Research Article

Cloning and Expression of the Cellulase Gene from the King Oyster Mushroom, *Pleurotus eryngii.*

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Abstract

A gene encoding for cellobiohydrolase (*PEcbh*) from *P. eryngii* was cloned by using RT-PCR 3' and 5' RACE techniques. The result showed that the *PEcbh* was 1377 bp nucleotide sequence encoded for 459-deduced amino acid. Analysis of predicted protein revealed that PEcbh consisted of a glycosyl hydrolase family 7 domain but lacked of cellulose binding domain, a calculated molecular weight of 49.3 kDa and a p*I* of 5.3. The *PEcbh* was cloned into pET28a (+) to obtain a recombinant pET/*PEcbh* and expressed in *E. coli* BL21 (DE3). The optimal conditions of *PEcbh* expression were 0.2 mM IPTG, 1 h induction time at 18^0C and 4 h post-induction time. The CMCase activity could be detected, but at a low activity. This is probably due to a lack of the cellulose binding domain in PEcbh. Expression of PEcbh in *E. coli* BL21 (DE3) and Rosetta (DE3) were compared and the results indicated that CMCase activity in Rosetta (DE3) was higher than in BL21 about 2 times.

***Key Words*:** *Pleurotus eryngii*; Cellobiohydrolase; Glycosyl hydrolase family 7; Protein expression; Cloning

Introduction

A King oyster mushroom (*Pleurotus eryngii*) belongs to genus *Pleurotus*. Its natural habitat is on the dead root of the weed *Eryngium campestre* (Gyorfi and Hajdu, 2007). *P.eryngii* is one of the popular mushrooms due to its flavour, texture and longer shelf life than other oyster mushrooms. *P. eryngii* can be cultivated on several cellulose substrates such as log, but sawdust are common used (Stamets, 1993). Nowadays, the mushroom has been commercially cultivated worldwide, especially in Europe, Asia and North America. Industrial cultivation of this species began in Japan in 1955 which the production is 60 tons and increased to 29,000 tons in 2003. In China, the production increased from 7,300 tons in 2001 to 114,100 tons in 2003. In US, the production reached 85 tons in 2004 (Ohga and Royse, 2004; Rodriguez Estrada and Royse, 2007).

Many filamentous fungi, especially basidiomycetes, are able to produce cellulases to degrade cellulose substrates to glucose, which are necessary for their growth (Kuforiji and Fasidi, 2008; Sánchez, 2009). Cellulases can be divided into three groups: endo-cellulase (endo-1, 4-β-glucanase, EC 3.2.1.4), exo-cellulase (exo-β-1, 4-glucanase, EC 3.2.1.91), and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21). Endo-cellulase and exo-cellulase catalyze the hydrolysis of β-1, 4-glucosidic linkages of internal and non-reducing end of crystalline cellulose chain, respectively. Then, β-glucosidase hydrolyzes disaccharide and tetrasaccharide into glucose (Shimada, 1991). Many cellulase genes from bacteria and fungi have been cloned and characterized. An organization of cellulase gene consists of catalytic domain and cellulose-binding domains (CBD) which both domain are separated by linker peptide. CBD is used to bind with cellulose, so they are essential for specific activities of the enzymes on soluble and insoluble cellulosic substrates. The catalytic domain hydrolyzes the glycosidic bond with retention or inversion of configuration. In addition, cellulase can be devided into families based on amino acid sequence of catalytic domain (Gilkes et al., 1991; Henriksson et al., 1997).

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Table 1 Nucleotide sequence of oligonucleotides used for cloning of the *PEcbh* by 3' and 5' RACE techniques.

Primer	Nucleotide sequence	Annealing temperature (^{0}C)
cbhIF1	TGYGAYTCICARTGYCCICGIGA	52
cbhIR2	GARTCIAGCCAIAGCATRTTIGC	52
cbhI3RACE	GTTTTGAGTATCTGGGACGA	55
cbhGSP1	CTTCCCAGATATCCATCTCA	55
cbhGSP2	TATCACGTGGACATTGAGAA	55
cbhGSP4	CGATGTTGGTGTAGTACGG	55

Many reports described cellulase gene from *Pleurotus* spp., but not from *P. eryngii*. In this study, cellobiohydrolase gene from *P. eryngii* (*PEcbh*) grown in medium containing cellulose was cloned by using RT-PCR, 3' RACE and 5' RACE techniques. The expression of *PEcbh* was studied by using *E .coli* system under T7-lac hybrid promoter (pET28a).

