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ORIGINAL PAPER

Cloning and expression of two genes coding endo-β-1,4-glucanases from *Trichoderma asperellum* PQ34 in *Pichia pastoris*

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Two genes coding endo-β-1,4-glucanases were cloned from *Trichoderma asperellum* PQ34 which was isolated from Thua Thien Hue province, Vietnam. The expression of these genes in *Pichia pastoris* produced two enzymes with molecular masses of approximately 46 kDa (about 42 kDa of enzymes and 4 kDa of signal peptide). The effects of induction time and temperature, inducer concentration, and culture medium on the endo-β-1,4-glucanase activity were investigated. The results showed that the highest total activities of two endo-β-1,4-glucanases were approximately 4.7×10^{-8} kat (from Glu1-TA gene) and 7.3×10^{-8} kat (from Glu2-TA gene) occurred after 4 days of induction using 25 mL L^{-1} methanol at 30 °C when the yeast cells were cultured in a YPL medium.

Keywords: endo-β-1,4-glucanase, expression, Glu1-TA, Glu2-TA, *Trichoderma asperellum*

Introduction

Endo-β-1,4-glucanase (EC 3.2.1.4) (also known as cellulase, endoglucanases, 1,4-β-glucanase, carboxymethyl cellulase, endo-1,4-β-D-glucanase, β-1,4-endoglucan hydrolase, and celludextrinase) randomly cleaves internal β-1,4-D-glycosidic linkages in cellulose chains, releasing smaller fragments of random lengths. These fragments are then processed by cellobiohydrase, progressively releasing cellobiose units from the non-reducing end of the molecule (Watanabe & Tokuda, 2001). Accordingly, endo-β-1,4-glucanases are widely used in the food-processing, and textile industries and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they have even found pharmaceutical applications.

Endo-β-1,4-glucanase genes were previously isolated from *Bacillus polymyxa* and *Bacillus circulanst* (Baird et al., 1990), *Aspergillus aculeatus* (Pauly et al., 1999), *Orpinomyces* PC-2 (Jin et al., 2011), *Bacillus subtilis* (Ko et al., 2012), vermicompost (Yasir et al., 2013), *Trichoderma reesei* (Penttilä et al., 1987; Saloheimo et al., 1994; Okada et al., 1998; Nakazawa et al., 2008), *Trichoderma viridae* (Liu et al., 2004; Huang et al., 2010; Li et al., 2011a, 2011b, 2012), and *Trichoderma ouroviridae* (Şahin et al., 2013). The heterologous expression of endo-β-1,4-glucanase has also been performed in different hosts such as *Pichia pastoris* (Palomer et al., 2004; Bai et al., 2010; Shumiao et al., 2010; Jin et al., 2011), *Saccharomyces cerevisiae* (Hong et al., 2001; Saloheimo et al., 1994; Sakamoto et al., 1995), *Aspergillus niger* (Rose & van Zyl, 2002), and *Escherichia coli* (Baird et al., 1990; Cazemier et al., 1999; Huang et al., 2005). However, according to Sreekrishna et al. (1997), *P. pastoris* (methylotrophic yeast) is an ideal host organism for the heterologous expression of recombinant proteins which are controlled by the alcohol oxidase 1 (AOX1) promoter.

To the best of our knowledge, the heterologous expressions of endo-β-1,4-glucanases from *Trichoderma asperellum* have not been reported to date. In the present work, the cloning and expression of two genes coding endo-β-1,4-glucanases from *T. asperel-*

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lum PQ34 which were isolated from Thua Thien Hue province (Vietnam) in *P. pastoris* were performed to produce a large quantity of these recombinant enzymes for future applications in the food-processing industry.

Experimental

T. asperellum PQ34 was isolated as described previously (Loc et al., 2011). *E. coli* TOP10 and *P. pastoris* GS115 were used as transformation hosts. The plasmids of pGEM-T Easy (Promega, Madison, WI, USA) and pPICZ-B (Invitrogen, Carlsbad, CA, USA) were used as cloning and expression vectors, respectively. A Luria–Bertani (LB) medium with 50 μg mL^{-1} of ampicillin or 25 μg mL^{-1} of zeocin was used for selection of the transformed *E. coli* cells. A yeast extract–peptone–dextrose–sorbitol (YPDS) medium with 100 μg mL^{-1} zeocin was used for selection of the transformed *P. pastoris* cells.

