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Construction of novel *Trichoderma orientalis* EU7-22, strain with improved β-glucosidase activity by cellobiohydrolase I promotor optimization from *Trichoderma reesei*

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ABSTRACT

An upstream region of cellobiohydrolase I gene promoter was modified from *Trichoderma reesei* to enhance heterologous gene expression. The base pairs from −677 to −724 were deleted indicating glucose repressor binding sites. *Trichoderma reesei* is considered an important workplace for homologous/heterologous genes expressions under the modified *cbh1* promoter. However, constitutive expression is desirable in some cases over inducible expression that leads to production of undesirable cellulase components as byproduct. Here we report that the reporter β-glucosidase gene (*bgl1*) from *Aspergillus niger* BE-2 was cloned and recombined with *cbh*1 promoter followed by terminator *trpC* cloned from fungal expression vector, pUR5750. The expression cassette was ligated to binary vector and transferred into the host strain EU7-22 via *Agrobacterium tumefaciens* mediated transformation (ATMT) using hygromycin B resistance gene as the screening marker. Four transformants Bgl-1, Bgl-2, Bgl-3 and Bgl-4 were screened. Compared with the host strain EU7-22, the enzyme activities of filter paper (FPA) and β-glucosidase (BG) of transformant Bgl-3 increased by 39% and 47.1% under repression condition respectively. The expression level of Bgl-3 was found 7.9 folds increase over host strain EU7-22. Our results indicate that *cbh*1 is a strong promoter and has successfully driven over-expression of *bgl1* gene in EU7-22 under repression condition and has surprisingly improved the efficiency of heterologous expression of cellulase genes in EU7-22.

1. Introduction

Lignocellulosic biomass represents a wide range of polysaccharides as renewable resource which can be used to produce environment-friendly biofuels, chemicals, polymers and materials (Kamm and Kamm, 2007). Lignocellulosic biomass conversion to fermentable sugars mainly depends on the degradation capacity of a range of cellulose degradation enzymes secreted by several fungi, including *Aspergillus*, *Penicillium, Trichoderma*, and *Acremonium* (Kumar et al., 2008; Kovacs et al., 2009). The cellulose degradation enzyme compound contains three kinds of fundamental divisions of enzymes: endoglucanases (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidases (EC3.2.1.21) all work together to destroy cellulose into glucose (Sipos et al., 2010). However, when filamentous fungi yield large amount these enzymes, it was regulated by carbon catabolite repression (CCR) that mainly control carbon assimilation (Portnoy et al., 2011; Takashima et al., 1996; Cubero and Scazzocchio, 1994). It is necessary, for efficient gene expression in filamentous fungi, that the expression of the target gene be enhanced by use of an active promoter derived either from the host or related species. The cbh promoter from *T. reesei* has been considered the strongest promoter and is generally used to construct high-efficiency expression vectors to yield homologous and heterologous proteins (Liu et al., 2008). Several mutant strains can produce cellulases (40 gL^{-1}) and major cellulase in which *cbh* accounts for approximately 50% of all secreted proteins (Durand et al., 1988). Thus, *cbh*1 promotor has been considered the strongest promoter in *T. reesei* and is generally used to construct highly efficient expression vectors to yield homologous and heterologous proteins (Nyyssonen et al., 1995; Penttila et al., 1987). *Trichoderma reesei* is considered an important the filamentous fungi to secrete industrialized cellulose degradation enzymes. After all, still a competitive system needs to improve recombinant cellulase expressions. For this desire, we made *cbh*1 promotor optimization to construct a hyper expression system in EU7-22.

Several types of saccharides as cellulose, sophorose, lactose and

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others are used as inducers of *cbhI* promoter to induce expression of the target protein and is regulated by catabolic repression After all, these kinds of inducers also enhance the expression of cellulase components, such as cellobiohydrolases, endo-β-glucanases and xylanases (Xu et al., 2000; Mach and Zeilinger, 2003; Seiboth et al., 2007).

The production of cellulose degradation enzymes is mainly coordinated at transcription level in fungi. Both the pathway related coordination, such as repression and induction, and wide-tirretory coordination discipline are managing at transcription level, along with regulatory portion by carbon source, including the carbon catabolite opressors (CREI/CreA) from *T. reesei* and *Aspergillus*, the cellulase activator (Ace2) from *T. reesei*, and a CCAAT box-binding protein in filamentous fungi (Kato, 2005). The cellulose degradation genes are reported to repressed in glucose existance in *T. reesei* by carbon catabolite repressors CREI and CreA of *Aspergillus*; these genomic regions are waking up in cellulose derivatives existance. A total of three presumed CREI binding sites exist in −674 to −724 regions of the promotor *cbh1* are directly engaged in the glucose inhibition (Ilmen et al., 1996; Takashima et al., 1996). The cbh1 promoter at approximately −783 have 5′-GGCTAATAA-3′ sequence to which Ace2 binds, causing improved effect of cellulase genes including (*cbh1*, *cbh2*, *egl1*, and *egl2*) and *xyn2* in cellulose-induced cultures (Aro et al., 2001).