Materials and methods

Strains and culture condition

Pleurotus eryngii was maintained on potato dextrose agar (PDA; criterion) medium at 25^{0}C by periodic transfer. *P. eryngii* was grown in liquid culture containing 0.2% of carboxymethyl cellulose, CMC (Lee et al., 2001) and incubated at 25^{0}C for 18 days without shaking.

The plasmid pET28a (+), *E.coli* strains BL21 (DE3) and Rosetta (DE3) kindly provided by Dr. Sarin Chimnaronk were used for the expression of recombinant *PEcbh*. *E. coli* BL21 (DE3) was grown in LB medium while, *E. coli* Rosetta (DE3) was cultured in LB medium containing 34 µg/ml chloramphenicol. The culture was incubated at 37^{0}C with shaking at 180 rpm.

RNA Isolation and RT-PCR

The mycelium was filtered from liquid culture and washed three times with deionized water, then frozen immediately in liquid nitrogen and grounded to a fine powder with mortar and pestle. Total RNA was extracted by using TRIZOL™ LS Reagent (Gibco BRL). The mRNA was used as the starting material to perform RT-PCR. For first-strand cDNA synthesis, 1 µg of RNA was subjected to 20-µl reaction volumes containing 10 µM AP primer, 5X MMuLV-RT buffer, 10 mM dNTP, 40 U/µl RNase inhibitor, 200 U/µl M-MuLV RT, and 20 mg/ml RNaseH. Subsequently, two degenerate primers (cbhIF1 and cbhIR2; Jia et al., 1999) were used to amplification of cellobiohydrolase gene from *P. eryngii.* Amplification conditions were: 1 cycle of 94^{0}C for 3 min, 39 cycles of 94^{0}C for 30 s, 52^{0}C for 30 s, and 72^{0}C for 1 min; then a final extension at 72^{0}C for 10 min before storage at 4^{0}C (Jia et al., 1999). The PCR product was subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

RACE PCR

The mRNA isolated from the cellulose-grown mycelium was used as the starting material to isolation of cellobiohydrolase gene. 3' and 5' Rapid Amplification of cDNA Ends (RACE; Invitrogen) techniques were used to obtain full length cellulase gene. For 3' RACE, first strand cDNA was synthesized with reverse trascriptase by using the adapter primer (AP). The original mRNA template was degraded with RNase H, which was specific for RNA: DNA heteroduplex molecules. The first amplification of cDNA was performed by using AUAP and cbhI3RACE primers (Table 1).

For 5' region, first strand cDNA was synthesized with reverse trascriptase by using the cbhGSP1 primer (Table 1). The original mRNA template was degraded with RNase H. A tail of cDNA was added by using TdT and dCTP. The amplification of dC-tailed cDNA was accomplished by using the Abridged Anchor Primer and cbhGSP2 (Table 1).Subsequently, the PCR product was reamplified by using AUAP and cbhGSP4 primers (Table 1).

Cloning, sequencing and bioinformatic analysis

The PCR products were purified from agarose gel, cloned into pCR8/GW/TOPO vector (Invitrogen) and transformed to *E. coli* strain Mach1™-T1R. Transformants were selected by resistance to spectinomycin. Plasmid was extracted and sequenced. These sequencing information were analyzed with database by BLAST search program (NCBI). The deduced amino acid sequence alignment was performed by using ClustalW. Conserved Domain Architecture Retrieval Tool (CDART) was used to detect domain in amino acid sequence. Molecular weight and isoelectric point (p*I*) were predicted

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online by Compute p*I*/Mw tool in Expasy. The signal peptide was analyzed using neural networks (NN) model in SignalP 4.0.

