Effects of the Transcription Factor Ace2 from Trichoderma Reesei on Cellulase and Hemicellulase Expression in Trichoderma Orientalis EU7-22

Yuyu Li
Xiamen University
Yong Xue
Xiamen University
Jian Liu (jianliu@xmu.edu.cn)
Xiamen University https://orcid.org/0000-0003-0696-0793
Lihui Gan
Xiamen University
Minnan Long
Xiamen University

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Abstract

Trichoderma orientalis (T. orientalis) EU7-22 has a complete cellulase system and shows a remarkable enzyme activity with high potential in the industry. Ace2 is an important transcriptional factor for cellulase and hemicellulase expression in *Trichoderma reesei (T. reesei)*. However, the *ace2* gene can not be found in the genome of *T. orientalis*. Researches show that the mechanism of cellulase transcriptional regulation in *T. orientalis* keeps high similarity with *T. reesei* up till now. So, in this study, the *ace2* of *Trichoderma reesei* QM9414 was heterologous expressed in *T. orientalis* EU7-22. As a result, xylanase activity and β-glucosidase activity of *ace2* heterogeneous expression strains are improved and total cellulase activity is decreased. The result of qPCR is in accordance with enzyme activities. This study provides a reference for an in-depth study on transcriptional regulation mechanisms of *T. orientalis*.

1. Introduction

As a renewable resource, lignocellulosic biomass, which typically consists of cellulose, hemicellulose and lignin, are plentiful and widely distributed on the earth. Cellulose and hemicellulose, as the main components of leaves, stalks and wood of plants, could become a substitute for the starch if suitably treated for making because they can be degraded to fermentable sugars, e.g. oligosaccharides, glucose, xylose, etc. So the bio-ethanol from lignocellulosic feedstocks is called “second generation” [1]. However, the cost and the efficiency of the enzymes capable of breaking cellulose down has always been a problem of the biofuel industry. Nowadays, the most popular enzyme producer is which can produce whole cellulase system, including endoglucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91), β-glucosidase (BG, EC 3.2.1.21), lytic polysaccharide and so on[3, 4].

To enhance the efficiency of cellulase production, plenty of methods have been tried, including site-directed mutagenesis, transcriptional regulation factors engineering, etc.([5, 6]). In this study, EU7-22 is reserved in our lab and obtained from *T. orientalis* XC-9 by a complex mutation [7, 8]. It keeps high similarity with *T. reesei*. By comparing the ability to produce different cellulase of EU7-22 with that of other common strains producing cellulase used in the industry, Long concluded that EU7-22 has a complete cellulase system and shows a remarkable enzyme activity with high potential in the industry[8]. Transcriptional regulation of filamentous fungi represents a logical engineering target to improve cellulase production. Researches on transcriptional regulation factors have been launched widely[3]. A suite of transcriptional regulation factors plays an important role in the production of cellulase in fungi. Some transcriptional factors regulating the expression of cellulase- and xylanase-encoding genes have also been identified in EU7-22. In *T. reesei*, it is known that the obvious difference of cellulase production between the more efficient strain Rut-C30 and the less efficient strain QM9414 is the truncation of Cre1. Cre1 shuts down the transcription of its target genes when glucose exists [9]. In EU7-22, Cre1 is also one of the master regulators which is a C2H2 type transcription factor and participates in carbon catabolite repression. The Cre1 disrupted strain shows both a higher basal level and a higher induced level of cellulase production[10]. Another typical repressor of cellulase expression is Ace1 [11] , and deletion of *ace1* increased the expression of all the crucial cellulase genes and two xylanase genes in the certain

induced cultures. In the basic research, it is also represented as a repressor in EU7-22. In addition to transcriptional repressor Cre1 and Ace1, some transcriptional activators have also been studied, such as Ace3 and Xyr1[7, 12-14]. It has been proven that overexpression of Xyr1 leads to a higher expression of cellulases and abolishes their catabolite repression in the presence of glucose. Besides, when two important transcription activators Xyr1 and Ace3 were overexpressed in the EU7-22 strain, the cellulase production was greatly improved[7]. In Ace3 as a Zn(II)2Cys6 type transcriptional factor binds the promoters of many cellulase genes, the cellulose response transporter gene *crt1* and transcription factor–encoding genes, including *xyr1*[12]. Xyr1 is also a Zn(II)2Cys6 type transcriptional activator, and it binds to a GGCTAA motif arranged as an inverted repeat in the *xyn1* promoter [15]. They are both responsible for the activation of lots of genes involved in the degradation of cellulose and xylan. By comparing the DNA sequence of QM9414, *T. orientalis* EU7-22 keeps high similarity with its cellulase transcriptional regulators as well[16].

However, Ace2 is one of the most special cellulase transcriptional regulators existing in *T. reesei* but it cannot be found in *T. orientalis*. The N-terminal part of the deduced Ace2 protein has a typical zinc binuclear cluster DNA-binding domain of the fungal type (Zn(II)2Cys6), first characterized in *S. cerevisiae*[17]. In *T. reesei*, Aro.N et al. indicated that Ace2 binds in vitro to the GGCTAATAA site in the *cbh1* promoter. This site also contains the proposed binding sequence of the xylanase activator XlnR from *Aspergillus niger*[18]. By knocking out *ace2* in *T. reesei* strain ALKO2221, the cellulase and hemicellulase activities both drastically decreased with reduced transcript levels of lignocellulolytic enzymes. Stricker also found that both Xyr1 and Ace2 can bind the complete motif of Xyn2 promoter consisting of a GGGTAA-element and a CCAGCC-element. The △ace2-strain showed faster initial inducibility when induced by xylan and xylobiose although final levels of xylanase activity of the parental strain were higher[19]. Some relevant studies were given by Xiong et al. They have successfully transformed the Ace2 transcription factor encoding gene in *T. reesei* and gotten five genetically engineered strains. Among which, the result displayed that only one has exhibited a significant increase in enzyme activity, but the other four showed lower total cellulase activities than the parental strain *T. reesei* QM9414[20]. Also, Ace2 was not subject to carbon catabolite repression, because in the △cre1 strain no increased expression levels of *ace2* gene were observed.[21]

In this study, on considering the high similarity between these two fungi, Ace2, as a cellulase transcriptional activator, may contribute to the promotion of cellulase production if introduced to EU7-22. However, the result of heterologous expressed *ace2* in EU7-22 is beyond expectation. Some of the cellulase and hemicellulase activities increased comparing to the parent strain, but some of the enzymic activities did not increase, even decreased. It provides the potential in the applications for differentiated purposes. This study reflected the complexity of mechanisms of regulation in vivo and interspecific difference between *T. reesei* and *T. orientalis*.

