Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters

Junxin Li†, Juan Wang†, Shaowen Wang, Miao Xing, Shaowen Yu and Gang Liu*

**Abstract**

**Backgrounds:** The fungus *Trichoderma reesei* is an important workhorse for expression of homologous or heterologous genes, and the inducible *cbh1* promoter is generally used. However, constitutive expression is more preferable in some cases than inducible expression that leads to production of unwanted cellulase components. In this work, constitutive promoters of *T. reesei* were screened and successfully used for high level homologous expression of xylanase II.

**Results:** The transcriptional profiles of 13 key genes that participate in glucose metabolism in *T. reesei* were analyzed by quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR). The results indicated that the mRNA levels of *pdc* (encoding pyruvate decarboxylase) and *eno* (encoding enolase) genes were much higher than other genes under high glucose conditions. Recombinant *T. reesei* strains that homologously expressed xylanase II were constructed by using the promoters of the *pdc* and *eno* genes, and they respectively produced 9266 IU/ml and 8866 IU/ml of xylanase activities in the cultivation supernatant in a medium with high glucose concentration. The productivities of xylanase II were 1.61 g/L (with the *pdc* promoter) and 1.52 g/L (with the *eno* promoter), approximately accounted for 83% and 82% of the total protein secreted by *T. reesei*, respectively.

**Conclusions:** This work demonstrates the screening of constitutive promoters by using RT-qPCR in *T. reesei*, and has obtained the highest expression of recombinant xylanase II to date by using these promoters.

**Keywords:** *Trichoderma Reesei*, Xylanase, Pyruvate decarboxylase, Enolase, Quantitative real-time PCR

**Backgrounds**

*Trichoderma reesei* is an attractive host for the expression of homologous and heterologous proteins because of its ability to secrete large amounts of hydrolytic enzymes [1-3]. It has been reported that highly productive *T. reesei* strains are able to produce and secrete up to 100 g/L of protein in optimal culture conditions, and the main ingredients are cellulases [4]. Of the secreted proteins in *T. reesei*, cellobiohydrolase I (CBHI) dominates, accounting for approximately 50%-60% of the total secreted proteins [5,6]. Since CBHI is encoded by single copy of *cbh1*, the *cbh1* promoter is considered to be strong and has been used to produce various kinds of homologous or heterologous proteins [1,7-10].

The *cbh1* promoter is an inducible promoter. It is induced by several kinds of saccharides, such as cellulose, sophorose, lactose, etc., and regulated by catabolic repression. When the *cbh1* promoter is used for protein expression, an inducer (or inducers) has to be added to trigger the expression of the target genes. However, such inducers also promote the expression of cellulase components, such as celllobiohydrolases, endo-β-glucanases, xylanases, etc. [11-13]. Unselective expression of cellulase components leads to contamination of target proteins with an excess of irrelevant proteins, and increases the difficulty for protein purification. Furthermore, extracellular proteases, which are deleterious to the yield of protein expression, might be produced simultaneously with cellulase induction [4].

In contrast, recombinant protein production mediated by constitutive promoters in *T. reesei* is more selective. Constitutive promoters drive gene expression without...
inducers. Unlike inducible promoters which are repressed by glucose, most of constitutive promoters are active in a glucose-rich medium. As cellulyases whose formation is repressed with high concentration of glucose account for 90%-95% of the T. reesei extracellular proteins [14], application of constitutive promoters can effectively reduce the accumulation of irrelevant proteins. Furthermore, in the case of constitutive expression, synthesis of extracellular proteases, which may digest the expressed products, is also inhibited, at least partly, by high glucose concentration [15,16]. Several constitutive promoters of T. reesei, such as the tef1 and pki promoters, and the promoter of an unidentified cDNA1, have been employed for recombinant protein production [15,17]. However, the efficiency of these promoters is relatively low. Efforts have been made to convert the cbh1 promoter into a constitutive promoter by mutating the sequences therein that are responsible for catabolic repression, and the modified cbh1 promoters that are constitutively active have been obtained. However, the protein expression level is about ten times lower in the presence of glucose than those obtained on a sorbitol-sophorose medium with the wild type cbh1 promoter [18].