Construction of expression vector and protein expression

The *PEcbh* was amplified by using *Bam*HI-PEcbhF primer (5'-TTGGATCCTCCACACTAGCAGCAATG-3') and *Hind*III-PEcbhR primer (5'-TTAAGCTTCCG CTATTATTTCCCCAC-3'). The PCR product was cloned to TOPO vector for obtained pTOPO/*PEcbh*. This plasmid and pET28a (+) were double digested by *Bam*HI and *Hind*III at 37^{0}C for 3 h. The digested products were ligated by T4 ligase at 22^{0}C for 1 h. The recombinant vector was transformed to *E. coli* BL21 (DE3) and selected positive colonies on LB agar which containing 50 µg/ml kanamycin.

For protein expression, a single colony bacteria was inoculated into 50 ml of LB broth which containing 50 µg/ml kanamycin, 1% glucose and incubated at 37^{0}C for overnight. 5% of overnight culture was inoculated to 50 ml of LB broth which containing 50 µg/ml kanamycin and 1% glucose. *PEcbh* was induced by add isopropyl-β-D-thiogalactopyranoside (IPTG) to medium. Cells were harvested by centrifugation at 8000 rpm for 10 min at 4^{0}C. Cell pellet was washed and resuspended by 0.05 M Sodium acetate buffer pH 5.0. The cell lysate was performed by sonication until obtained clear solution and centrifuge at 13000 rpm for 10 min at 4^{0}C. The soluble protein was used as crude enzyme for determination of CMCase activity and protein content.

In optimization for protein expression in *E. coli* BL21 (DE3), the culture conditions studied was temperature (18, 25 and 37^{0}C), the concentration of inducer, IPTG (0.2, 0.5 and 1 mM), the induction time (1, 3, 4 and 5 h), and the post-induction time (2, 4, 6, 8 and 10 h). To study the effect of host strains, BL21 (DE3) and Rosetta (DE3), for protein expression, the culture condition was 0.2 mM IPTG, 1 h induction time at 18^{0}C and 4 h post-induction time.

CMCase Assay

For CMCase activity, carboxymethylcellulose (CMC) was used for substrate and determined the amount of reducing sugar by using Nelson-Somogyi method (Somogyi, 1952). The reaction mixture consisted of 0.25 ml of 1% of CMC in 0.05 M Sodium acetate buffer pH 5.0 and 0.25 ml of crude enzyme and incubated at 45^{0}C for 120 min. The reaction was added with 0.25 ml of alkaline copper tartrate reagent and boiling for 10 min. Then, 0.25 ml of arsenomolybdate reagent and 1.5 ml of water was added and mixed. The reactions were placed at room temperature for 10 min and measuring at 620 nm. The protein content was determined by Bradford method (Bradford, 1976). One unit of enzyme activity was defined as the amount of enzyme that produced nmole of glucose in 1 min per ml per mg of total protein at optimal condition.

Results

Isolation of cellulase gene from *P. eryngii*

To isolate a cellobiohydrolase gene, *P. eryngii* mycelium was cultured in liquid media containing 0.2% of CMC or glucose as the sole carbon source. From the both cultures, RNA was extracted from the mycelium and used as a template for RT-PCR reaction. Two degenerate primers were used to amplify a cellulase gene from *P. eryngii*. These primers were designed from conserved region of known cellulase sequences (Jia et al., 1999). The result showed that *PEcbh* was expressed only in the mycelium grown in medium containing cellulose but not in medium containing glucose (Figure 1). The PCR product was sequenced and aligned to compare with other cellulase genes in database. The results showed that the 642 bp nucleotide sequence obtained was a part of cellobiohydrolase gene (corresponding to position 571-1214 in Figure 2) and similar to cellulase genes form other fungi and mushrooms. Subsequently, the 3' and 5' RACE technique was used to find the unknown sequence in 3' and 5' region of *PEcbh*. The 1377 bp nucleotide sequence was cloned (accession number LC034188). Analysis of *PEcbh* revealed that PEcbh was composed of 459-deduced amino acid, an estimated molecular weight of 49 kDa with an isoelectric point (p*I*) of 5.0. Conserved Domain Architecture Retrieval Tool (CDART) detected glycosyl hydrolase family 7 domain (Figure 2) but it lacked of cellulose binding domain. In addition, signal sequence was found (corresponding to amino acid position 1-16 in Figure 2), this indicated that PEcbh was extracellular enzyme.