2. Materials And Methods

2.1. Microorganism strains

T. reesei QM9414 (ATCC 26921) was used for cloning *ace2* gene, promoter (Peno) and terminator (Teno) of *eno* gene. *T. orientalis* EU7-22 was used as the parental strain for transformation throughout this study.

2.2. Culture media

Minimum medium (MM medium) is used for strain growth consisting of 20.0 g/L glucose, 15.0 g/L KH_2PO_4, 5.0 g/L $(NH_4)_2SO_4$, 0.6 g/L $CaCl_2$, 0.6 g/L $MgSO_4$ and 10 mL/L trace element solution (The solution containing 0.2 g/L $CoCl_2$, 0.5 g/L $FeSO_4{\cdot}7H_2O$, 0.14 g/L $ZnSO_4{\cdot}7H_2O$ and 0.16 g/L $MnSO_4{\cdot}H_2O$). The pH of MM medium is 4.8. The solid MM medium(SMM medium) is MM medium adding with 2% agar. The SMM medium supplemented with 44.7 g/L KCl is the MK medium which is used in the process of protoplast regeneration. The medium for growth observation is SMM medium with different carbon sources, including avicel (MA), cellobiose(MB), CMC-Na (MC), glycerol(MG), lactose(ML), xylose (MX) and xylan from bagasse (MY). The strain was cultured in an induction medium (ABP medium) at pH 5.2 for cellulase production containing 10.0 g/L wheat bran, 20 g/L avicel, 5.0 g/L peptone, 2.5 g/L KH_2PO_4, 0.5 g/L $CaCl_2$, 0.5g/L $MgSO_4$ and 10 mL/L trace element solution.

2.3. Construction of *ace2* expression cassette

The heterogeneous expression of *ace2* cassette was constructed as follows. The primers PA2eno-1 and PA2eno-3, as well as TA2eno-4 and TA2eno-5 were used to amplify Peno and Teno from *T. reesei* QM9414 genomic DNA. Ace2 gene (GenBank accession no. CP016233.1) using primers ace2-F and ace2-R was also amplified from *T. reesei* QM9414 genomic DNA by PCR. The hygromycin resistance cassette was cloned from pUR5750 plasmid containing hygromycin resistance gene (hygromycin phosphotransferase, *hph*) which was preserved in our laboratory using primers PgpdA-hph-F and hph-TgtrpC-R. Then, the *ace2* expression cassette could be obtained by fusion PCR with primers PA2eno-2 and TgtrpC-2 using the fragments mentioned above as the templates[22]. After that, primer hph-F and hph-R were used to verify the existence of the hygromycin resistance in the transformants, and then they were chosen after 96 h fermentation under the inducing condition of ABP medium. Sequences of all primers are given in Table1.

The DNA polymerases used in this study includes PrimeSTAR® HS DNA Polymerase (Takara, Japan) for amplifying 1-2kb gene segments, PrimeSTAR® Max DNA Polymerase (Takara, Japan) for synthesizing 3kb-10kb gene segments, and the 2×Taq Plus Master Mix (Dye Plus) (Vazyme, China) for the PCR of verifying transformants.

Table 1. Sequences of all primers

Primers	Sequence（5′-3′）
PA2eno-1	GGAGGGATATAGACTATGGCTCATG
PA2eno-2	GTGATTCCGTCCTGGATTGC
PA2eno-3	CATGCTTGCCGGAGGTCCATTTTGAAGCTATTTCAGGTGGCTG
ace2-F	ATGGACCTCCGGCAAGCAT
ace2-R	TCACTTCAGCAGTCTGGCACTG
TA2eno-4	CAGTGCCAGACTGCTGAAGTGAATGGCCACGAGAGACAACTACCTAT
TA2eno-5	GATGGCGTCGTTGATGTTTCG
PgpdA-hph-F	CGAAACATCAACGACGCCATCGATCTTTCGACACTGAAATACGTC
hph-TgtrpC-R	AAGAAGGATTACCTCTAAACAAGTG
TgtrpC-2	GTGCATTCTGGGTAAACGACTC
hph-F	GAATCGGTCACTACACTACATGGC
hph-R	CGCCCAAGCTGCATCATCGAA

2.4. Fungal transformation

The method of protoplast transformation (obtaining the transformants) follows Xue et al [7].

2.5. Measurement of enzyme activity

in MM medium. Then after 18h, 5% of the culture was incubateded into ABP culture for fermentation. The fermentation broth was collected by centrifugation every 24 h till 120 h. The FPase, CMCase, pNPCase, pNPGase, xylanase and pNPXase were measured and they represent the total cellulase, endoglucanases, exoglucanases, β-glucosidase, xylanase and xylosidase activity, respectively [23]. The definition of one unit of enzyme activity follows the report [7].

2.6. Measurement of total soluble protein

Total soluble protein was measured as the manufactory's instructions indicated by a Modified Bradford Protein Assay Kit (Sangon, China).

