

# Enhancing cellulase production in *Trichoderma reesei* RUT C30 through combined manipulation of activating and repressing genes

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**Abstract** To investigate whether enzyme production can be enhanced in the *Trichoderma reesei* industrial hyper-producer strain RUT C30 by manipulation of cellulase regulation, the positive regulator Xyr1 was constitutively expressed under the control of the strong *T. reesei* *pdC* promoter, resulting in significantly enhanced cellulase activity in the transformant during growth on cellulose. In addition, constitutive expression of *xyr1* combined with downregulation of the negative regulator encoding gene *ace1* further increased cellulase and xylanase activities. Compared with RUT C30, the resulting transformant exhibited 103, 114, and 134 % greater total secreted protein levels, filter paper activity, and CMCase activity, respectively. Surprisingly, strong increases in *xyr1* basal expression levels resulted in very high levels of CMCase activity during growth on glucose. These findings demonstrate the feasibility of improving cellulase production by modifying regulator expression, and suggest an attractive new single-step approach for increasing total cellulase productivity in *T. reesei*.

**Keywords** *Trichoderma reesei* · Cellulase · RNAi · Xyr1 · Ace1

## Introduction

*Trichoderma reesei* (anamorph of *Hypocrea jecorina*) is one of the most efficient producers of cellulolytic and xylanolytic enzymes used in a broad range of industrial applications, including the textile, food and feed, and pulp and paper industries [4]. Owing to increasing production demand for biofuels from lignocellulose, current research on *T. reesei* is focused on improving cellulase production, with the aim of reducing the relatively high cost of this process [7]. Strain improvement has previously been achieved using classical mutagenesis and screening procedures [18]. The availability of sophisticated gene manipulation methods and recent sequencing of the *T. reesei* genome [14] have raised the possibility of introducing molecular genetic methods into such improvement programs—for example, by upregulating activators and/or downregulating repressors of the cellulase genes. To accomplish this goal, however, a sound understanding of cellulase regulation is essential.

The major cellulase and hemicellulase genes in *T. reesei* are regulated in a coordinated manner by available carbon sources [8, 13]. Cellulose, lactose, and other materials induce expression of cellulase and hemicellulase genes, while glucose acts as a repressing carbon source. Several genes encoding regulators of cellulase and hemicellulase expression have been isolated. These regulators include the carbon catabolite repressor Cre1 [26], the repressor Ace1 [22], the activator Ace2 [2], the CCAAT binding complex Hap2/3/5 [30], and the activator Xyr1 [21]. In addition, the *T. reesei* protein methyltransferase LAE1 was recently

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shown to control cellulase gene expression [24]. The regulator Xyr1 is an ortholog of *Aspergillus niger* XlnR [31], a member of the zinc binuclear cluster protein family that binds with both the 5'-GGCTAA-3' motif and several common 5'-GGC(A/T)<sub>3</sub>-3' motifs found in the 5'-upstream region of Xyr1-regulated genes [5]. Xyr1 is a general and essential transcription factor controlling expression of the major cellulolytic and xylanolytic genes, regardless of inducer (xylose, xylobiose, sophorose, and lactose) and mode of expression (basal, derepression, and induction) [16, 27]. Mach-Aigner et al. [12] have constructed a recombinant strain expressing the *xyr1* gene under control of the *T. atroviride nag1* (*N*-acetyl- $\beta$ -D-glucosaminidase-encoding) promoter. In this strain, a slightly earlier beginning of xylanase formation and altered transcription profile of genes encoding for enzymes associated with degradation of (hemi)celluloses were observed [12, 20]. However, the promoter of *nag1* they used is not much stronger than the native *xyr1* promoter, and thus the final enzyme activity in this strain was not significantly enhanced [10]. On the other hand, Seiboth et al. [24] overexpressed the *xyr1* gene under the control of the constitutive *pki1* promoter in QM9414, which resulted in increased cellulase formation. However, the efficiency of the *pki1* promoter is still low compared with strong constitutive promoters, such as the *T. reesei pdc* gene (encoding pyruvate decarboxylase), which was recently identified and used to generate very high expression levels of recombinant xylanase II [11]. It is unknown whether an increase in constitutive expression of *xyr1* could further improve enzyme production in and industrial production strain improved greatly by mutagenesis and screening programs.

The mutant strain *T. reesei* RUT C30, developed using several rounds of random mutagenesis, exhibits outstanding levels of protein secretion and high cellulolytic activity [18]. In our study, constitutive *xyr1* expression under the control of the strong constitutive *pdc* promoter was introduced into RUT C30 to investigate whether increased *xyr1* expression can enhance enzyme production in this industrial production strain. To further improve its cellulase production ability, *xyr1* overexpression and RNAi-mediated gene silencing of the cellulase repressor-encoding gene *ace1* were simultaneously introduced into RUT C30.

## Materials and methods

### Strains and culture conditions

*Escherichia coli* Top10F' (Invitrogen, Carlsbad, CA, USA) was used as the host strain for recombinant plasmid construction. The hypercellulolytic mutant strain *T. reesei* RUT C30 (ATCC 56756), used as a parent strain

throughout this study, was maintained on potato dextrose agar (PDA). To study cellulase and xylanase gene expression in response to different carbon sources, mycelia were precultured on a rotary shaker (220 rpm) at 28 °C for 48 h in 250-ml Erlenmeyer flasks containing 50 ml of MM medium [8] with 1 % (w/v) glycerol as a carbon source. Conidia at a final concentration of  $1 \times 10^8$  l<sup>-1</sup> were used as inoculum. Pre-grown mycelia were washed, and equal amounts were then resuspended in MM medium containing either 2 % (w/v) glycerol, glucose, or cellulose (Avicel PH-105, FMC BioPolymer, Philadelphia, PA, USA) as carbon sources. Incubation was continued for 8 and 48 h; mycelia were then harvested by filtration, washed twice with distilled water, and used for RNA extraction.

