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Engineering *Trichoderma reesei* Rut-C30 with the overexpression of *egl1* at the *ace1* locus to relieve repression on cellulase production and to adjust the ratio of cellulolytic enzymes for more efficient hydrolysis of lignocellulosic biomass

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ABSTRACT

Cellulose hydrolysis is a synergetic process performed sequentially by different cellulolytic enzymes including endoglucanases, exoglucanases and glucosidases. *Trichoderma reesei* has been acknowledged as the best cellulase producer, but cellulase production by *T. reesei* through submerged fermentation is costly due to intensive energy consumption associated with the process for mixing and aeration, since non-Newtonian fluid properties are developed with mycelial growth. Therefore, engineering the ratio of cellulolytic enzymes in the cocktail for more efficient cellulose hydrolysis is an alternative strategy for reducing cellulase dosage and thus saving cost in enzyme consumption for cellulose hydrolysis. In this study, *T. reesei* QS305 with high endoglucanase activity was developed from *T. reesei* Rut-C30 by replacing the transcription repressor gene *ace1* with the coding region of endoglucanase gene *egl1*. Compared to *T. reesei* Rut-C30, *T. reesei* QS305 showed 90.0% and 132.7% increase in the activities of total cellulases and endoglucanases under flask culture conditions. When cellulase production by *T. reesei* QS305 was performed in the 5-L fermentor, cellulases activity of 10.7 FPU/mL was achieved at 108 h, 75.4% higher than that produced by *T. reesei* Rut-C30. Moreover, cellulases produced by *T. reesei* QS305 were more efficient for hydrolyzing pretreated corn stover and Jerusalem artichoke stalk.

1. Introduction

Lignocellulosic biomass is composed predominantly of cellulose, which needs to be hydrolyzed into glucose as feedstock for microbial fermentation to produce aimed products. Although cellulose can be hydrolyzed by chemical catalysis using either acid or alkali, enzymatic hydrolysis by cellulases under mild conditions without by products is more preferred (Sweeney and Xu, 2012). *Trichoderma reesei* has been acknowledged as the best cellulases-producer, and most strains for cellulase production in laboratory and industry have been derived from the fungal species, in particular from *T. reesei* Rut-C30 which was previously termed as a hyper-cellulases producer (Peterson and Nevalainen, 2012). However, cellulases produced by *T. reesei* are still too expensive, and not efficient for cellulose hydrolysis, making the sugar platform based on the enzymatic hydrolysis of the cellulose component one of the bottlenecks for the biorefinery of lignocellulosic biomass.

The reasons for high cost with cellulases are due to the unique characteristics of submerged fermentation of *T. reesei* and cellulose hydrolysis by cellulases (Singhania et al., 2010). On the one hand, both the growth of *T. reesei* and cellulase production by the species are aerobic, but the fermentation broth is developed as a non-Newtonian fluid quickly with high viscosity as mycelia grow, making the mixing and aeration very energy-intensive (Gabelle et al., 2012). On the other hand, cellulose hydrolysis is a synergetic process performed sequentially by different cellulolytic enzymes including endoglucanases, exoglucanases and glucosidases, and the ratio of these enzymes in the cocktail produced by *T. reesei* is not optimal (Bischof et al., 2016), which consequently requires cellulase to be supplemented at high dosage for cellulose hydrolysis. The whole genome sequencing of *T. reesei* Rut-C30 revealed that at least 200 genes encode glycoside hydrolases (GHs) and 17 of them have been biochemically determined to encode functional cellulases, including eight endoglucanases (EGs), two cellobiohydrolases (CBHs) and seven β-glucosidases (BGLs), which act

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0168-1656/

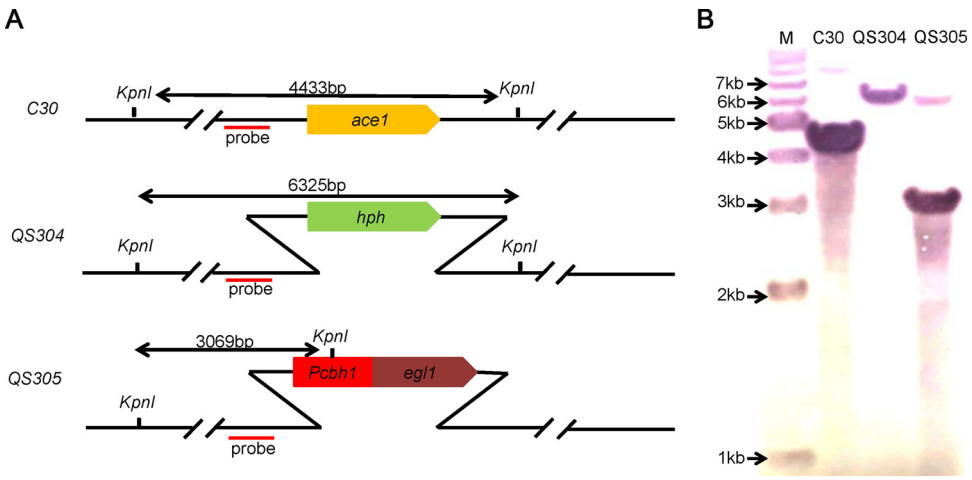

Fig. 1. Deletion of *ace1* from *T. reesei* Rut-C30 and integration of *egl1* into the *ace1* loci by homologous recombination (A) and the verifications of the gene deletion and integration by Southern blot analysis (B).