In contradiction, engineered protein generation mediated by fundamental promoters in *T. orientalis* is choosier. Constitutive promoters lead protein expression without using of inducers. They are usually active in glucose enriched media while inducible promotors are commenly repressed by glucose. Cellulase gene expression is repressed by high concentration of glucose which account for 90%–95% of the *T. orientalis* extracellular proteins (Gusakov, 2011) therefore, application of constitutive promoters can effectively reduce the accumulation of undesirable proteins. Further, synthesis of extracellular proteases may digest the expressed products in the case of constitutive expression, is also reported to inhibit up to some extent using high glucose dose (Nakari-Setala and Penttila, 1995; Delgado-Jarana et al., 2000). Several constitutive promoters of *T. reesei* as *tef1, pki* and the promoter of an unidentified cDNA1 have been employed for recombinant protein production (Nakari-Setala and Penttila, 1995; Kurzatkowski et al., 1996). After all, the capacity of these mentioned promoters is relatively depressed. A try has been attempted to change the *cbh1* promoter into a constitutive promoter from *T. reesei* to *T. orientalis* EU7-22 by mutating sequence modification for catabolic repression, and the modified *cbh1* promoters that are constitutively active have been obtained.

This research reveals the selection of greater constitutive promoter and homologous over-expression of β-glucosidase in EU7-22. The promoter, reporter and terminator genes were used to construct expression cassettes and was ligated to the binary vector to construct pUR5750-bgl1 which was then transformed into the parental strain EU7-22 via *Agrobacterium Tumefacien* mediated transformation. The recombinant *T. reesei* strains were cultivated in a modified Mandels medium and surprisingly a high yield of recombinant β-glucosidase was obtained in comparison to the receipt EU7-22 under repressing condition.

2. Material and methods

2.1. Strains and culture conditions

The cellulase-producing strain *Trichoderma orientalis* EU7-22 was identified (accession no. KC751873) and sequence of tef1 (accession no. KC751874) are screened to be used as a host cell. Later it was stored at −80 °C in a 20% glycerol solution until use. Potato dextrose agar (PDA) slant was used for reactivation just before inoculation followed by incubation in liquid PDA on rotary shaker (30 °C, 34–36 h, 180 rpm). The culture was then transferred into submerged fermentation medium with 10% (*v/v*) inoculation quantity (106 spores mL^{-1}). For vector construction and propagation, *Escherichia coli* strain DH5@ was used. Transformation was mediated by *Agrobacterium tumefaciens* AGL1 and was grown either on Luria-Bertani (LB) broth (50 μg/mL kanamycin) or repression medium (RM) additives with a concentration of 0.2 mM aceto-syringone (IMAS). PDA media was used including hygromycin B (100 μg/ml), 0.2 mM cefotaxime and 20% Triton-X100 as additives for selection of transformants.

2.2. DNA and RNA manipulation

Induction media was used to culture the mycelia for isolation of RNA according to the protocol used by Verwoerd and his team (Verwoerd et al., 1989). To avoid contamination problem, RNase-free DNase I was handled in this study. Spectrophotometer was used for analysis of intensification of RNA. The Genomic DNA was obtained from all mycelia based on the method described by Penttila and his group (Penttila et al., 1987). The final concentration of genomic DNA was reported about 700 ng that was diluted to 200 ng for experimental use. Standard measurement was used to handle RNA and DNA material (Sambrook et al., 1989).

2.3. Construction expression vectors pUR5750-bgl1

For expression vector construction, the modified *cbh*1 promoter (1539 bp) was amplified from genomic DNA of *Trichoderma reesei* QM6a by PCR with pcbh1-F&R primers (contain *Xba*I and *Pst*I site). The *bgl1* gene (2583 bp) was amplified from *A. niger* BE-2 mRNA by RT-PCR with primer bgl1-F&R containing *Pst*I and *Not*I-*Xho*1-*Hind*III site (Table 1). Total RNA was isolated by Trizol reagent (Invitrogen) according to the manufacturer's instruction. The quality and quantity of RNA was assessed by spectrophotometer (Biophotometer, eppendorf) and cDNA was synthesized using M-MLV reverse transcriptase (Takara, Japan). The gene fragments encoding *bgl1* was amplified from cDNA by PCR using gene-specific primers (Gene-Bank accession No. KJ724529). While *trpC* terminator (740bp) was amplified from plasmid pUR5750 with primer TtrpC-F&R (containing *Not*I and *Xho*I site). The plasmid pUR5750 was used to construct the binary expression vector whereas as hygromycin B resistant gene (*hph*) was used as a marker for screening *T. orientalis* transformant. As shown in Fig. 1 pUC19 was used as

Table 1
Primers used to amplify genes by polymerase chain reaction.

