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CLONING AND EXPRESSION OF PUTATIVE BETA-XYLOSIDASE B FROM *PHANEROCHAETE CHRYSOSPORIUM*

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SUMMARY

White-rot fungus *P. chrysosporium* has been reported to produce complex hemicelluloses degradation enzymes including β-xylosidases. Genes encode for these enzymes have been predicted, namely β-xylosidase A and β-xylosidase B, however, the functional of these genes have not been characterized. This study aimed to clone a putative cDNA encoding for β-xylosidase B (*PcXylB*) from *P. chrysosporium* BKM-F-1767. Sequence analysis indicated the gene consisted of 981 nucleotides, coding for 326 amino acids. N-terminal containing 18 amino acids was identified as secretion signal peptide. The predicted secondary structure of *PcXylB* was composed of 27 beta strands, while three catalytic size residues Asp37, Asp152, and Glu221 were found to be general base, pKa module, and general acid, respectively. *PcXylB* was constructed with expression vectors pPICZA and pPICZαA and integrated into *P. pastoris* genome using electroporation method. The rPcXylB secretion of recombinant pPICZA-*PcXylB P. pastoris* was driven by intrinsic secretion signal while pPICZαA-*PcXylBα P. pastoris* was promoted of α-secretion signal from *Saccharomyces cerevisiae*. The rPcXylB secrection was carried out by adding methanol to final concentration of 1 % for every 12 h. The recombinant pPICZA-*PcXylB* constructed *P. pastoris* did not secret rPcXylB. Western dot-blot analysis showed the difference on recombinant protein released among of twenty pPICZαA-*PcXylBα* transformants. Free-cell medium culture of recombinant *P. pastoris* on the fourth day cultivation was harvested to purify enzyme using anti his-tag column. Purified rPcXylB exhibited a single band of approximately 33 kDa on SDS-PAGE. The present study was first report on the cloning and expression of putative *P. chrysosporium* β-xylosidase B.

Keyword: *xylosidase, P. chrysosporium, P. pastoris, expression, rPcXylB.*

INTRODUCTION

Hemicellulose, the second abundant plant natural component after cellulose, generally accounts for 15-35 percent of plant dry weight. Hemicellulose is composed of xylans, manans, xyloglucans, glucomannans, and β-(1→3, 1→4)-glucans. Among them, xylans are major hemicelluloses which constitute up to 50 percent dry weight in some tissues of grasses and cereals (Gírio *et al.*, 2010; Scheller, Ulvskov, 2010). Xylans are mainly consisted of $_{D}$-xylopyranosyl units linked by β-1,4-glycosidic bonds with various side chains such as 4-*O*-methyl-$_{D}$-glucuronic acid and acetic acid in hardwood, $_{L}$-arabinofuranose residues in non-acetylated softwood, or *p*-cumaric acid and ferulic acid in grasses. The degradation of xylans are usually required a synergistic action of different enzyme classes including endo-xylanase which randomly hydrolyzes the internal β-1,4-glycosidic backbone, yielding short xylooligomers, β-xylosidase acts as exo-type to cleave xylooligomers into single xylose units, while α-L-arabinofuranosidase and esterase remove the side groups (Shallom, Shoham, 2003).

β-xylosidase is classified into seven groups of glycoside hydrolases family, named 3, 30, 39, 43, 52, 54, and 116. β-xylosidase is widely produced by hemicelluloses utilization microorganisms such as fungi, bacteria, and yeast (Sunna A, Antranikian G, 1997). β-xylosidase has been reported to enhanced xylanase activity in the synergistic action because β-xylosidase prevents xylanase, cellulase inhibition by hydrolysis end product (Poutanen, Puls, 1988; Qing, Wyman, 2011). This enzyme is cell-associated in most bacteria and yeast, but it is freely found in the culture media of some fungi (Guerfali *et al.*, 2008; Sunna A, Antranikian G, 1997).