### Construction of the Ppdc-xyr1-Tpdc cassette

Promoter and terminator regions of the *pdc* gene were acquired from the *T. reesei* genome sequence (<http://genome.jgi-psf.org/Trire2/Trire2.home.html>). The *pdc* promoter (Ppdc, 1305 bp, scaffold 8:106110–107414 bp), the *pdc* terminator (Tpdc, 721 bp, scaffold 8:103465–104185 bp), and the *xyr1* gene (GenBank accession no. AF479644) were PCR-amplified from *T. reesei* QM9414 genomic DNA using primers Ppdc-F and Ppdc-R, Tpdc-F and Tpdc-R, and *xyr*-F and *xyr*-R (Table 1), respectively. Amplified Ppdc, *xyr1*, and Tpdc fragments were fused by overlap extension PCR using primers Ppdc-F and Tpdc-R, generating a *xyr1* expression cassette. This expression cassette was cloned into a pMD 18-T Vector (Takara, Dalian, China), yielding the plasmid pPDC-XYR (Fig. 1a). This plasmid was inserted into the genome of *T. reesei* RUT C30 for constitutive expression of the *xyr1* gene.

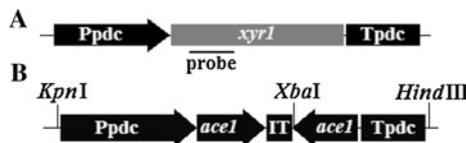
### Construction of the RNAi vector

The RNAi vector for silencing the *T. reesei ace1* gene consisted primarily of an expression cassette for an intron-harboring hairpin RNA (ihpRNA) (Fig. 1b). The *pdc* promoter (Ppdc), the *pdc* terminator (Tpdc), two 507-bp *ace1* fragments (*ace1*<sub>sense</sub> and *ace1*<sub>anti</sub>) covering the *ace1* open reading frame from 1,780 to 2,286 bp, and a 249-bp spacer fragment (IT), which included intron 2 of the *T. reesei eg2* gene were PCR-amplified using *T. reesei* QM9414 genomic DNA as a template. Primers used, and their targets, were as follows: Ppa-F and Ppa-R (for amplification of Ppdc, adding a *KpnI* site to the 5' end), Tpa-F and Tpa-R (for Tpdc amplification, adding a *HindIII* site to the 3' end), ace-F and ace-R (for *ace1*<sub>sense</sub> amplification), acea-F and acea-R (for *ace1*<sub>anti</sub> amplification, adding an *XbaI* site to the 5' end), and it-F and it-R (for IT amplification, adding an *XbaI* site to the 3' end). The amplified Ppdc, *ace1*<sub>sense</sub>, and IT fragments were fused by overlap extension PCR using

**Table 1** Primers used for construction of the *xyl1* expression cassette and RNAi vector

Primers	Sequences (5'–3') <sup>a</sup>	Notes
Ppdc-F	ATGAAAGGAGGGAGCATTCTTCGAC	For construction of the <i>xyl1</i> expression cassette
Ppdc-R	<u>AGCGACGGAGAGGATTGGACAACATGATTGTGCTGTAGCTGCGCTGCTTT</u>	
xyr-F	<u>AAAGCAGCGCAGCTACAGACAATCATGTTGTCCAATCCTCTCCGTCGCT</u>	
xyr-R	<u>CTACCCGGTCAGACTTCATGCCGGGTTAGAGGGCCAGACCGTTCCGTTA</u>	
Tpdc-F	<u>TAACGGAACCGGTCTGGCCCTCTAA</u> CCCGGCATGAAGTCTGACCGG	For construction of the RNAi vector for silencing the <i>ace1</i> gene
Tpdc-R	TTGCCTTTTGAGCTGGCGTCTT	
Ppa-F	CGGGGTACCATGAAAGGAGGGAGCATTCTTCGAC	
Ppa-R	<u>CCGTAGGTAGCGAGCCAATCGATTGTGCTGTAGCTGCGCTGCTTT</u>	
aces-F	<u>CAGCGCAGCTACAGACAATCGATTGGCTCGCTACCTACGG</u>	
aces-R	<u>TACAGCCAAAGTCAAAAACCCGTTGAAGATGTCGGGCTGTG</u>	
it-F	<u>CACAGCCCGACATCTTCAACGGGTTTTGACTTTGGCTGTA</u>	
it-R	CTAGTCTAGAGTTCTTCAACGGAGGATAAA	
acea-F	CTAGTCTAGAGTTGAAGATGTCGGGCTGTG	
acea-R	<u>CCGGTCAGACTTCATGCCGGGATTGGCTCGCTACCTACGG</u>	
Tpa-F	<u>CCGTAGGTAGCGAGCCAATCCCGGCATGAAGTCTGACCGG</u>	
Tpa-R	ACCCAAGCTTTTGCCTTTTGAGCTGGCGTCTT	

<sup>a</sup> Restriction sites are *underlined*; overlapping regions in primers are *double underlined*



**Fig. 1** Schematic representation of the *xyl1* expression cassette (a) and RNAi vector (b). Ppdc: *Trichoderma reesei* pdc promoter; *ace1* (right arrow): 507-bp *ace1* coding sequence; IT: 249-bp spacer fragment including intron 2 of the *T. reesei* *eg2* gene; *ace1* (left arrow): antisense strand of the 507-bp *ace1* coding sequence; Tpdc: *T. reesei* pdc terminator. The line indicates the probe for Southern blot

primers Pp-F and it-R, and the resulting fusion fragment was inserted into pUC19 using the above-mentioned restriction sites, generating the recombinant plasmid pPpdc-aces-IT. Amplified *ace1*<sub>anti</sub> and Tpdc fragments were also fused by overlap extension PCR using primers acea-F and Tp-R. The fusion fragment was inserted into plasmid pPpdc-aces-IT, generating the RNAi plasmid pPDC-ACEi.