This study describes the screening of strong constitutive promoters and homologous over-expression of xylanase II (XYNII) with these promoters in T. reesei QM9414. The T. reesei strain is cultivated in a glucose containing medium, and the transcriptional profile of 13 key genes related to glucose metabolism is analyzed by using quantitative real-time reverse-transcription polymerase chain reaction (RT-qPCR). The promoters and the terminators of two genes (pdc, encoding pyruvate decarboxylase; and eno, encoding enolase) with high expression level are used to construct expression cassettes that consist of XYNII, which are then transformed into the parental strain T. reesei QM9414. The recombinant T. reesei strains are cultivated in a modified Mandels medium, and extremely high yields of recombinant XYNII are obtained. The highest xylanase activity in the culture supernatant of the recombinant strain is 9266 IU/ml, which is the highest recombinant expression of XYNII achieved to date.

Results

Evaluation of constitutive promoter activities in different glucose concentrations

The genes that are responsible for glucose utilization are usually regarded as housekeeping genes, and their expression is driven by constitutive promoters. However, as previously described, the expression of these genes in T. reesei might still be affected by glucose concentration [19].

13 critical genes that participate in glucose metabolism according to Chambergo et al. [19] were selected and their transcriptional profiles were quantitatively analyzed by using RT-qPCR. These genes include those encoding glyceraldehyde-3-phosphate dehydrogenase (gpd), pyruvate decarboxylase (pdc), enolase (eno), alcohol dehydrogenase (adh), triose phosphate isomerase (tpi), aldolase (fba), pyruvate kinase (pyk), citrate synthase (cit), α-ketoglutarate dehydrogenase (kdh), aldehyde dehydrogenase I (ald1), aldehyde dehydrogenase II (ald2), pyruvate dehydrogenase (pda), and glucokinase (gkl), which participate in glycolysis and the tricarboxylic acid cycle. Samples were taken at 24 h, 44 h and 84 h of cultivation, when the residue concentration of glucose was 15.6 g/L (about 85 mM), 10.3 g/L (about 56 mM) and 0 g/L, respectively. The results indicated that the expression levels of two genes (pdc and eno) decreased in accordance with glucose exhaustion, the expression levels of six genes (pyk, ald2, cit, gkl, ald1 and fba) increased with glucose exhaustion, while those of four genes (gpd, tpi, pda and kdh) were relatively stable (Figure 1). The expression level of the adh gene changed irregularly during glucose exhaustion, possibly due to other factors that influenced the transcription of this gene. To construct a constitutive expression system, the promoters that are active at high glucose concentration might be preferable, for recombinant protein production in rapidly growing cells is more active. The formation of proteases is also somewhat repressed in high-level glucose cultivation [15,16]. Therefore, according to an analysis of the relationship between the transcriptional efficiency of the 13 genes and glucose concentration, the promoters of pdc, eno, gpd, tpi, pda and kdh appear to be proper candidates for the construction of a constitutive expression system.

Selection of strong constitutive promoters

The relative expression levels of the genes in T. reesei QM9414 that grows at different glucose concentrations are shown in Figure 2. The expression levels of pdc, eno, gpd are obviously higher than the other genes, especially when T. reesei grows at high glucose concentrations. For instance, the mRNA level of pdc is approximately 1,373 times higher than that of ald2 when the glucose concentration is 85 mM. The expression level of the gpd gene is the highest among the 13 genes at low glucose concentrations. The mRNA levels of gpd are 39 times higher (at a glucose concentration of 56 mM) and 22 times higher (at a glucose concentration of 0 mM) than those of pyk, whose promoter has been applied in the construction of a constructive expression system and proven to be relatively weak [17]. Since the expression levels of pdc and eno increased with glucose concentration, their promoters might be stronger than that of gpd at higher glucose concentrations. Actually, at a glucose concentration of 85 mM, the expression level of pdc had already surpassed gpd, and eno had the tendency to do so at higher glucose concentrations. Therefore, the promoters
of pdc and eno were selected for the construction of constitutive expression cassettes, and the gpd promoter was selected as the reference. The promoter and the terminator regions of these genes were acquired from the genome sequence of T. reesei (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Ppdc, the promoter of pdc, is located from 106110 bp to 107643 bp in scaffold 8; Tpdc, the terminator of pdc, is located from 103156 bp to 104185 bp in scaffold 8. Peno, the promoter of eno, is located from 102421 bp to 103910 bp in scaffold 4; Teno, the terminator of eno, is located from 99879 bp to 100865 bp in scaffold 4. Pgpd, the promoter of gpd, is located from 1561323 bp to 1562759 bp in scaffold 1; and Tgpd, the terminator of gpd, is located from 1564087 bp to 1564995 bp in scaffold 1.