The white-rot fungus *Phanerochaete chrysosporium* is known to have capacity to completely hydrolyze biomass by producing an enzyme complex including lignin peroxidases, cellulases and hemicellulases. The genome sequence

and computational analysis indicated its putative hemicellulases system involves in endo-xylanase, β-xylosidase, endo-arabinanase, α-1,5-L-arabinofuranosidase, β-mananase, β-manosidase and acetyl xylan esterase (Martinez *et al.*, 2004; Wymelenberg *et al.*, 2006), which are found on the secreted protein matrix in the culture medium containing lignocellulosic biomass (Adav *et al.*, 2012).

MATERIAL AND METHODS

Strains, plasmids and medium cultures

The white rot fungus *P. chrysosporium* BKM-F-1767 was obtained from the Korean Collection for Type Culture and maintained in medium as described by Tien (Tien, Kirk, 1988). *Escherichia coli* Top10 was used for general cloning. Recombinant vectors were transferred into *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA). Gene expression was carried out by recombinant *P. pastoris* cultured in YP medium (1% yeast extract, and 2% peptone) containing 1 % methanol.

Polymerase chain reaction (PCR) product was ligated with pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced. The *P. pastoris* expression vectors pPICZC and pPICZαC (Invitrogen, Carlsbad, CA, USA) were constructed with *PcXylB*. The standard gene and protein manipulation were carried out as described by Sambrook and Russell (Sambrook, Russell, 2003).

Total cDNA synthesis

Total *P. chrysosporium* mRNA was isolated using the Oligotex mRNA Mini kit (Qiagen, Valencia, CA, USA). cDNA library was synthesized using the SMARTer PCR cDNA Synthesis kit (Clontech, Mountain View, CA, USA).

Construction and transformation of the recombinant plasmid

The nucleotide sequence of the putative *PcXylB* was obtained from the *P. chrysosporium* genome database of the DOE Joint Genome Institute website at http://genome.jgi-psf.org/Phchr1/Phchr1.home.html as predicted by Wymelenberg (Wymelenberg *et al.*, 2006). The cDNA of *PcXylB* including the signal peptide was amplified by PCR using the forward PcXylB-F 5′-CTCGAGATGTTCCCATCGGTGA TCAC-3′ and reverse PcXylB-R 5′-TACGTACGTGAAGTGCACAGTCTTGC-3′ primers. The PCR products as respected size was excised and purified from 0.8% agarose gel, then ligated to pGEM-T Easy Vector. Three recombinant plasmids were randomly isolated and sequenced. The *PcXylB* gene was excised using *Xho*I and *Sna*BI, then inserted into the pPICZA vector, resulting in pPICZA-*PcXylB*.

To cloning the gene into pPICZαA, the forward primer PcXylBαF 5′-GAATTCGCGGCGATACAGAAGCGC-3′ and the reverse primer PcXylBαR 5′-TCTAGAGTGAAGTGCACAGTCTTGCC-3′ were used for the gene amplification. Then, PCR product, *PcXylBα*, was cloned into the pGEM-T Easy Vector, excised with *Eco*RI and *Xba*I. The gene restricted by *Eco*RI-*Xba*I was purified from agarose gel, and inserted into pPICZαA to generate pPICZαA-*PcXylABα*. The presence of the inserts in recombinant vectors was confirmed both by PCR and restriction enzyme digestion. A sequence analysis was performed on pPICZA-*PcXylB* and pPICZαA-*PcXylBα* using AOX1 primer as manufacture's recommendation.

The pPICZA-*PcXylB* and pPICZαA-*PcXylBα* were linearized by *Pme*I and transferred into *P. pastoris* GS115. The transfomants were screened on YPD agar plates containing 100 μg ml^{-1} zeocin and 1 M sorbitol at 30°C. The presence of *PcXylB* in recombinant *P. pastoris* genomes was confirmed by PCR using AOX1 primers.

Expression and production of recombinant PcXylB (rPcXylB)

The rPcXylB production of twenty positive *P. pastoris* transformants were screened by dot blot analysis using his-tag antibody as described by Vasu *et al* (Vasu *et al.*, 2012). The *P. pastoris* transformant secreting the highest recombinant protein was seeded in 5 ml YPD medium at 30°C and 200 rpm for 24 hrs, then, transferred to 50 ml of fresh YP medium containing 1% glycerol. The cultivation was continued at 30°C and 180 rpm for overnight. The cell pellets were harvested by centrifugation at 2000 rpm for 5 min, and resuspended in 100 ml YP medium supplemented of 1 % methanol for induction at 25°C, 180 rpm.