Gene	Primer sequence (Underlined restriction site) 5 → 3	Restriction sites
Pcbh1-F	TCTAGAAGTTGTGAAGTCGGTAATC	*Xba*1
Pcbh1-R	CTGCAGGATGCCAGTCCGCGGTTGA	*Pst*1
Bgl1-F	CTGCAGATGAGGTTCACTTTGATCGA	*Pst*1
Bgl1-R	GCGGCCGCCTCGAGAAGCTTTTAGTGAACAGTAGGCAGAGAC	*Not* I-*Xho*1-*Hind*III
TtrpC-F	GCGGCCGCAGTAGATGCCGACCGCGG	*Not* I
TtrpC-R	CTCGAGCCTCCTGTCAATGCTGGC	*Xho*I
M13-F	CGCCAGGGTTTTCCCAGTCACGAC	
M13-R	GAGCGGATAACAATTTCACACAGG	
Hph-F	CGACAGCGTCTCCGACCTGA	
Hph-R	CGCCCAAGCTGCATCATCGAA	

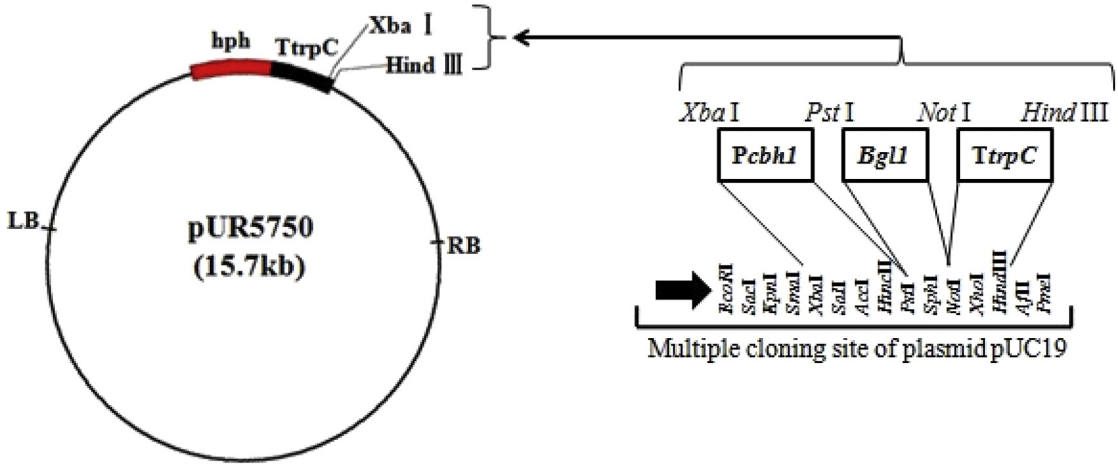

Fig. 1. Schematic illustration of the binary expression vectors pUR5750-Bgl1 construction.

intermediate plasmid for construction of expression vector pUR5750-bgl1.

2.4. Agrobacterium-tumefaciens-mediated-transformation in EU7-22

A. tumefaciens AGL1 containing binary vector (pUR5750-bgl1) was grown at 28 °C for 48hrs in LB medium supplemented with kanamycin (50 μg/mL). The AGL1 cells were diluted to optical density (OD_{600} = 0.15) in induction medium (IMAS). Cells were grown for an additional 8 h unless an OD at 660 nm of 0.4 ahead blending with the same amount of EU7-22 spore solutions (1 × 106 conidia per mL). This mixture (200 μL plate^{-1}) was spread onto an IMAS agar plate for co-cultivation (Modified IMAS containing 5 mM glucose) and was incubated at 25 °C for 48hrs. The PDA medium having additives hygromycin B (100 μg/mL), 0.2 mM cefotaxime and 20% Triton-X100 was poured onto IMAS agar plate, sequentially incubated at 30 °C for 3–6 days and the visible putative transformants were chosen for further analysis.