**Fungal transformations**

Protoplast transformation of *T. reesei* RUT C30 was performed as previously described [17] with the following modifications. Lysing enzymes (10 mg ml<sup>-1</sup>) from *T. harzianum* (Sigma-Aldrich, Brøndby, Denmark, Cat. No. L1412) in 1 mol l<sup>-1</sup> MgSO<sub>4</sub> were used for protoplast formation. For overexpression of *xyl1*, *T. reesei* RUT C30 was co-transformed with equal amounts of plasmids (5 μg each) pPDC-XYR and pAN7.1. To combine *xyl1* overexpression and *ace1* downregulation, *T. reesei* RUT C30 was

co-transformed with equal amounts of plasmids (5 μg each) pPDC-XYR, pPDC-ACEi, and pAN7.1. The plasmid pAN7.1 was used as an assisting plasmid to confer hygromycin B resistance on the transformants. The transformants were selected on PDA plates containing 100 μg ml<sup>-1</sup> hygromycin B. The conidia of the candidate transformants were further spread onto hygromycin B selection plates to form single colonies. The single colonies were screened by genomic DNA PCR analysis to verify that *xyl1* and/or *ihpRNA* expression cassettes were integrated into the genome of the transformants.

**Southern-blot analysis of transformants**

Fungal genomic DNA was isolated with a fungal DNA extraction kit (Sangon, Shanghai, China). Southern hybridization and detection was carried out with the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Basel, Switzerland). To determine the copy number of the *xyl1* expression cassettes, the genomic DNA (5 μg) of PX41, PXAi20 was digested with *EcoRI*. The 581-bp probe for the *xyl1* gene was amplified with the primers S-XYR-F (TCCAGGATGGTCCAGAGG) and S-XYR-R (ATCGCACGCCAAAGACA), using plasmid pPDC-XYR as template.

**Nucleic acid isolation**

Mycelia in a liquid culture were harvested by centrifugation, frozen in liquid nitrogen, and stored at -86 °C.

Genomic DNA was extracted as previously described [23]. Total RNA was isolated from frozen mycelia using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To remove genomic DNA, RNA extracts were treated with DNase I (Fermentas, Vilnius, Lithuania). The quantity and quality of extracted RNA was assessed on a GeneQuant 1300 spectrophotometer (Biochrom, Cambridge, UK) and by agarose gel electrophoresis.

#### Quantitative reverse-transcription PCR

Approximately 500 ng of total RNA was subjected to reverse transcription using an RT-PCR kit (Takara, Dalian, China), which contained a blend of oligo(dT) and random hexamer primers. Quantitative PCR (qPCR) was performed in an ABI Prim 7300 System (Applied Biosystems, Carlsbad, CA, USA). Each 20- $\mu$ l reaction volume contained 2  $\mu$ l of template (1:60 dilution of the reverse transcriptase (RT) reaction product), 10  $\mu$ l of 2  $\times$  SYBR Premix Ex *Taq* (Takara), 300 nmol l<sup>-1</sup> forward and reverse primers (Table 2), and nuclease-free water. The PCR protocol consisted of 30 s of initial denaturation at 95 °C, followed by 40 cycles of 5 s at 95 °C and 31 s at 60 °C. A melting curve analysis was performed after each run to check PCR product specificity. Primers were designed so that the amplicon length was 100–200 bp and complementary sequences within primers and mismatches were avoided. In addition, half of the primer hybridized to the 3' end of one exon and the other half to the 5' end of the adjacent exon, or, alternatively, the primer flanked a region containing at least one intron.

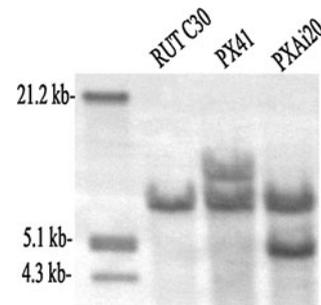
All PCRs were carried out in triplicate within a plate. The data, obtained using an ABI Prism 7300 Sequence Detection System, were analyzed according to the standard curve method for relative quantization (Applied Biosystems User Bulletin #2). Gene expression levels in the different samples were determined using a standard curve and normalized with an endogenous control, *sar1*, as described previously [25]. Means  $\pm$  standard deviations of replicates are shown in Figs. 2, 4, and 5. To allow better comparisons, for each transcript, the lowest ratio measured among strains and conditions was set to 1, and all other ratios were related to it.