Enzyme purification

To purify, cell-free supernatants on the fourth day of culture were collected by centrifugation at 2,000 rpm for 5 min and filtered through 0.45 μm

filters. The supernatant was mixed with 10× binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole; pH 7.4) at ratio 9:1. Ni^{2+} charged his-tag column (Histrap-GE Healthcare, Picataway, NJ, USA) was used to separate rPcXylB using the ÄKTA fast protein liquid chromatography purification system. rPcXylB was eluted with an elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole; pH 7.4). The purified rPcXylB was dialyzed with cellulose dialysis tubing membrane (Sigma, St. Louis, MO, USA) in distilled water at 4°C overnight. Protein concentration was measured by the Bradford method, using a Thermo Scientific Protein Assay kit (Rockford, IL, USA) with serum albumin as the standard. Molecular mass of purified rPcXylB was determined by SDS-PAGE as standard procedure.

RESULTS AND DISCUSSION

The sequence of putative β-xylosidase was searched on *P. chrysosporium* RP78 genome sequence database. We found two predicted sequences related with β-xylosidase, hence named *PcXylA* and *PcXylB*. These putative enzymes were classified to glycoside hydrolase family 43, which *PcXylA, and PcXylB* contain 598 and 326 amino acids, respectively. *PcXylA* is composed of 10 exons and 9 introns, whereas *PcXylB* contains 5 exons and 4 introns. There was no similarity between these two putative enzymes. Base on the predicted cDNA *PcXylB* sequence, we designed the primers to amplify this gene from cDNA library. Figure 1 shows a stronger DNA band of approximately of 950 base pairs from PCR using PcXylB-F and PcXylB-R primers on agarose gel, which corresponded to the expected size. Sequence analysis indicated the putative *PcXylB* consists of 981 nucleotides, coding for 326 amino acids. The *PcXylB* perfectly matched with *P. chrysosporium* RP78 β-xylosidase, where both nucleotide and amino acid sequences shared 99% identity. The full-length cDNA nucleotide sequence of *PcXylB* was deposited in GenBank (accession no. JX625153).

The function of *PcXylB* was predicted by homology modeling with crystal structure protein database using Phyre server (Kelley, Sternberg, 2009). *PcxylB* highly matched with β-xylosidase of *Bacillus subtilis* (PDB 1YIF), *Geobacillus stearothermophilus* (PDB 2EXI), *B. halodurans* (PDB 1YRZ), and *Clostridium acetobutylicum* (PDB 1YI7) structures, suggesting *PcXylB* might be a β-xylosidase. A secretion signal peptide of 18 amino acids at N-terminus was found using SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). While there is no predicted glycosylation site or fungus carbohydrate binding module was identified on *PcXylB*. The predicted secondary structure of *PcXylB* was composed of 27 beta strands (Fig. 2). Three catalytic size residues Asp37, Asp152, and Glu221 were found to be general base, pKa module, and general acid, respectively which similar to member of glycoside hydrolase family 43 (Pons *et al.*, 2004).

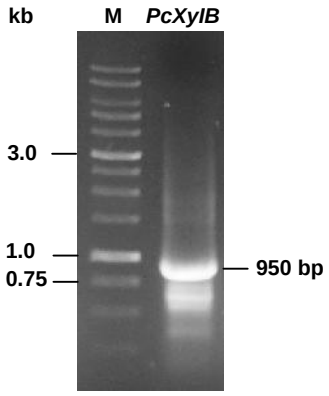

Figure 1. Agarose gel electrophoresis of amplified *PcXylB* gen from cDNA using PcXylB-F and PcXylB-R primers. M: 1 kb DNA ladder marker; *PcXylB*: *PcXylB* gene obtained from cDNA. The strongest observed band which corresponds to *PcXylB* gene was calculated to be approximately 950 bp.