Application of strong promoters in homologous xyn2 expression
To evaluate the transcriptional activities of the promoters screened by RT-qPCR, xyn2 was selected as a reporter, since it is convenient and standardized to perform xylanase activity assays. This gene encodes XYNII, a small xylanase of T. reesei that has been expressed in various hosts [20-22]. The xyn2 expression cassettes, Ppdc-xyn2-Tpdc, Peno-xyn2-Teno, and Pgpd-xyn2-Tgpd, were respectively mixed with pAN7-1, and the mixtures were used to transform T. reesei QM9414.

About 25% of the transformants that were resistant to hygromycin B contained the co-transformed expression cassette. The transformants that exhibited the highest xylanase productivity for each expression cassette were designated as T. reesei pxyn2, T. reesei exyn2, and T. reesei gxyn2, respectively. The expression cassettes in the transformants were PCR amplified, with the genomic DNA as the template (Figure 3), and the PCR amplified products were sequenced and proven to be correctly constructed. Southern blot analysis showed that all of these transformants had a single-copy insert of the expression cassette. The expression cassettes might be inserted into the genome of T. reesei randomly, for the endogenous pdc, eno and gpd cassettes remained intact in the transformants (Figure 3).

Then, T. reesei pxyn2, T. reesei exyn2, and T. reesei gxyn2, which respectively expressed xyn2 under the control of promoters Ppdc, Peno, Pgpd, were cultivated for recombinant XYNII production. The parent strain, T. reesei QM9414, was selected as the control. The activity of xylanase in the cultivation supernatant of the three transformants all reached the peak at 168 h of incubation (Figure 4). T. reesei pxyn2 exhibited the highest productivity of recombinant XYNII, with 9266 IU/ml of xylanase activity in the culture supernatant, while T. reesei exyn2 produced 8866 IU/ml of xylanase activity, which is slightly lower than that of T. reesei pxyn2.
**Discussion**

The strong *cbh1* promoter has been used frequently for heterologous or homologous protein expression in *T. reesei*. However, this promoter needs induction and is at least partly regulated by catabolite repression. Several
constitutive promoters, such as the *tef1* and *pyk* promoters, have been used to drive recombinant protein production in *T. reesei*, but their transcriptional activities are fairly low when compared with the *cbh1* promoter [15,17]. In general, there is still a lack of constitutive promoters for *T. reesei*, either for heterologous protein production or for genetic manipulation. In yeast, there are a number of research works dealing with promoter finding and optimization, and many effective promoters have been characterized, such as the GAP promoter in *Pichia pastoris* and the *TEF1* promoter in *Saccharomyces cerevisiae* [23-25]. This report has analyzed the transcriptional efficiency of the promoters of 13 key genes that participate in glucose metabolism through RT-qPCR. The results indicate that the *pdc* promoter (Ppdc), the *eno* promoter (Peno), and the *gpd* promoter (Pgpd) are more active in glucose containing medium.

Glucose concentration influences the transcriptional efficiency of the promoters in different ways, i.e., the transcriptional efficiency of Ppdc and Peno dramatically increases at high glucose concentrations, while that of Pgpd only increases slightly.

To verify the actual ability of these promoters in triggering recombinant protein production, expression cassettes of *xyn2* with the promoters were constructed and transformed into *T. reesei* QM9414, respectively. The recombinant strains, *T. reesei pxyn2*, *T. reesei exyn2* and *T. reesei gxyn2* for the *pdc*, *eno* and *gpd* promoters, respectively, were cultivated in a modified Mandels medium with a glucose concentration of 7%. The *T. reesei pxyn2* strain presented the highest ability to produce recombinant protein, while the *T. reesei gxyn2* strain had a lower ability. This result is consistent with the data of the expression levels of the corresponding genes initiated by these promoters as analyzed through RT-qPCR. Although the mRNA level of *pdc* is lower than *gpd* at low glucose concentrations, it increases faster with glucose concentration, and at 85 mM of glucose concentration, the mRNA level of *pdc* is 2.1 times higher than the *gpd*. The mRNA level of *eno* also shows the trend to increase faster with glucose concentration, and it is reasonable that the productivity of recombinant xylanase of *T. reesei exyn2* is 8866 IU/ml, which is also much higher than *T. reesei gxyn2*. As indicated in
The results reveal that the pdc promoter and the eno promoter are highly active, especially in medium with high concentration of glucose. These promoters have great potential in driving recombinant gene expression in T. reesei. Two recombinant strains, T. reesei pxyn2 and T. reesei exyn2, that respectively contained the xyn2 expression cassettes constructed with the promoters of pdc and eno, exhibit high productivity of recombinant XYNII in medium with high concentration of glucose. In addition, recombinant XYNII is the dominant protein in the culture supernatant, and the cellulase activity produced is negligible. The approach of producing recombinant proteins in T. reesei with the promoters high functional on glucose could be widely applied in industrial enzyme production.