#### Enzyme activity assays

Total soluble protein concentration in the culture supernatant was assayed using Bradford reagent (Sangon Biotech, Shanghai, China). Xylanase activity was measured following [3], using birch xylan (Sigma-Aldrich, Brøndby, Denmark) as the substrate. Filter paper activity (FPA) and

**Table 2** qPCR primers used in this study

Primers	Sequences (5'–3')
<i>sar1</i> -F	TGGATCGTCAACTGGTCTACGA
<i>sar1</i> -R	GCATGTGTAGCAACGTGGTCTTT
<i>ace1</i> -F	GGACGAGGAGGAGATTATG
<i>ace1</i> -R	GTGAGTCTTCTCGTGCTT
<i>xyr1</i> -F	CCATCAACCTTCTAGACGAC
<i>xyr1</i> -R	AACCTGCAGGAGATAGAC
<i>cbh1</i> -F	CCGAGCTTGGTAGTTACTCTG
<i>cbh1</i> -R	GGTAGCCTTCTTGAACAGT
<i>egl1</i> -F	CGGCTACAAAAGCTACTACG
<i>egl1</i> -R	CTGGTACTTGCGGGTGAT
<i>bg1</i> -F	AGTGACAGCTTCAGCGAG
<i>bg1</i> -R	GGAGAGGCGTGAGTAGTTG
<i>xyn2</i> -F	CCGTCAACTGGTCCAAC
<i>xyn2</i> -R	ACACGGAGAGGTAGCTGTT



**Fig. 2** Southern-blot analysis of the transformants PX41, PXAi20. The genomic DNA (5  $\mu$ g) was digested with *EcoRI* and hybridized with the amplified 581-bp probe for the *xyr1* gene. PX41: recombinant strain constitutively expressing *xyr1* under the control of the *pdv* promoter; PXAi20: recombinant strain with both *xyr1* overexpression and *ace1* downregulation

carboxymethyl cellulase assay for  $\beta$ -1,4-endo-glucanase activity were measured according to the method of Ghose [6]. One unit of enzyme activity was defined as the amount of enzyme releasing 1  $\mu$ mol of product per minute.

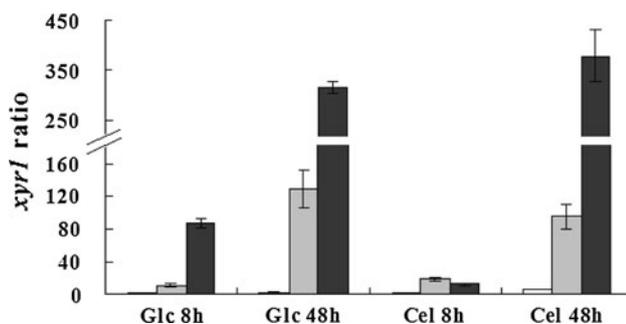
## Results and Discussion

#### Construction of a strain with *xyr1* overexpression

Xyr1 is an essential regulatory protein responsible for activation of major cellulolytic and xylanolytic enzymes in *T. reesei* [27]. Recently, Portnoy et al. [19] observed greatly increased *xyr1* basal expression, compared with strain QM9414, in the industrial hyperproducer strain CL847. Regulation of *xyr1* expression is thus known to have a significant impact on the ability of *T. reesei* to

produce cellulose- and hemicellulose-degrading enzymes. To produce the regulator Xyr1 in *T. reesei* under the control of the strong constitutive promoter Ppdc, the cassette Ppdc-*xyr1*-Tpdc was constructed by linking the coding region of the *xyr1* gene to 1.3 kb of the *pdc* promoter and 0.7 kb of the *pdc* gene 3' flanking region comprising the transcription terminator. The expression cassette was co-transformed into *T. reesei* RUT C30 with plasmid pAN7.1. Upon screening of 40 randomly selected transformants by PCR, 27 showed successful integration of the *xyr1* expression cassette. According to qPCR analysis, the *xyr1* expression levels in five randomly selected transformants carrying the *xyr1* expression cassette were much higher than those in RUT C30 (data not shown). One of them, the transformant PX41 with the highest *xyr1* expression levels, was selected for the following studies. Southern-blot analysis showed that PX41 contained a single-copy insert of the *xyr1* expression cassette (Fig. 2).

In agreement with previous data [19], *xyr1* expression in *T. reesei* RUT C30 was induced by cellulose (Fig. 3) in addition to lactose. Relative transcript abundance of *xyr1* in the transformant PX41 was very high as early as 8 h after transfer into glucose or cellulose medium, whereas *xyr1* expression was relatively low in the parent strain RUT C30 (Fig. 3). After 48 h, differences in *xyr1* transcript abundance were even more profound. The relative transcript abundance of *xyr1* in PX41 compared with RUT C30 was 48- or 16-fold higher in glucose or cellulose cultivations, respectively. These observations indicate that strongly elevated *xyr1* expression levels were obtained in PX41 using the strong promoter Ppdc under both catabolite repressing and inducing conditions.



**Fig. 3** Expression ratio of *xyr1* to steady-state *sar1* reference gene in strains RUT C30 (open bars), PX41 (shaded bars), and PXAi20 (filled bars) pre-grown with glycerol as a carbon source and then transferred into glucose (Glc) or cellulose (Cel) medium for 8 or 48 h. RUT C30: parental strain; PX41: recombinant strain constitutively expressing *xyr1* under the control of the *pdc* (encoding pyruvate decarboxylase) promoter; PXAi20: recombinant strain with both *xyr1* over-expression and *ace1* downregulation. Error bars indicate standard deviations