2.7. RNA-extraction, cDNA-synthesis, and real-time PCR

RNA samples from transformant and parental strain for analyses of gene expression were prepared from mycelium powder obtained by grinding the filtrated biomass from shake flasks in liquid nitrogen. Then samples were lysed and cleaned up by using TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Japan). In the procedure of isolation of total RNA, the materials pipette tips and columns are dealt with diethylpyrocarbonate treated water and the mortars are dry-heat sterilized at 180℃ for 60 min to ensure

there is no RNA lytic enzyme. Then 5× All-In-One MasterMix Kit (Vazyme, China) is used for synthesizing cDNA (reverse transcription).

The primers for amplifying genes encoding cellulases and regulatory factors used for real-time quantitative PCR are listed in Table 2.

Table 2. The primers for qPCR

Primers	Sequence（5’-3’）
actin-YGF	TCCATCATGAAGTGCGAC
actin-YGR	GTAGAAGGAGCAAGAGCAGTG
cbh I-YGF	ATCGGCTTCGTCACGCAATC
cbh I-YGR	ACGCCACCATCCGCATCCA
cbh II-YGF	GACAAACCTCGGCACTCC
cbh II-YGR	GACCAGCGTCCAGATACATT
eg I-YGF	CAGGGCTTCTGCTGTAATGAG
eg I-YGR	TTG AACTGGGTGATGATGGTG
eg II-YGF	GCTCCGCCAGAATAACCG
eg II-YGR	CAGCCA ACATAGCCAAGATAGAC
bgl 1-YGF	ATCACCTACCCGCCTTCA
bgl 1-YGR	TCTCGTCGTCGGATGTTG
xyn I-YGF	CGTCAACACGGCGAACCA
xyn I-YGR	CGGTGATGGAAGCAGAGCC
bxl1-YGF	TGTTCGCACAAGCAACGCT
bxl1-YGR	ACTCGTCGGTGTTCAAGGCT
xyr1-YGF	TGCTTGACGACGACGACTTGT
xyr1-YGR	ACGCCGTAGAAGAAGGGCAT
cre1-YGF	TCTACGGCTCCTTCTTCTC
cre1-YGR	ACAAGTTCCTCAGACTCGG
ace1-YGF	TGATGAGGGCTTTGACGAGTC
ace1-YGR	GGTTGAAGATGTCGGGCTGT
ace2-YGR	GACAAGAAGCTCAGGTGTC
ace2-YGF	ACTGTGTTCATGGCTGTG
ace3-YGF	TGCTGAGGGTGATGAACGAG
ace3-YGR	GGGTGAATCCTGGTTGCGAT

Quantitative PCR was performed on fluorescence quantitative thermocycler (ABI, USA). Amplification reactions were performed using the Universal SYBR qPCR Master Mix (Vazyme, China). Data analysis

was performed using the $2^{-\Delta\Delta CT}$ method. As a reference gene, we used *actin* which shows stable transcript levels across different conditions [24].

The RT-qPCR protocols are initially denatured at 95℃, followed by 45 cycles of 15 s at 95℃, 15 s at 59℃ and 15 s at 72℃. All reactions were repeated for three times in 20 μL-mixtures including 1 μL forward primer, 1 μL reverse primer, 1 μL cDNA(10-fold diluted) as a template and 10 μL Universal SYBR qPCR Master Mix. Each run included a blank (sterile bi-distilled water instead of the sample). All samples were analyzed in three independent biological experiments with three RT-qPCR replicates in each assay.

3. Results And Discussion

3.1. Construction of *ace2* recombinant transformants

The *ace2* cassette has been successfully synthesized by fusion PCR. The four gene segments were amplified as previously described and shown in Fig. 1. The lanes1-4 represent the Peno (1669 bp), *ace2* (1026 bp), Teno (986 bp) and hygromycin resistance cassette (3936 bp) respectively. According to Li et al., the promoters of enolase (*eno*) were found under the condition of glucose while the transcript levels of the gene was much higher than others[25].

3.2. Screening of recombinant transformants

The strains were screened out from the of PDA medium with 100 μg/mL hygromycin B and 0.01% tritonX -100. The *hph* gene of the screening strains was amplified with the primer hph-F and hph-R, and 9 recombinant strains were obtained through 4 times of transforming operation named 0-1, 0-2, 0-3, 0-4, 0-5, 1-1, 1-2, 2-9 and 3-1. The highest enzyme activity of EU7-22 was judged at the fourth-day of fermentation. FPA activity were quantitated as 0.86, 0.97, 1.08, 1.01, 0.51, 0.88, 1.00, 0.93 and 0.93 IU/mL, and the total cellulase activity all decreased compared with the parental strain (1.15 IU/mL) after the fermentation for 4 days. Three strains (0-2, 1-1 and 2-9) with an average performance of total cellulase activity were selected for further study.

3.3. Enzyme activity of transformants and total

According to Fig. 4, in ABP inducing medium, the FPase activities of the *ace2* heterologous expression strain were lower than that of the parental strain, and the recombinant strains had reductions by 4.27%, 13.06%, and 6.65%, respectively. By measuring the CMCase activity, it is noticed that the expression of *ace2* gene reduced the expression of endocellulase activity of *T. orientalis*, and the endocellulase activities reduced by 7.01%, 21.50%, and 20.14%, respectively. The CBH activity of the 0-2 strain increased by 20.69%, and the enzyme activities of 1-1 and 2-9 were basically the same as the original strain.

β-glucosidase activity was improved, and the enzyme activities of the three strains (0-2, 1-1 and 2-9) increased by 45.81%, 28.40% and 20.13%. The activity of xylanase was also improved, and the enzyme activities of the three strains (0-2, 1-1 and 2-9) increased by 23.71%, 7.74% and 10.70%. The β-xylosidase

activity. The β-xylosidase activity of three strains (0-2, 1-1 and 2-9) reached their peak values on the third day, decreased by 38.91%, 17.01%, and 56.08%, respectively. These results indicated that the expression of *ace2* in *T. orientalis* largely improved the β-glucosidase activity and xylanase activity. In *T. reesei*, deletion of *ace2* gene resulted in reduced expression levels of cellulases on Solka floc cellulose[18, 19] and overexpression of *ace2* gene exhibited significant increase in [20]. However, in contrast with the claim of Ace2 activating the cellulase production, most of the cellulase activities decreased after expressing *ace2* in EU7-22. Based on *ace2* expression, the species differences between *T. reesei* and *T. orientalis* are significant.

