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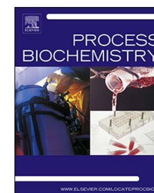

Optimization of acetyl xylan esterase gene expression in *Trichoderma reesei* and its application to improve the saccharification efficiency on different biomasses

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ABSTRACT

Trichoderma reesei is an important microbe in biomass hydrolysis but is characterized by its inability to remove acetylated glucomannan. To improve the saccharification efficiency of *T. reesei*, an acetyl xylan esterase (AXE) gene from *Aspergillus oryzae* was successfully overexpressed by fusing with xynlanase 2 (Xyn2) from *T. reesei* as a carrier protein. The production of AXE in the selected transformant T2 was 11.04 U/ml, which was approximately 74% higher than that of the parent strain. The molecular mass of recombinant AXE (rAXE) on SDS-PAGE was about 35 kDa. The saccharification efficiency of rAXE from *T. reesei* Rut-C30 was better than that of the parent strain and resulted in approximately 23.2%, 22.7%, and 19.4% higher yield of reducing sugar in paddy straw, corn stover plus paddy straw mix (1:1 ratio), and corn stalk (delignified) hydrolysis, respectively. This result demonstrates that the expression of heterogenic accessory enzymes in *T. reesei* may be an alternative approach to improve the ability of the enzyme complex to decompose lignocellulose.

1. Introduction

Hemicellulose is the second most abundant renewable polysaccharide in nature, which is composed of β-1,4-linked D-xylose polymers with random substitutions of arabinose, mannose, galactose, ferulic acid, acetic acid, and glucuronic acid with glycosidic and ester bonds [1]. Three xylanases, *exo*-xylanase, *endo*-xylanase, and β-xylosidase, are responsible for the complete hydrolysis of xylan to xylose. However, the acetylation of xylan has been reported to inhibit the action of *exo*-xylanase, which led to decreased xylose yield during biomass hydrolysis [2,3]. Recent research reported that the combined use of xylanases and other accessory enzymes, e.g., α-arabinofuranosidase, α-methylglucuronidase, acetyl xylan esterase (AXE), and ferulic acid esterase, results in enhanced hydrolysis of xylan and cellulose [2,4–8]. Among the different accessory enzymes, AXE is an important enzyme that can remove acetylated xylan and thereby increase the accessibility of xylan for other endo/exo-xylanase enzymes. AXEs from several fungi [9–13] and bacteria [14–18] have been characterized. *Trichoderma reesei* is one of the most important filamentous fungi, which is widely used in industry for its ability to secrete large quantity of cellulase and hemicellulase enzymes for biomass hydrolysis [19,20]. However, AXE activity is insufficient in *T. reesei* culture, compared with the cellulase and xylanase activity [21]. Moreover, the AXE of *T. reesei* is active only on polymeric xylan and not on glucomannan, whereas acetylglucomannan esterase from *A. orazye* is most active toward polymeric glucomannan and acetyl groups of xylan [22]. Heterologous expression of AXE is the only way to meet the industrial requirements and improve production yield of AXE for the efficient hydrolysis of lignocellulose biomass. The ester group present in plant cell walls is highly resistant to microbial enzymes [2,3,23]. A recent study reported that the addition of AXE 1 to *Myceliophthora heterothallica* enzyme mixture resulted in increased sugar yield during saccharification of sugar beet pulp [24]. Maeda et al. [25] reported that cocktail use of crude enzymes from *Penicillium funiculosum* and *Trichoderma harzianum* significantly improved biomass hydrolysis. However, the production cost of enzymes is higher when using a cocktail of enzymes from two different microbes; thus, engineering AXE in an industrial cellulase-producing microbe is more appropriate to improve its biomass saccharification efficiency.