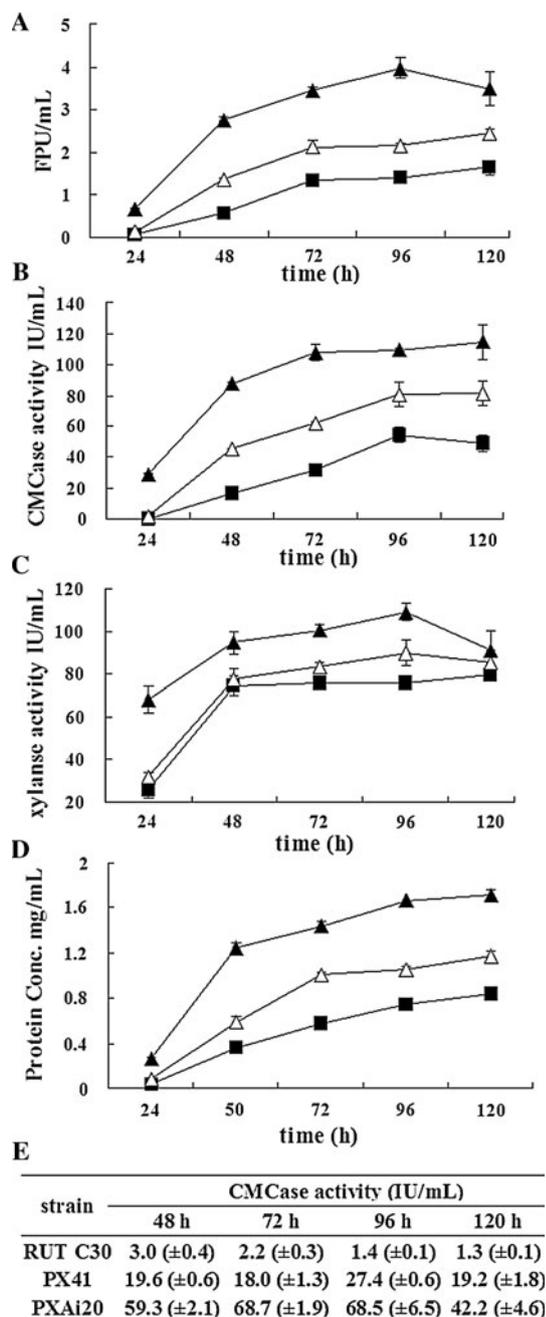
*xyr1* overexpression leads to significant increases in enzyme production

To investigate the effects of *xyr1* overexpression on cellulase expression and protein production, cellulase and xylanase activity and amounts of extracellular protein produced by the *xyr1*-overexpressing transformant (PX41) and the parent strain were measured from culture supernatants under inducing conditions. In addition, the effects of *xyr1* overexpression on cellulase production were studied in glucose-based cultivations.

During growth on cellulose, PX41 secreted larger amounts of total proteins than did strain RUT C30 (Fig. 4d). In addition, clearly higher FPA and CMCCase activity were observed from cultures of PX41 than from cultures of RUT C30 (Fig. 4a, b). After 5 days of cultivation, total secreted protein amounts, FPA, and CMCCase activity of transformant PX41 were 64, 55, and 107 % higher, respectively, than those of the parent strain; this indicates that increased basal expression of *xyr1* in RUT C30 under the control of the strong constitutive promoter Ppdc leads to significant enhancement of cellulase activity and protein production. Xylanase activity in cellulose cultivations was not increased significantly in PX41 compared with RUT C30, however (Fig. 4c). Very high levels of CMCCase activity were detected in PX41 in glucose-based cultivations, while only very minute levels of CMCCase activity were observed in RUT C30 (Fig. 4e). This is surprising because RUT C30 carries a truncated mutant of the *cre1* gene encoding a carbon catabolite repressor, thus losing the repression mechanism [9].

To determine whether expression of primary cellulase genes was indeed elevated in PX41 and to study the role of Xyr1 in cellulase and xylanase expression in the presence of different carbon sources, strains PX41 and RUT C30 were pre-grown on glycerol and then transferred into liquid minimal media containing a different carbohydrate—either glycerol, glucose, or cellulose—as the sole carbon source. mRNA levels of the major cellulase genes (*cbh1*, *egl1*, and *bgl1*) and one xylanase (*xyn2*) were monitored by qPCR of samples collected 8 and 48 h after transfer.

In liquid culture media containing cellulose as the sole carbon source, cellulase genes in PX41 were highly expressed as early as 8 h after transfer into the inducing culture medium, whereas in the parent strain the expression of cellulase genes was barely detectable (Fig. 5c). This suggests that the cellulase genes were induced earlier and at a higher level owing to increased *xyr1* basal expression. These data correlate well with enzyme activity measurements. Interestingly, *xyn2* was induced earlier and to a higher level of expression in PX41 than in RUT C30 during growth on cellulose, but at later times expression levels were almost identical in the two strains. This result is



**Fig. 4** Enzyme activities and extracellular protein production at different times in strains RUT C30 (filled square), PX41 (open triangle), and PXAi20 (filled triangle) pre-grown with glycerol as a carbon source and then transferred to cellulose (a–d) or glucose (e). Filter paper (FPA), CMCCase, and xylanase activities were measured using filter paper, sodium carboxymethyl cellulose (CMC-Na), and birch xylan as substrates, respectively. **a** FPA; **b** CMCCase activity; **c** xylanase activity; **d** extracellular protein production; **e** CMCCase activity in glucose-based cultivation. Averages from three parallel cultivations of each strain are shown with standard deviations

congruent with xylanase activity measurements from cellulose cultures (Fig. 4c). The observed change in the *xyn2* transcription profile over time may be due to the fact that,

