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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 e□cient hydrolysis of lignocellulosic biomass

Qing-Shan Meng[a], Chen-Guang Liu[b], Xin-Qing Zhao[b,*], Feng-Wu Bai[a,b,*]

[a] School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116024, China
[b] State Key Laboratory of Microbial Metabolism and School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

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ABSTRACT

Cellulose hydrolysis is a synergetic process performed sequentially by di□erent 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 e□cient 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 e□cient 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 e□cient 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 di□erent 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

* Corresponding authors at: State Key Laboratory of Microbial Metabolism and School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China.
E-mail addresses: xqzhao@sjtu.edu.cn (X.-Q. Zhao), fwbai@sjtu.edu.cn (F.-W. Bai).

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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 e□cient 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 e□cient 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 du f v1Tq1qT1jqfl

were applied for each PCR reaction. The relative transcription of genes was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Batch culture within fermentor for cellulase production

Spores grown on the malt extract agar plate was washed with sterilized water, and 10^8 spores/mL was transferred into 250 mL Erlenmeyer flasks containing 50 mL medium composed of 5 g/L glucose and 10 g/L corn steep liquor for mycelia to grow as seed, which was cultured for 24 h at 28 °C and 180 rpm. The mycelial seed culture was inoculated at 10% (v/v) into the 5-L fermentor (Shanghai BaoXing Bio-Engineering CO., Ltd) containing 3 L MM-based medium supplemented with 2% Avicel, 2% wheat bran, 1.5% soybean cake flour and 0.2% Tween-80. The pH for the submerged fermentation was controlled at 4.8 through automatic addition of ammonia water. The dissolved oxygen (DO) was controlled above 20% by manipulating the agitation speed and the aeration rate automatically. Antifoamer (DOWFAX DF103, DOW Chemical Co., Ltd., USA) was added into the fermentor automatically to prevent foam development. The temperature was controlled at 28 °C by pumping cooling water. Samples were collected intermittently to measure cellulose consumption, mycelial growth, cellulase production and secretion of extracellular proteins.

2.8. Hydrolysis of pretreated lignocellulosic biomass

Corn stover (CS) is a typical agricultural residue, which has been intensively studied for biorefinery, particularly for producing cellulosic ethanol. Jerusalem artichoke (JA) is a potential energy crop (Li et al., 2016), and its stalk is an emerging lignocellulosic biomass. Therefore, CS and JA stalk were selected to evaluate the performance of raw cellulases produced by *T. reesei* QS304 and QS305, which were pretreated in 5 L flask by 2% (w/v) NaOH with solid uploading of 10% (w/v) through autoclaving at 121 °C for 90 min. Then the alkali-pretreated corn stover (APCS) and Jerusalem artichoke stalk (APJAS) were washed by water to remove alkali and dried at 45 °C for 48 h in an oven to constant mass. The chemical compositions of APCS and APJAS were determined following the NREL analytical protocol for cellulose, hemicelluloses, lignin and ashes (Sluiter et al., 2008).

The amount of 2.5 g APCS or APJAS was hydrolyzed in 50 mL citrate buffer (50 mM, pH4.8) at 50 °C and 150 rpm by crude cellulases produced by the batch culture of *T. reesei* QS304, QS305 and Rut-C30 at the dosage of 30 mg/g substrate. Glucose released from the enzymatic hydrolysis was analyzed by HPLC at an interval of 12 h according to previously described conditions (Li et al., 2016). Glucose yield was calculated as follows:

$$\text{Glucose yield} = \frac{\text{Glucose (mg)}}{\text{Substrate (mg)} \times \text{Cellulose content (\%)} \times 1.1} \times 100\%$$

3. Results and discussion

3.1. Design for ace1 delection and egl1 overexpression

The hygromycin-resistant cassette and *egl1* over-expression cassette were engineered into the *ace1* locus of *T. reesei* Rut-C30, respectively, through homologous recombination intermediated by *A. tumefaciens* AGL-1 (Fig. 1A) for the deletion of *ace1* and overexpression of *egl1*. The