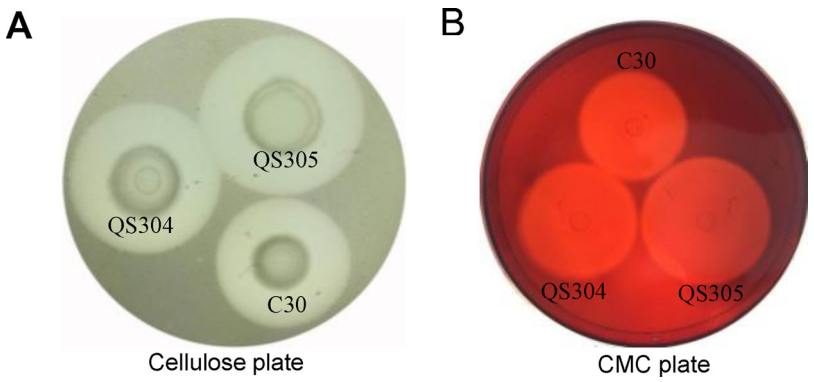

Fig. 2. Qualitative evaluation for the production of cellulases (A) and endoglucanases (B) by *T. reesei* QS304, QS305 and Rut-C30.

synergistically to ultimately degrade cellulose into glucose (Li et al., 2017a). EGs randomly hydrolyze cellulose chains at internal amorphous regions, generating ends for CBHs to attack progressively to release cellobiose, which is further hydrolyzed into glucose by BGLs (Kubicek et al., 2009). Four major cellulases including CBHI (Cel7A), CBHII (Cel6A), EGI (Cel7B) and EGII (Cel5A) represent up to 90-95% of total proteins secreted by *T. reesei*, in which CBHI comprises 50-60% (Merino and Cherry, 2007), and EGI accounts for 5-10% only (Miettinen-Oinonen and Suominen, 2002). EGI has broad substrate specificity, releasing reducing ends by hydrolyzing amorphous cellulose, hydroxyethyl cellulose and carboxymethyl cellulose as well as xylans (Klemanleyer et al., 1996). What's more, a high EGI ratio has been found to be a prerequisite for more efficient conversion of various substrates (Billard et al., 2012). The strong promoter *cbh1* has been frequently used for directing the expression of heterologous or homologous proteins in *T. reesei* (Ma et al., 2011; Li et al., 2017b). Therefore, we reason that EGI could be overexpressed in *T. reesei* under the direction of *cbh1* to enhance EGI production, and consequently adjust the ratio of cellulolytic enzymes in the cocktail for more efficient hydrolysis of the cellulose component in lignocellulosic biomass.

In *T. reesei*, cellulase biosynthsis is controlled by various regulatory factors, including at least four transcriptional activators (XYR1, ACE2, ACE3 and the HAP2/3/5 complex) and two repressors (ACE1 and CRE1) (Kubicek et al., 2009). The transcriptional factors ACEI and CRE1 were identified based on their ability to bind onto the *cbh1* promoter region of *T. reesei*, and consequently repress the expression of genes encoding cellulases (Aro et al., 2003; Ilmen et al., 1998). Deletion of *cre1* exhibited severe growth defect, and induction of *xyr1* and *ace2* required CRE1 (Nakari-Setala et al., 2009; Portnoy et al., 2010). However, *ace1* deleted strains grew better on cellulose-based medium due to the relief of its repression on *xyr1* transcription (Aro et al., 2003; Mach-Aigner et al., 2008). As a result, *ace1* disruption would be a useful strategy for strain engineering to improve cellulase production by *T. reesei*.

In this study, *T. reesei* Rut-C30 was engineered with the overexpression of the EGI encoding the gene *egl1* at the *ace1* locus to relieve its repression on cellulase synthesis, and in the meantime adjust the ratio of cellulolytic enzymes by enhancing the production of EGI for more efficient hydrolysis of the cellulose component in lignocellulosic biomass, which was evaluated by hydrolyzing pretreated biomass. We hypothesized that such a strategy would be more effective than engineering *egl1* into other sites from the viewpoint of the metabolic trade-off with host cells.

2. Materials and methods

2.1. Strains, media and culture conditions

Escherichia coli DH5α was used for plasmid propagation, which was cultivated in flasks with the Luria-Bertani (LB) medium in a shaker at 37 °C and 200 rpm. *Agrobacterium tumefaciens* AGL-1 was used for *T. reesei* transformation, which was grown in flasks with the LB medium in a shaker at 28 °C and 200 rpm. In case of need, kanamycin (50 μg/mL) was supplemented into the medium.

T. reesei Rut-C30 (ATCC 56,765) and transformants were cultured on malt extract agar plates (malt extract 3% and agar 2%) at 28 °C for 5–7 d to produce conidia. For analysis of cellulase production, *T. reesei* strains inoculated with 1×10^6 spores/mL were cultured with 250 mL