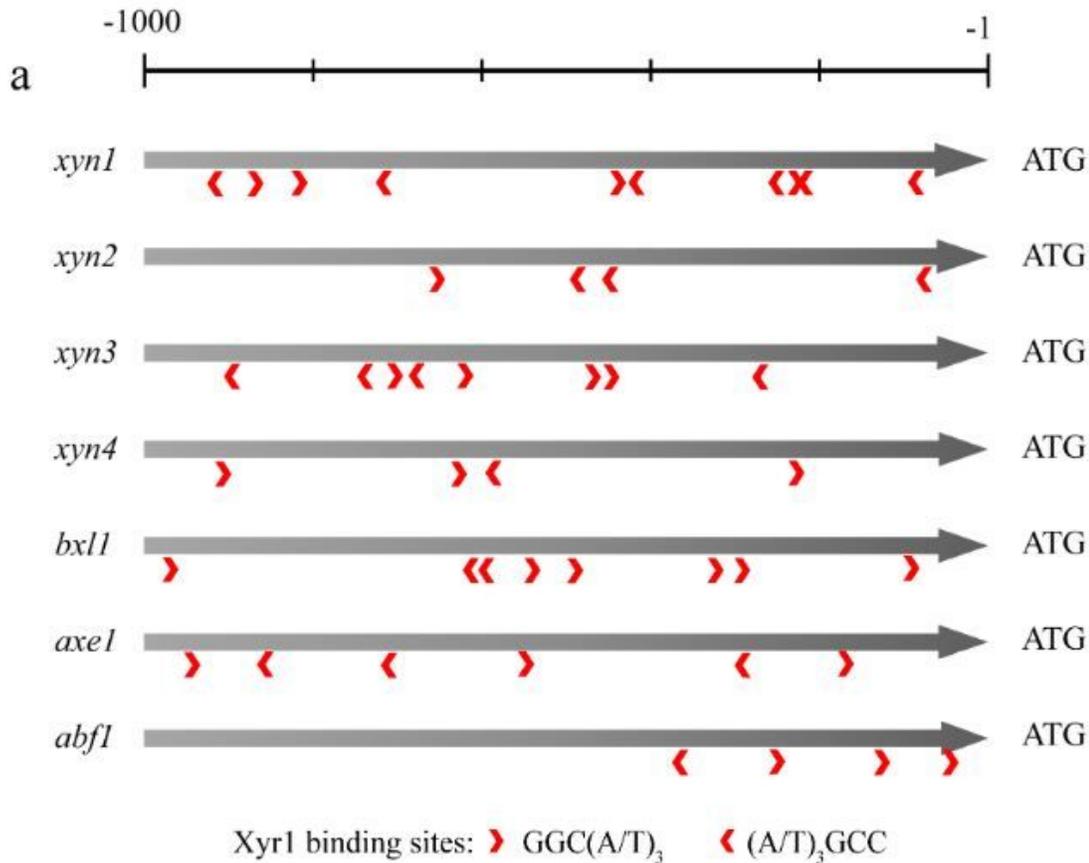
**Figure 1**

Overexpression of XYNII under *cbh1* promoter in *T. reesei*. a, an overview of the *xyn2* overexpression cassette constructed as described in materials and methods. b, SDS-PAGE analysis of the supernatant of Rut-C30, C30/*xyn2* and C30/*xyn2*Δ*cbh1* in Avicel or glucose medium. Lane 1-3: Rut-C30, C30/*xyn2* and C30/*xyn2*Δ*cbh1* in 2% Avicel medium; lane 4-5: C30/*xyn2* and C30/*xyn2*Δ*cbh1* in 3% glucose medium, equal volume of supernatant was loaded in each lane. c-e, xylanase and FPase analysis of Rut-C30, C30/*xyn2* and C30/*xyn2*Δ*cbh1* in Avicel or glucose medium.