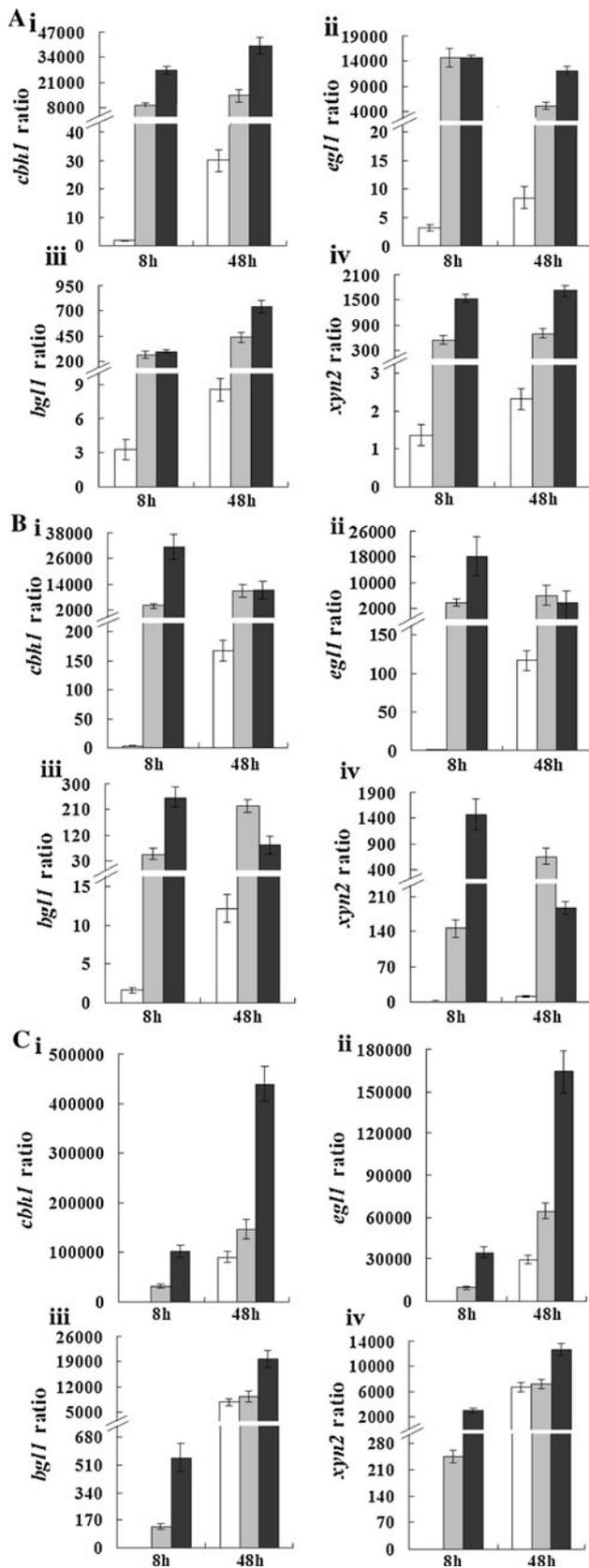
in addition to Xyr1, the regulator Ace2, which is not overexpressed in transformant PX41, acts both as an antagonist of early induction and as an enhancer of continuous *xyn2* expression [28].

Consistent with observed CMCCase activity, the relative transcript abundance of cellulase and xylanase genes in PX41 in glycerol and glucose media (Fig. 5a, b) was already very high at the earliest measured time point, indicating derepression and activation of cellulase and xylanase gene expression under non-inducing conditions. This implies that disruption of *cre1* alone is insufficient for extensive cellulase expression, and that Xyr1 is essential for extensive activation of cellulase expression under both inducing and repressing conditions. These findings are consistent with the observation of Nakari-Setälä et al. [15] that deletion of *cre1* only slightly improved protein production in the hypercellulolytic strain VTT-D-80133. In contrast to results observed with cellulose, *bgl1* and *xyn2* transcript levels were consistently higher in PX41 than in RUT C30 when grown on glycerol or glucose; this indicates that gene regulation was somewhat different for these two genes depending on whether inducing or non-inducing conditions were used.

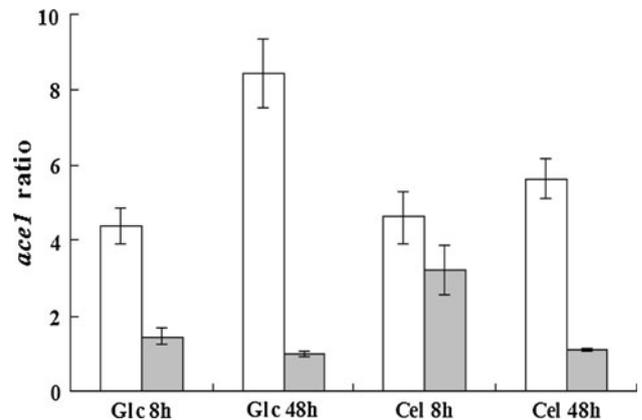
#### Construction of a strain with both *xyr1* overexpression and *ace1* downregulation

Deletion of the *ace1* repressor in *T. reesei* has been shown to increase expression of the major cellulase and hemicellulase genes in sophorose- and cellulose-induced cultures (Aro et al. [1]). In addition, RNAi-mediated gene silencing of *T. koningii ace1* enhanced production of total proteins, cellulase, and xylanase under inducing conditions [29]. Gene transcription and protein production are often due to the synergistic action of different transcriptional factors. To explore whether enzyme production in RUT C30 could be further improved by simultaneous overexpression of *xyr1* and silencing of *ace1*, we constructed a strain with these features by co-transforming an *xyr1* expression cassette and an *ace1* silencing vector with the aid of the plasmid pAN7.1.