After complete disruption and homogenisation of the biomass of *T. asperellum* PQ34, the total RNA was isolated using the InviTrap® Spin Plant RNA Mini Kit (Stratec Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions.

First strand cDNA was synthesised using the First Strand cDNA Synthesis Kit (#K1612, Fermentas, Waltham, MA, USA) of a final volume of 20 μL with approximately 5 μg of total RNA, 0.5 μg of oligo$(dT)_{18}$ primer, 4 μL of 5 × reaction buffer, 20 unit of RiboLock™ ribonuclease inhibitor, 2 μL of 10 mM dNTP mix and 40 units of M-MuLV reverse transcriptase.

The PCR amplifications for endo-β-1,4-glucanase genes were performed in a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) using PCR master mix (#M7502, Promega, Madison, WI, USA). The amplification reaction contained 6 μL of master mix, 10 pmol of each primer, 2.5 μL of cDNA solution, and distilled water to make up a final volume of 12 μL. The thermal cycling programme used for PCR was 5 min at 95 °C followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min. After the last cycle, an extension of 10 min at 72 °C was applied.

Specific primers of endo-β-1,4-glucanase genes (Table 1) were designed based on the genomic database of *T. asperellum* CBS 433.97 (http://genome.jgi.doe.gov/Trias1/Trias1.home.html).

PCR products were purified from agarose gel by Wizard®SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, USA) and ligated to pGEM-T Easy vector (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The inserts were then sequenced by the fluorescent dideoxy terminator method on 3130 ABI system (Applied Biosystems, Foster City, CA, USA).

The genes of *T. asperellum* PQ34 were digested from the recombinant pGEM-T Easy vector by *Eco*RI and *Not*I and purified using Wizard® SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, USA) after electrophoresis on 8 g L^{-1} agarose gel. The genes were then ligated into *Eco*RI and *Not*I sites downstream of the AOX1 promoter of pPICZ B vector (Invitrogen, Carlsbad, CA, USA) and transformed into chemically competent *E. coli* TOP10 cells. The pPICZ B/endo-β-1,4-glucanase vectors were characterised using restriction analysis with *Sac*I.

Subsequently, the recombinant pPICZ B vectors were transformed into *P. pastoris* (Invitrogen, Carlsbad, CA, USA) by the electroporation method, following the manufacturer's instructions. The pPICZ B vector is designed for native expression of the interesting protein in *P. pastoris*. It contains the zeocin-resistant gene for transformant selection in yeast.

P. pastoris cells containing the recombinant vector with the coding sequence of endo-β-1,4-glucanase genes were grown in a shaker with a rotation speed of 250 min^{-1} at 30 °C, to an OD600 nm value of 1, then methanol was added to a final concentration of 10 mL L^{-1} for induction of the expression of these

Table 1. Oligonucleotide primers used in PCR amplification for endo-β-1,4-glucanase genes

No.	Primers	Nucleotide sequences
1	TA1-F	GAATTATGATCAACAACAAGGCTG
	TA1-R	GCGGCCGCCTAACGAGCAAGGCAAAGC
2	TA2-F	GAATTATGAATAAGCCCATGGCCC
	TA2-R	GCGGCCGCCTATGCGCGGGAGAAGCAA

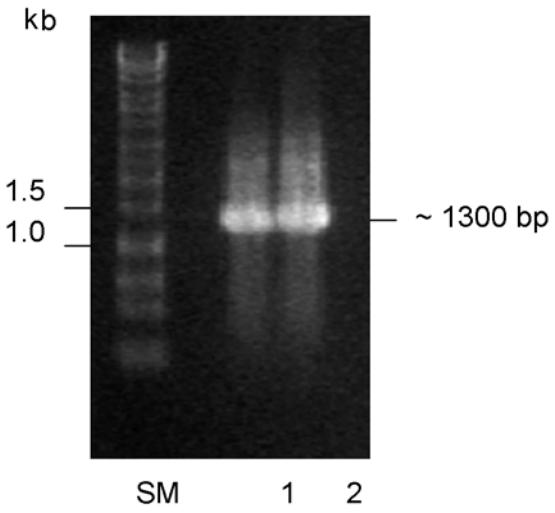

Fig. 1. PCR amplification with TA1-F/TA1-R and TA2-F/TA2-R primers. SM: DNA size marker (1 kb), 1: PCR product of TA1-F/TA1-R primers, 2: PCR product of TA2-F/TA2-R primers.