**Figure 2**

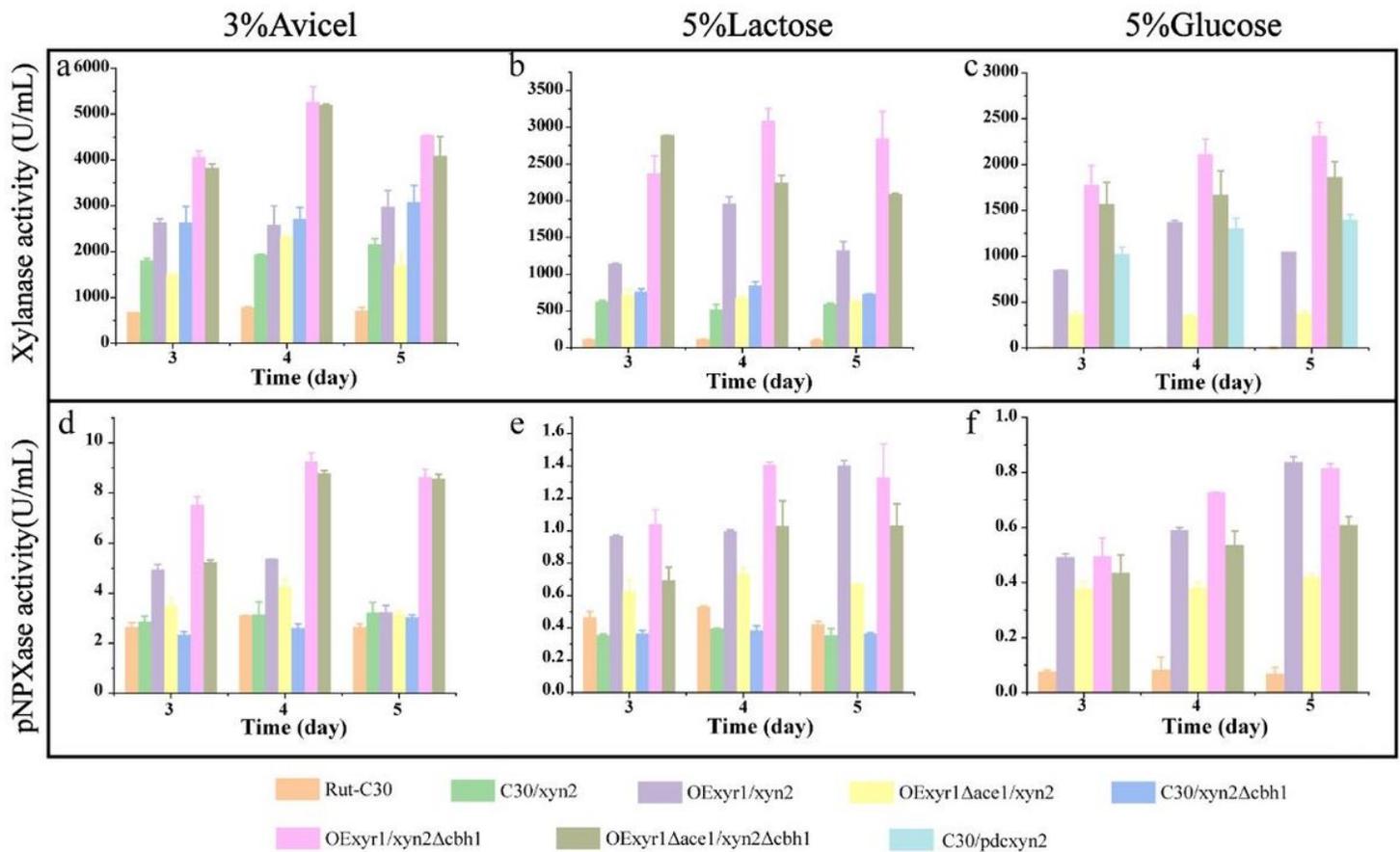
Effects of XYR1 and ACE1 on the FPase, xylanase and pNPXase activities in *T. reesei*. a, the construction of double crossover and single crossover recombinant strains described in materials and methods. b-d, the FPase, xylanase and pNPXase activities of the recombinant strains OExyr1 and OExyr1Δace1 in 3% Avicel medium respectively. e-f, the FPase and xylanase activities in 5% glucose medium.