Materials and methods

Strains, plasmids, and cultivation conditions

Escherichia coli (E. coli) Top10F (Invitrogen, USA) was used for plasmid construction and maintenance. T. reesei QM9414 (ATCC 26921) was used as a parental strain throughout the study. The E. coli strain was cultivated in LB medium, in which ampicillin (100 μg/ml, Invitrogen) was supplemented when necessary. The T. reesei strain was maintained on potato dextrose agar (PDA), and for liquid cultivation, it was grown in Mandels medium that contained an appropriate concentration of glucose [31]. The recombinant T. reesei strains were selected on PDA agar supplemented with hygromycin B (100 μg/ml), and for recombinant xylanase production, the strains were cultivated in modified Mandels medium supplemented with 7% glucose, 5% soybean powder, and 1% peptone. The E. coli and T. reesei strains were routinely cultured at 37°C and 28°C, respectively. Plasmid pUC19 was used for the construction of xyn2 expression cassettes. Plasmid pAN7-1 which contained the hygromycin B resistant cassette was used as an assisting plasmid for the transformation of T. reesei [32].

RNA extraction and cDNA synthesis

About $10^7$ T. reesei spores collected from a PDA plate grown for 5 days were inoculated into a 2-liter flask that contained 400 ml of Mandels medium with glucose at a final concentration of 1.8% (100 mM). They were then grown at 28°C and 250 r/min. Samples were taken at 24, 44 and 84 hours. Mycelia were harvested by centrifuge, frozen in liquid nitrogen and stored at -80°C. The glucose concentration in the samples was measured by using the 3, 5-dinitrosalicylic acid (DNS) method [33]. The total RNA of the samples was extracted by using a Universal Plant Total RNA Extraction Kit (BioTekes Corporation, China). To remove the genomic DNA, the RNA preparations were treated with DNase I (Fermentas, Canada). The quantity and quality of the extracted
RNA were assessed on a GeneQuant 1300 spectrophotometer (Biochrom Ltd., England) and by agrose gel electrophoresis. The synthesis of the complementary DNA (cDNA) from 1.0 μg of the total RNA per reaction (20 μl) was carried out by using a PrimeScript reagent kit (TaKaRa).

Quantitative real-time PCR
Quantitative real-time PCRs (RT-qPCRs) were performed in an ABI Prim 7300 System (Applied Biosystems, USA). Each reaction mixture contained 2 μl of template (1:60 dilution of the reverse transcription (RT) reaction product), 10 μl of SYBR Premix Ex Taq 2× (TaKaRa), 300 nmol/L of forward and reverse primers (Table 1), and nuclease-free water with a final volume of 20 μl. The PCR protocol consisted of 30 s of initial denaturation at 95°C, followed by 40 cycles of 5 s at 95°C and 31 s at 60°C. A melting curve was performed after each run to check the PCR product specificity. All PCRs were carried out in triplicate on a plate. Data obtained by using the ABI Prim 7300 Sequence Detection System were analyzed as described in the Applied Biosystems User Bulletin #2. The expression levels of the genes in the glucose metabolism were normalized with an endogenous control, sar1, as previously described by Steiger et al. [34]. The means ± standard deviations of replicates are shown in the figures.