Commercial productions of cellulases and hemicellulases are mainly from filamentous fungi, of which, *T. reesei* (telemorph *Hypocrea jecorina*) is being widely used as a workhorse of cellulases and hemicellulases production [26]. Cellobiohydrolase I (CBHI) accounts for 50–60% of the total secreted protein of *T. reesei* [27]. Its promoter, Pcbh1, which is the strongest measured promoter, has been widely used

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for the over-expression of homologous and heterologous proteins in *T. reesei* [28,29]. To date, only a few fungal AXEs from *Aspergillus oryzae*, *Aspergillus awamori*, *Volvariella volvacea,* and *Coprinopsis cinerea* have been functionally expressed in *Pichia pastoris* [6,30–32]. In the present work, we used for the first time an engineered Pcbh1 promoter from our previous studies [29] for the heterologous expression of AXE gene from *A. oryzae*, one of the most widely used fungi in industry, in *T. reesei*. We purified AXE and analyzed its efficacy in various biomass hydrolyses. We also tested how the expression of heterologous AXE in *T. reesei* would improve its ability to decompose different lignocellulose biomass.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sangon (Shanghai, China). Miniprep plasmid DNA purification kit and DNA Gel Extraction kit were purchased from Axygen (Hangzhou, China). Restriction enzymes and prestained protein markers were purchased from Thermo Fisher Scientific and GenStar (Waltham, MA, USA; Beijing, China). *p*-Nitrophenyl acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cloning was performed using ClonExpress MultiS One Step Cloning kit from Vazyme (Nanjing, China). Protein concentration was tested using Bio-Rad DC Protein Assay kit (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as standard. MEGAscript T7 kit was used for *in vitro* RNA transcription (Invitrogen, Vilnius, Lithuania).

2.2. Strain and culture conditions

A. oryzae RIB 40 (NBRC 100959) was used as the source organism for obtaining the AXE gene (cDNA). *T. reesei* Rut-C30 (ATCC 56765) was used as the recipient for the heterologous expression of AOAXE. All fungal strains were maintained on potato dextrose agar (PDA). Plasmids were propagated in *Escherichia coli* Top 10 competent cells (Thermo Fisher Scientific, Waltham, MA, USA) overnight at 37 °C with 200 rpm on an orbital shaker in Luria-Bertani (LB) broth containing 100 mg/ml kanamycin as a selection marker. *Agrobacterium tumefaciens* AGL1 [33] was propagated in LB medium at 28 °C with 200 rpm on an orbital shaker for 36 h, and this AGL strain was used as a T-DNA donor for the transformation of AXE gene into *T. reesei* Rut-C30. For genomic DNA isolation, *A. oryzae* and *T. reesei* Rut-C30 were cultured in Sabouraud's dextrose broth (SDB) for 2 days, and the mycelia were harvested by centrifugation at 12,000 × *g* for 5 min and used for the extraction of genomic DNA by using the Genomic DNA Extraction kit from Takara (Dalian, China).

2.3. Vector construction

The vector backbone used for constructing the plasmid was binary vector pCambia1300 (CAMBIA, Canberra, Australia). The T-DNA binary vector was constructed using pCambia1300 as a recipient. First, *hph* gene coding for hygromycin B phosphotransferase (under control of the *Aspergillus nidulans trpC* promoter and terminator) was introduced into the plasmid to generate pXBthg vector [34]. The engineered *cbh1* promoter Pcbh1M2 [29], *xyn2* gene (Genome.jgi.:124931), and the signal peptides coding sequences of CBHI and XYNII (named *spcbh1* and *spxyn2*, respectively) were amplified using the primer pairs Pcbh1F, Pcbh1R, Xyn2F, Xyn2R, Spcbh1F, Spcbh1R, Spxyn2F, and Spxyn2R, respectively (Table 1) using *T. reesei* Rut-C30 genomic DNA as the template. The primers AoaxeF and AoaxeR (Table 1) were designed to amplify the coding region of an AXE gene from *A. oryzae* (GenBank: AB167976) genomic DNA by fusion PCR along with the attachment of 6 × His-tag at the C-terminal of AXE. Then AOAXE was separately fused with *spcbh1*, *spxyn2*, and *xyn2* genes using Spcbh1F, Spxyn2F, Xyn2F, and AoaxeR primers. The primers TtrpcF and TtrpcR (Table 1) were designed to amplify *trpC* terminator (TtrpC) from *A. nidulans* genomic DNA as the template. Then all the fragments were assembled in pXBthg vector at the XbaI cloning site by using MultiS One Step cloning kit from Vazyme (Nanjing, China) resulting in pXBthg-spcbh1-AXE, pXBthg-spxyn2-AXE, and pXBthg-xyn2-AXE (Fig. 1). These vectors were further transformed into *A. tumefaciens* AGL1 for *Agrobacterium*-mediated transformation of AXE into *T. reesei* Rut-C30.