**Figure 3**

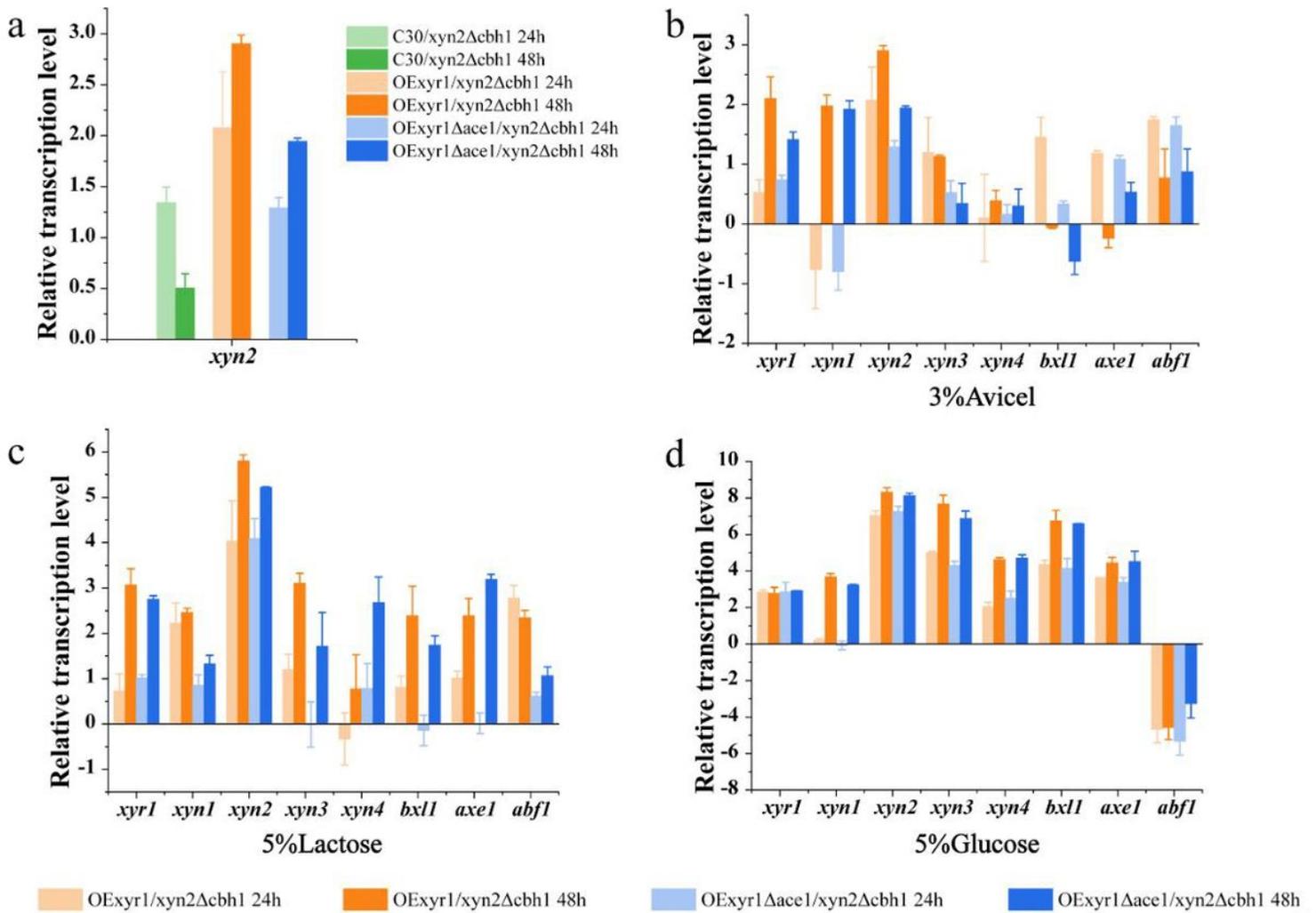
Transcription level of xylanolytic genes in OExyr1 and OExyr1Δace1. a, XYR1 binding sites in the promoter of xylanolytic genes, 1000 bp upstream of ATG was chosen for analysis. Red arrows indicate the direction of binding sites. The promoter sequence was acquired from the *T. reesei* genomic database <https://genome.jgi.doe.gov/Trire2/Trire2.home.html>. b, the relative transcription level of several xylanolytic genes. The data was represented with  $\log_2(\text{target strain}/\text{Rut-C30})$ . Rut-C30 was used as the

reference strain and the transcription level of *sar1* was used as an endogenous control. All the strains were cultured with 3% Avicel inducing medium, and the experiments were conducted with three biological repetitions.



**Figure 4**

The xylanase and pNPXase activities of the recombinant strains in different mediums. a,d, the xylanase and pNPXase activities of the recombinant strains cultured in 3% Avicel inducing medium. b,e, the xylanase and pNPXase activities of the recombinant strains cultured in 5% lactose medium. c,f, the xylanase and pNPXase activities of the recombinant strains cultured in 5% lactose medium.