The extracellular protein of strain (0-2, 1-1 and 2-9) reduced by 14.32%, 21.5% and 8.5% when compared with the parent strain EU7-22. It kept consistent with the result of decreasing.

3.4. Quantitate PCR analysis

After cultivation on the solid PDA and MM medium, there is a significant difference in growth phenotype between 0-2 and EU7-22. Considering that the insertion site may have an impact on the of 0-2, so 1-1 was chosen for detailed research later.

The result indicated the *ace2* transcript can be detected in the strain 1-1 but absent in EU7-22. The $\triangle$CT of *ace2* is 2.41 compared to the CT value of the actin gene. This demonstrated that *ace2* was successfully expressed in the recombinant strain 1-1. The genes encoding cellulase and hemicellulase in the strain 1-1 were not significantly increased(Fig. 6). The value of $2^{-\triangle\triangle CT}$ of *bgl1* is 1.20 and xyn1 is 1.79, which is kept in line with the cellulase activities described before. The level of *xyn2* transcription is too low to be detected.

The values of $2^{-\triangle\triangle CT}$ of *cre1*, *ace1*, *ace3* and *xyr1* is 0.54, 0.73, 0.57 and 0.37, respectively. According to the result of qPCR, we provide the evidence that *ace2* has a certain role in the induction of the major cellulase and xylanase genes of EU7-22. The transcriptional quantity of *cre1* and *ace1* encoding cellulase transcription repressor Cre1 and Ace1 decreased. The amount of *ace3* and *xyr1* transcription of 1-1 was decreased. According to the lost enzyme activities of the *ace3* disrupted strain in EU7-22[26], it can be deduced that in *ace2* expression strain 1-1, Ace3 plays an influential role.

3.5. Phenotype and growth rate analysis

The result demonstrated that expression of *ace2* in EU7-22 scarcely affected the conidia formation after cultured for 72 h (Fig. 7a).

The strain 1-1 spread slower than EU7-22 on all medium except on MB(cellobiose) and MX(xylose) which coincided with the result of increased β-glucosidase activity and xylanase activity (Fig. 7b). It can be deduced that the decreased total caused the slower speed of the utilizing avicel.

Conclusions

In conclusion, Ace2 is responsible for the change of cellulase activities on the ABP medium as a cellulase transcriptional activator when expressed in *T. orientalis* EU7-22. Xylanase activity and β-glucosidase activity of *ace2* heterogeneous expression strains are improved and total cellulase activity is decreased. The result of qPCR is accordance with enzyme activities. However, the presence of several putative binding sites for Ace2 in a promoter affects enzyme activities significantly and the expression of other regulatory factors in vivo. It provides the potential in the applications for differentiated purposes and its effect on the induced fermentation still requires further studies. This study provides a reference for an in-depth study on cellulase transcriptional regulation mechanisms of *T. orientalis*.

Declarations

Ethical Approval

No related Ethical issues.

Consent to Participate

The authors promise that the work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.

Consent to Publish

The authors promise that if the manuscript is accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the Publisher. There are no conflicts of interest to declare.

Authors Contributions

Yuyu Li performed the experiment and wrote the paper.

Yong Xue conceived the idea of the study.

Jian Liu contributed to supervision, fund supporting and revised the manuscript.

Lihui Gan contributed to supervision and analyzed the data.

Minnan Long helped perform the analysis with constructive discussions.

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Competing Interests

None.