The deduced amino acid sequence of PEcbh was compared with amino acid sequences from database and showed similarity to cellulases from other mushroom species such as *Pleurotus* sp. 'Florida' (*cbhI-I*, CAK18913) by 98%, *Polyporus arcularius* (*cel1*, BAF80326) by 75%, *Volvariella volvacea* (*cbhI-I*, AAT64006) by 70%, *Flammulina velutipes* (*cel7A*, BAJ07534) by 69%, *Neolentinus lepideus* (*cbhI*, ADN88316) by 68%, and *Volvariella volvacea* (*cbhI-II*, AAT64007) by 66% (Figure 3). Hence, the results indicated that the obtained gene was a cellobiohydrolase.

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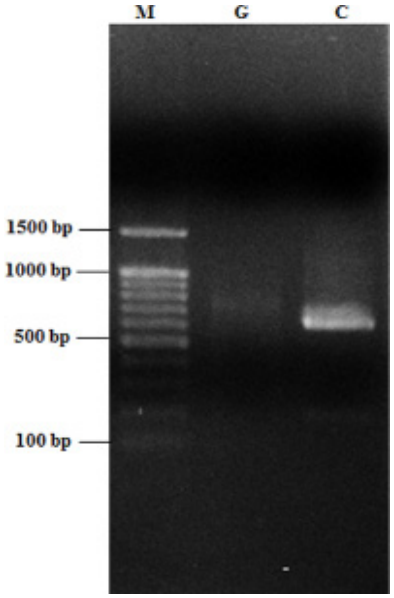

Figure 1 RT-PCR of cellulase gene fragment. RNA was extracted from *P. eryngii* mycelium which cultured in liquid media containing either glucose (G) or cellulose (C) as a sole carbon source. M is a DNA molecular weight marker.

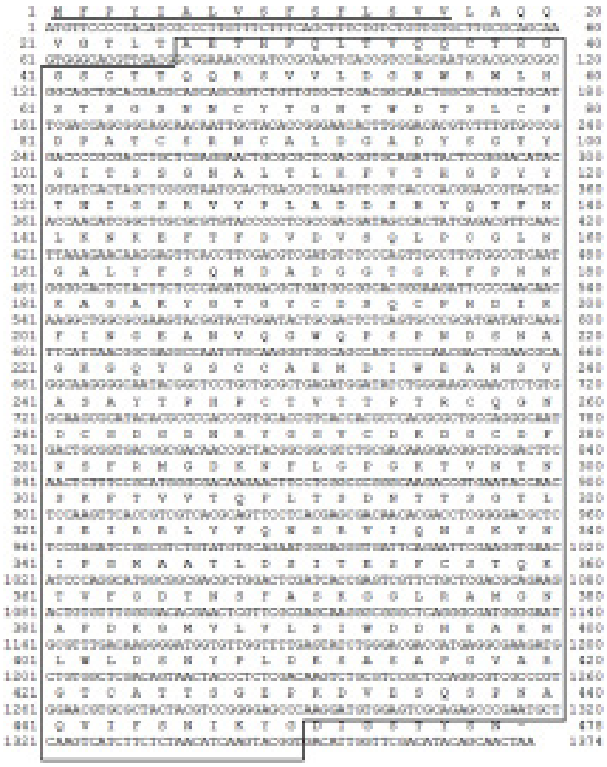

Figure 2 Nucleotide and deduced amino acid sequence of *PEcbh* from *P. eryngii*. The DNA sequence was showed in bottom row. The amino acid sequence was showed in top of nucleotide sequence. The boxed represent the conserved glycosyl hydrolase family 7 domain. The putative signal sequence was underlined.

