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Promoting cellulase and hemicellulase production from *Trichoderma orientalis* EU7-22 by overexpression of transcription factors Xyr1 and Ace3

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ABSTRACT

The construction of hyper-production strains of cellulase is the prerequisite for the production of biofuels or biochemicals. *Trichoderma orientalis* EU7-22 with complete cellulase system shows the potential for cellulase production in industrial scale. To improve the cellulase production, two crucial transcription activators Xyr1 and Ace3 were constitutively overexpressed in EU7-22 strain. Cellulase, xylanase and protein secretion were significantly improved in the recombinant strain dxyA-8 under inducing culture, which were 2.34, 0.68 and 1.06 folds higher than those of EU7-22, respectively. Moreover, the FPase and CMCase activities were up to 2.55 IU/mL and 90.38 IU/mL with glucose as carbon source, which were 2.12 and 1.95 folds higher than those of EU7-22 under inducing condition, respectively. Reducing sugar released from pretreated spartina that hydrolyzed by crude enzyme from dxyA-8 had achieved 24% improvement. Therefore, overexpression of these transcription factors effectively promotes the production of cellulase and hemicellulase of *Trichoderma orientalis* EU7-22.

1. Introduction

Lignocellulosic biomass, mainly including cellulose, hemicellulose and lignin, is the most abundant renewable resource on the earth (Rubin, 2008). Biofuels and biochemicals produced from lignocellulosic biomass through biorefinery are environmentally friendly compared with those from fossil fuels, which has received great attention (Kawaguchi et al., 2016). Enzymatic hydrolysis of lignocellulose to fermentable sugars is one of the key steps for biorefinery (Chandel et al., 2018; Guo et al., 2018). However, the major bottleneck of the process is the high cost of cellulase, and development of highly efficient cellulase-producing strains is of great interest to lignocellulosic biorefinery (Taha et al., 2016). Filamentous fungi such as *Trichoderma* spp., *Aspergillus* spp., *penicillium* spp. and *Sclerotium spp*., which can produce complex mixture of cellulase to degrade the cellulose synergistically, are mainly considered for commercial exploitation (Sajith et al., 2016). The strain *Trichoderma orientalis* EU7-22 (*Hypocrea orientalis* EU7-22) preserved in our lab was bred from strain XC-9 by physiochemical mutagens and identified as *Trichoderma spp*. by Chuannan Long (Long et al., 2013). It can secret abundant cellulase and hemicellulase (Chuannan et al., 2012; Long et al., 2017). Comparison of cellulase activities among *Trichoderma orientalis*, *Trichoderma reesei*, *Trichoderma koningii*, *Aspergillus niger* and *Penicillium decumbens*, the strain EU7-22 has higher cellulase activities and also possessed of integrated cellulase system (Chuannan et al., 2012). According to our observation, the strain EU7-22 grows faster than *Trichodema reesei* Rut-30 on PDA plats and have a good fermentation capacity to secrete cellulase and hemicellulase. So, *Trichoderma orientalis* can be an alternative for cellulase production in industrial scale.

Production of cellulase in fungi is coordinately regulated by a suit of transcription factors (Amore et al., 2013). Carbon catabolite repression (CCR) mediated by the transcription factor Cre1/CreA under glucose and other easily metabolized carbon sources suppresses the expression of a majority of cellulase and hemicellulase genes in the cellulolytic fungi (Glass et al., 2013). Deletion of *cre1* gene in the *Trichoderma reesei* QM6a and *Trichoderma orientalis* EU7-22, the activities of cellulase and xylanase in mutant strains were dramatically increased under glucose condition (Long et al., 2018; Nakari-Setala et al., 2009). Another transcription repressor is Ace1 which represses the production of cellulase and hemicellulase in *T. reesei* and *T. koningii* (Aro et al., 2003; Wang, 2012). Deletion of the *ace1* gene improved the expression of all the main cellulase and hemicellulase genes in sophorose- and cellulose-induced cultures (Aro et al., 2003). Addition to the transcription repressors, the cellulase expression depends on some transcription activators including Ace2 (Aro et al., 2001), the CCAAT binding complex Hap2/3/5 (Brakhage et al., 1999), Xyr1/XlnR (Stricker et al., 2008) and

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Ace3 (Häkkinen et al., 2014). The Xyr1 is the essential for the expression of cellulase and hemicellulase genes in *Aspergillus niger* and *T. reesei* (Mach-Aigner et al., 2008; Raulo et al., 2016). The deletion of the Xyr1 of *T. reesei* abolished the majority of cellulase and hemicellulase encoding genes expression and the cellulase and hemicellulase activites were almost undetectable under the inducing culture (Stricker et al., 2006). Meanwhile, the same phenomenon was happened in *Trichoderma orientalis* when we deleted the *xyr1* gene (data unpublished). The Ace3 is another master regulator of cellulase expression in the *Trichoderma reesei* and *Trichoderma orientlais*. Deletion of *ace3* in *Trichoderma ressei* totally abolished cellulase expression and reduced xylan degrading enzyme expression (Häkkinen et al., 2014). Also, the cellulase and hemicellulase activities dramatically decreased and remained a little after deleting the *ace3* gene in *Trichoderma orientlais* EU7-22 (Liu et al., 2018).

The transcription of *xyr1* was increased in the strain overexpressed *ace3* and decreased in the strain deleted *ace3*, however, the transcription of *xyr1* was not completely abolished in the strain deleted *ace3* (Häkkinen et al., 2014). Meanwhile, the transcription of *ace3* in the *xyr1* deletion strain was slight decreased compared to the parental strain (dos Santos Castro et al., 2016). Hence, it is speculated that the two key transcriptional regulators of Xyr1 and Ace3 act as synergetic manner to activate the expression of genes encoding cellulase and hemicellulase.

Cellulase hyper-production strains and production of cellulase preparations with lower cost carbon source are desirable for the economic transformation of lignocellulose to biofuels and biochemicals. Genetic modification regulators are thought to be a direct and promising strategy for improving cellulase production in filamentous fungi, and constitutive overexpression of transcriptional activators is one of the effective ways to increase cellulase production (Ellila et al., 2017; Häkkinen et al., 2014; Nakari-Setala et al., 2009; Wang, 2012). The selection of promoters plays a key role in imposing effect on the expression of transcription activators that are overexpressed in host strains and further influences the cellulase production (Wang et al., 2013). The promoters of pyruvate decarboxylase (*pdc*) and enolase (*eno*) were screened under glucose condition while the transcript levels of the two genes were dramatically higher than others under the condition of high glucose concentration (85 mmol/L), specially (Li et al., 2012).

Above all, Xyr1 and Ace3 were simultaneously constitutively overexpressed in *Trichoderma orientalis* EU7-22 with promoters of *pdc* and *eno* to investigate whether the two factors can synergistically improve the production of cellulase in *Trichoderma orientlis* and obtain a mutant highly producing cellulase under the conditions of cellulose or glucose.