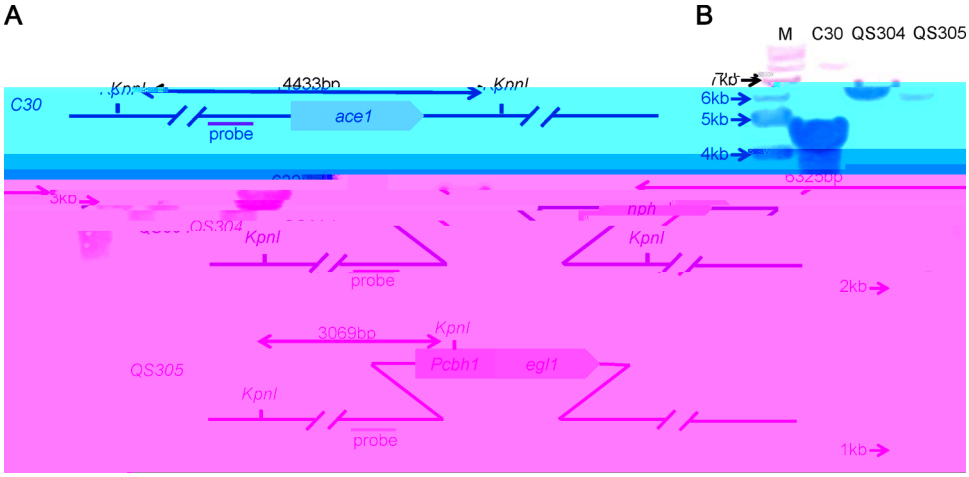

Fig. 1. Deletion of *ace1* from *T. reesei* Rut-C30 and integration of *egl1* into the *ace1* loci by homologous recombination (A) and the verifications of the gene deletion and integration by Southern blot analysis (B).

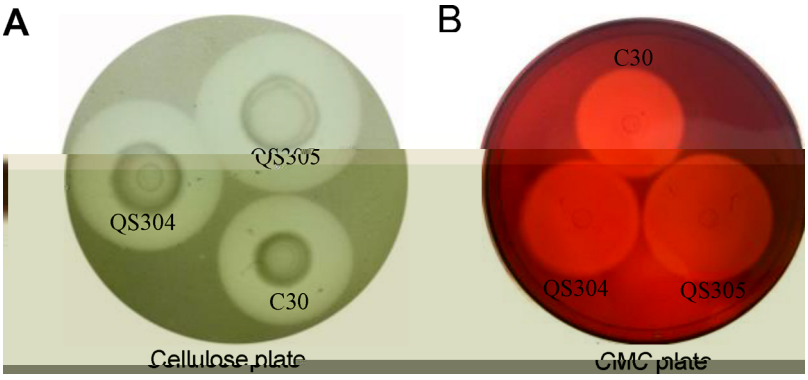

Fig. 2. Qualitative evaluation for the production of cellulases (A) and endoglucanases (B) by *T. reesei* QS304, QS305 and Rut-C30.

transformants *T. reesei* QS304 and QS305 were selected under the selective pressure to study the impact of these genetic modifications on cellulase production by *T. reesei*. The deletion of *ace1* from *T. reesei* Rut-C30 and the integration of *egl1* into the *ace1* locus were verified by the Southern-blot analysis of the DNA fragments between the 2 restriction sites cut by KpnI: 4,433 bp fragment with *ace1* from *T. reesei* Rut-C30, 6,325 bp fragment with *hph* from *T. reesei* QS304 and 3,069 bp fragment with the promoter region of *egl1* from *T. reesei* QS305 (Fig. 1B).

3.2. Cellulase production by T. reesei QS304 and QS305

Compared to *T. reesei* Rut-C30, larger transparent zones were developed by *T. reesei* QS304 and QS305 on the cellulose plate (Fig. 2A), indicating that more cellulases were produced due to the relief of the repression of ACE1. On the other hand, the largest de-colored zone was developed by *T. reesei* QS305 on the CMC plate due to its enhanced production of endoglucanase caused by the overexpression of *egl1* (Fig. 2B).