Construction of Ppdc-xyn-Tpdc expression cassette
The promoter of the pdc gene (Ppdc, 1,534 bp upstream fragment starting from the start codon of pdc), the xyn2 gene, and the terminator of the pdc gene (Tpdc, 1,030 bp downstream fragment starting from the stop codon of pdc) were PCR amplified by using the genomic DNA of T. reesei QM9414 as the template. The primers Ppdc-F and Ppdc-R were used for amplification of Ppdc, and a HindIII restriction site was added to the 5’-end of the amplified product. The primers xyn-p-F and xyn-p-R were used for amplification of xyn2 (GenBank accession number: U24191.1), and a PstI site was added to the 3’-end. The signal peptide coding sequence was included in the amplified product. The primers xyn-p-F and xyn-p-R, and the resulting fusion fragment were fused by overlapping extension PCR through the use of primers Ppdc-F and Ppdc-R, and the amplified product. The primers xyn-p-F and xyn-p-R, and the resulting fusion fragment were fused by overlapping extension PCR through the use of primers Ppdc-F and Ppdc-R, and the resulting fusion fragment was inserted into pUC19 by using the restriction sites HindIII and PstI, which generated recombinant plasmid pUC19-Ppdc-xyn2. Finally, the Tpdc fragment was inserted into plasmid pUC19-Ppdc-xyn2 by using PstI and XbaI sites, which generated plasmid pUC19-Ppdc-xyn2-Tpdc. This plasmid was used for the transformation of T. reesei QM9414 and the recombinant expression of XNYII. The primers are listed in Table 2. The restriction enzymes, DNA polymerase and T4 DNA ligase were purchased from TaKaRa.

Construction of Peno-xyn-Teno expression cassette
This procedure is similar to the construction of the Ppdc-xyn-Tpdc expression cassette. The eno promoter (Peno, 1,490 upstream fragment starting from the start codon of the eno gene), xyn2 gene, and eno terminator (Teno, 987 bp downstream fragment starting from the stop codon of the eno gene) were PCR amplified from T. reesei QM9414 genomic DNA with the primers listed in Table 2. Restriction sites were added to the fragments as needed. The amplified Peno and xyn2 fragments were fused by overlapping extension PCR through the use of primers Peno-F and xyn-e-R, and the resulting fusion fragment was inserted into pUC19. Finally, Teno was

| Name | Sequence (5’–3’) | Amplification target |
|------|------------------|---------------------|
| sar-1 | tggatcgtcaactgttgctacga | sar1 cDNA |
| sar-2 | gcattctagcaacacgtgtcttt | |
| gpd-F | gtgctgcctcacaacctctcc | gpd cDNA |
| gpd-R | ttgcggtggcgcaacgaaatg | |
| pdc-F | tcaactcgcgtgcctctt | pdc cDNA |
| pdc-R | caagaaaccctccatcgcac | |
| kdh-F | agggagagacgagacatatacc | kdh cDNA |
| kdh-R | gttcaaggacagtgggaag | |
| pda-F | aacctccctgtgctgttggc | pda cDNA |
| pda-R | ttctccgtgttagtacggtca | |
| eno-F | aqcttcgcggcatctgctctgct | eno cDNA |
| eno-R | aacctgatggcttggtcggg | |
| ald1-F | ggaagaagccgaagcttggagtgat | ald1 cDNA |
| ald1-R | tggagaatgtggggtgatg | |
| glk-F | aagcccaacgctgtgattaaag | glk cDNA |
| glk-R | tgtgcggccagctgctacag | |
| pyk-F | cttcgcgcaagcagacaggac | pyk cDNA |
| pyk-R | acatcggttggtggcaagtggt | |
| cit-F | gcgaacctctgctctatcttcg | cit cDNA |
| cit-R | ggtcactaaaagggcactaaca | |
| adh-F | cttccacagagggcaacagt | adh cDNA |
| adh-R | tgggtcgatagctgcgtgatcg | |
| fba-F | cctgtgcggatctgcgact | fba cDNA |
| fba-R | gggatcggcttccacctcgt | |
| tpi-F | aacgagaagcactgcggtaac | tpi cDNA |
| tpi-R | gagctgtcataaggccagg | |
| ald2-F | gagggaacagcttgaggca | ald2 cDNA |
| ald2-R | gcagtgacaggaggaagttc | |
inserted into the plasmid, which generated the expression plasmid pUC19-Peno-xyn2-Teno.

**Construction of Pgpd-xyn-Tgpd expression cassette**

For construction of the Pgpd-xyn-Tgpd expression cassette, the *gpd* promoter (Pgpd, 1,437 bp upstream fragment starting from the start codon of the *gpd* gene), *xyn2* gene, and *gpd* terminator (Tgpd, 805 bp downstream fragment starting from the stop codon of the *gpd* gene) were PCR amplified from *T. reesei* QM9414 genomic DNA with the primers listed in Table 2. Restriction sites were added to the fragments as needed. The amplified Pgpd and *xyn2* fragments were fused by overlapping extension PCR through the use of primers Pgpd-F and *xyn-g*-R, and the resulting fusion fragment was inserted into pUC19. Finally, Tgpd was inserted into the plasmid, which generated the expression plasmid pUC19-Pgpd-xyn2-Tgpd.