2. Methods

2.1. Microorganism strains and culture condition

The parental strain, *Trichoderma orientalis* EU7-22, and its transformants in this paper are cultured on PDA plates for 7 days to obtain fresh conidia. The minimum medium (MM medium, pH = 5.5) for hyphal growth contains 20 g/L glucose, 5 g/L $(NH_4)_2SO_4$, 15 g/L KH_2PO_4, 0.6 g/L $MgSO_4$, 0.6 g/L $CaCl_2$ and 10 mL 100 × trace element solution. The 100 × trace element consists of 0.5 g/L $FeSO_4{\cdot}7H_2O$, 0.16 g/L $MnSO_4{\cdot}H_2O$, 0.14 g/L $ZnSO_4{\cdot}7H_2O$ and 0.2 g/L $CoCl_2$.The medium for transformation (MK medium) is MM medium supplemented with 2% agar and 0.6 mol/L KCl. The solid medium for hyphal morphology observation is MM medium adding 2% agar and the glucose is substituted for different carbon source, including xylose (MX), lactose (ML), xylan from bagasse (MY), Avicel (MA) and CMC-Na (MC). The fermentation medium (1 L, initial pH = 5.2), is composed of 3% glucose or 2% Avicel and 1% wheat bran, 0.5% tryptone, 0.25% KH_2PO_4, 0.05% $MgSO_4$, 0.05% $CaCl_2$, 0.4% (v/v) Tween80 and 10 mL 100× trace element solution. All the percentages of materials and chemicals in the medium represent the mass-volume ratio unless otherwise specified. The equal amount mycelia inoculated in fermentation medium for cellulase production and mRNA extraction was pre-cultured for 36 h in 100 mL MM medium, which were collected through vacuum drum filtration with G5 sintered glass filter and washed twice with fermentation culture without inducer. All plates were cultured in an incubator at 30 °C, all liquid cultures were incubated in a rotary shaker at 30 °C and 180 rpm. Samples were collected and measured at the time points indicated in the text.

2.2. Fungal transformation

Fresh cultured strains on PDA were inoculated into 50 mL MM medium and cultured for 42 h. The mycelia were collected with three layers of lens paper and washed twice with solution A1(0.6 mol/L KCl, 0.05 mol/L citrate acid, pH 5.8). Solution A1 with 10 mg/mL snailase (Sangon Biotech, China), 10 mg/mL cellulase from *Trichoderma reesei* (Sigma-Aldrich, USA), 1 mg/mL lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, USA) and 1 mg/mL zymolase-20 T from *Arthrobacter luteus* (MP Biomedicals, USA) was employed to degrade the mycelia cell wall at 32 °C, 120 rpm for 1.5 to 2.0 h. After being degraded, the mixture was added with equal volume of solution A2 (0.6 M KCl), filtered through three layers of lens paper, and then centrifuged at 4000 rpm and 4 °C for 15 min. The supernatant was discarded and the sediment was resuspended with 2 mL solution A2, and then centrifuged at 4000 rpm and 4 °C for 10 min. The supernatant was discarded and the precipitate was resuspended with 0.5 mL solution A3 (0.6 mol/L KCl, 0.05 mol/L $CaCl_2$), centrifuged at 4000 rpm and 4 °C for 15 min. Finnally, the supernatant was discarded and the precipitate was resuspended with proper volume of A3 solution.

The transformation system, consisting of 10 μL DNA fragment (concentration ≥ 100 ng/μL), 50 μL solution A4 (0.6 mol/L KCl, 0.05 mol/L $CaCl_2$ and 25% PEG6000) and 100 μL protoplast suspension, was placed on ice for 25 min. Then 1 mL solution A4 was added to the system and kept for another 25 min at room temperature. Thereafter, the solution mixture was added to MK medium at 50 °C for plate pouring. After 3 days of cultivation, another layer of MM medium was added with hygromycin B (hygB: 100 μg/mL) or geneticin (G418: 300 μg/mL) and poured above MK medium at 50 °C. After another 4–6 days of cultivation, the transformants were obtained and further streaked onto selection plates to form single colonies for removing the effect of heterokaryon.

2.3. Overexpression of the xyr1 and transformants selection

The promoter (P*pdc*) and terminator (T*pdc*) of *pdc* gene, encoding pyruvate decarboxylase, were PCR-amplified from *T. reesei* QM9414 genomic DNA using primers Ppdc-F and Ppdc-xyR, as well as Tpdc-xyF and Tpdc-1R (Table 1). *xry1* gene (GenBank accession no. KC793261) was amplified from *T. orientalis* EU7-22 genomic DNA using primers xyr-F and xyr-R. The *hygB* cassette was amplified from pUR5750 plasmid using primers PpgdA-pxyP2 and TxtrpC-P6. The *xyr1* expression cassette was obtained by fusion PCR (Ppdc-1F and TgtrpC-xyP5) using the four fragments above as the templates (Szewczyk et al., 2006). The *xyr1* expression cassettes were transformed into *T. orientalis* EU7-22 to obtain the *xyr1* overexpression transformants. After that, the transformants were verified by genomic DNA PCR with primer pairs (hph-F and hph-R), and then the transformant with the highest FPase activity after 4 d fermentation under cellulase inducing condition was selected (Fig. 1a).

2.4. Overexpression of the ace3 and transformants selection

The promoter (P*eno*) and terminator (T*eno*) of *eno* gene, encoding enolase, were amplified from *T. reesei* QM9414 genomic DNA using the primers Peno-1 and Peno-3, as well as Teno-4 and Teno-5 (Table 1). The

Table 1
Primers used for construction of the *xyr1* expression cassette and *ace3* expression cassette.

Primers	Sequences (5′–3′)[a]	Notes
Ppdc-F	AGTCGGACCTGCTGAGCTTC	For construction of the *xyr1* expression cassette
Ppdc-1F	GAGGACTTCCAGGGCTACTTG	
Ppdc-xyR	GACGGAGAGGATTGGACAACATGATTGTGCTGTAGCTGCGCT	
Tpdc-xyF	AACCGGTCTGGCCCTCTAACCCGGCATGAAGTCTGACC	
Tpdc-1R	TGGACGCCTCGATGTCTTCC	
xyr-F	ATGTTGTCCAATCCTCTCCGTC	
xyr-R	TTAGAGGGCCAGACCGGTT	
PgpdA-pxyP2	GAAGACATCGAGGCGTCCAGATCTTTCGACACTGAAATACGTC	
TgtrpC-xyP5	AAGAAGGATTACCTCTAAACAAGTGT	
TxtrpC-P6	CACAGGAAACAGCTATGACCATG	
hph-F	CGACAGCGTCTCCGACCTGA	For PCR verification of *xyr1* overexpression transformants
hph-R	CGCCCAAGCTGCATCATCGAA	
Peno-1	GGAGGGATATAGACTATGGCTCATG	For construction of the *xyr1* expression cassette
Peno-2	GTGATTCCGTCCTGGATTGC	
Peno-3	GGGGGAGTAGCGCAGCATTTTGAAGCTATTTCAGGTGGCTG	
Teno-4	ACGTCTAATACCGTTGTTGGCTAAATGGCCACGAGAGACAACTACC	
Teno-5	TGGCGTCGTTGATGTTTCG	
ace3-F	ATGCTGCGCTACTCCCCCGTCT	
ace3-R	TTAGCCAACAACGGTATTAGACGTATTCCTC	
PgpdA-eaP2	CGAAACATCAACGACGCCAGATCTTTCGACACTGAAATACGTC	
TtrpC-rhsR	CCCAAGCTTATTTAAATAAGAAGGATTACCTCTAAACAAGTG	
G418-F	ATGATTGAACAAGATGGATTGCA	For PCR verification of ace3 overexpression transformants
G418-R	TCAGAAGAACTCGTCAAGAAGGC	

[a] Overlapping regions for fusion PCR are underline.

ace3 gene (GenBank accession no. MG720022.1) of *T. orientalis* EU7-22 was amplified from its genomic DNA with primers ace3-F and ace3-R. The *G418* selected cassette was amplified from the pUC-G418 plasmid with primers PgpdA-eaP2 and TtrpC-rhsR. The amplified Peno, Teno, *ace3* and *G418* cassette fragments were fused by PCR using primers Peno-2 and TgtrpC-xyP5 to generate *ace3* expression cassettes, and then it was transformed into the dxyr-1 mutant. The single colonies with G418 resistance were verified by genomic DNA PCR with primers G418-F and G418-R, and the mutant with the highest FPase activity was selected (Fig. 1 b). The DNA polymerases used in this paper were high fidelity enzymes from Takara, including PrimeSTAR® HS DNA Polymerase or PrimeSTAR® Max DNA Polymerase, except for the PCR of verifying transformants using the 2 × Taq Plus Master Mix (Dye Plus) from Vazyme Biotech.