The production of major cellulolytic enzymes and extracellular proteins by *T. reesei* QS305 and QS304 was further quantitatively evaluated in flasks under submerged culture conditions, and the experimental results are shown in Fig. 3. Activities of 2.75, 20.47, 0.74 and 0.35 IU/mL were detected at 6 d for FPase, CMCase, *p*NPCase and *p*NPGase produced by *T. reesei* QS304, which increased 51.9%, 58.1%, 34.5% and 34.6%, respectively, compared to that of 1.81, 12.95, 0.55 and 0.26 IU/m produced by *T. reesei* Rut-C30, indicating the deactivation of *ace1* substantially improved cellulase production by *T. reesei*. Meanwhile, more extracellular proteins of 7.28 mg/mL were produced by *T. reesei* QS304, compared to that of 6.42 mg/mL produced by *T. reesei* Rut-C30. On the other hand, activities of 3.42, 27.04 and 1.28 IU/mL were detected at 6 d for FPase, CMCase and *p*NPCase produced by *T. reesei* QS305, which increased 24.3%, 32.1% and 73.0%, respectively, compared to that produced by *T. reesei*

glucosidase as that produced by *T. reesei* Rut-C30 was achieved at 7 d.

Based on detected activities of the cellulolytic enzymes and extracellular proteins, we calculated specific enzyme activities for the cellulase cocktail produced by *T. reesei* QS304, QS305 and Rut-C30, and no significant difference was observed in the profiles of the specific enzyme activities (Supplementary Fig. 2S), indicating that the improved production of extracellular proteins was due to the enhanced production of the cellulolytic enzymes rather than impurities.

3.3. Transcription analysis for T. reesei QS304 and QS305

In order to explore molecular mechanism underlying enhanced cellulase production by *T. reesei* QS304 and QS305, vigorously growing mycelia were sampled at 24 h and 48 h, and transcription of genes encoding major cellulases as well as regulating factors and accessory proteins for cellulose hydrolysis was analyzed, and compared to that detected in *T. reesei* Rut-C30 (Fig. 4).

While genes *cbh1*, *cbh2*, *egl1*, *egl2* and *bgl1* encode primary cellulolytic enzymes CBHI, CBHII, EGI, EGII and BGL1, genes *ace2*, *ace3*, *xyr1*, *vib1* and *bglr* encode regulators for the synthesis of the aforementioned cellulases (Druzhinina and Kubicek, 2017; Nitta et al., 2012). Although xylanases do not hydrolyze cellulose directly, they hydrolyze xylan in hemicelluloses to disrupte the complex composed of cellulose, hemicelluloses and lignin, exposing cellulose for hydrolysis by cellulolytic enzymes. Therefore, the expression of *xyn1* and *xyn2* encoding major xylanases (Törrönen et al., 1992) was studied together with those genes encoding the primary cellulolytic enzymes. In addition, a few proteins encoded by *swo1*, *cip1*, *cip2* and *Cel61a* have been identified as accessory proteins that involve in the enzymatic hydrolysis of cellulose through their role in the disruption of cellulose fibers (Kim et al., 2014; Li et al., 2007).

For *T. reesei* QS304 with *ace1* deletion, improved transcription of 3.02, 2.94 and 8.03 folds was observed earlier at 24 h for *bgl1*, *xyn1* and *xyn2*. On the other hand, the expression of regulating factors was also enhanced, particularly at the early stage, since the relative expression of 2.75, 2.57, 4.64, 2.88 and 2.18 folds was observed for *ace2*, *ace3*, *bglr, xyr1* and *vib1* at 24 h. ACE2, ACE3 and BglR are regulators for the transcription of genes encoding cellulolytic enzymes, and XYR1 works as a regulator for the expression of xylanases in *T. reesei* (Stricker et al., 2006). Although the regulation of VIB1 on cellulase synthesis is uncertain to a large extent, it was reported that the deletion of *vib1* from *T. reesei* compromised its cellulase production (Ivanova et al., 2017). These results indicated that the deletion of *ace1* improved cellulase production by *T. reesei* through releasing its repression directly on the genes encoding cellulolytic enzymes and also indirectly on the genes encoding regulating factors.