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Query  1      ATGATCAACAACAAGGCTGCACTGCTATTTGCGGCCTATGCTGGAGTGAGTGGTGTTGTG  60
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61451  1      ATGATCAACAACAAGGCTGCACTGCTATTTGCAGCCTATGCTGGAGTGAGTGGTGTTGTG  60

Query  61     GCACAGCAGCAGACCACCTGGGGACAGTGCGGAGGGCAAGGCTACGGAGGTCCAACAAAT  120
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61451  61     GCACAGCAGCAGACCACCTGGGGACAGTGCGGAGGGCAAGGCTACGGAGGTCCAACAAAT  120

Query  121    TGTGTTTCTGGAACGGCCTGCTCAACATTAAATTCTTACTATGCTCAATGCGTCCCTGCT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61451  121    TGTGTTTCTGGAACGGCCTGCTCAACATTAAATTCTTACTATGCTCAATGCGTCCCTGCT  180

Query  181    ACCGGCATAGTCACCAGTACCACCAGAGCTACAGCAACGTCAACCGTGACAAAGTCAACA  240
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61451  181    ACCGGCATAGTCACCAGCACCACCAGAGCTACAGCAACGTCAACCGTGACAAAGTCAACA  240

Query  241    GTGACAACGTCAGCCAGTGCGTCAGCAAGTGCATCTCCGCCTCCCGTTGGCTCAGGCACC  300
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61451  241    GTGACAACGTCAGCCAGTGCGTCAGCAAGTGCGTCTCCGCCTCCCGTTGGCTCAGGCACC  300

Query  301    CAGTTTGCCGGTATCAACATTGCTGGATTCGACTTTAGCTGCTCTACTGACGGAACTTGC  360
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61451  301    CAGTTTGCCGGTATCAACATTGCTGGATTCGACTTTAGCTGTTCTACTGACGGAACTTGC  360

Query  361    AACTTGAACGGTGCCTACCCGCCGCTGAAGAATTACGACGGCGCAAATAACTATCCAGAT  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61451  361    AACTTGAACGGTGCCTACCCGCCGCTGAAGAATTACGACGGCGCAAATAACTATCCAGAT  420

Query  421    GGCGTTGGGCAGATGCAGCATTTCGTTAAAGACGACGGATTCAATATCTTCCGTCTCCCT  480
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61451  421    GGCGTTGGGCAGATGCAGCATTTCGTTAAAGACGACGGATTCAATATCTTCCGTCTCCCT  480

Query  481    GTCGGTTGGCAGTATTTAGTCAACGGTACTCTTGGCGGTACCTTGAACCCTACCAATATA  540
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61451  481    GTCGGTTGGCAGTATTTAGTCAATGGTACTCTTGGCGGTACCTTGAACCCTACCAATATA  540

Query  541    GGCTATTACGATCAACTTGTCCAAGGATGCCTGGCCACGGGTGCATACTGCATCATTGAT  600
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61451  541    GGCTATTACGATCAACTTGTTCAAGGATGCCTGGCCACGGGTGCATACTGCATCATTGAT  600

Query  601    ATCCACAACTATGCTCGCTGGAATACCGGAATCATTGGCCAAGGCGGCCCTACAAACGCC  660
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61451  601    ATCCACAACTATGCTCGCTGGAATACCGGAATCATTGGCCAAGGCGGCCCTACAAACGCC  660

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61451  661    CAGTTCGTTGATGTTTGGACCCAACTGGCTACCAAATACGCCTCCGAGTCGAAGATTTGG  720

Query  721    TTTGGCGTAATGAATGAGCCACACGACGTCAACATCACAACCTGGGCGGCCACCGTGCAG  780
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61451  721    TTTGGCGTAATGAATGAGCCACACGACGTCAACATCACAACCTGGGCGGCCACCGTGCAG  780