2.5. Phenotypic observation

Conidia suspension (1 μL, about 10^5 spores) of strains were spotted on different carbon source mediums as described above and incubated at 30 °C for 3 days. Then the diameter of colonies in the plates were measured. All the analysis was performed in triplicates.

2.6. Biomass measurement

Equal amount hyphae of strains were added into 50 mL fermentation medium with 3% (w/v) glucose as carbon source and mycelia were collected at 72, 96 and 120 h and dried at 80 °C to constant weight for biomass measurement.

2.7. Enzyme activity assays

Supernatants of fermentation broth were collected by centrifugation to remove the residual medium and mycelia. Total soluble protein concentration in the culture supernatant was quantified by a Modified Bradford Protein Assay Kit (Sangon Biotech, China), following the manufactory's instructions. FPase, CMCase and xylanase activities were determined by 50 mg Whatman No. 1 filter paper (Hangzhou, China), 6.67 mg/mL sodium carboxymethyl cellulose solution (800–1200 mPa·s, pH 4.8, Sinopharm Chemical ReagentCo., Ltd, China) and 10 mg/mL bagasse xylan solution (pH 4.8, Shanghai Yuanju Biological Technology Co., Ltd, China) as the substrates, respectively. The enzyme reaction was performed in 0.05 mol/L citrate buffer at 50 °C for 60, 30 and 10 min, respectively, using DNS method to quantify the released reducing sugar (GHOSE, 1987). The CMCase, *P*NPCase, *P*NPGase and FPase activities represent the EGs, CBHs, BGs and total cellulase activity, respectively (Dashtban et al., 2010). The *p*NPCase, *p*NPGase and *p*NPXase activities were measured in the above-used citrate buffer at 50 °C for 30 min with 50 mmol/L *p*NPC, *p*NPG and *p*NPX (Sigma, USA) as the substrate. One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol reducing sugar or *p*NP per min under the assayed conditions.

2.8. Quantitative reverse-transcription PCR

The collected and washed mycelium were starved for 2 h in a carbon-free fermentation medium, and then the starved 0.5 g mycelium was transformed into a fermentation medium containing 2% Avicel and 1% wheat bran, and incubate for another 8 or 24 h. The total RNA extraction and cDNA synthesis using the RNAiso Plus reagent (TaKaRa, Japan) and 5× All-In-One MasterMix with AccuRT Genomic DNA Removal Kit (abm, American) were performed according to the manufactory's instructions. Quantitative PCR (qPCR) analysis was performed by a QuantStudio™ real-time PCR system with ChamQTM Univerasl SYBR qPCR Master Mix (Vazyme, China) according to the manufactory's instruction, in which the primers used for qPCR analysis of genes encoding cellulases, hemicellulases and regulatory factors were shown in Table 2. The CT data of qPCR were analyzed by comparative cycle threshold (CT) method ($(2^{-\Delta\Delta CT})$) for relative quantification. Expression of the *actin* gene was chosen as an endogenous control for all experiments. All the experiments were performed in triplicates.

2.9. Enzymatic saccharification of pretreated biomass

$NaOH/H_2O_2$ pretreated spartina (NPSA), provided by Fujian Vocational College of Bio-engineering, was used as the substrate for enzymatic saccharification. NPSA hydrolysis was performed by the crude enzymes which were produced in the fermentation under inducing condition. The hydrolysis system including 0.6 g pretreated NPSA (dry weight), 3 mL crude enzymes, 0.3 mL 2% prolin300, and 26.7 mL

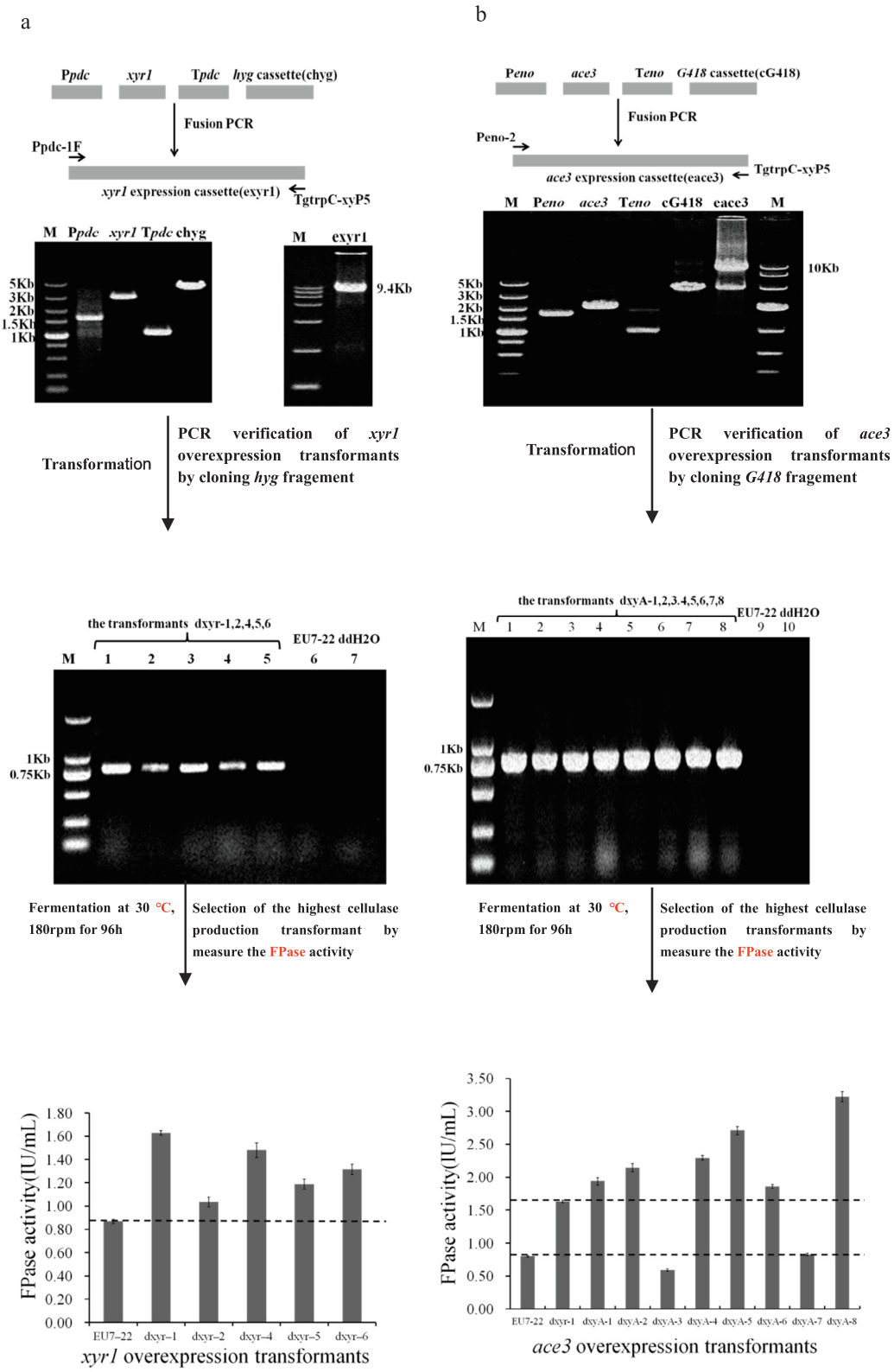

Fig. 1. Schematic diagram of the construction of *xyr1* overexpression strains and *ace3* overexpression mutants. (a) The construction of *xyr1* overexpression strains and selection of the strain with highest FAP activity. The *hygB* fragment of 811 bp was detected in *xyr1* overexpression strains with primer pair hph-F and hph-R. (b) The construction of *ace3* and *xyr1* overexpression strains and selection the strain with the highest FAP activity. The *G418* gene of 795 bp was verified by PCR in *ace3* overexpression transformants with primer pair G418-F and G418-R.