2.5. Molecular analysis of the transformants

Single colonies of four visible transformants (Bgl-1, Bgl-2, Bgl-3, Bgl-4) were grown on screening medium and then diverted to PDA agar plate containing hygromycin B. As shown in Fig. 2 the two positive transformants (3 and 4) were cultured positively. The two transformants were cultured on PDA agar plate without hygromycin B three times. To determine the stability of the transformant the monoconidial cultures were transferred to 100 μg/mL hygromycin B for. Genomic DNA of transformant was extracted from available mycelia according to the established protocol described by (Penttila). The transformants were confirmed by PCR via using primer hph-F&R for 811 bp and pcbh1-F & TtrpC-R for 4900 base pairs sequences respectively.

2.6. Enzymatic analysis

A total of 2% treated *Miscanthus* cellulose extracted with 2% NaOH and 1% H_2O_2 for 2 h at 60 °C for the inducing medium (pH 5.2). It was incubated on a rotary shaker (30 °C, 4 days, 180 rpm) in 250-mL Erlenmeyer flask. Crude enzyme was centrifuged at 6000 rpm for 10 min) to get rid of the cells and other tight objective. After centrifugation total enzyme activity of the surface liquid was analyzed. Total activity of filter paper shortly called (FPA) was determined according to the protocol used by Ghose and his team (Ghose, 1987) D-glucose's standard curve was used as a reference. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μM of reducing sugar per minute and expressed as IU mL^{-1}. β-glucosidase (BG) activity was assayed as described by Saha and his group (Saha, 2004). *p*-nitrophenol (*p*NP) reagent was used for standard graph as a reference. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol *p*NP per minute in the reaction and expressed as IU mL^{-1}. Bradford protein assay kit (Sangon Biotech Co. Ltd., Shanghai, China) was used to measure intensification of crude enzymes.

2.7. Transcription analysis

A total of 200 mg of mycelium was chopped to like a dust using liquid nitrogen and shipped to a 50 mL tube at ice surface. Total RNA was extracted from the mycelium of *T. orientalis* and transformants by Trizol reagent (Takara). The quality of the extracted RNA was analyzed by gel electrophoresis and spectroscopy was used for the measurement of mRNA intensification. PrimeScript® RT reagent Kit (Takara) was used for reverse transcription. The mRNA expression levels of *bgl1* were figured out as compared with the expression of 18S rRNA gene by Real-Time PCR (ABI StepOnePlus) with primers BGLYG-F&R, 18s-F&R.

3. Results and discussions

3.1. Over-expression of β-glucosidase

To investigate the over expression under *cbh*1 promoter, β-glucosidase gene (*Bgl1*) from *A. niger* BE-2 was used as a reporter gene. An expression vector (pUR5750-Bgl1) was designed and targeted into *T. orientalis* EU7-22 by *Agrobacterium tumefaciens* mediated transformation (ATMT). A total of four transformants were detectable after culturing 3–6 days on PDA selecting plates. The four transformants were grown by inoculation to new plate containing 100 μg/mL hygromycin B.

Transformants were grown on PDA plates to test mitotic stability. After three successive generations, the transformants were inoculated on PDA plates containing 100 μg/mL hygromycin B. All transformants grown well that showed their mitotic stability. A total of four visible transformants with hygromycin B-resistant were isolated and designated as Bgl-1, Bgl-2, Bgl-3 and Bgl-4. The amplification of 811 bp fragment by PCR with gene specific primer hph-F&R revealed that the *hph* gene was delivered with success into the four transformants genome as shown in Fig. 3A. To test whether the *cbh*1 promoter, *bgl1* gene and *trpC* terminator were synchronously integrated into the genomes, 4900 bp gene expression cassette (pcbh1-*Bgl1*-*trpC*) fragment was amplified with primer pcbh1-F & TtrpC-R (Fig. 3B). Data indicated that only Bgl-2 and Bgl-3 transformants were successfully integrated into the genomes as shown in Fig. 3B, lanes 3 and 4. The host strain EU7-22 without gene expression cassette fragment was amplified and was used