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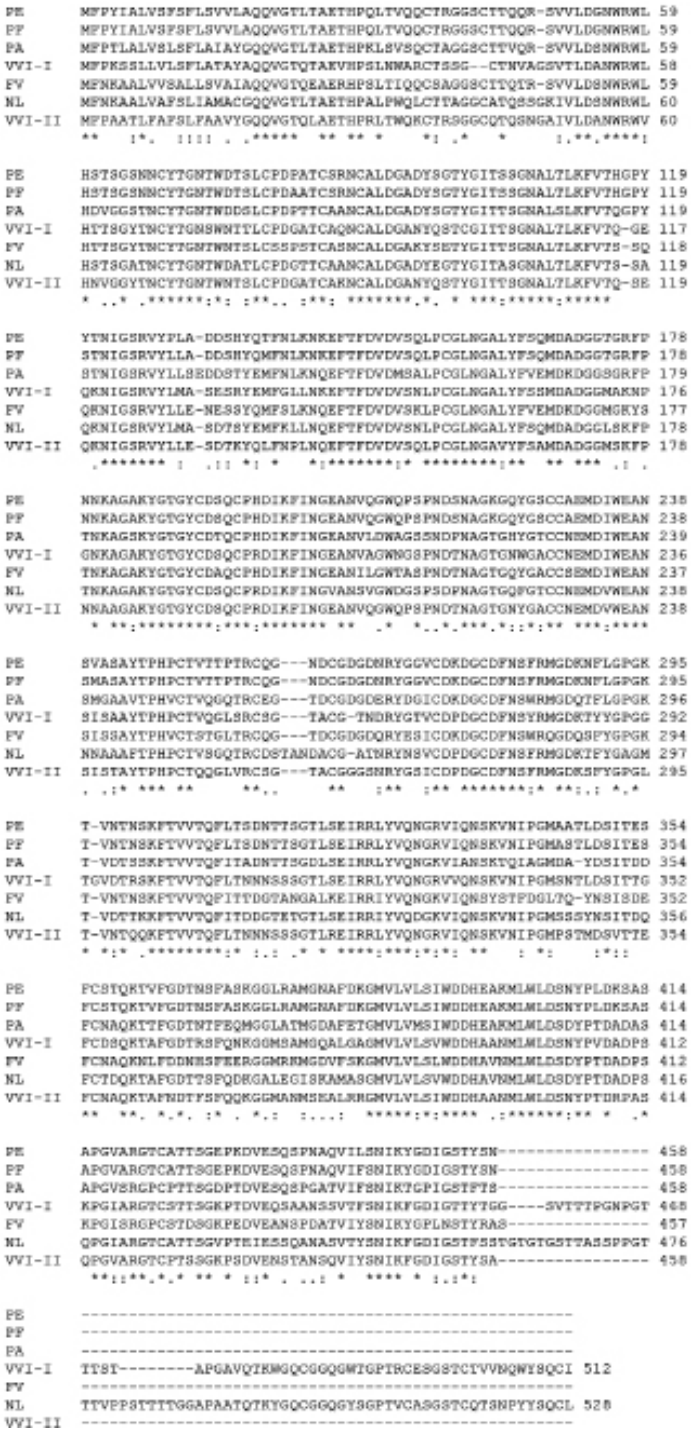

Figure 3 Amino acid sequence alignment of the PEcbh from *P. eryngii* with cellulase from other fungal performed using ClustalW. Abbreviations: PF, *Pleurotus* sp. 'Florida'; PA, *Polyporus arcularius*; VV, *Volvariella volvacea*; FV, *Flam mulina velutipes*; NL, *Neolentinus lepideus*; PE, *P. eryngii. Asterisks* (*) denote the conserved amino acids in all cellulase proteins. Single (.) and double (:) dots indicate positions with conserved and semiconserved substitutions, respectively.

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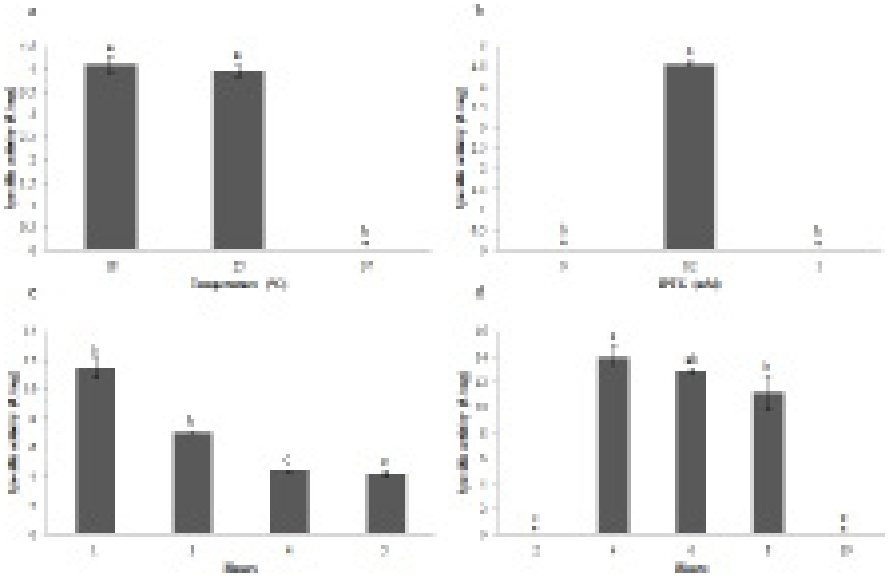