Table 2
qPCR primers used in this study.

Primers	Sequences(5′-3′)
actin-YGF	TCCATCATGAAGTGCGAC
actin-YGR	GTAGAAGGAGCAAGAGCAGTG
cbh I-YGF	ATCGGCTTCGTCACGCAATC
cbh I-YGR	ACGCCACCATCCGCATCCA
cbh II-YGF	GACAAACCTCGGCACTCC
cbh II-YGR	GACCAGCGTCCAGATACATT
eg I-YGF	CAGGGCTTCTGCTGTAATGAG
eg I-YGR	TTG AACTGGGTGATGATGGTG
eg II-YGF	GCTCCGCCAGAATAACCG
eg II-YGR	CAGCCA ACATAGCCAAGATAGAC
bgl 1-YGF	ATCACCTACCCGCCTTCA
bgl 1-YGR	TCTCGTCGTCGGATGTTG
xyn I-YGF	CGTCAACACGGCGAACCA
xyn I-YGR	CGGTGATGGAAGCAGAGCC
Bxl1-YGF	TGTTCGCACAAGCAACGCT
Bxl1-YGR	ACTCGTCGGTGTTCAAGGCT
xyr1-YGF	TGCTTGACGACGACGACTTGT
xyr1-YGR	ACGCCGTAGAAGAAGGGCAT
Cre1-YGF	TCTACGGCTCCTTCTTCTC
Cre1-YGR	ACAAGTTCCTCAGACTCGG
Ace1-YGF	TGATGAGGGCTTTGACGAGTC
Ace1-YGR	GGTTGAAGATGTCGGGCTGT
Ace3-YGF	TGCTGAGGGTGATGAACGAG
Ace3-YGR	GGGTGAATCCTGGTTGCGAT

citrate buffer (pH 4.8) was incubated at 50 °C and 150 rpm. The hydrolysates were sampled by every 12 h, boiled for 5 min and centrifuged at 8000 rpm until the concentration of reducing sugar reached at a steady state. The reducing sugar of supernatant was determined by DNS method.

3. Results and discussion

3.1. Construction of strains with xyr1 overexpression

Xyr1 is the essential regulator responsible for the activation of cellulase and hemicellulase expression in *Trichoderma spp* (da Silva Delabona et al., 2017; Mach-Aigner et al., 2008). In *T. reesei* Rut-30, overexpression of xyr1 with strong constitutive promoter P*pdc*, the promoter of pyruvate decarboxylase gene of *T. reesei* QM9414, notably increased the cellulase production under inducing conditions (Wang et al., 2013). We have deleted the *xyr1* of *Trichoderma orientalis* EU7-22 and the cellulase and hemicellulase activities of mutant strain was barely detected in induced culture (data unpublished), so the Xyr1 also is the master regulator responsible for the cellulase and hemicellulase genes expression in *T. orientalis*. To improve the production of cellulase of *T. orientalis*, the *xyr1* expression cassette including the encoding region of *xyr1* gene in *T. orientalis* EU7-22, 1.5 kb of *pdc* promoter region of *T. reesei* QM9414, 1.3 kb of *pdc* terminator region of *T. reesei* QM9414 and *hygB* cassette cloned from pUR5750 plasmid was constructed and transformed into *T. orientalis* EU7-22 (Fig. 1 a). The obtained 5 transformants were verified by amplifying the *hygB* gene fragment. After 4 days of cultivation, the FPase activities of them were 87, 17, 70, 37 and 51% higher than that of the parental strain EU-22, respectively. The strain with the highest FPase activity for further genetic modification was designated as dxyr-1 (Fig. 1a).

3.2. Construction of strains with both xyr1 and ace3 overexpression

Ace3 regulator is considered to be another essential activator for cellulase expression in *T. reesei* and the deletion of *ace3* gene almost abolished the cellulase activity of *T. reesei*. The transcription levels of *xyr1* increased in the strain overexpressed *ace3* and decreased in the strain deleted *ace3*, but the *xyr1* transcription did not totally abolish in the strain deleted *ace3* (Häkkinen et al., 2014). This implies that the Ace3 regulating the cellulase expression is realized partially by reducing the *xyr1* expression and other mechanisms is existed to activate the cellulase expression by this regulator. Based on above description, the *xyr1* and *ace3* perhaps have synergistic effects on the improvement of the cellulase production. Meanwhile, the deletion of *ace3* in *T. orientalis* EU7-22 dramatically decreased the cellulase production (Liu et al., 2018). In order to further increase the production of cellulase in *T. orientalis*, the *ace3* expression cassette was constructed and transformed into dxyr-1 strain (Fig. 1 b). Eight transformants in total were selected and verified by amplifying the *G418* gene. The constitutive promoter of enolase in *T. reesei* QM9414 was screened under glucose condition and cellobiohydrolase I controlled by this promoter were efficiently expressed under the condition of induction or repression in *T. reesei* QM6a. (Li et al., 2012; Linger et al., 2015). Therefore, the promoter may strongly promote the *ace3* expression in these transformants. The FPase activities of the transformants cultured under inducing medium for 4 days were measured and compared with EU-22 and dxyr-1 strains. The FPase activities in six of them were higher than that of dxyr-1 strain, which increased by 19, 32, 41, 66, 14 and 98%, respectively (Fig. 1b). This result indicates that the *ace3* overexpression based on *xyr1* overexpression can further improve the cellulase production of *T. orientalis*. The strain with the highest FPase activity for further study was named as dxyA-8.

3.3. Cellulase and hemicellulase activity analysis of the strain dxyA-8

Cellulases including endoglucanases (EGs), cellobiohydrolases (CBHs) and β-glucosidases (BGs) act in a synergistic manner to degrade the cellulose into glucose (Dashtban et al., 2009). In lignocellulose biomass, the cellulose microfibers are imbedded in xylan and highly cross-linked to shape network, so addition of hemicellulase can synergistically hydrolyze the lignocellulose (Huang et al., 2018). Two key transcription factors *xyr1* and *ace3* were overexpressed in this study to make it clear whether this manipulation can improve the production of cellulase and hemicellulase in *T. orientalis*. The cellulase and xylanase activities of dxyA-8 which constitutively overexpressed *xyr1* and *ace3* genes were obviously improved in comparison to those of the parental strain EU7-22 during the fermentation (Fig. 2). The levels of FPase, CMCase, *P*NPCase, *P*NPGase, xylanase activities and extracellular total protein in dxyA-8 were approximately 2.34, 1.23, 1.64, 0.68, 0.65 and 1.06 folds higher than those of the parental strain EU7-22 at 96 h. However, β-xylosidases activity (*P*NPXase) of dxyA-8 was lower than that of EU-22 at initial 3 days, but became higher from the 4th day (Fig. 2 f). β-Xylosidase is the essential components of hemicellulase and releases xylose from *xylo*-oligosaccharides (Ye et al., 2017). The *xylo*-oligosaccharides which was produced by the hydrolysis of lignocellulose inhibited the cellulase activity, thus the efficiency of enzymatic hydrolysis decreased (Xin et al., 2015). It turned out to be that the production of β-xylosidases production both from the strains of dxyA-8 and EU7-22 were deficient, hence, improving the β-xylosidases production of dxyA-8 is considered to be the next strategy to increase the efficiency of lignocellulose hydrolysis by *T. orientalis*.