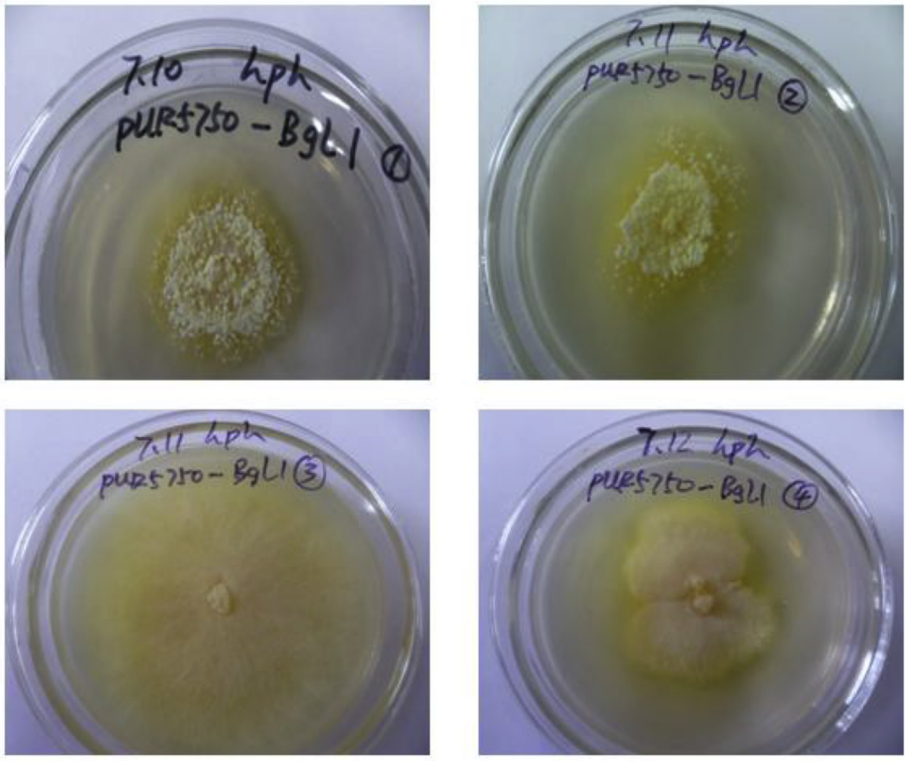

Fig. 2. The morphology of Transformants of *Trichoderma orientalis* Bgl-1, Bgl-2, Bgl-3 and Bgl-4 on PDA medium solid plates. Plates 3 & 4 are transformants.

as the control (Fig. 3B, lane 1). The agrobacterium mediated transformation protocol was an impressive and smooth approach for transformation but the T-DNA integration into the host genome appeared to be a random event (Lee et al., 2008; Michielse et al., 2005) and the truncation of the inserted T-DNA would be possible. These phenomena also occurred in *Fusarium oxysporum* (Mullins et al., 2001) and *Aspergillus awamori* (De-Groot et al., 1998).

3.2. Comparative analysis of cellulase production

The positive transformants of *Trichoderma* orientalis Bgl-2 and Bgl-3 were compared to host strain of EU7-22 in the preliminary analysis of cellulase production. The crude enzyme solution was measured FPA and BG activity. The BG and FPA activity of Bgl-2 decreased significantly (0.26 IU/mL) in comparison with the host strain EU7-22 with FPA and BG activities of 0.49 IU/mL while Bgl-3 transformants produced increased FPA and BG activities of 0.67 IU/mL and 0.86 IU/mL respectively. Therefore Bgl-3 transformants were further analyzed.

To enhance cellulase production, pretreated *Miscanthus* cellulose was used as induction. Activities on filter paper (FPA) and β-glucosidase (BG) of host EU7-22 were 0.49 $IUmL^{-1}$ and 0.49 $IUmL^{-1}$ respectively where as those of Bgl-3 strain were 0.67 $IUmL^{-1}$ and 0.86 $IUmL^{-1}$, respectively (Fig. 4A). The FPA and BG activities of transformant Bgl-3 increased by 39.6% and 47.1% under repressing conditions respectively as compared with strain EU7-22, which owed to the overexpression of β-glucosidase gene. The mRNA expression level of *bgl1* was evaluated by qRT-PCR. The concentration of mRNA expression for *bgl1* in EU7-22 strain was set as 1 in induced cellulase production. The relative mRNA expression of *bgl1* transcripts in strain Bgl-3 increased by 7.9 times (Fig. 4B). These results showed that the expression of β-