**Protoplast transformation of T. Reesei**

Protoplast transformation of *T. reesei* was performed by using the polyethylene glycol method as described in Punt et al. [32]. Lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich) were used in the *T. reesei* protoplast preparation. For the transformation, the expression cassettes were released from the plasmids through digestion with appropriate restriction enzyme pairs, then purified, and mixed with equal amounts of plasmid pAN7-1. The mixture was then used for co-transformation of *T. reesei* protoplasts. Candidate transformants were streaked twice on PDA plates that contained 100 μg/ml of hygromycin B, and then transferred to PDA plates to form conidia. For each expression cassette, twenty single colonies were selected for cultivation in Mandels medium supplemented with 4% glucose, and the activity of xylanase in the supernatant was analyzed. For each expression cassette, the single colony that exhibited the highest productivity was selected for further study.

**Southern blot hybridization**

The chromosomal DNA was extracted and purified by the phenol/chloroform method. The DNA was digested with *SacI* and *BamHI*, fractionated on 0.7% (w/v) agarose gels and then transferred to nylon membranes (Roche). High-stringency probing was carried out at 50 °C overnight using digoxigenin (DIG)-labeled DNA probes, which were produced by amplifying a 494 bp fragment of the *T. reesei* *xyn2* gene with the primers Probe-F (aatccgtggctgtggaggaag) and Probe-R (tgcgtgcggtaaatgctgta) and labeled with digoxigenin DNA labeling mix (Roche). Chromogenic signal detection was done with the detection system from Roche Molecular Biochemicals. NBT/BCIP was used as the Chromogenic substrate.

---

**Table 2 Sequences of primers used for construction of expression cassettes**

| Name | Sequence (5′–3′) | Amplification target |
|------|-----------------|---------------------|
| Ppdc-F | ccaacctgatacctcaggctcttg (HindIII) | pdc promoter |
| Ppdc-R | ggagctgaagagacagcatgttcgtctgtagtgcgt | |
| xyn-p-F | agccgaactcacacacacacatgcctcccttcacctccct | *xyn* coding sequences |
| xyn-p-R | gcaactgaacactacacacacacaggaagagacgcc (PstI) | |
| Tpdc-F | gcaacttagcccggtcgatagtctgacc (PstI) | pdc terminator |
| Tpdc-R | gtctagatgacgctctagttcat (XbaI) | |
| Peno-F | gcaactcagttccgctctgattgc (BamHI) | eno promoter |
| Peno-R | agagagctgaagagacagcatgttcgtctgtagtgcgt | |
| xyn-e-F | gcaacctgaattcctcagacagcatgttcgtctgtagtgcgt | *xyn* coding sequences |
| xyn-e-R | ggcgtaccacacacacacacaggaagagacgcc (KpnI) | |
| Teno-F | ggcgtaccacacacacacacaggaagagacgcc (KpnI) | eno terminator |
| Teno-R | ggaattctcgtctgctgttcgtctgtagtgcgt | |
| Pgpd-F | gcaaccgttgacgcaagaggaagagacgcc (HindIII) | *gpd* promoter |
| Pgpd-R | gcggttgacgcaagaggaagagacgcc (HindIII) | |
| xyn-g-F | caattcqcagataacacacaggtccctcctctccctctcctcct | *xyn* coding sequences |
| xyn-g-R | gcaactcagttccgctctgattgc (XbaI) | |
| Tgpd-F | gcaactcagttccgctctgattgc (XbaI) | *gpd* terminator |
| Tgpd-R | gctctagattacggctctgattgc (XbaI) | |

*Restriction sites are underlined by single lines, and overlapping regions for overlap extension PCR are indicated by double lines. F: forward primer, R: reverse primer.*
Enzyme assays and protein analysis

For recombinant xylanase production, about 10^5 spores of the recombinant T. reesei strains were inoculated into 30 ml of Mandels medium, and maintained at 28°C and 250 r/min for 48 h. Then, 1.5 ml of the above culture was transferred into 30 ml of a modified Mandels medium, and maintained at 28°C and 250 r/min for about 192 h. In the modified minimal medium, the glucose concentration was raised from 2% to 7%, concentration of peptone from 0.5% to 1%, and 5% soybean powder was added. Xylanase activity was assayed as described in Bailey et al. with birchwood xylan (Sigma-Aldrich) as the substrate [35]. The culture supernatant was assayed for total cellulase activity as previously described in Bailey et al. with birchwood xylan (Sigma-Aldrich) as the substrate [35]. The amount of protein in the SDS-PAGE bands was estimated by densitometry through the use of a Furi FR-200A ultra-violet analyzer (Furi Tech, China).