The promoters of *pdc* and *eno* were screened by analyzing the transcriptional levels of 13 key genes that participated in glucose metabolism in *T. reesei* under the conditions of different concentrations of glucose and the recombinant *T. reesei* strains that homologously expressed xylanase II with the two promoters produced very high xylanase acitivities in a medium with high glucose concentration (Li et al., 2012). So the dxyA-8 strain was cultured under the condition of 3% (w/v) glucose to check whether the cellulase and hemicellulase of high activities can be detected. The results (Fig. 2) show that the levels of FPase CMCase, *P*NPCase, *P*NPGase, xylanase activities and extracellular total protein in strain dxyA-8 were approximately 31.63, 47.19, 39.39, 2.33, 6.16 and 2.53 folds higher than those of the parental strain under the condition of 3% glucose at 4d. Surprisingly, FPase activity, CMCase activity and protein concentration of dxyA-8 under the condition of 3%

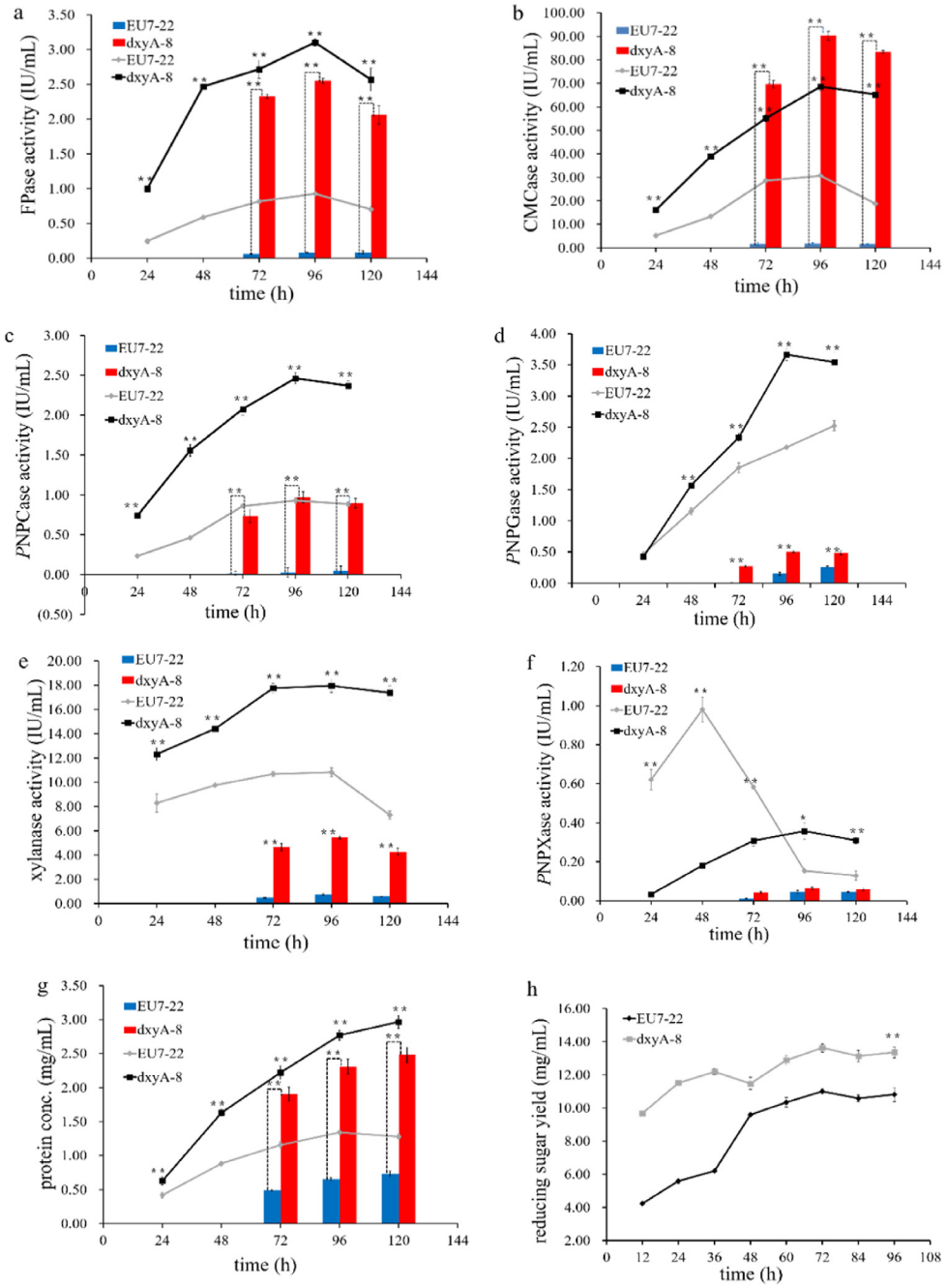

Fig. 2. Activities of FPase, CMCase, *P*NPCase, *P*NPGase, xylanase and *P*NPXase and concentrations of secreted protein from *T. orientalis* EU7-22 and dxyA-8 using inducing or glucose medium and reducing sugars yield in the hydrolysis of NPSA by enzyme of *T. orientalis* EU7-22 and dxyA-8. (a), (b), (c) and (d) are the activities for FPase, CMCase, *P*NPCase and *P*NPGase, respectively. (e) and (f) are the xylanase and *P*NPXase activities of *T. orientalis* EU7-22 and dxyA-8. (g), total secreted protein from *T. orientalis* EU7-22 and dxyA-8. (h), reducing sugar yield in the hydrolysis of NPSA by equal volum of supernatant produced by *T. orientalis* EU7-22 and dxyA-8 using inducing medium for 4 days. Line chart and column diagram of a, b, c, d, e and g were measured using inducing or glucose medium, respectively. The values show the mean of three biological replicates and the error bar indicates the standard deviation. * ($p < 0.05$) and ** ($p < 0.01$) indicate statistical significance calculated by students' test.

glucose were about 2.12, 1.95 and 0.72 folds higher than those of the parental strain EU7-22 under inducing condition at 96 h (Fig. 2 a, b, and g). The FPase acitvity of dxyA-8 under the condition of 3% glucose was 2.55 IU/mL, up to about 83% of FPase activity of dxyA-8 under inducing culture. The CMCase activity of dxyA-8 under the condition of 3% glucose was up to 90.38 IU/mL and even 32% higher than that under inducing condition. These results indicated that overexpression of xyr1 and ace3 in *T. orientalis* largely relieved the CCR effecting the cullulase production under the condition of glucose (Fig. 2).