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Figures

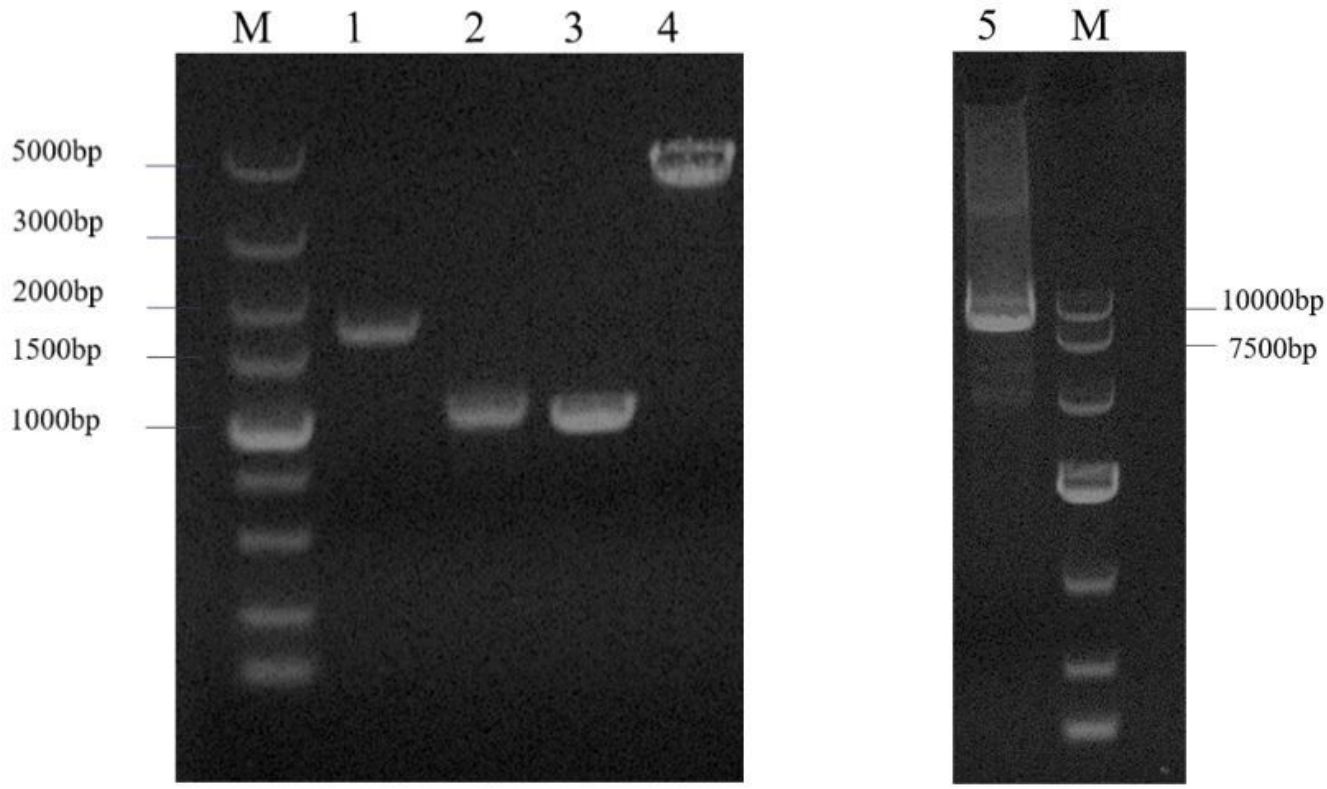


Figure 1

The electrophoretogram of four segments and integrated segments. M means marker, the 1, 2, 3, 4 and 5 represent the promoter, the terminator of eno, hygromycin resistance cassette and ace2 expression cassette, respectively.

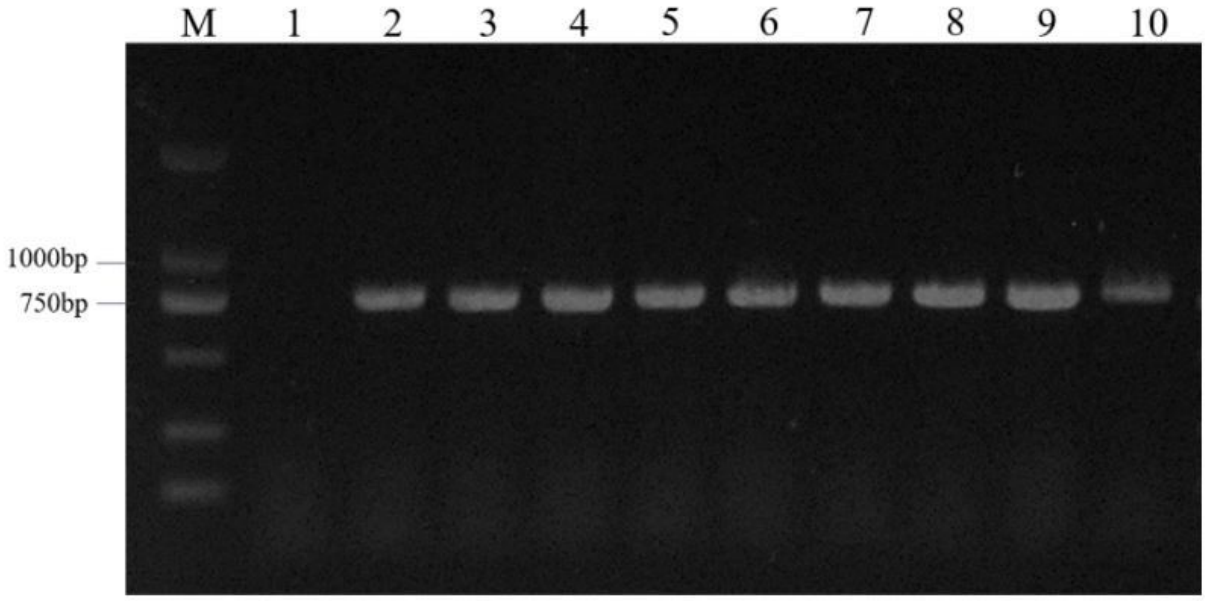


Figure 2

The verification of the transformants by amplifying the partial hph gene. (M) marker, the templates of the PCR are (1) EU7-22, (2) 0-1, (3) 0-2, (4) 0-3, (5) 0-4, (6) 0-5, (7) 1-1, (8) 1-2, (9) 2-9 and (10) 3-1.