Based on the results of PCR screening used to check for successful integration of both features (data not shown), the transformant PXAi20 was selected for use in further studies. qPCR was used to monitor *xyr1* and *ace1* transcript levels in PXAi20. Under glucose-based cultivation, *xyr1* basal expression levels were strongly elevated in PXAi20 compared with PX41 and RUT C30 (Fig. 3). Forty-eight hours after transfer of mycelia to glucose or cellulose media, *xyr1* mRNA levels were 1.4- and 3.0-fold higher in PXAi20 than in PX41, respectively. Transcript abundance of *ace1* in PXAi20 was also analyzed using qPCR. Under glucose or cellulose cultivation, *ace1* mRNA was less



◀ **Fig. 5** Expression ratio of (i) *cbhI*, (ii) *egII*, (iii) *bgII*, and (iv) *xynII* to the steady-state *sarI* reference gene in strains RUT C30 (open bars), PX41 (shaded bars), and PXAi20 (filled bars) pre-grown with glycerol as a carbon source and then transferred to **a** glycerol, **b** glucose, or **c** cellulose for 8 or 48 h. RUT C30: parental strain; PX41: recombinant strain constitutively expressing *xylI* under the control of the *pdC* promoter; PXAi20: recombinant strain with both *xylI* over-expression and *aceI* downregulation. Error bars indicate standard deviations



**Fig. 6** Expression ratio of *aceI* to steady-state *sarI* reference gene in strains RUT C30 (open bars) and PXAi20 (filled bars) pre-grown with glycerol as a carbon source and then transferred to glucose (Glc) or cellulose (Cel) for 8 or 48 h. RUT C30: parental strain; PXAi20: recombinant strain with both *xylI* over-expression and *aceI* downregulation. Error bars indicate standard deviations

abundant in PXAi20 than in RUT C30 (Fig. 6); this suggests that partial silencing of *aceI* in PXAi20 was achieved through expression of the inverted repeat construct under repressing or inducing conditions. These results demonstrate that simultaneous *xylI* overexpression and *aceI* downregulation occurred in PXAi20. When *aceI* was silenced, *xylI* expression levels further increased under both repressing and inducing conditions, indicating that Ace1 negatively affects *xylI* transcription. This observation is consistent with the known presence of two Ace1-binding sites in the *xylI* structural gene upstream regulatory sequence as well as previously published data indicating that disruption of *aceI* elevates *xylI* transcription levels in RUT C30 [12].

Further increased enzyme production by both *xylI* overexpression and *aceI* downregulation

To study the combined effects of *xylI* overexpression and *aceI* downregulation on enzyme production, PXAi20 was cultured in glucose and cellulose media as described previously for PX41 and RUT C30. As early as 24 h after

transfer into cellulose media, cellulase and xylanase activities were clearly induced to higher levels in PXAi20 compared with PX41 and RUT C30 (Fig. 4). After 5 day of cultivation, FPA, CMCase activity, and secreted protein levels were 43, 41, and 45 % higher, respectively, in PXAi20 compared with PX41. Interestingly, downregulation of *ace1* led to clearly higher levels of xylanase activity in PXAi20 compared with PX41 under cellulose cultivation (Fig. 4c).

Surprisingly, further increases in CMCase activity (about 1.2- to 2.8-fold higher) were detected in PXAi20 compared with PX41 under glucose-based cultivation (Fig. 4e). The overall results show that a combination of *xyl1* overexpression and *ace1* downregulation was more effective in boosting enzyme production in *T. reesei* RUT C30 than was *xyl1* overexpression alone.

We also studied the effect of combined *xyl1* overexpression and *ace1* downregulation on cellulase and xylanase gene expression in PXAi20. PXAi20 was grown in glycerol, glucose, and cellulose cultures in the same manner as for PX41 and RUT C30. In media containing cellulose as the sole carbon source, cellulase and xylanase gene expression was significantly higher in PXAi20 than in PX41 (Fig. 5c). In contrast to PX41, *xyn2* expression levels were clearly higher in PXAi20 than in RUT C30 under cellulose cultivation; this result implies that Ace1 negatively regulates, either directly or indirectly, *xyn2* expression in RUT C30, and is consistent with previous findings that deletion of the *ace1* gene leads to increased *xyn2* expression in *T. reesei* QM9414 [1]. The above results accord well with the enzyme activity data. Similarly, at the early time point in glycerol and glucose media, cellulase and xylanase genes were expressed at a higher level in PXAi20 compared with PX41 (Fig. 5a, b); 48 h after transfer into glucose media, *cbh1* and *egl1* expression levels in PXAi20 resembled those in PX41, while *bgll* and *xyn2* expression levels were lower in PXAi20 than in PX41.

These findings suggest that Xyr1 expression alone is sufficient for extensive cellulase induction in RUT C30 and indicate that increased *xyl1* expression increases cellulase gene expression and consequently cellulase activity.

## Conclusions

In this study, we improved cellulase production in hyper-producer strain RUT C30 by altering cellulase regulator expression. The positive regulator Xyr1 was constitutively expressed under the strong *T. reesei* *pdc* promoter in RUT C30, resulting in significant increases in cellulase activity and levels of corresponding mRNAs. Constitutive expression of *xyl1* combined with downregulation of the

repressor-encoding gene *ace1* further increased cellulase and xylanase activities in RUT C30. These findings form the basis for an attractive new single-step approach for increasing total cellulase activity in *T. reesei*.

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**Conflict of interests** The authors declare that they have no competing interests.