Query  781    CTCGTTGTTACTGCGATCCGCAATGCGGGTGCCACCTCACAATACATCTCACTGCCGGGT  840
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61451  781    CTCGTTGTTACTGCGATCCGCAATGCGGGTGCCACCTCACAATACATCTCACTGCCGGGT  840

Query  841    ACTGACTGGCAATCTGCTGGAAGCATCATATCCGATGGTGGTGCAGCGGCTTTGGGTGCC  900
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61451  841    ACTGACTGGCAATCTGCTGGAAGCATCATTTCCGATGGTGGTGCAGCGGCTTTGGGTGCC  900

Query  901    ATCACCAATCCTGATGGCTCAAAGACCAACTTGATTTTCGATGTGCACAAGTACTTGGAC  960
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
61451  901    ATCACCAATCCTGATGGCTCAAAGACCAACTTGATTTTCGATGTGCACAAGTACTTGGAC  960
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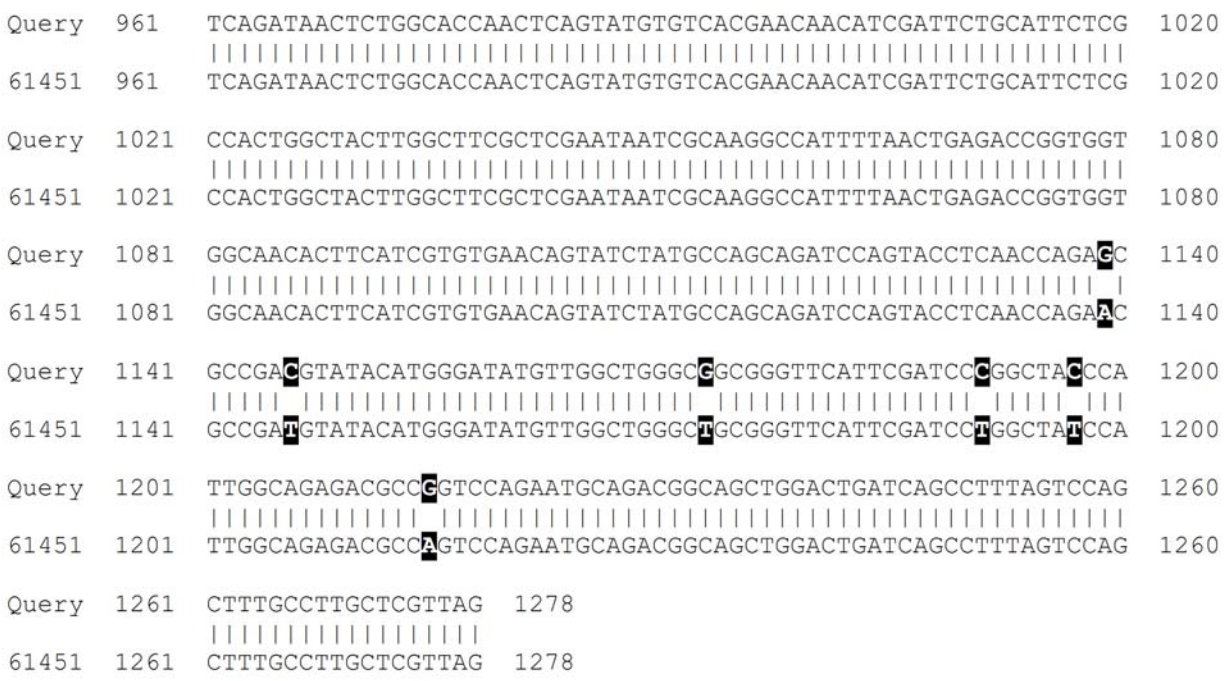

Fig. 2. Multiple alignment of Glu1-TA gene (accession no.: KJ188167 from NCBI) and endo-β-1,4-glucanase gene (protein ID: 61451 from database http://genome.jgi.doe.gov/Trias1/Trias1.home.html).

Fig. 3. Amino acid sequences of Glu1-TA gene and endo-β-1,4-glucanase gene (protein ID: 61451).