Competing interests

The authors declare that they have no competing interests

Author’s contributions

GL and JW designed the experiments and wrote the manuscript. XJL performed most of the experiments. SWW participated in RT-qPCR experiments and fungal transformation. MX participated in enzyme and protein concentration assay. SWY participated in construction of expression cassettes. All authors read and approved the final manuscript.

Acknowledgements

This work was partly supported by the National Natural Science Foundation of China (No. 31070044), Shenzhen Municipal Science and Technology Basic Research Program (JC201005280559A), and Shenzhen Municipal Science and Technology key projects of Basic Research Program (JC201005250041A).

Received: 9 February 2012 Accepted: 7 June 2012

Published: 18 June 2012

References

1. Wang BB, Xia LW. High efficient expression of cellulase gene from Aspergillus niger in the cells of Trichoderma reesei. Biotes Technol 2011, 102 (6):4568–4572.
2. Rahman Z, Shida Y, Furukawa T, Suzuki Y, Okada H, Ogasawara W, Morikawa Y. Evaluation and characterization of Trichoderma reesei cellulase and xylanase promoters. Appl Microbiol Biotechnol 2009, 82(5):899–908.
3. Nevalainen KMH, Teo VJ, Bergquist PL. Heterologous protein expression in filamentous fungi. Trends Biotechnol 2003, 23(9):468–474.
4. Schuster A, Schmoll M. Biology and biotechnology of Trichoderma. Appl Microbiol Biotechnol 2010, 87(3):787–799.
5. Markov AV, Gusakov AV, Kondratyeva EG, Okunev ON, Bekkarevich AO, Sinitsyn AP. New effective method for analysis of the component composition of enzyme complexes from Trichoderma reesei. Biochem (Moscow) 2005, 70(6):657–662.
6. Marguet A, Hahn-Hagerdal B, Edlund M, Slade R, Monot F. New improvements for lignocellulose ethanol. Curr Opin Biotechnol 2009, 20 (3):372–380.
7. Zou G, Shi S, Jiang Y, van den Brink J, de Vries RP, Chen L, Zhang J, Ma L, Wang C, Zhou Z. Construction of a cellular hyper-expression system in Trichoderma reesei by promoter and enzyme engineering. Microb Cell Fact 2012, 11:21.
8. Joosten V, Lokman C, van den Hondel CAMJ. Punt PJ. The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi. Microb Cell Fact 2003, 2:11.
9. Bergquist P, Teo V, Gibbs M, Cefnericky A, de Faria F, Azevedo M, Nevalainen H. Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts. Extremophiles 2002, 6:177–184.
10. Liu T, Wang T, Li X, Liu X. Improved heterologous gene expression in Trichoderma reesei by cellulobiodegradation I gene (cbh1) promoter optimization. Acta Biochim Biophys Sin (Shanghai) 2008, 40(2):158–165.
11. Xu J, Nagawa M, Okada H, Morikawa Y. Regulation of xyn3 gene expression in Trichoderma reesei PC-3-7. Appl Microbiol Biotechnol 2000, 54 (3):370–375.
12. Mach RL, Zellinger S. Regulation of gene expression in industrial fungi: Trichoderma. Appl Microbiol Biotechnol 2003, 60(3):515–522.
13. Seiboth B, Gamauf C, Pail M, Hartl L, Kubicek CP. The D-xylene reductase of Hypoecia jecora is the major aldose reductase in pentose and D-galactose catabolism and necessary for beta-galactosidase and cellulase induction by lactose. Mol Microbiol 2007, 66(4):890–900.
14. Gusakov AV. Alternatives to Trichoderma reesei in biofuel production. Trend Biotechnol 2011, 29(9):419–425.
15. Nakari-Selata T, Penttila M: Production of Trichoderma reesei cellulases on glucose-containing media. Appl Environ Microbiol 1995, 61(10):3650–3655.
16. Delgado-Jarana J, Pintor-Toro J, Benitez T. Overproduction of β-1,6-glucanase in Trichoderma harzianum is controlled by extracellular acidic proteases and pH. Biochim Biophys Acta 2000, 1481(2):289–296.
17. Kurzatkowski W, Törönen A, Filipek J, Mach RL, Herzog P, Sawka S, Kubicek CP. Glucose-induced secretion of Trichoderma reesei Xylanases. Appl Environ Microbiol 1996, 62(8):2859–2865.