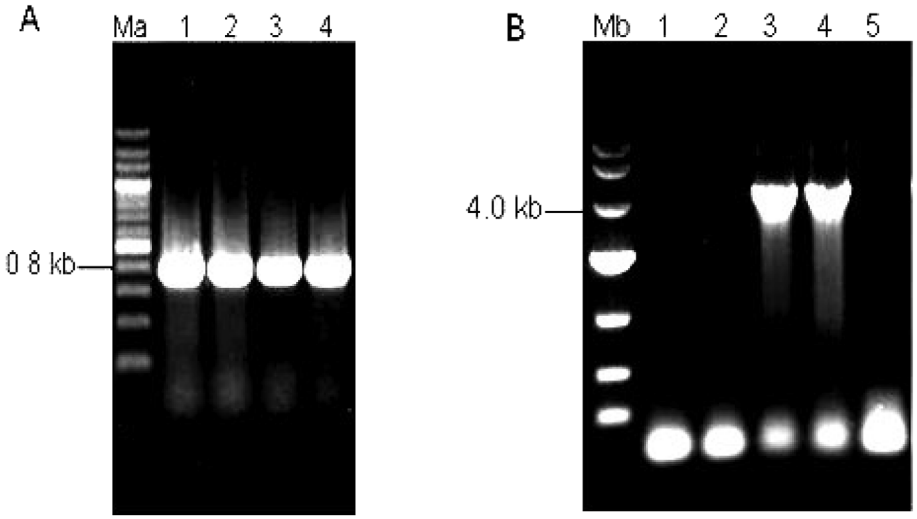

Fig. 3. Molecular analysis of integration of T-DNA into *T. orientalis* (A: PCR analysis of the phosphotransferase gene (*hph*) using specific primers hph-F& R to amplify an 811 bp fragment of transformants Lanes 1–4: transformants Bgl-1, Bgl-2, Bgl-3 and Bgl-4, respectively; Lane M: 200 bp DNA Marker. B: PCR analysis of the gene expression cassette (pcbh1-*bgl1*-T*trp*C) using specific primers pcbh1-F & T*trpC*-R to amplify a 4900 bp fragment of transformants. Lane 1: host strain EU7-22; Lanes 2–5: transformants Bgl-1, Bgl-2, Bgl-3 and Bgl-4. Lane M: DNA Marker).

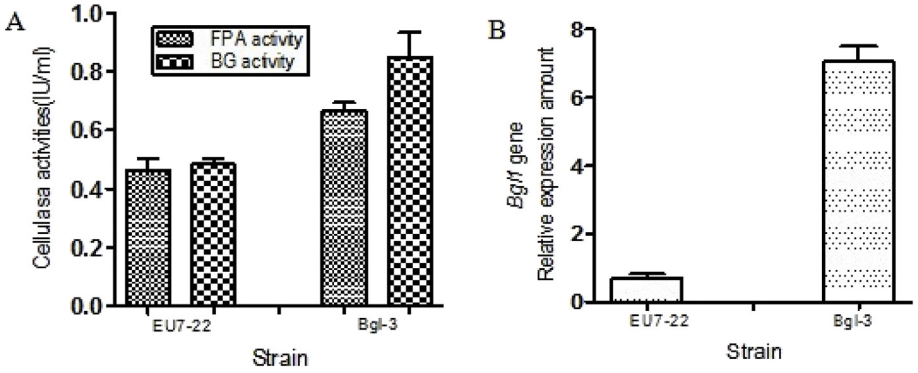

Fig. 4. (A) Analysis of FPA and BG activities of host strain EU7-22 and transformants Bgl-3. (**B**) Analysis of *bgl1* gene relative expression amount of host strain EU7-22 and transformants Bgl-3.

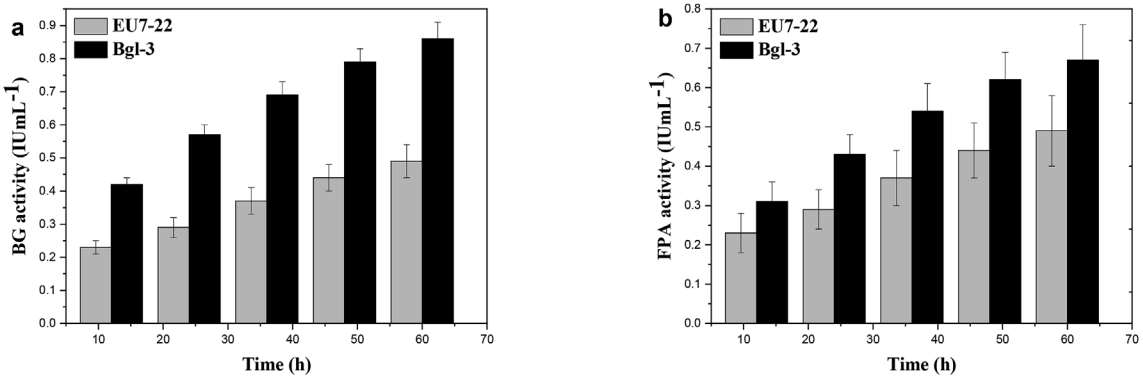

Fig. 5. Time course analysis of BG and FPA activities. Time course analysis of BG activity of host strain EU7-22 and transformants Bgl-3 (a). Time course analysis of FPA activity of host strain EU7-22 and transformants Bgl-3 (b).