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Query  1      ATGAATAAGCCCATGGCCCCGTTGCTGCTCGCTGCCACGCTTATGGCAAGCGGTGCTATT  60
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356270 1      ATGAATAAGCCCATGGCCCCGTTGCTGCTCGCTGCCACGCTTATGGCAAGCGGTGCTATT  60

Query  61     GCACAGACACAAACTGTTTGGGGACAATGTGGAGGTACAGGCTACAGCGGTCCGACAAAC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
356270 61     GCACAGACACAAACTGTTTGGGGACAATGTGGAGGTACAGGCTACAGCGGTCCGACAAAC  120

Query  121    TGTGCTTCTGGTTCTGCATGCTCAACACTTAACCCCTATTACGCCCAGTGCATTCCCGGC  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
356270 121    TGTGCTTCTGGTTCTGCATGCTCAACACTTAACCCCTATTACGCCCAGTGCATTCCCGGC  180

Query  181    GCAACCAGCTTCACTACATCGACTACCTCGACCAAACCACCAGGTTCTGGGTCAACCACA  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
356270 181    GCAACCAGCTTCACTACATCGACTACCTCGACCAAACCACCAGGTTCTGGGTCAACCACA  240

Query  241    ACATCTTCAGCTTCCGCACCAACTGGCTCTGGTCAGACGCGATTTGCTGGCATTAACATT  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
356270 241    ACATCTTCAGCTTCCGCACCAACTGGCTCTGGTCAGACGCGATTTGCTGGCATTAACATT  300

Query  301    GCTGGATTCGACTTTGGCTGCACAACTGATGGAACCTGTGTTACTTCGCAAGTATATCCA  360
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356270 301    GCTGGATTCGACTTTGGCTGCACAACTGATGGAACCTGTGTTACTTCGCAAGTATATCCA  360

Query  361    CCATTGAAAAACTTTGATGGTGCGAACAACTATCCAGATGGTATTGGTCAGATGCAGCAC  420
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356270 361    CCATTGAAAAACTTTGATGGTGCGAACAACTATCCAGATGGTATTGGTCAGATGCAGCAC  420

Query  421    TTTGTCAATAATGATAAATTAAACATCTTCCGTCTACCTGTTGGGTGGCAATATCTTGTT  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
356270 421    TTTGTCAATAATGATAAATTAAACATCTTCCGTCTACCTGTTGGGTGGCAATATCTTGTT  480

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Query  721    CATGATGTGGATATTAACACTTGGGCCACGACTGTGCAAGCCGTTGTTACCGCCATCCGT  780
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Query  781    AACGCGGGTGCCACGACGCAATTCATTTCACTACCAGGAACCGATTATCAGTCTGCCGGA  840
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Query  841    AACTTCCTTAGCGATGGCAGTTCCACTGCCTTATCTCAAGTGAAGAACCCGGATGGTTCA  900
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Query  901    ACAACGAATTTGATCTTTGATCTCCACAAATACCTTGACTCAGATAATTCTGGCACTTCC  960
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Query  961    ACAACTTGCGTCACTAACAACATCGCTACCGCATTCCAGCCTGTCGCAACCTGGCTTCGC  1020
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Query  1021   CAGAACAAACGCCAAGCTATTCTGACCGAAACTGGTGGCGGCAACACTCAGTCATGCATT  1080
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Query  1081   CAGGACGTGTGCCAACAGAACCAGTTCCTCAACCAAAACTCTGACGTTTTCCTCGGTTAC  1140
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Query  1201   AACGGAAACACTTGGACTGACACTGCTCTGGCAGCAGCTTGCTTCTCCCGCGCAT  1255
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356270 1201   AACGGAAACACTTGGACTGACACTGCTCTGGCAGCAGCTTGCTTCTCCCGCGCAT  1255
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Fig. 4. Multiple alignment of Glu2-TA gene (accession no.: KJ188168 from NCBI) and endo-β-1,4-glucanase gene (protein ID: 356270 from database http://genome.jgi.doe.gov/Trias1/Trias1.home.html).

genes. The culture was then allowed to grow for a further 5 days and the cells were harvested by centrifugation at 15000g for 5 min at 4℃. Sonication was carried out for 10 min to break the cells and recombinant endo-β-1,4-glucanases were obtained using a phosphate buffer (pH 7). The expression levels of