3.4. Regulation of genes encoding major cellulase, hemicellulase and transcription factors by overexpression of ace3 and xyr1

It is worth of examining whether changes in enzyme production in dxyA-8 are caused by regulation of the genes encoding cellulase, hemicellulase and transcriptional regulators at the transcriptional level. Therefore, transcripts of major cellulases, hemicellulases, and transcriptional regulatory genes were studied by qRT-PCR analysis. *T. orientalis* EU7-22 and dxyA-8 were precultured in MM medium for 36 h, starved for 2 h under the no carbon source condition, and incubated in fermentation medium with Avicel and wheat bran for 8 and 24 h, respectively. The result indicated the transcription abundance of genes encoding major cellulase and xylanase in dxyA-8 was up-regulated significantly compared to that of the parental strain EU7-22 in both post-8 h and post-24 h induction (Fig. 3a). The $\log_2$ ratios (dxyA-8/EU7-22) of *cbh1*, *cbh2*, *eg1*, *eg2*, and *bgl1* reached 2.92, 4.43, 3.58, 4.18 and 2.14 at 48 h, respectively, which is consistent with the aforementioned cellulase activities. The $\log_2$ ratio (dxyA-8/EU7-22) of gene *xyn1* was 0.26 at 8 h and increased to 1.13 at 24 h (Fig. 3a). The transcription quantity of *bxl1* of dxyA-8 at 8 h was one-sixth of that from EU7-22, but the transcription quantities of *bxl1* of dxyA-8 and EU7-22 were almost the same at 24 h. This variation is consistent with the development of

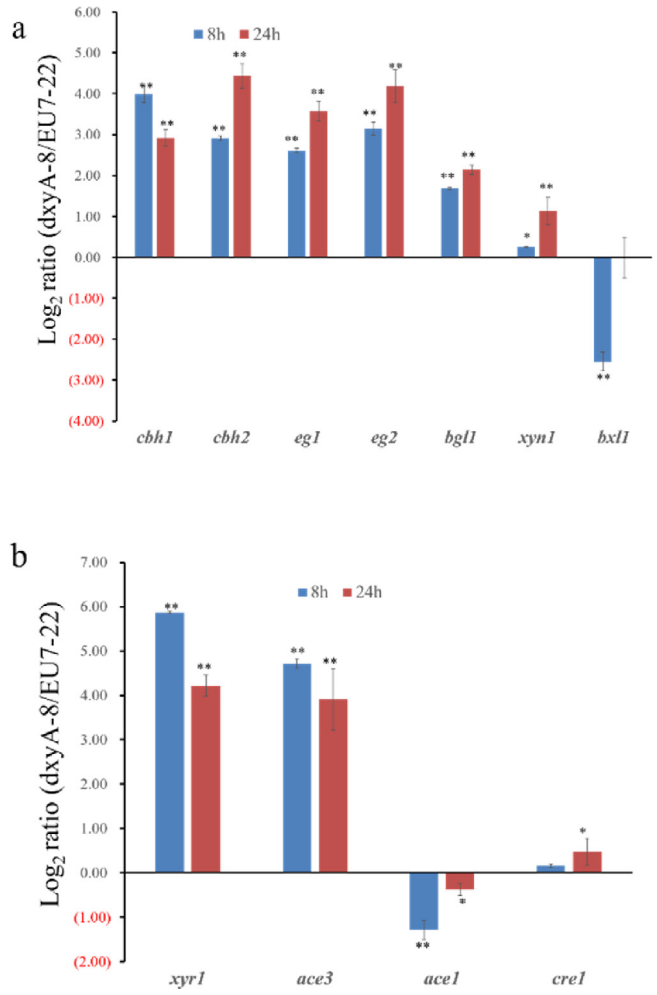

Fig. 3. Transcription analysis for genes encoding major cellulase, hemicellulase and transcription regulators of *T. orientalis* EU7-22 and dxyA-8 under the inducing condition. (a), comparison of transcription level for main genes encoding cellulase and hemicellulase. (b), comparison about transcriptional quantities of major transcription regulator genes for cellulase expression. Transcription levels of genes in *T. orientalis* EU7-22 and dxyA-8 were analyzed by qPCR. Transcription level of *actin* was used as the endogenous control. The values show the mean of three biological replicates and the error bar indicates the standard deviation. * ($p < 0.05$) and ** ($p < 0.01$) indicate statistical significance calculated by students' test.

*P*NPXase activity during the inducing culture. It indicated that overexpression of *xy1* and *ace3* affected the transcription of β-xylosidases in *T. orientalis*.

Xyr1 and Ace3 both act as the key transcription factors that activate the cellulase and xylanase production in *T. orientalis*. After the *xyr1* and *ace3* genes were constitutively overexpressed with the promoters of *pdc* and *eno* in *T. orientalis* EU7-22, the transcription levels of *xyr1* and *ace3* were substantially up-regulated in both post-8 h and post-24 h induction (Fig. 3b). The transcription quantity of regulator gene *ace1* in dxyA-8 was half of that in EU7-22 after the 8 h induction and still lower than that of EU7-22 after 24 h induction. The Ace1 act as a transcription repressor of cellulase and xylanase production in *Trichoderma* spp., so the deletion of *ace1* or RNA interference on *ace1* contributes to improve the cellulase and xylanase production (Aro et al., 2003; Fang & Chen, 2018; Wang, 2012). Lower level of *ace1* transcription was one of reasons to improve the cellulase and hemicellulase production of dxyA-8. The hyper-production of cellulase can release more glucose from Avicel and wheat bran in short time and cause the carbon metabolic repression, so the levels of *cre1* transcription of dxyA-8 was higher than that of EU7-22 after 24 h induction (Fig. 3b). The deletion of *cre1* in *T. orientalis* EU7-22 can improve cellulase and hemicellualse production dramatically in the glucose repression medium (Long et al., 2018). Therefore, the disruption the *cre1* and *ace1* of dxyA-8 can further increase the cellulase and hemicellulase production of *T. orientalis*.

3.5. Phenotype and growth rate analysis of EU7-22 and dxyA-8

To compare the phenotype and growth rate between the cellulase hyper-production strain dxyA-8 and its parental strain EU7-22, the equal spores of them were inoculated on plates with different carbon source mediums including glucose, xylose, lactose, xylan, Avicel and CMC-Na and cultured for 72 h at 30 °C. The result (Fig. 4a) demonstrated that overexpression of *ace3* and *xyr1* in *T. orientalis* slightly affected the conidia formation. The spread rates of dxyA-8 were almost the same to EU7-22 on MX and ML medium, a little slower on MY, MA and MC medium (Fig. 4b), and dramatically slower on MM (Fig. 4b). The result showed that overexpression of *ace3* and *xyr1* in *T. orientalis* can slow down the growth rate of mutant strain when using the glucose as carbon resource. So we further investigated the growth kinetics of dxyA-8 and EU7-22 with 3% glucose fermentation medium. The biomass of dxyA-8 was only approximately half of the EU7-22 from the 3th day to the 5th day (Fig. 4c), but as aforementioned, the corresponding cellulase and hemicellulase activities were extremely higher than that of EU7-22.

3.6. Improved reducing sugar release from NPSA using the crude enzyme produced by the recombinant strain dxyA-8

The equal volume of crude enzyme produced by EU7-22 and dxyA-8 under inducing condition for 4 days was used for hydrolyzing NPSA to evaluate the efficiency of cellulase (Fig. 3 h). Reducing sugars in the hydrolysate released by cellulase of dxyA-8 reached 12.89 mg/mL at 72 h, which increased about 24% compared with that of EU7-22. The efficiency of hydrolyzing pretreated biomass depends on the component of enzymatic cocktails (Reyes-Sosa et al., 2017). Overexpression *ace3* and *xyr1* in *T. orientalis* improved the cellulase and hemicellulase production and altered the diversity of enzymatic cocktails. In consequence, the hydrolysis of NPSA by crude enzyme produced by mutant strain dxyA-8 was more efficient than that of the parental strain EU7-22.