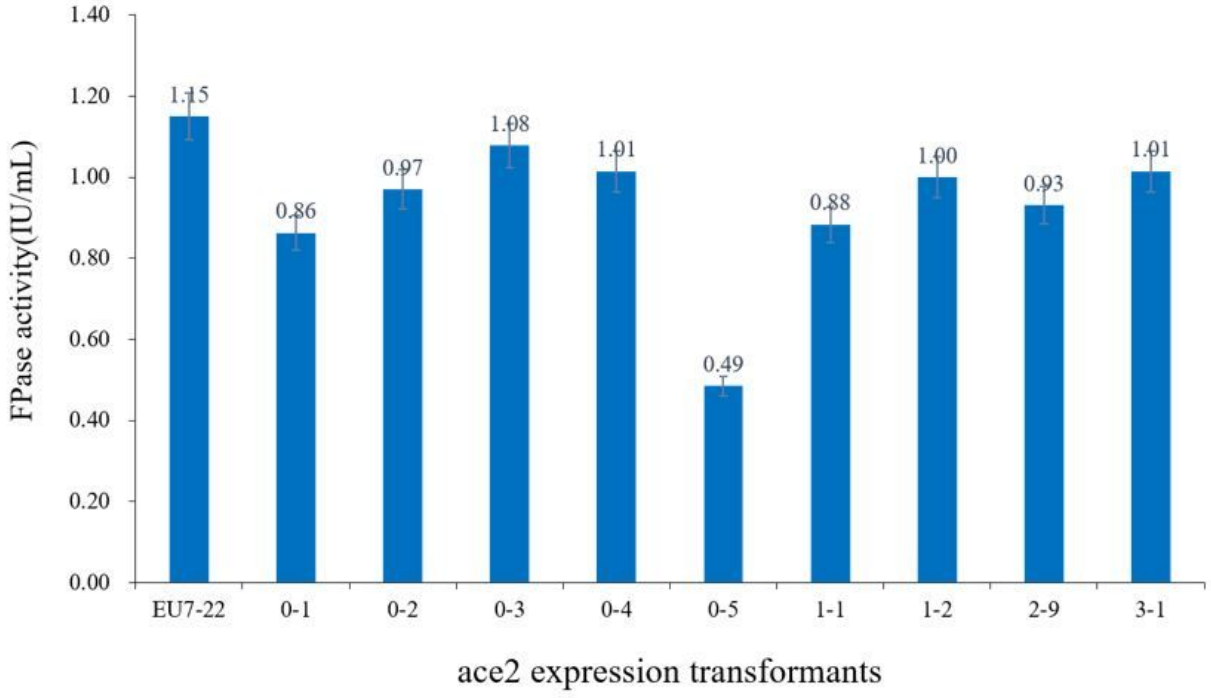


Figure 3

Activities of FPase from EU7-22 and transformants using the inducing medium

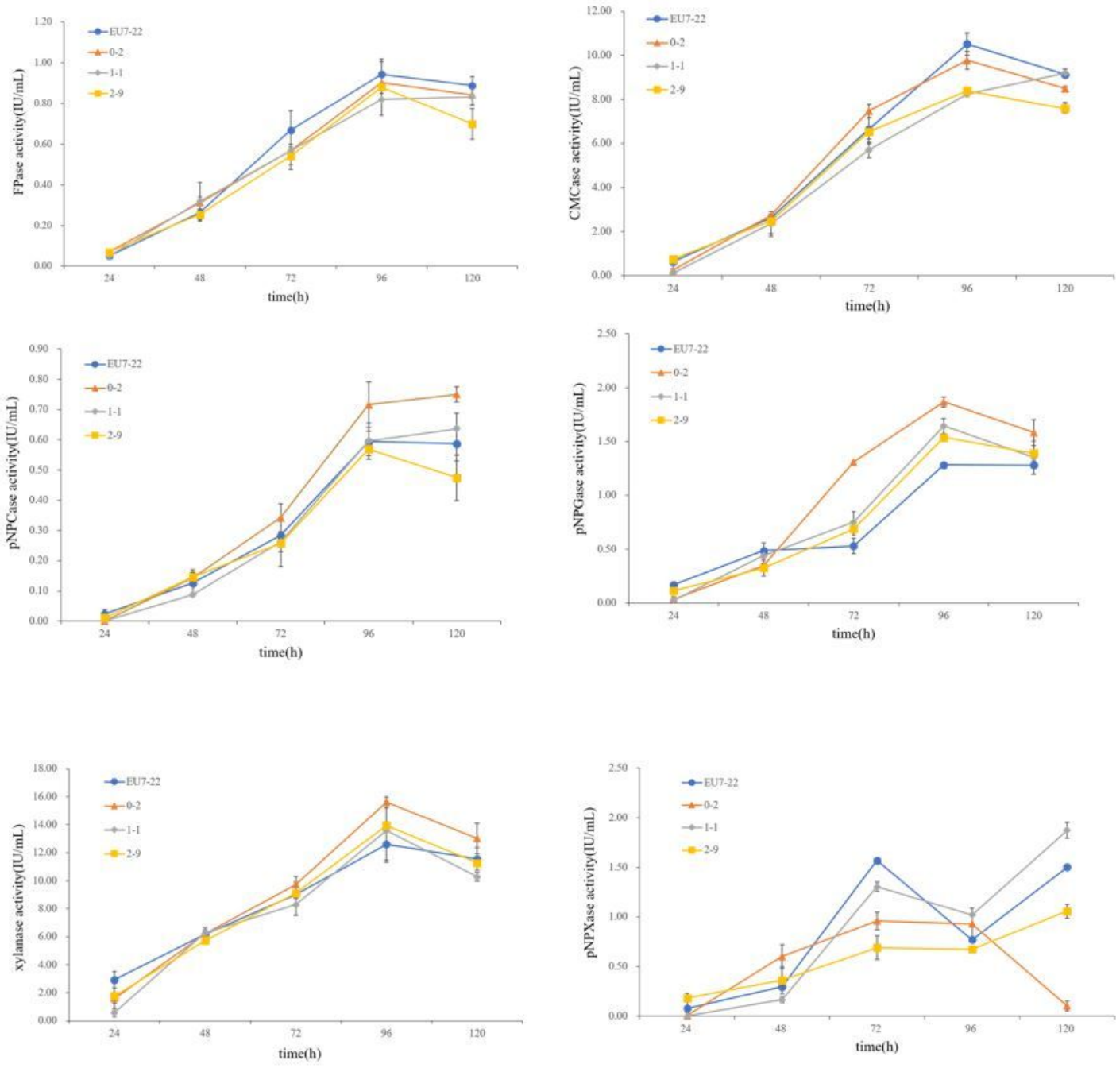


Figure 4

Activities of FPase, CMCase, pNPCase, pNPGase, xylanase and pNPXase of EU7-22 and transformants (0-2, 1-1 and 2-9) using the inducing medium