The constructions of pPICZA-*PcXylB* and pPICZαA-*PcXylBα* were transferred into *P. pastoris* GS115 using electroporation method. The transformants were screened on YPDS supplementation with Zeocin. Ten colonies from each plasmid were randomly selected to check the presence of *PcXylB* gene. As the results, the PCR products of transformants were observed of two bands, which correspond to AOX1 gene of *P. pastoris* and pPICZαA-*PcXylBα* genes (Fig. 3) indicated pPICZαA-*PcXylBα* was successfully homologous integrated into *P. pastoris* genome. Similarly, we also obtained the transfomants of pPICZA-*PcXylB* in *P. pastoris* genome.

In view of expression of recombinant *P. pastoris*, twenty positive colonies of each transfomant were selected. Colonies were cultured for 24 h in YPD medium for biomass production, then the pellets were harvested and replaced by YP medium, induction by supplementation with 1 % methanol for 2 days.

Western dot-blot analysis was carried out for expression determination using 5 µl free-cell supernatants. The recombinant xylanase C was used as positive control (Huy *et al.*, 2011), while negative control was *P. pastoris* GS115. The results indicated nineteen recombinant pPICZαA-*PcXylBα* colonies were efficiently secreting recombinant putative β-xylosidase. Among them, number fourth colony showed the highest hybridization significant (Fig. 4). However, the recombinant construction pPICZA-*PcXylB* did not displayed hybridization significant, resulting in an unsuccessful secretion by intrinsic signal peptide. There have been reported the expression of *P. chyrososporium* genes using intrinsic secretion signal (Gu *et al.*, 2003; Huy *et al.*, 2011; Wang *et al.*, 2004). The signal peptide is responsible for the translocation of the pro-protein into the endoplasmic reticulum and is subsequently cleaved by signal peptidase during the translocation process, and then the pro-protein is transported to the Golgi where the pro-region is cleaved by Kex2 protease to release the mature protein (Daly, Hearn, 2005). An incorrect process at this step may result in a cell that does not release rPcxylB. Thus, the intrinsic *PcXylB* signal peptide does not have an efficient protein translocation processing in *P. pastoris*.

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Sequence    MFPSVITTSVLFLPLVAGAAIQKRGITGPVITSNFPDPSFVKGLDGTWYAFSTNSGGKHV60
Structure   ........................ββ...ββ......βββββ...βββββββββ.....β

Sequence    PVANSPDFITWTLTGADALPTVGAWSTGGDVWAPDVIQRPDGKFVMYYSADQNGNGGKHC  120
Structure   βββββ......βββ.βββ............βββββββββ...βββββββ.........ββ

Sequence    IGAAVSDTAHGPYTPEPTALSCNTAQGGAIDPAGFVDTDGTVWVVWKIDGNSIGHGGNCN  180
Structure   βββββββ.......ββ..ββββ.......ββ..ββββ.....βββββ.............

Sequence    NGVPPIVSTPIMLQQLAADGITPIGSPVQILDRSDADGPLVEAPSLVKVNGVYILFFSSN  240
Structure   .........βββββββ.....ββ....βββββ.......βββββββββββ..βββββββ..

Sequence    CYSGGLYDTSYATADNIKGPYTKAQAPNAPLLQTGTPYSQLYSPGGLDVGPGGVNVLFHA  300
Structure   ......βββββββββ.......ββ.......βββ.......ββ....βββ....ββββββββ

Sequence    DKGTTADVRQLYAGQIKVTGKTVHFT
    326
Structure   .........βββββββββββ..ββββ.
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Figure 2. Deduced amino acid sequence and predicted secondary structure of *PcXylB*. The arrow indicated the signal peptide cleaving site. The closed inverted triangles show the three conserved catalytic residues. The secondary structure containing 27 beta strands was generated flowing three dimension structure of *B. subtilis* (PDB 1YIF).