Figure 4 Effect of induction conditions on CMCase activity from *E. coli* BL21-pET/*PEcbh*. The effect of (a) The temperature for induction, (b) The concentration of IPTG, (c) The induction time, (d) The post-induction time. The asterisk (*) was defined as no reaction or low reaction which $OD_{620} < 0.1$. The results showed the average of three experiments. Error bars represent standard deviation. Bars with different alphabets indicated values that are significantly different at $p < 0.05$.

Construction of expression vector and protein expression

To construct the recombinant plasmid, the *PEcbh* was inserted into pET28a (+). The recombinant pET/*PEcbh* was transformed to *E. coli* strains BL21 (DE3) and Rosetta (DE3). The positive transformants (BL21-pET/*PEcbh* and Rosetta-pET/*PEcbh*) were checked by amplifying *PEcbh* and cut with *Bam*HI/*Hind*III (data not shown).

The optimization of expression conditions

In the expression of *E.coli* system, there are several factors which involving in the expression of protein. Thus, the optimal condition for expression of *PEcbh* in *E.coli* BL21 (DE3) was studied. Growth of BL21 (DE3) was determined in LB medium containing 1% glucose at 37^0C, 180 rpm. The various growth stages were determined; lag phage (OD_{600} = 0.2) was at 1 h, exponential phase (OD_{600} = 0.6) was at 3 h, late-exponential phase (OD_{600} = 0.8) was at 4 h and stationary phase (OD_{600} = 1.0) was at 5 h. The effect of temperature for induction was evaluated by adding 0.2 mM IPTG at 4 h of growth and then incubated at various temperatures (18, 25 and 37^0C) for 8 h. The activity was detected when *E. coli* was cultured at 18 and 25^0C but not at 37^0C. However, the highest activity was observed when induced at 18^0C (Figure 4a). Then, the effect of IPTG concentration was determined by adding IPTG (0, 0.2, and 1 mM) at 4 h of growth and induced plasmid at 18^0C for 8 h. CMCase activity was detected only in 0.2 mM IPTG condition (Figure 4b). To study the effect of induction time, the activity was induced by adding 0.2 mM IPTG at various times of growth (1, 3, 4, 5 h) and incubated at 18^0C for 8 h. The highest activity was obtained when induced at 1 h. However, the activity was decreased when extending time (Figure 4c). To determine the post-induction time, the cultured was grown for 1 h and 0.2 mM IPTG was added afterward. The cultures were incubated at 18^0C and collected at various times (2, 4, 6, 8, 10 h). The highest activity was detected after induction for 4 h and decreased when induced for 6 and 8 h (Figure 4d). In conclusion, the optimal conditions of *PEcbh* in BL21 (DE3) were found to be as follows: the temperature for induction was 18^0C; IPTG concentration was 0.2 mM; the induction time was during lag phase (1 h); the post-induction time was 4 h, respectively. The specific activity of CMCase was 13.961 U/mg of total protein (Figure 4d). The negative controls (BL21 (DE3) either with or without pET28a) was performed, but no activity could be detected. The recombinant protein (PEcbh) expression was also analyzed by SDS-PAGE. However, the result showed no differnt pattern of protein band in *E.coli* BL21 (DE3), *E.coli* BL21-pET/*PEcbh* and *E.coli*

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BL21-pET/*PEcbh* which induced with IPTG (data not shown). It is probably due to the low expression of PEcbh which not sufficient to detected on SDS-PAGE.