When *egl1* was integrated into the *ace1* locus (*T. reesei* QS305), the expression of *egl1* was substantially enhanced to 12.92 folds at the early stage of 24 h, which consequently contributed to the production of endoglucanases characterized by the increased CMCase activity. On the other hand, the down-regulated expression of 2.17 folds was observed for *bgl1* at 24 h, which was in accordance with the decreased pNPGase activity. In addition, an enhanced expression of 2.13, 2.64, 2.07 and 1.81 folds was observed at 24 h for *ace2*, *ace3*, xyr1 and *vib1*, indicating that the production of endoglucanases might be specifically regulated by these factors.

Swollenin is another accessory protein that synergistically enhances endoxylanase activity by disrupting the complex composed of cellulose, hemicelluloses and lignin, and thus indirectly enhancing the hydrolysis of cellulose by cellulases (Andberg et al., 2015). Cellulose induced proteins (CIP1 and CIP2) are also important for efficient hydrolysis of cellulose by cellulases (Li et al., 2007). Lytic polysaccharide monooxygenases (LPMOs) encoded by *Cel61a* is assumed to act on the surface of crystalline cellulose fibrils, making them more accessible for cellulases to hydrolyze the cellulose component (Kim et al., 2014). Therefore, the transcription of genes encoding these accessory proteins was evaluated. For *T. reesei QS304*, up-regulation was observed at 24 h for *cip1*, *cip2* and *Cel61a*. Particularly, the expression of *Cel61a* was substantially enhanced by 3.94 and 3.12 folds, respectively, in *T. reesei* QS304 and QS305. No doubt, the enhanced production of these accessory proteins would facilitate cellulose hydrolysis by cellulases, although mechanism underlying this phenomenon is still unclear.

3.4. Cellulase production through submerged culture

Cellulase production by *T. reesei* QS304 and QS305 with cellulose as substrate was performed in the 5-L fermentor. The major challenge in cellulase production through submerged culture is low enzyme titer and prolonged fermentation time, which together compromise cellulase