**Figure 5**

Transcription level of xylanolytic genes in OExyr1/*xyn2*Δ*cbh1* and OExyr1Δ*ace1*/*xyn2*Δ*cbh1*. a, the relative transcription level of *xyn2* in recombinant strains. b-d, the relative transcription level of xylanolytic genes in OExyr1/*xyn2*Δ*cbh1* and OExyr1Δ*ace1*/*xyn2*Δ*cbh1* cultured on Avicel, lactose and glucose. The data was represented with  $\log_2(\text{target strain/Rut-C30})$ . The strains were all cultured in the corresponding medium and sampled at 24 h and 48 h respectively.

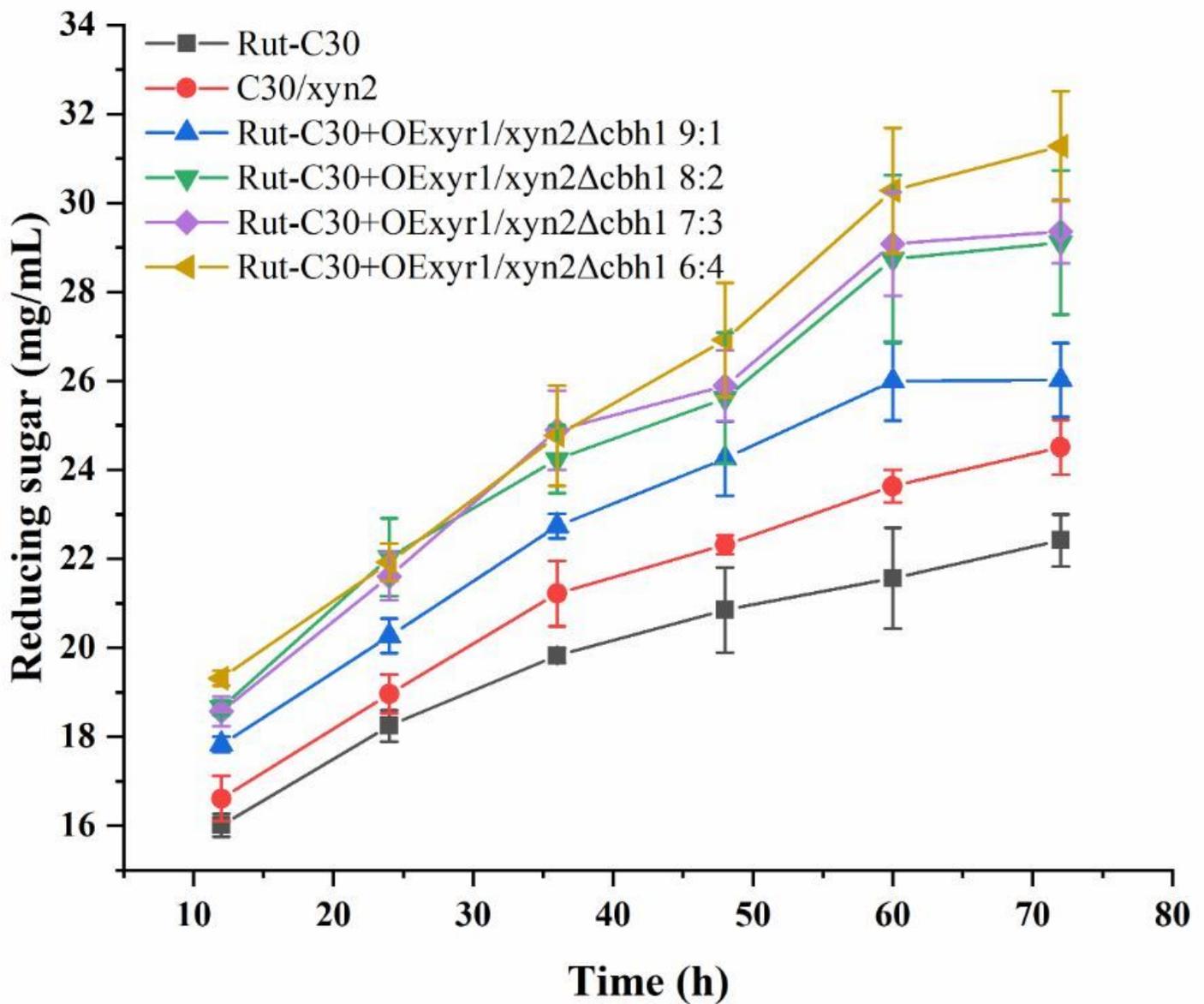


Figure 6

Saccharification of APCS with enzyme cocktails produced in xylanase hyper producing recombinants. The experiments were conducted as described in materials and methods. Three individual biological repeats were performed.

## Supplementary Files

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