The effect of *E. coli* host strains to protein expression

To study the effect of *E.coli* host strains to protein expression, PEcbh was expressed under T7 promoter by using pET28a (+). *E. coli* strains BL21 (DE3) and Rosetta (DE3) were used as host. The protein expression was performed in optimal conditions. The results showed that CMCase activity in BL21 (DE3) was 9.466 U/mg of total protein which lower than Rosetta (DE3) was 15.291 U/mg of total protein. The results suggested that strain of *E. coli* was influence to the expression of protein in *E. coli* system at the same condition.

Discussion and conclusion

Cellulases are important enzyme for degradation of cellulose substrates by mushrooms. The cellulase genes form *Pleurotus* spp. were cloned but not from *P. eryngii.* This is the first report which cloned cellobiohydroase gene from *P. eryngii*. The *PEcbh* cDNA contained 1377 bp nucleotide sequences. The predicted open reading frame encoded for 459 amino acids. The predicted molecular weight and p*I* of the protein was 49 kDa and 5.0, respectively. In addition, there was signal peptide which involved with the secretion of protein. The result showed that *PEcbh* was similar to cellulase gene from other fungi and mushrooms. This gene contains catalytic domain which was grouped in glycosly hydrolase family 7. However, it lacked cellulose binding domain. For RT-PCR analysis, *PEcbh* was induced by cellulose but inhibited by glucose, probably due to carbon catabolic repression (Mathew et al., 2008). The expression pattern was agreed with previous studies such as *cbhI* and *cbhII* from *Volvariella volvacea* (Jia et al., 1999); *cel7A* and *cel6B* from *Lentinula edodes* (Lee et al., 2001; and *cbhI* from *Penicillium chrysogenum* (Hou et al., 2007).

In previous studies, *E. coli* system was used to express of recombinant protein. The cellulase genes from other fungi were successfully expressed in *E.coli* such as Exo2b from *Trichoderma reesei* (Geng et al., 2012), *celA celB* and *celC* from *Neocallimastix patriciarum* (Xue et al., 1992), EGI from *Trichoderma viride* (Kwon et al., 1999), CEL6B and CEL7A from *Lentinula edodes* (Taipakova et al., 2011). There are several factors which effect to the expression of protein such as temperature for induction, concentration of IPTG, induction time and the post-induction time. In this study, the optimal conditions of protein expression were determined. The *PEcbh* was expressed in *E. coli* BL21 (DE3) by using pET28a (+) under T7 promoter. The highest CMCase activity was obtained when 0.2 mM IPTG was added at lag phase of growth and induction at 18^0C for 4 h.

The specific activity of CMCase was 13.961 U/mg, suggesting that *PEcbh* can function although it contained only catalytic domain and lacked cellulose binding domain. Generally, the cellulase gene consists of two domains i.e. catalytic domain and binding domain. However, some cellulase gene bears merely catalytic domain which called core enzyme such as *Cel1* form *Claviceps purpurea* (Muller et al., 1997) and *cbhI-II* from *Vovariella volvacea* (Ding et al., 2006). These genes can function to hydrolysed substrate but the activity was lower than complete enzyme (Irwin et al., 1998). The cellulose binding domain improved facility in degradation of cellulose (Lui and Hu, 2012). Thus, the gene which only containing catalytic domain has binding capacity and activity lower than complete enzyme (Stahlberg et al., 1991). According to the adsorption isotherms of CBHI core from *Trichoderma reesei* was reduced when compared with intact enzyme (Nidetzky et al., 1994).

For routine protein expression, BL21 (DE3) is most frequently used. However, there are different of codon usage in prokaryote and eukaryote which is a translation problem in protein expression. The derivative of BL21 (DE3), Rosetta (DE3) is designed to enhance the protein expression of eukaryotic which contains tRNA for rare codon on compatible plasmid under control of their native promoter (Terpe, 2006; Tegel et al., 2011). Therefore the expressions of *PEcbh* from *P. eryngii* in *E. coli* strain BL21 (DE3) and Rosetta (DE3) were compared. The result showed that CMCase activity in Rosetta (DE3) was higher than BL21 (DE3) about 2 times.

In this work, CMCase activity of PEcbh was detected but at a low activity. This is probably due to the absent of post transcriptional modification (i.e. glycosylation), secretion, cellular compartmentalization and eukaryotic chaperones in *E. coli* system. The protein expression in eukaryotic system may be dissolved this problem. In further study, we will shed the light on the expression of *PEcbh* in yeast system.

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