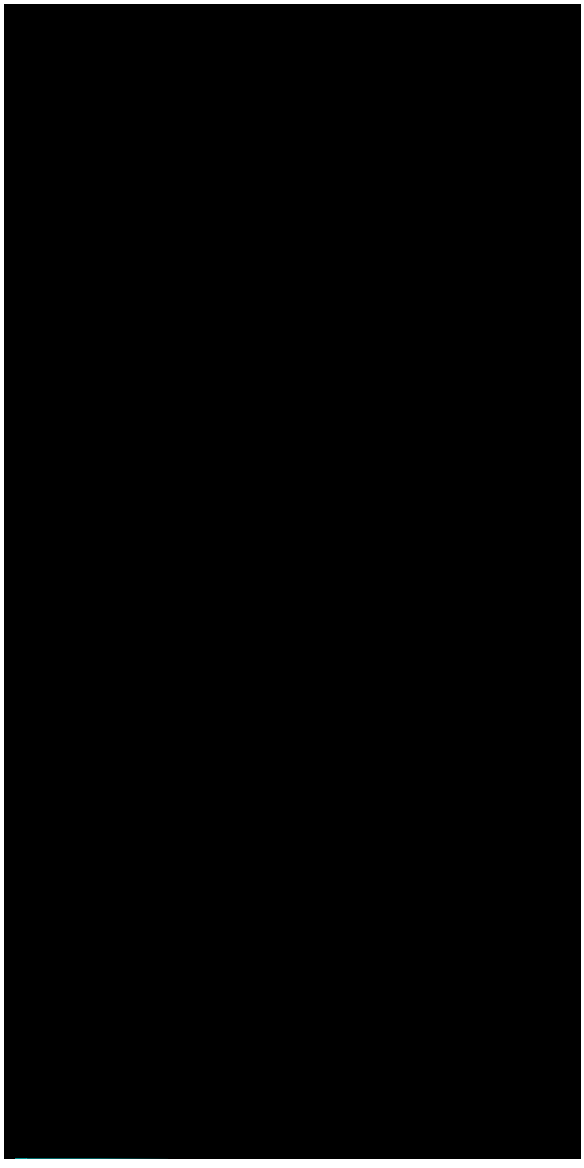

Fig. 4. Transcription analysis of genes encoding major cellulolytic enzymes (A), regulators for cellulase synthesis (B) and accessory proteins for cellulose hydrolysis (C) for *T. reesei* QS304 and QS305 with that detected in *T. reesei* Rut-C30 as the reference. Gene transcription with both the mutants and the control were normalized to the transcription of their internal control gene *tef1*, and R represents the ratio of the transcription with targeted genes detected in the mutants over that detected in the control. The data are the means of the triplicate with standard deviations.

Fig. 5. Cellulase production by *T. reesei* QS304, QS305 and Rut-C30 under submerged culture conditions in the 5-L fermentor. Averages of the duplicate are shown with standard deviations.

productivity of the fermentors, and substantially increase energy consumption, since both mycelial growth and cellulase production are aerobic, and the fermentation broth is characterized by non-Newtonian fluid properties, particularly its high viscosity developed as mycelia grow, making aeration and mixing extremely energy-intensive.

As can be seen in Fig. 5, *T. reesei* QS305 consumed cellulose more quickly at the early stage, and grew better with more biomass accumulated. As a result, cellulases activity of 10.7 IU/mL was achieved at 108 h, increased 35.4% and 75.4%, respectively, compared to that of 7.9 IU/mL and 6.1 IU/mL achieved by *T. reesei* QS304 and Rut-C30, and productivity of the fermentor was increased to 99.1 IU/L/h for cellulase production by *T. reesei* QS305, compared to 73.1 IU/L/h and 56.5 IU/L/h achieved for celllulases production by *T. reesei* QS304 and Rut-C30. We calculated specific activities (IU/g mycelial biomass) for cellulases produced by *T. reesei* QS304 and QS305, which were increased 37.3% and 50.3%, respectively, at 108 h compared to cellulases produced by *T. reesei* Rut-C30. Xylanase production was also significantly increased for *T. reesei* QS305, with maximal xylanase activity of 430.5 IU/mL achieved at 108 h, which was increased by 13% and 38% compared to that of 381.2 IU/mL and 311.6 IU/mL produced by *T. reesei* QS304 and Rut-C30. The improved xylanase production would facilitate xylan hydrolysis directly and cellulose hydrolysis by cellulases indirectly for

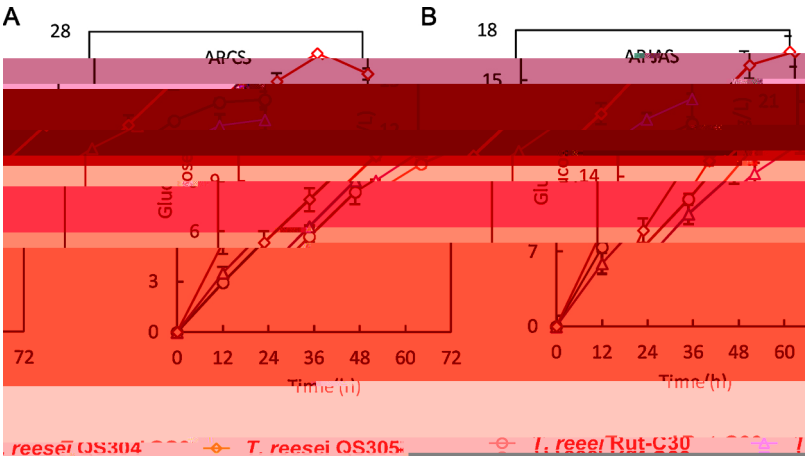

Fig. 6. Hydrolysis of APCS (A) and APJAS (B) by cellulases produced by *T. reesei* QS305, QS304 and Rut-C30. Data are represented as the mean of the triplicate with standard deviations.