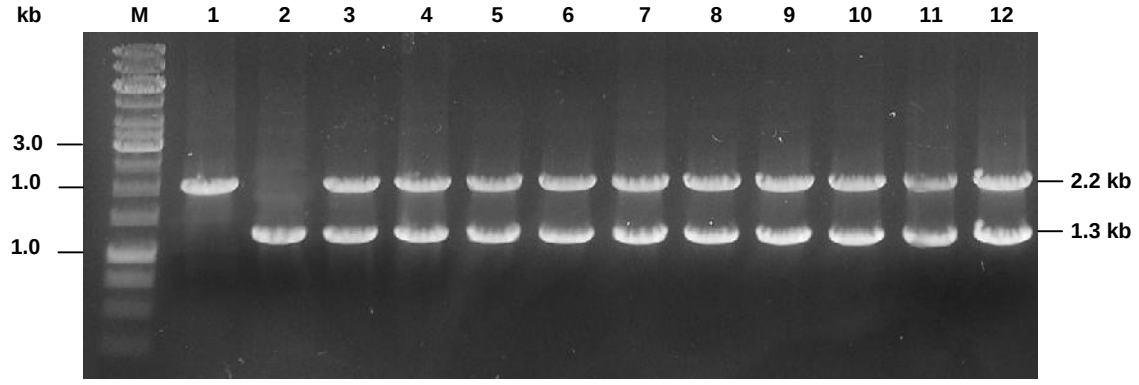

Figure 3. The colony PCR for confirmation of pPICZαA-*PcXylBα* construct. M: 1 kb DNA standard molecular marker; 1: *P. pastoris* genome; 2: pPICZαA-*PcXylBα* recombinant vector; 3-12: the recombinant *P. pastoris* with pPICZαA-*PcXylBα*.

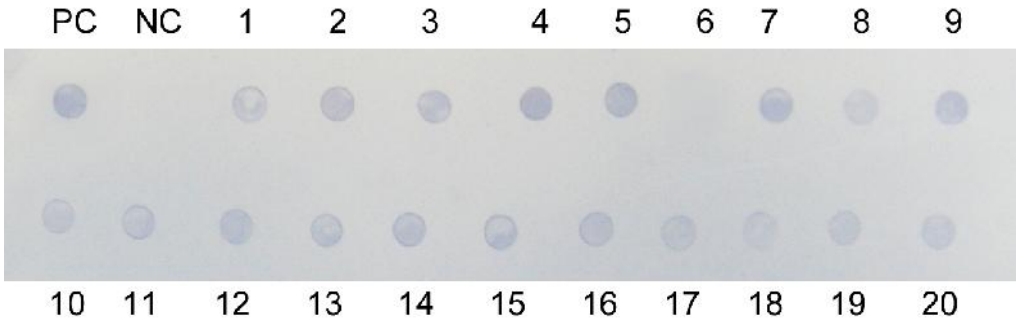

Figure 4. Western dot-blot analysis of *P. pastoris* expressed protein using his-tag antibody. PC: free-cell supernatant of recombinant xylanase C from recombinant *P. pastoris* (Huy *et al.*, 2011); NC: free-cell supernatant from *P. pastoris* culture; 1-20: twenty transformants of pPICZαA-*PcXylBα P. pastoris*.

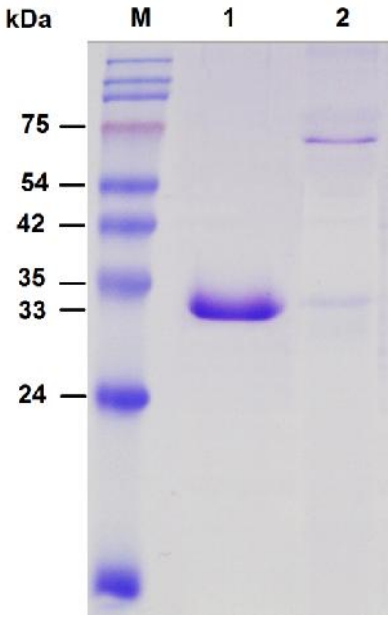

Figure 5. SDS-PAGE analysis of purified recombinant β-xylosidase (rPcXylB). M: molecular mass markers; 1: purified rPcXylB; 2: free-cell supernatant of recombinant rPcXylB from *P. pastoris* culture. The molecular weight of rPcXylB was estimated to be 33 kDa.