The integration site may affect the expression level of regulators, leading to the variation of cellulase production among transformants (Zhang et al., 2018). So after transformation, the mutants were further screened to get the strain with the highest cellulase activity. Moreover, the integrated fragment of expression cassette in the genome may disrupt the key transcription factors of cellulase production or genes encoding cellulase, leading to decrease the cellulase activity in the transformants, such as dxyA-3 and dxyA-7 strains (Fig. 1b).

The dxyA-8 strain with overexpressed *xyr1* and *ace3* genes showed high cellulase activity. The FPase activity was up to 2.55 IU/mL in the fermentation using 3% glucose medium with Erlenmeyer flask, thus the dxyA-8 strain has high great potential for industrial scale production of cellulase preparations. The insoluble cellulosic materials are the most effective natural inducer for cellulase production by fungi, but their insolubility causes lots of problems during industrial scale production (Li et al., 2017). So the soluble inducer lactose is the prior selection for cellulase production in industry. In this study, as a substitute cheaper than lactose, the glucose can be another option for cellulase production in industrial scale. For the cellulase production using glucose as the carbon source in industrial scale, the dxyA-8 should be furtherly investigated for genetic modifications, such as release the CCR by the disruption of *cre1*, the deletion of *ace1*, the constitutive overexpression of *bgl1*, and so on.

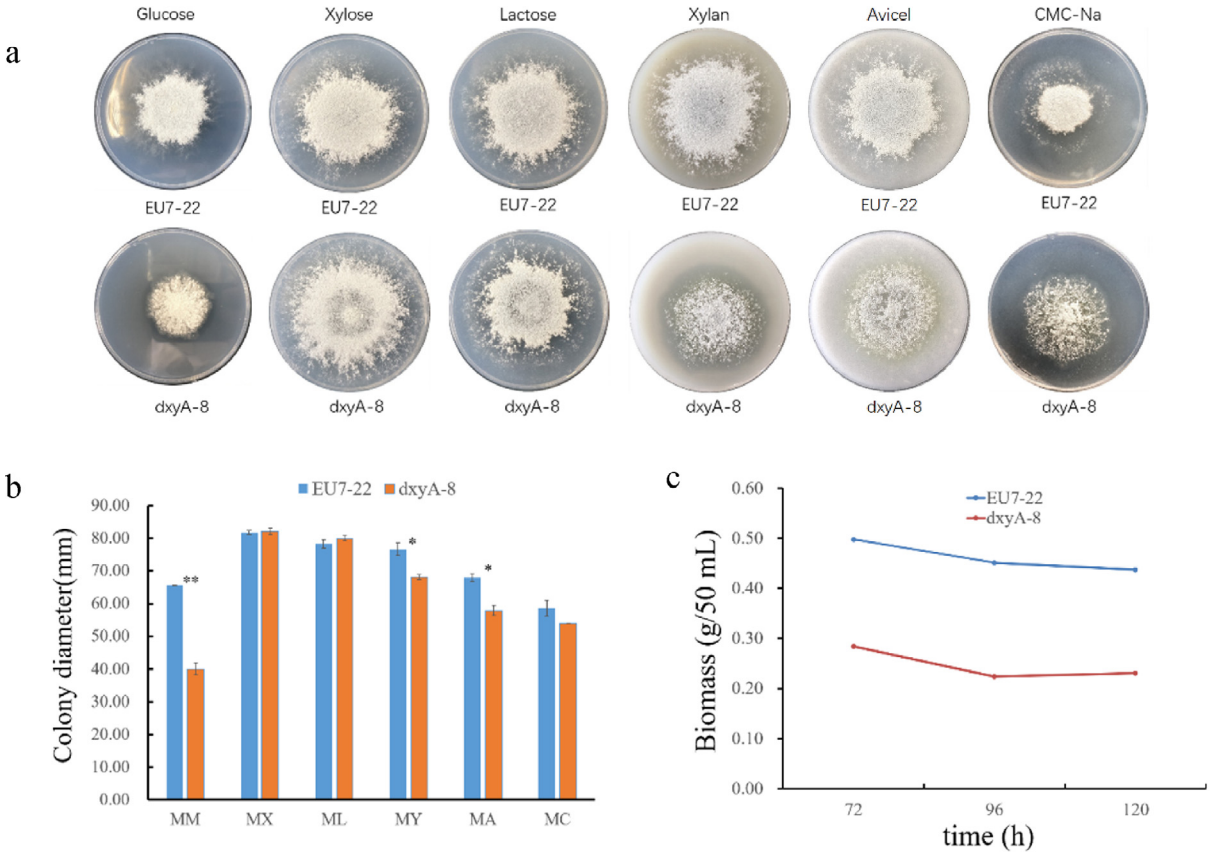

Fig. 4. Colony morphology (a) and spread rate (b) of EU7-22 and dxyA-8 on difference carbon resource medium plates for 3 days and biomass (c) of them cultured in 3% glucose fermentation medium.

4. Conclusion

The simultaneous overexpression of *xyr1* and *ace3* substantially improves cellulase production in *T. orientalis* EU7-22 under both cellulosic inducer and glucose condition. Reducing sugar released from NPSA by the crude enzyme of dxyA-8 under inducing condition was achieved by 24% improvement. The FPase activity of dxyA-8 was up to 2.55 IU/mL with glucose as the carbon source and it shows great potential for the cellulase production in industrial scale.

Declaration of 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.