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Query  1    METNKPMETAPLLLAATLMETASGAIAQTQTVWGQCGGTGYSGPTNCASGSACSTLNPYY  60
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Query  61   AQCIPGATSFTTSTTSTKPPGSGSTTTSSASAPTGSGQTRFAGINIAGFDFGCTTDGTCV  120
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Query  181  LGYYDQLVQGCLATGAYCIVDIHNYARWNGAIIGQGGPTNAQFTNLWTQLATKYASQSKI  240
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Query  421  ACFSRA  426
356270 421  ACFSRA  426
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Fig. 5. Amino acid sequence of Glu2-TA gene and endo-β-1,4-glucanase gene (protein ID: 356270).

enzymes were evaluated by sodium dodecyl sulphate (SDS)-120 g L^{-1} polyacrylamide gel electrophoresis (PAGE). The gels were then stained with Coomassie Blue R-250 and the images were analysed using the Quality One program (version 4.1, BioRad).

The endo-β-1,4-glucanase activity was assayed using Iqbal's method with a slight modification. The reaction was performed by mixing 300 μL of carboxyl methyl cellulose in 0.05 M acetate buffer (pH 5) with 150 μL of crude enzyme solution. After incubation at 50 ℃ for 30 min, the reaction was terminated by adding 600 μL of 3,5-dinitrosalicylic acid then boiled for 5 min. The enzyme activity was measured at 540 nm wavelength with the glucose standard curve. One unit of total enzyme activity was defined as the amount of enzyme that liberated 1 mol of glucose in 1 s under the conditions described above (Zhekova et al., 2012).

Results and discussion

Cloning endo-β-1,4-glucanase genes

Fig. 1 shows that two DNA fragments of approximately 1300 bp were amplified from the genome of *T. asperellum* PQ34, suggesting that endo-β-1,4-glucanase genes might be present in them. These results also indicate that two primer pairs of TA1-F/TA1-R and TA2-F/TA2-R were specific for the amplification reactions.

To confirm the PCR of the endo-β-1,4-glucanase genes from *T. asperellum* PQ34 (named Glu1-TA and Glu2-TA), the PCR products were cloned into a pGEM-T Easy vector and their sequences were analysed. The sequencing profiles indicated that the Glu1-TA and the Glu2-TA had 1278 and 1255 nucleotides, respectively. The BLAST search showed that the nucleotide and the amino acid sequences of Glu1-TA and Glu2-TA genes from *T. asperellum* PQ34 exhibited 99 % and 100 % homology to the endo-β-1,4-glucanase genes from *T. asperellum* CBS 433.97 (respective protein IDs of 61451 and 356270 from the database http://genome.jgi.doe.gov/Trias1/Trias1.home.html) (Figs. 2–5).

The Glu1-TA and Glu2-TA genes were recovered from the recombinant pGEM-T Easy vector by restriction and ligated into the pPICZ B expression vector, employing *E. coli* TOP10 competent cells. The pPICZ B/endo-β-1,4-glucanase vectors were then subjected to digestion with *Sac*I. The restriction produced

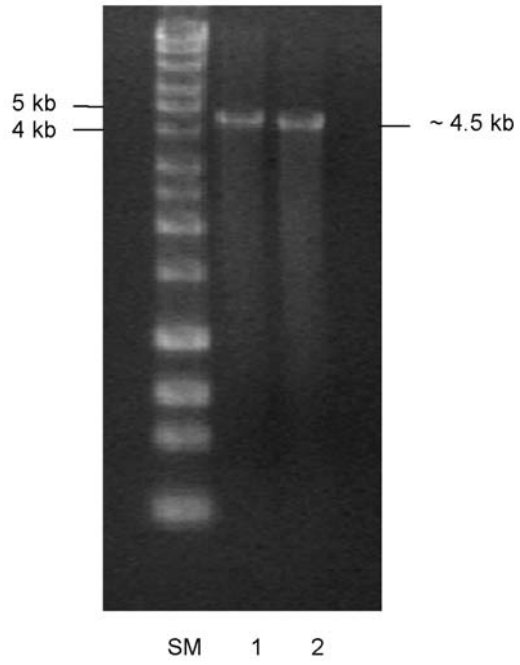

Fig. 6. Digestion by *Sac*I for pPICZ B/endo-β-1,4-glucanase vectors. SM: DNA size marker (1 kb), 1: pPICZ B/Glu1-TA vector, 2: pPICZ B/Glu2-TA vector.