The rPcxylB was further purified by anti-his-tag column. The molecular mass of purified rPcxylB was determined by SDS-PAGE, showing a single band around 33 kDa (Fig. 5). rPcXylB molecular mass was very closed to theoretical molecular mass of the fusion mature PcXylB, c-*myc*- and his-*tag* tail of 34.7 kDa. The methylotrophic *P. pastoris* yeast is a useful system to express milligram-to-gram quantities of proteins for both basic laboratory research and industrial purposes. Moreover, the glycosylation system of *P. pastoris* is less efficient than that of other heterologous expression systems such as *S. cerevisiae*; therefore, it secretes a native recombinant protein or a less hyperglycosylated form (Daly, Hearn, 2005). Similarly, recombinant *P. pastoris* released rPcXylB as its native form which might have the same function as *P. chrysosporium* produced.

CONCLUSION

In the present study, we reported the cloning and expression of a putative β-xylosidase from *P. chrysosporium*, which play an important role in the biomass degradation processing. rPcXylB were efficiently produced by recombinant *P. pastoris* without glycosylation and further purified. Although, the biochemical properties of this enzyme are still unknown, however, the characterization may be easy carried out using purified rPcXylB on specific substrate such as *p*-nitrophenyl-β-D-xylopyranoside. Moreover, the characterization and application on biomass degradation in the synergistic action with other hemicellulases such as xylanaseC should be further topic of research.

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TẠO DÒNG VÀ BIỂU HIỆN BETA-XYLOSIDASE B TỪ *PHANEROCHAETE CHRYSOSPORIUM*

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TÓM TẮT

Nấm sợi trắng *P. chrysosporium* từ lâu đã được biết đến có khả năng tiết ra tổ hợp enzyme thủy phân hemicelluloses như β-xylosidase. Hai gene mã hóa cho enzyme này đã được dự báo với tên gọi β-xylosidase A và B. Tuy nhiên, chức năng của các gene này đến nay vẫn chưa được xác định. Mục đích của nghiên cứu này là tạo dòng đoạn cDNA giả định mã hóa cho enzyme β-xylosidase B (*PcXylB)* từ *P. chrysosporium* BKM-F-1767 đã được tạo dòng. Phân tích trình tự nucleotide cho thấy gene có 981 nucleotide, mã hóa cho chuỗi polypeptide với 326 amino acid. Chuỗi polypeptide gồm 18 amino acid được xác định đóng vài trò tín hiệu tiết ở đầu tận cùng N. Dự đoán cấu trúc bậc hai của protein được mã hóa bởi gene *PcXylB* cho thấy nó được cấu thành bởi 27 chuỗi beta, trong đó ba vị trí xúc tác Asp37, Asp152 và Glu221 đóng vài trò như base, pKa và acid module. Gene *PcXylB* đã được gắn vào vector biểu hiện pPICZA and pPICZαA và được dung hợp với genome của *P. pastoris* bằng phương pháp điện biến nạp. Sự tiết enzyme tái tổ hợp được cảm ứng bằng cách bổ sung methanol đến nồng độ cuối 1 % trong mỗi 24 h. Thể tái tổ hợp *P. pastoris* và pPICZA-*PcXylB* không tiết ra protein tái tổ hợp rPcXylB. Phân tích biểu hiện bằng kỹ thuật Western dot-blot cho thấy sự khác nhau về mức độ biểu hiện của hai mươi thể tái tổ hợp pPICZαA-*PcXylBα*. Dịch nuôi cấy đã loại bỏ tế bào nấm ở ngày thứ tư được sử dụng để tinh sạch enzyme tái tổ hợp sử dụng cột anti his-tag. Một band protein có khối lượng phân tử khoảng 33 kDa xuất hiện trên điện di đồ của rPcXylB tinh sạch. Đây là nghiên cứu đầu tiên về tạo dòng và biểu hiện gene giả định mã hóa cho β-xylosidase B từ *P. chrysosporium*.

Từ khóa*: xylosidase, P. chrysosporium, P. pastoris, biểu hiện, rPcXylB.*

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