Acknowledgments

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References

Amore, A., Giacobbe, S., Faraco, V., 2013. Regulation of cellulase and hemicellulase gene expression in fungi. Curr. Genomics 14, 230–249.
Aro, N., Ilmen, M., Saloheimo, A., Penttila, M., 2003. ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. Appl. Environ. Microb. 69 (1), 56–65.
Aro, N., Saloheimo, A., Ilmen, M., Penttila, 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.
Brakhage, A.A., Andrianopoulos, A., Kato, M., Steidl, S., Davis, M.A., Tsukagoshi, N., Hynes, M.J., 1999. HAP-Like CCAAT-binding complexes in filamentous fungi implications for biotechnology. Fungal Genet. Biol. 27, 243–252.
Chandel, A.K., Garlapati, V.K., Singh, A.K., Antunes, F.A.F., da Silva, S.S., 2018. The path forward for lignocellulose biorefineries: bottlenecks, solutions, and perspective on commercialization. Bioresource Technol. 264, 370–381.
Chuannan, L., Yijin, C., Xiaobing, W., Jian, L., Minnan, L., 2012. Cloning and sequence analysis of cellulase genes from *Hypocrea orientalis* EU7-22. Biotechnol. Bull. 11, 110–117.
da Silva Delabona, P., Rodrigues, G.N., Zubieta, M.P., Ramoni, J., Codima, C.A., Lima, D.J., Farinas, C.S., da Cruz Pradella, J.G., Seiboth, B., 2017. The relation between xyr1 overexpression in *Trichoderma harzianum* and sugarcane bagasse saccharification performance. J. Biotechnol. 246, 24–32.
Dashtban, M., Maki, M., Leung, K.T., Mao, C., Qin, W., 2010. Cellulase activities in biomass conversion: measurement methods and comparison. Crit. Rev. Biotechnol. 30 (4), 302–309.
Dashtban, M., Schraft, H., Qin, W., 2009. Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int. J. Biol. Sci. 5 (6), 578–595.
dos Santos Castro, L., de Paula, R.G., Antoniêto, A.C.C., Persinoti, G.F., Silva-Rocha, R., Silva, R.N., 2016. Understanding the role of the master regulator XYR1 in *trichoderma reesei* by global transcriptional analysis. Front. Microbiol. 7, 175.
Ellila, S., Fonseca, L., Uchima, C., Cota, J., Goldman, G.H., Saloheimo, M., Sacon, V., Siika-Aho, M., 2017. Development of a low-cost cellulase production process using *Trichoderma reesei* for Brazilian biorefineries. Biotechnol. Biofuels 10, 30.
Fang, C., Chen, X., 2018. Potential biocontrol efficacy of *Trichoderma atroviride* with cellulase expression regulator ace1 gene knock-out. 3. Biotech 8, 302.
Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.
Glass, N.L., Schmoll, M., Cate, J.H., Coradetti, S., 2013. Plant cell wall deconstruction by ascomycete fungi. Annu. Rev. Microbiol. 67, 477–498.
Guo, H., Chang, Y., Lee, D.J., 2018. Enzymatic saccharification of lignocellulosic biorefinery: research focuses. Bioresource Technol. 252, 198–215.
Häkkinen, M., Valkonen, M.J., Westerholm-Parvinen, A., Nina Aro, M.A., Vitikainen, M., Penttilä, M., Saloheimo, M., Pakula, T.M., 2014. Screening of candidate regulators for cellulase and hemicellulase production in *Trichoderma reesei* and identification of a factor essential for cellulase production. Biotechnol. Biofuels 7, 14.
Huang, C., Zhao, C., Li, H., Xiong, L., Chen, X., Luo, M., Chen, X., 2018. Comparison of different pretreatments on the synergistic effect of cellulase and xylanase during the enzymatic hydrolysis of sugarcane bagasse. RSC Adv. 8 (54), 30725–30731.
Kawaguchi, H., Hasunuma, T., Ogino, C., Kondo, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. Curr. Opin. Biotech. 42, 30–39.
Li, C., Lin, F., Zhou, L., Qin, L., Li, B., Zhou, Z., Jin, M., Chen, Z., 2017. Cellulase hyper-

production by *Trichoderma reesei* mutant SEU-7 on lactose. Biotechnol. Biofuels 10, 228.
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 (1), 84–93.
Linger, J.G., Taylor 2nd, L.E., Baker, J.O., Vander Wall, T., Hobdey, S.E., Podkaminer, K., Himmel, M.E., Decker, S.R., 2015. A constitutive expression system for glycosyl hydrolase family 7 cellobiohydrolases in *Hypocrea jecorina*. Biotechnol. Biofuels 8, 45.
Liu, F., Xue, Y., Liu, J., Gan, L., Long, M., 2018. ACE3 as a Master Transcriptional Factor Regulates Cellulase and Xylanase Production in *Trichoderma orientalis* EU7-22. BioResources 13 (3), 6790–6801.
Long, C., Cheng, Y., Cui, J., Liu, J., Gan, L., Zeng, B., Long, M., 2018. Enhancing cellulase and hemicellulase production in *Trichoderma orientalis* EU7-22 via knockout of the creA. Mol. Biotechnol. 60 (1), 55–61.
Long, C., Cheng, Y., Gan, L., Liu, J., Long, M., 2013. Identification of a genomic region containing a novel promoter resistant to glucose repression and over-expression of beta-glucosidase gene in *Hypocrea orientalis* EU7-22. Int. J. Mol. Sci. 14 (4), 8479–8490.
Long, C., Liu, J., Gan, L., Zeng, B., Long, M., 2017. Optimization of Xylanase production by *Trichoderma orientalis* using corn cobs and wheat bran via statistical strategy. Waste Biomass Valori. 10 (5), 1277–1284.
Mach-Aigner, A.R., Pucher, M.E., Steiger, M.G., Bauer, G.E., Preis, S.J., Mach, R.L., 2008. Transcriptional regulation of xyr1, encoding the main regulator of the xylanolytic and cellulolytic enzyme system in *Hypocrea jecorina*. Appl. Environ. Microb. 74 (21), 6554–6562.
Nakari-Setala, T., Paloheimo, M., Kallio, J., Vehmaanpera, J., Penttila, M., Saloheimo, M., 2009. Genetic modification of carbon catabolite repression in *Trichoderma reesei* for improved protein production. Appl. Environ. Microb. 75 (14), 4853–4860.
Raulo, R., Kokolski, M., Archer, D.B., 2016. The roles of the zinc finger transcription factors XlnR, ClrA and ClrB in the breakdown of lignocellulose by *Aspergillus niger*. AMB Express. 6, 5.
Reyes-Sosa, F.M., Lopez Morales, M., Platero Gomez, A.I., Valbuena Crespo, N., Sanchez Zamorano, L., Rocha-Martin, J., Molina-Heredia, F.P., Diez Garcia, B., 2017. Management of enzyme diversity in high-performance cellulolytic cocktails. Biotechnol. Biofuels 10, 156.
Rubin, E.M., 2008. Genomics of cellulosic biofuels. Nature 454 (7206), 841–845.
Sajith, S., Priji, P., Sreedevi, S., Benjamin, S., 2016. An overview on fungal cellulases with an industrial perspective. J. Nutr. Food Sci. 06 (1) 1000461.
Stricker, A.R., Grosstessner-Hain, K., Wurleitner, E., Mach, R.L., 2006. Xyr1 (xylanase regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. Eukaryot. Cell 5 (12), 2128–2137.
Stricker, A.R., Mach, R.L., de Graaff, L.H., 2008. Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina (Trichoderma reesei*). Appl. Microbiol. Biot. 78 (2), 211–220.
Szewczyk, E., Nayak, T., Oakley, C.E., Edgerton, H., Xiong, Y., Taheri-Talesh, N., Osmani, S.A., Oakley, B.R., 2006. Fusion PCR and gene targeting in *Aspergillus nidulans*. Nat. Protoc. 1 (6), 3111–3120.
Taha, M., Foda, M., Shahsavari, E., Aburto-Medina, A., Adetutu, E., Ball, A., 2016. Commercial feasibility of lignocellulose biodegradation: possibilities and challenges. Curr. Opin Biotech. 38, 190–197.
Wang, S.-W., 2012. Improving Cellulase Production in *Trichoderma koningii* Through RNA Interference on *ace1* Gene Expression. J. Microbiol. Biotechnol. 22 (8), 1133–1140.
Wang, S., Liu, G., Wang, J., Yu, J., Huang, B., Xing, M., 2013. Enhancing cellulase production in *Trichoderma reesei* RUT C30 through combined manipulation of activating and repressing genes. J. Ind. Microbiol. Biot. 40 (6), 633–641.
Xin, D., Sun, Z., Viikari, L., Zhang, J., 2015. Role of hemicellulases in production of fermentable sugars from corn stover. Ind. Crop. Prod. 74, 209–217.
Ye, Y., Li, X., Cao, Y., Du, J., Chen, S., Zhao, J., 2017. A beta-xylosidase hyper-production *Penicillium oxalicum* mutant enhanced ethanol production from alkali-pretreated corn stover. Bioresource Technol. 245 (Pt A), 734–742.
Zhang, F., Zhao, X., Bai, F., 2018. Improvement of cellulase production in *Trichoderma reesei* Rut-C30 by overexpression of a novel regulatory gene Trvib-1. Bioresource Technol. 247, 676–683.