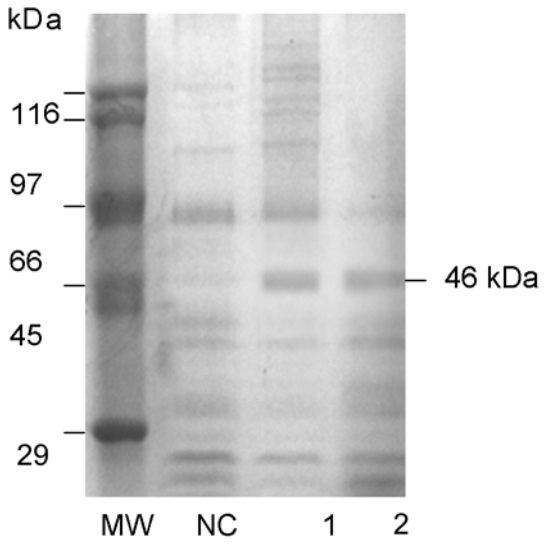

Fig. 7. Expressions of endo-β-1,4-glucanases in yeast cells were induced by methanol. WM: protein mass marker. NC: un-induced yeast cells. 1 and 2: expression of enzymes from Glu1-TA and Glu2-TA genes after 4 h of induction, respectively.

DNA fragments of approximately 4.5 kb in length (vector 3.3 kb and Glu1-TA 1257 bp or Glu2-TA 1278 bp) as expected, indicating that the genes were inserted in the vector (Fig. 6).

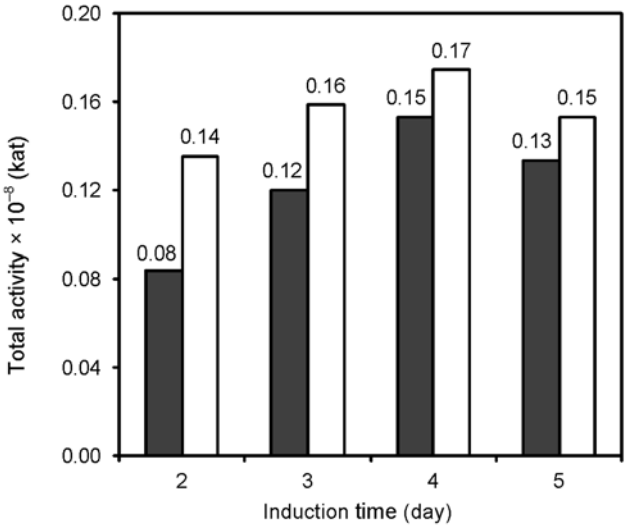

Fig. 8. Effect of induction time on total activities of two recombinant endo-β-1,4-glucanases Glu1-TA (■) and Glu2-TA (□).

Expression of endo-β-1,4-glucanases in Pichia pastoris

pPICZ B/endo-β-1,4-glucanase expression vectors, together with an empty pPICZ B vector as a control, were transformed into *P. pastoris*. The expressions of recombinant endo-β-1,4-glucanases were analysed using SDS-PAGE. The results showed that a band of approximately 46 kDa (about 42 kDa of enzyme and 4 kDa of signal peptide) was obtained in the culture induced with methanol (Fig. 7). For the un-induced cells, no band of the expected sizes was observed, suggesting that induction with methanol is required for the expression of endo-β-1,4-glucanase genes.

The literature contains some reports on the heterologous expression of different glucanase genes of *T. asperellum* as exo-β-1,3-glucanase (Bara et al., 2003; Marcello et al., 2010) or α-1,3-glucanase (Sanz et al., 2005). However, there are no data in the literature concerning the expression of the endo-β-1,4-glucanase from *T. asperellum* in *P. pastoris*.