18. Ilmén M, Ommela ML, Klemidal S, Keränen S, Penttilä M. Functional analysis of the cellulobiodegradation I promoter of the filamentous fungus Trichoderma reesei. Mol Cell Genet 1996, 253(3):303–314.
19. Chambers FG, Bonaccorsi ED, Ferreira AJ, Ramos AS, Ferreira JR, Abraham Neto J, Farah JP, El-Dony H. Elucidation of the metabolic fate of glucose in the filamentous fungus Trichoderma reesei using expressed sequence tag (EST) analysis. J Biochem 2002, 277(16):1938–1988.
20. La Grange DC, Pretorius IS, Van Zyl WH. Expression of a Trichoderma reesei β-xylanase gene (XYN2) in Saccharomyces cerevisiae. Appl Environ Microbiol 1996, 62(3):1036–1044.
21. He J, Yu B, Zhang KY, Ding XM, Chen DW: Expression of endo-1, 4-beta-xylanase from Trichoderma reesei in Pichia pastoris and functional characterization of the produced enzyme. BMC Biotechnol 2009, 9:56.
22. He J, Yu B, Zhang KY, Ding XM, Chen DW: Expression of a Trichoderma reesei β-xylanase gene in Escherichia coli and the activity of the enzyme on fiber-bond substrates. Protein Expr Purif 2009, 67(1):1–6.
23. Stadlmayr G, Mecklenbrauker A, Rothmüller M, Maurer M, Sauer M, Mattanovich D, Gasser B: Identification and characterization of novel Pichia pastoris promoters for heterologous protein production. J Biotechnol 2010, 150(4):519–529.
24. Partow S, Sluvers W, Bjorn S, Nielsen J, Maury J. Characterization of different promoters for designing a new expression vector in Saccharomyces cerevisiae. Yeast 2010, 27(2):155–164.
25. Waterham HR, Digan ME, Koutz PJ, Lai SW, Cregg JM: Isolation of the Pichia pastoris glyceroldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. Gene 1997, 186(1):337–44.
26. Xiong HR, von Weymarn M, Turunen O, Leisola M, Pastinen O: Xylanase production by Trichoderma reesei Rut C-30 grown on L-arabinose-rich plant hydrolysates. Bios Biotechnol 2005, 96(7):753–759.
27. Beg OK, Kappor M, Mahajan L, Hoondal GS: Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 2001, 56(3–4):326–338.
28. Bergquist P, Te’o V, Gibbs M, Cziferszky A, de Faria FB Fabricia Paula, Azevedo M, Nevalainen H: Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts. Extremophiles 2002, 6(3):177–184.
29. Nevalainen KMH, Te’o VSJ, Bergquist PL: Heterologous protein expression in filamentous fungi. Trend Biotechnol 2005, 23(9):468–474.
30. Ruanglek V, Sriprang R, Ratanaphan N, Tirawongsaroj P, Chantasigh D, Tanapongpipat S, Pootanakit K, Eurwilaichitr L: Cloning, expression, characterization, and high cell-density production of recombinant endo-1,4-β-xylanase from Aspergillus niger in Pichia pastoris. Enzyme Microb Technol 2007, 41(1–2):19–25.
31. Mandels M, Andreotti RE: Problems and changes in the cellulose to cellulase fermentation. Process Biochem 1978, 13(1):6–13.
32. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJM: Transformation of Aspergillus based on the hygromycin B resistance marker from Escherichia coli. Gene 1987, 56(1):117–124.
33. Miller GL: Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959, 31(3):426–428.
34. Steiger MG, Mach RL, Mach-Aigner AR: An accurate normalization strategy for RT-q PCR in Hypocrea jecorina (Trichoderma reesei). J Biotechnol 2010, 145(1):30–37.
35. Bailey MJ, Biely P, Poutanen K: Interlaboratory testing of methods for assay of xylanase activity. J Biotechnol 1992, 33(3):257–270.
36. Ghose TK: Measurement of cellulase activities. Pure Appl Chem 1987, 59(9):257–268.
37. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 259(5572):680–685.

Cite this article as: Li et al: Achieving efficient protein expression in Trichoderma reesei by using strong constitutive promoters. Microbial Cell Factories 2012 11:84.