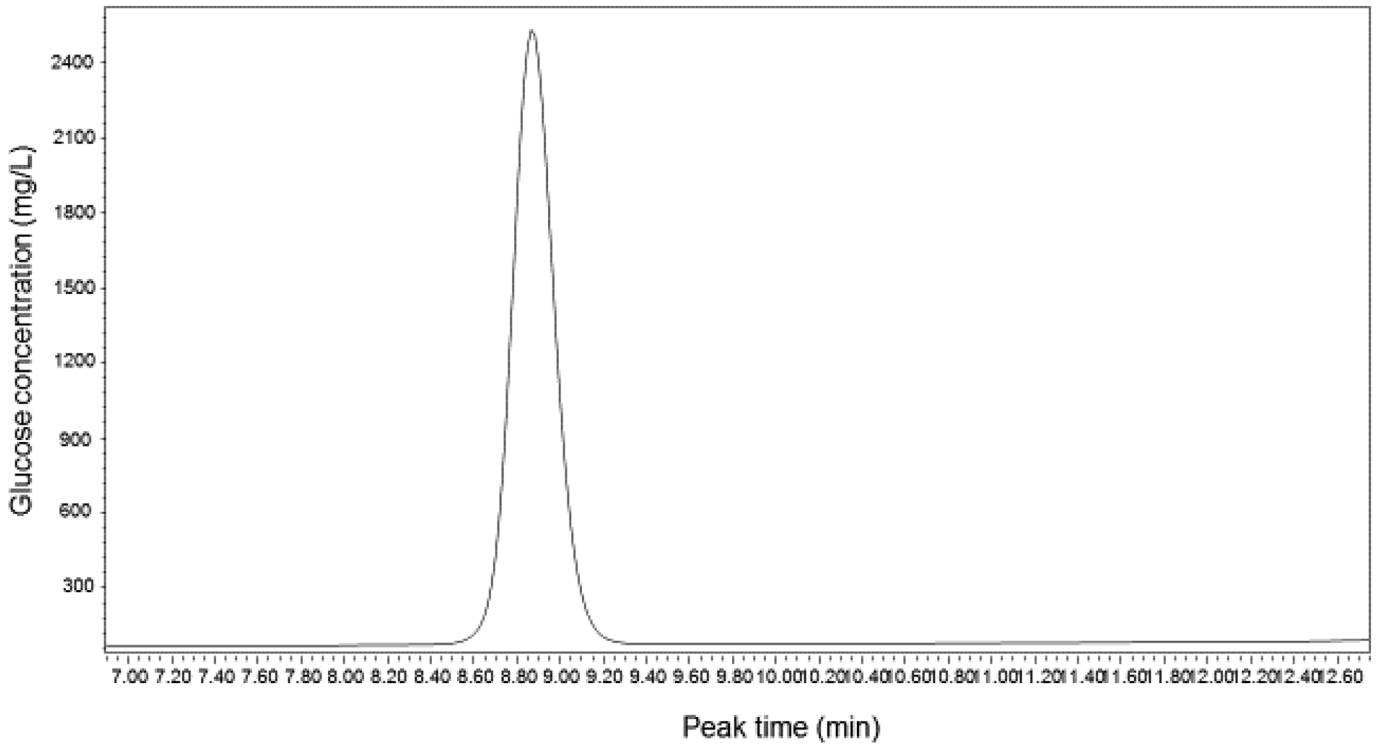

Fig. 6. HPLC analysis of the end products produced by enzymatic hydrolysis of miscanthus cellulose using enhance expressed β-glucosidase enzyme in EU7-22.

Table 2
Comparison of enzymatic hydrolysis of different pretreated substrates by various enzyme sources.