Effect of culture condition on endo-β-1,4-glucanase activity

A batch culture of transformed *P. pastoris* cells was transferred into 100-mL Erlenmeyer flasks for the endo-β-1,4-glucanase production. Fig. 8 displays the typical curves of enzyme production based on the OD at 540 nm. The expression of the endo-β-1,4-glucanases of Glu1-TA and Glu2-TA genes increased continuously from days 2 to 4 of cultures with maximum OD540 values of 0.71 and 0.73 in the extract, respectively; it then rapidly decreased on the 5th day. The OD540 values of two enzymes in the broths were minimal (data not shown). The total activities

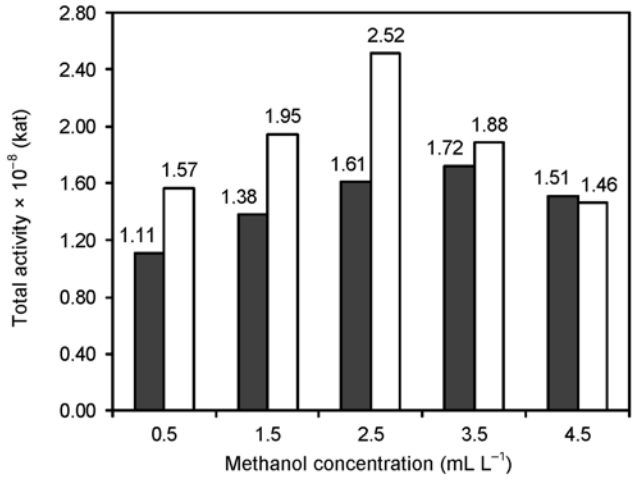

Fig. 9. Effect of methanol concentration on total activities of two recombinant endo-β-1,4-glucanases, Glu1-TA (■) and Glu2-TA (□).

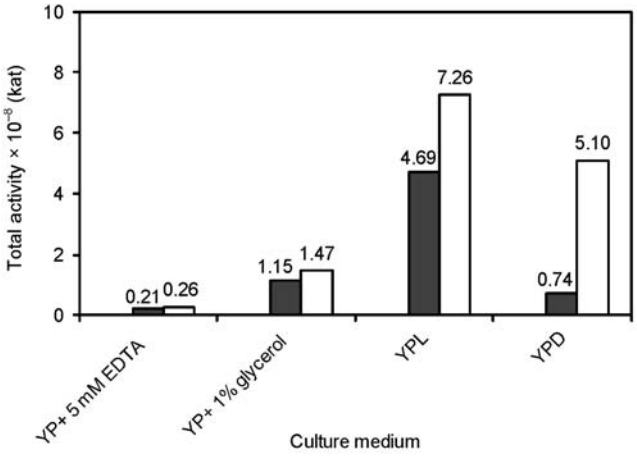

Fig. 10. Effect of culture medium on total activities of two recombinant endo-β-1,4-glucanases, Glu1-TA (■) and Glu2-TA (□). YP: yeast extract–peptone, YPL: yeast extract–peptone–lactose, and YPD: yeast extract–peptone–dextrose.

of enzymes which achieved the highest values corresponding to the OD540 were 0.15×10^{-8} kat and 0.17×10^{-8} kat. These enzymes were absent in the broth and the extract from the control (untransformed *P. pastoris*) culture.

The effects of inducer (methanol) concentration, induction temperature and culture medium on enzyme activity were investigated. The results showed that 25 mL L^{-1} methanol caused the highest expression of both enzymes, Glu1-TA and Glu2-TA, with total activities reaching values of 1.6×10^{-8} kat and 2.5×10^{-8} kat at 30 °C, respectively (Fig. 9). For Glu1-TA, the activities of 1.6×10^{-8} kat (25 mL L^{-1} methanol) and 1.7×10^{-8} kat (35 mL L^{-1} methanol) are not significantly different. The other levels of induction temperature (20 °C, 25 °C, and 35 °C) did not display positive influences for enzyme expression compared with a level of 30 °C (data not shown). Four different nutrient media were also tested with an induction of 25 mL L^{-1} methanol at 30 °C. Fig. 10 indicates that the YPL medium was the most suitable. The total activities of endo-β-1,4-glucanases achieved values of 4.7×10^{-8} kat (Glu1-TA) and 7.3×10^{-8} kat (Glu2-TA).

In conclusion, the experiments revealed that two endo-β-1,4-glucanases from *T. asperellum* PQ34 were expressed in *P. pastoris* cells. The transformed *P. pastoris* cells are relatively suitable, hence their applications can be further exploited once optimisation of the culture conditions is investigated in a bioreactor (e.g. pH, dissolved oxygen, agitation, etc.).

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