## References

- Aro N, Ilmén M, Saloheimo A, Penttilä M (2003) ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. *Appl Environ Microbiol* 69(1):56–65
- Aro N, Saloheimo A, Ilmén M, Penttilä M (2001) ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. *J Biol Chem* 276(26):24309–24314
- Bailey MJ, Biely P, Poutanen K (1992) Interlaboratory testing of methods for assay of xylanase activity. *J Biotechnol* 23(3):257–270
- Bhat MK (2000) Cellulases and related enzymes in biotechnology. *Biotechnol Adv* 18(5):355–383
- Furukawa T, Shida Y, Kitagami N, Ota Y, Adachi M, Nakagawa S, Shimada R, Kato M, Kobayashi T, Okada H, Ogasawara W, Morikawa Y (2008) Identification of the *cis*-acting elements involved in regulation of xylanase III gene expression in *Trichoderma reesei* PC-3-7. *Fungal Genet Biol* 45(7):1094–1102
- Ghose T (1987) Measurement of cellulase activities. *Pure Appl Chem* 59(2):257–268
- Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Liden G, Zacchi G (2006) Bio-ethanol: the fuel of tomorrow from the residues of today. *Trends Biotechnol* 24(12):549–556
- Ilmén M, Saloheimo A, Onnela ML, Penttilä ME (1997) Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Appl Environ Microbiol* 63(4):1298–1306
- Ilmén M, Thrane C, Penttilä M (1996) The glucose repressor gene *cre1* of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. *Mol Gen Genet* 251(4):451–460
- Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B (2009) Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. *Biotechnol Biofuels* 2(19):1–14
- Li J, Wang J, Wang S, Xing M, Yu S, Liu G (2012) Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters. *Microb Cell Fact* 11:84
- Mach-Aigner AR, Pucher ME, Steiger MG, Bauer GE, Preis SJ, Mach RL (2008) Transcriptional regulation of *xyl1*, encoding the main regulator of the xylanolytic and cellulolytic enzyme system in *Hypocrea jecorina*. *Appl Environ Microbiol* 74(21):6554–6562
- Mach RL, Zeilinger S (2003) Regulation of gene expression in industrial fungi: *Trichoderma*. *Appl Microbiol Biotechnol* 60(5):515–522
- Martinez D, Berka RM, Henrissat B et al (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat Biotechnol* 26(5):553–560

15. Nakari-Setälä T, Paloheimo M, Kallio J, Vehmaanperä J, Penttilä M, Saloheimo M (2009) Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. *Appl Environ Microbiol* 75(14):4853–4860
16. Nemoto T, J-i Maruyama, Kitamoto K (2009) Improvement of heterologous protein production in *Aspergillus oryzae* by RNA interference with alpha-amylase genes. *Biosci Biotechnol Biochem* 73(11):2370–2373
17. Penttilä M, Nevalainen H, Ratto M, Salminen E, Knowles J (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 61(2):155–164
18. Peterson R, Nevalainen H (2012) *Trichoderma reesei* RUT-C30—thirty years of strain improvement. *Microbiology* 158:58–68
19. Portnoy T, Margeot A, Seidl-Seiboth V, Le Crom S, Ben Chaabane F, Linke R, Seiboth B, Kubicek CP (2011) Differential regulation of the cellulase transcription factors XYR1, ACE2 and ACE1 in high and low cellulase producing strains of *Trichoderma reesei*. *Eukaryot Cell* 10(2):262–271
20. Pucher ME, Steiger MG, Mach RL, Mach-Aigner AR (2011) A modified expression of the major hydrolase activator in *Hypocrea jecorina* (*Trichoderma reesei*) changes enzymatic catalysis of biopolymer degradation. *Catal Today* 167(1):122–128
21. Rauscher R, Würleitner E, Wacenovskiy C, Aro N, Stricker AR, Zeilinger S, Kubicek CP, Penttilä M, Mach RL (2006) Transcriptional regulation of *xynI*, encoding xylanase I. *Hypocrea jecorina*. *Eukaryot Cell* 5(3):447–456
22. Saloheimo A, Aro N, Ilmén M, Penttilä M (2000) Isolation of the *ace1* gene encoding a Cys(2)-His(2) transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. *J Biol Chem* 275(8):5817–5825
23. Seiboth B, Hartl L, Pail M, Fekete E, Karaffa L, Kubicek CP (2004) The galactokinase of *Hypocrea jecorina* is essential for cellulase induction by lactose but dispensable for growth on D-galactose. *Mol Microbiol* 51(4):1015–1025
24. Seiboth B, Karimi RA, Phatale PA, Linke R, Hartl L, Sauer DG, Smith KM, Baker SE, Freitag M, Kubicek CP (2012) The putative protein methyltransferase LAE1 controls cellulase gene expression in *Trichoderma reesei*. *Mol Microbiol* 84(6):1150–1164
25. Steiger MG, Mach RL, Mach-Aigner AR (2010) An accurate normalization strategy for RT-qPCR in *Hypocrea jecorina* (*Trichoderma reesei*). *J Biotechnol* 145(1):30–37
26. Strauss J, Mach RL, Zeilinger S, Hartler G, Stöffler G, Wolschek M, Kubicek CP (1995) Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS Lett* 376(1–2):103–107
27. Stricker AR, Grosstessner-Hain K, Würleitner E, Mach RL (2006) Xyr1 (xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. *Eukaryot Cell* 5(12):2128–2137
28. Stricker AR, Trefflinger P, Aro N, Penttilä M, Mach RL (2008) Role of Ace2 (Activator of Cellulases 2) within the *xyn2* transcriptosome of *Hypocrea jecorina*. *Fungal Genet Biol* 45(4):436–445
29. Wang S-W, Xing M, Liu G, Yu S-W, Wang J, Tian S-L (2012) Improving cellulase production in *Trichoderma koningii* through RNA interference on *ace1* gene expression. *J Microbiol Biotechnol* 22(8):1133–1140
30. Wilson DB (2009) Cellulases and biofuels. *Curr Opin Biotechnol* 20(3):295–299
31. Zeilinger S, Schmoll M, Pail M, Mach RL, Kubicek CP (2003) Nucleosome transactions on the *Hypocrea jecorina* (*Trichoderma reesei*) cellulase promoter *cbh2* associated with cellulase induction. *Mol Genet Genomics* 270(1):46–55