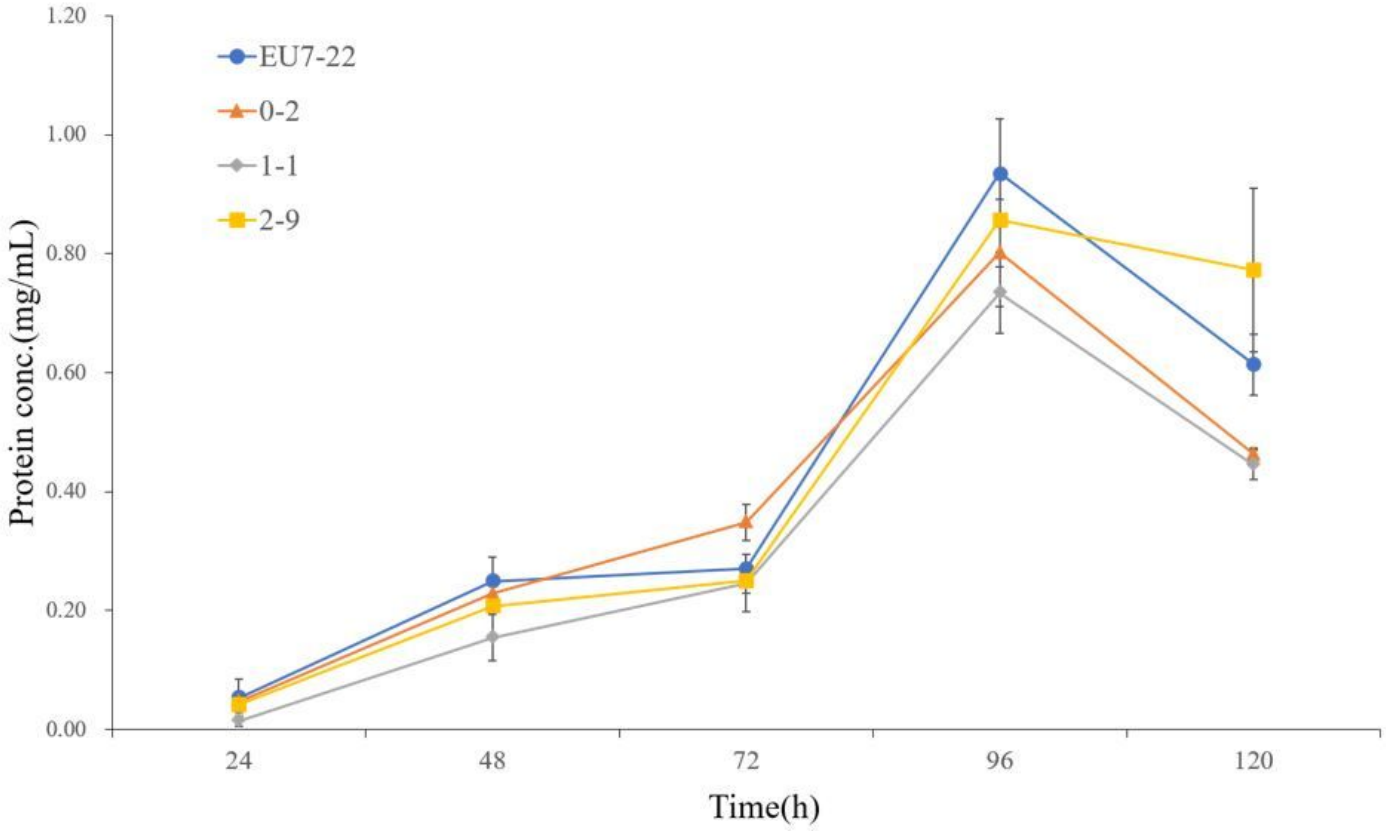


Figure 5

Protein concentrations from T. orientalis EU7-22 and transformants (0-2, 1-1 and 2-9) using the inducing medium