2.4. Transformation of AXE to T. reesei Rut-C30

Agrobacterium-mediated transformation was conducted as described previously by our group Ma et al. [34]. Seven-day-old conidia were collected from *T. reesei* Rut-C30 PDA plates by washing the plate with distilled water containing 0.02% Tween 80. *A. tumefaciens* AGL1 was grown in LB medium with kanamycin (100 mg/ml concentration) at 28 °C for 36 h, and then the cells were collected and diluted to 0.15 OD at 660 nm using an induction medium (IM) containing 0.2 mM acetosyringone. The cells were cultured again at 28 °C for 6–8 h to reach 0.6 OD value at 660 nm *T. reesei* Rut-C30 were co-cultured with *A. tumefaciens* cells by mixing equal volume of fungal conidia and bacterial cells (10^6/ml) and smeared on an IM plate containing 0.2 mM of acetosyringone. The cultures were incubated at 25 °C for 2 days and well-grown cultures were washed with 0.02% Tween 80 and plated onto PDA containing hygromycin (15 μg/ml) as a selection marker for transformants and 0.6 mM of cefotaxime to inhibit the growth of *A. tumefaciens*. After 4–7 days of incubation, hygromycin-resistant single-spore colonies were isolated from PDA, and the integration of AXE was confirmed by PCR using AoaxeF and AoaxeR primers (94 °C for 4 min, 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min and finally 72 °C for 5 min).

2.5. Deletion of acetyl xylan esterase 1 and 2 (AXE1 and AXE2) in Updc-Cas9 by CRISPR/Cas9 method

Our group previously established CRISPR/Cas9 gene expression in *T. reesei* Rut-C30 for the effective and precise editing of its genome, and we named the strain as Updc-Cas9 [35]. The protospacer adjacent motif (PAM) sequence of AXE1 (5′-GGTGGTCAGGCTTCATGTGG*cgg*-3′, PAM is shown in italics) and AXE2 (5′-GGTCGTTGCCCGCGGCAGCG*agg*-3′, PAM is shown in italics) gene CRISPR/Cas9 target site was identified (see Supplementary data). The final 127-bp sequences of gRNA for AXE1 and AXE2 were transcribed using the MEGAscript T7 *in vitro* transcription kit (Invitrogen). The donor DNA (dDNA) containing the 5′ and 3′ flanking sequences of AXE1 and AXE2 was amplified using primers listed in Supplementary data. The selection marker *ura5* gene cassette was obtained from our group, published by Liu et al. [35] (the *ura5* gene from *Penicillium oxalicum* controlled by the Pgpda promoter and Ttrpc terminator to form Pgpda-*poura5*-Ttrpc gene cassette). Assembling of AXE1 and AXE2 dDNA (5′ and 3′ flanking sequences) with *ura5* gene cassette into the pMD-18T vector was achieved using ClonExpress MultiS One Step Cloning kit from Vazyme. The generated vectors were propagated in *E. coli* Top 10 at 37 °C overnight, and plasmids were purified using the Miniprep Axygen Plasmid purification kit. The gRNA and the target sequences of AXE1 and AXE2 (dDNA) were co-transformed into *T. reesei* by protoplast transformation [35]. After 4–7 days of incubation at 28 °C, AXE1 and AXE2 deletions were verified by PCR (see Supplementary data).

2.6. Fermentation of T. reesei Rut-C30 for rAXE production

Seven-day-old actively grown *T. reesei* Rut-C30 conidial suspension (10^7/ml) was prepared using 0.02% Tween 80, and 0.5 ml spore suspension at a concentration of 10^7/ml was inoculated into a 50-ml flask containing 10 ml SDB. The flask was incubated at 28 °C for 48 h on an orbital shaker at 200 rpm. This actively grown culture was then transferred into a 50-ml flask containing 10 ml fermentation medium at

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