Source	Substrate	Reducing sugar(mg/g-substrate)	Reference
Trichoderma reesei RUT-C 30	Sunflower hull	468	Sharma et al. (2004)
Celluclast 1.5 L and Novozyme 188	Olive tree	288	Cara et al. (2006)
Cellulase (Sigma)	Bamboo culms	350	Zhang and Cai, 2008
Meicelase	Bamboo	488	Yamashita et al. (2010)
NS50013 (cellulase), NS50010 (b-glucosidase)	Cistus ladanifer (Rock rose)	313	Ferreira et al. (2009)
NS50013 (cellulase), NS50010 (b-glucosidase)	Cytisus striatus (Boom)	448	Ferreira et al. (2009)
Trichoderma atroviride TUB F-1505 and 1663	Spruce	245	Kovacs et al., 2009
Celluclast 1.5 L and Novozyme 188	Rice hull	154	Saha, 2004
Meicelase (*Trichoderma viride*)	Douglas fir	–	Lee et al. (2008)
Endoglucanase, xylanase and b-glucosidase (*L. sulphureus*)	Pinus densiflora	70.90	Lee et al. (2008)
Cellulase (Sigma)	Chinese willow (Salix babylonica)	–	
Cellulase (Sigma)	China-fir	–	
Mannaway 25 L and Gammanase	Palm kernel (Elaeis guineenis)	365	Cervero et al. (2010)
Spezyme CP and Novozyme 188	Balsam	125	Jensen et al. (2010)
Cellulase (*Agaricus arvensis*)	Poplar	293	Jensen et al. (2010)
Cellulase (*Aspergillus niger* BE-2)	Bamboo	170	Ali et al. (2015)
B-glucosidase (*Aspergillus niger* BE-2)	Miscanthus	230	This study

glucosidase promoted by the *p*-cbh1 is considered as an ideal promoter for expressing cellulase genes under repression condition.

Fig. 5a shows time course analysis of BG activity of host strain EU7-22 and transformants Bgl-3, while Fig. 5b shows time course analysis of FPA activity of host strain EU7-22 and transformants Bgl-3. The data shows the maximum BG activity after 60 h and almost 45% higher activity from the host strain. The FPA activity is also found at peak after 60 h and higher 20% than host strain.

3.3. Enzymatic hydrolysate analysis

We detected glucose, the single monomer present in the hydrolysates of pretreated miscanthus cellulose. The standard curve glucose intensification revealed fine linear graph (results not shown). As shown in Fig. 6, the time of retention for glucose was 8.40 min. On the base of the standard graph, the intensification of glucose in the hydrolysate was calculated, which was 2441 mg/L. Basically, glucose is the main targeted proportion obtained which can be converted to different kind of energy product including acetic acid, butyric acid, alcohol, lactic acid and many more using various types of microorganism such as yeast, anaerobic and aerobic bacteria. Reaction set up was optimized for enhanced experimental results. Table 2 provides a summary of the enzymatic hydrolysis of the different substrates and the obtained sugar levels using various improved microbial strains. Published reports revealed that optimization experimental set up with alkaline pretreatment lead an increased sugar level of 2.23 g/L, secreted by recombinant *T. reesei* ZM4-F3 cellulase (Zhang and Cai, 2008). A total reducing sugar level of 488 mg/g were reported from bamboo substrates using commercial cellulases (Yamashita et al., 2010). The cellulase that has been used for the saccharification of different lignocellulosic biomasses is generally obtained from a commercial source and the further addition of BGL is required (Sharma et al., 2004; Cara et al., 2006; Ferreira et al., 2009; Kovacs et al., 2009; Saha, 2004; Lee et al., 2008; Cervero et al., 2010; Jensen et al., 2010; Ali et al., 2015). In this research, a reasonable activity was recorded from over expressed cellulase in EU7-22 by a modified promotor of cbh1 from *T. reesei* without any addition of commercial β-glucosidases.

4. Conclusions

An ideal modified *cbh*1 promoter for expressing cellulase was successfully isolated from *Trichoderma reesei*. β-glucosidase ((*bgl1)* (2583 bp)), a reporter gene, was amplified from *A. niger* BE-2 mRNA by RT-PCR whereas T*trpC* (740bp), a terminator region, was amplified from fungal expression plasmid pUR5750. pUR5750 plasmid was used as a backbone to construct binary expression vector. The hygromycin B resistant gene (*hph*) was adopted as a selecting marker for screening EU7-22 transformats. The binary vector pUR5750-Bgl1 carrying *cbh*1 promoter was constructed and transferred into *T. orientalis* via ATMT for expressing *bgl1* gene. FPA and BG activity of transformant strain Bgl-3 was increased by 39.6% and 47.1% respectively when compared with the host strain EU7-22 under the repression condition. The transcription level of bgl1 gene was also analyzed. The relative expression of bgl1 of Bgl-3 strain was 7.9 times of host EU7-22 strain. It is concluded that promoter pcbh1 is a strong promoter. The results also indicated that *Agrobacterium* AGL1 is capable of mediating binary plasmid vector in transforming into filamentous fungi EU7-22.

Conflicts of interest

Both authors declare that there is no conflict of interest. They have read and approved the final manuscript.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101223.

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