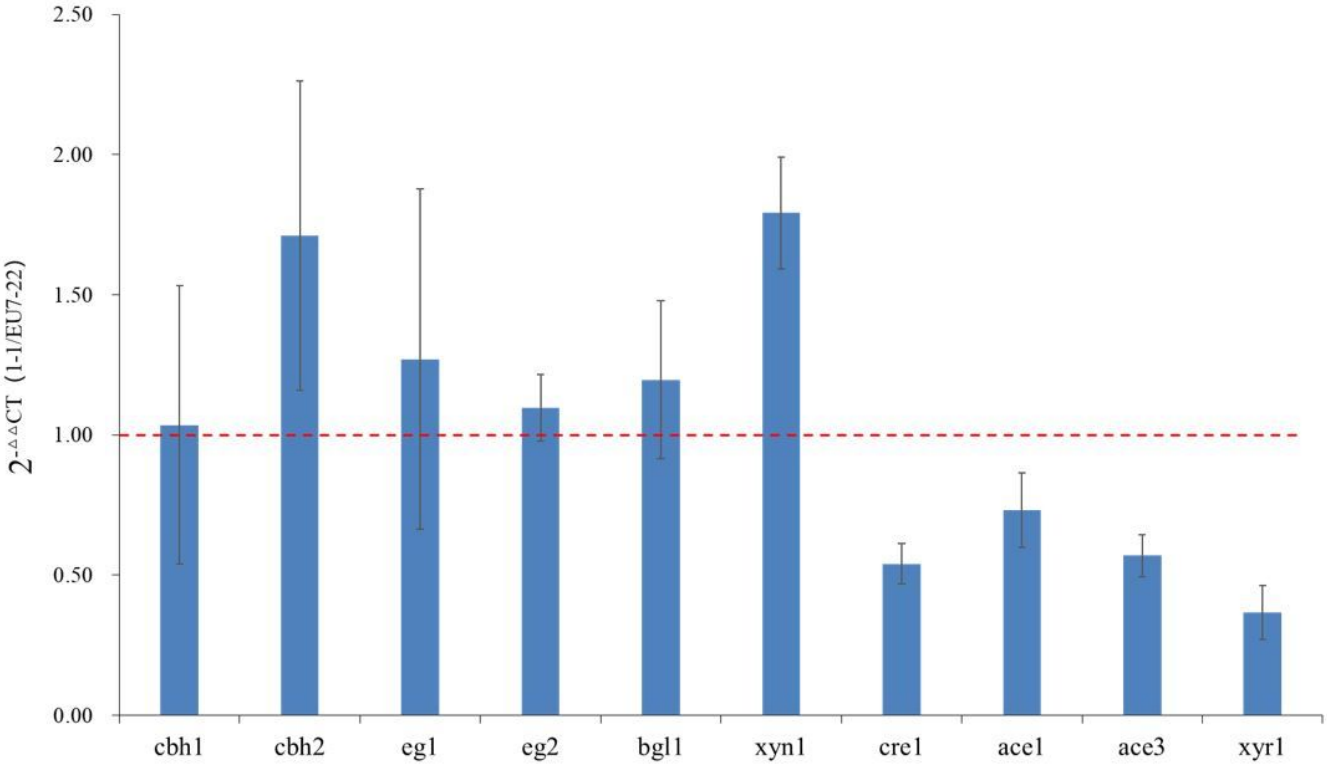
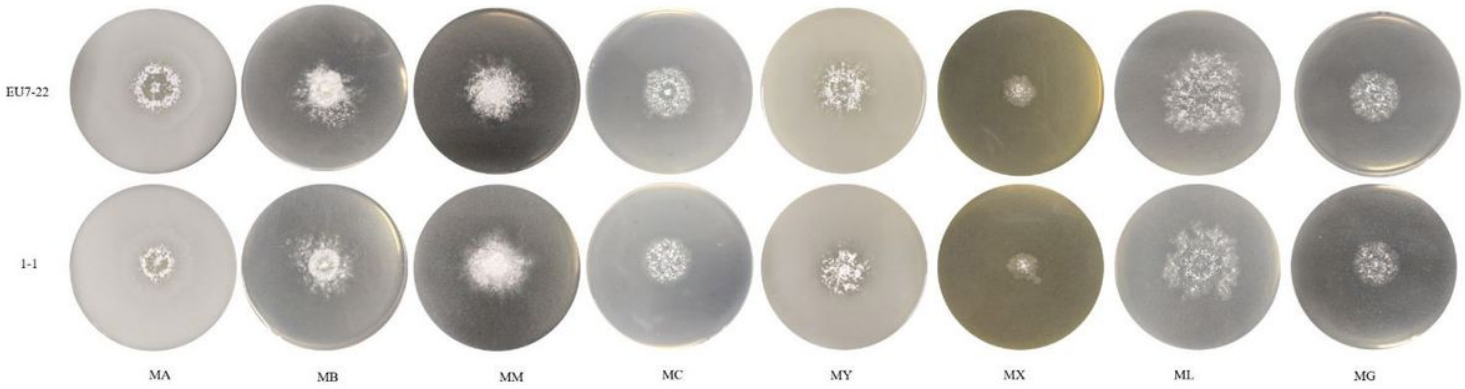
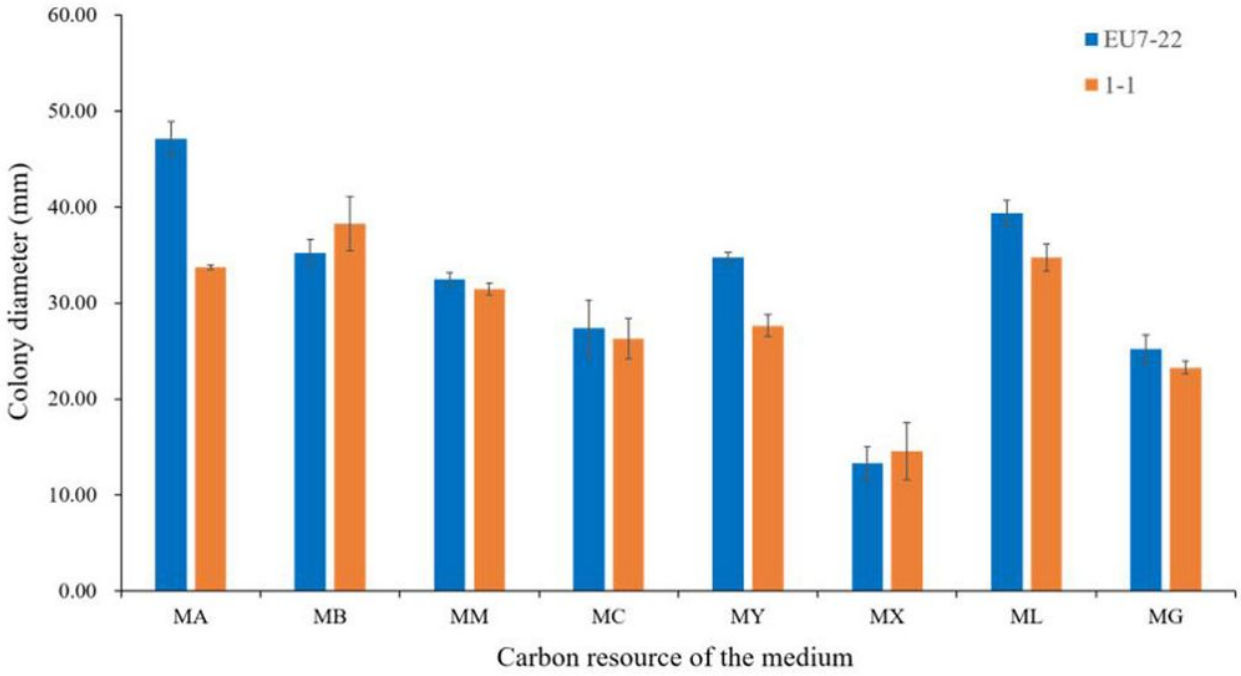


Figure 6

The transcription levels of the main genes at 24 h.



(a)



(b)

Figure 7

Colony morphology (a) and spread rate (b) of EU7-22 and 1-1 on different carbon resource medium cultured for 72 h.