lignocellulosic biomass pretreated by alkali with xylan remained. Due to enhanced production of cellulases and xylanase by *T. reesei* QS305, 25.2 g/L extracellular proteins were produced at 108 h in the fermentation broth, increased 57.5% and 83.9%, respectively, compared to that of 16.0 g/L and 13.7 g/L produced by *T. reesei* QS304 and Rut-C30.

3.5. Enzymatic hydrolysis of APCS and APJAS

In order to evaluate their hydrolytic performance, crude cellulases produced through batch culture were used to hydrolyze APCS and APJAS (Supplementary Table S3). Under same enzyme dosage conditions, 26.0 g/L and 16.7 g/L glucose was released from APCS and APJAS at 60 h and 72 h, respectively, by cellulases produced with *T. reesei* QS305, increased 35.4% and 20.1% as well as 21.5% and 34.6% compared to that of 19.2 g/L and 13.9 g/L as well as 21.4 g/L and 12.4 g/L released from the hydrolysis by cellulases produced with *T. reesei* QS304 and Rut-C30 (Fig. 6). Di□erence in glucose yield (80.7%, 59.6% and 66.4% for APCS and 63.8%, 53.13% and 47.4% for APJAS) observed in the hydrolysis by cellulases produced by *T. reesei* QS305, QS304 and Rut-C30 would be due to di□erent composition and structure of APCS and APJAS. However, improved cellulose hydrolysis and glucose yield were observed for cellulases produced by *T. reesei* QS305.

The relatively low activity of glucosidase in cellulases produced by *T. reesei* QS305 didn't a□ect the hydrolysis of APCS and APJAS significantly, since the genetic modification of *T. reesei* Rut-C30 didn't a□ect its glucosidase production. Although more glucosidase was produced by *T. reesei* QS304, lower production of endoglucanase and cellobiohydrolase compromised this advantage in hydrolyzing APCS and APJAS. These experimental results clearly indicated that cellulases produced by *T. reesei* Rut-C30 are short of endoglucanase to hydrolyze cellulose chains randomly, creating more ends for cellobiohydrolase to act on. It has been acknowledged that glucosidase is not enough in cellulases produced by *T. reesei* Rut-C30 (Karkehabadi et al., 2014), and overexpression of BGLs from di□erent sources could address this problem (Nakazawa et al., 2012; Dashtban and Qin, 2012). Alternatively, cellulases produced by *T. reesei* can be formulated by supplementing glucosidase commercially produced at low cost. Therefore, improvement of glucosidase activity, either by genetic modification of *T. reesei* QS305 or formulating its cellulases, will further improve synergistic hydrolysis of the cellulose component in lignocellulosic biomass.

4. Concluding remarks

This work demonstrated that disruption of *ace1* in *T. reesei* Rut-C30 and integration of *egl1* into the *ace1* locus for its overexpression improved cellulase production, particularly endoglucanase production. Cellulase titer of 10.7 IU/mL was achieved at 108 h for submerged culture of *T. reesei* QS305, increasing 75.4% compared to that of 6.1 IU/mL achieved by *T. reesei* Rut-C30, and cellulase productivity of the fermentor was increased to 99.1 IU/L/h. In addition, APCS and APJAS were hydrolyzed more e□ciently by crude cellulases produced by *T. reesei* QS305. Therefore, such a strategy would benefit cellulase production by *T. reesei* for more e□cient hydrolysis of the cellulose component in lignocellulosic biomass.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.09.001.

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