Cloning of AA9 Polysaccharide Monooxygenase gene AN3860 into pEX2B for expression in *Aspergillus oryzae*

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Abstract

Polysaccharide monooxygenases (PMOs) catalyze oxidative degradation of recalcitrant carbohydrate chains in cellulose, starch, and chitin. In biofuel industry, the conversion of rich lignocellulose source to fermentable sugars by hydrolytic cellulases can be synergistically boosted by cellulose-active PMOs (AA9 PMOs) found in a vast number of fungi that grow on biomass. *Aspergillus nidulans*, a filamentous fungus, possess a dozen of PMO-encoding genes, but only the AN3860 is expressed at a high level when cultured with wheat straw as the sole carbon source. Bioinformatic analysis indicates that AN3860 belongs to type 3 AA9 PMO subfamily that is capable of hydroxylating both C1 and C4 of the glycosidic linkages. Therefore, AN3860 may be a potential enzyme to improve cellulose hydrolysis efficiency, which has not been characterized. In this study, we describe the AN3860 cloning into *Aspergillus oryzae* AUT1-PlD. To facilitate the purification of AN3860, we added a CBM20 tag to its C-terminal. The recombinant vector was designed and constructed successfully. Simultaneously, we have obtained the clone of *A. oryzae* carrying the target gene by the ATMT method. Further expression optimization and characterization of AN3860 by both activity assays and spectroscopic techniques are underway.

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Keywords
AA9, AN3860, *Aspergillus oryzae*, cloning, Polysaccharide Monooxygenase.

1 Introduction

Bioenergy can be considered as a promising alternative to fossil fuels which are gradually becoming exhausted in the next 40-50 years [1]. Changing the usage of fossil fuels into bioenergy can reduce the negative impacts such as greenhouse gas emissions, global warming, and industrial pollution from fossil fuels burning. Today's technology in the fuel ethanol industry mainly bases on sugar fermentation from starch, especially cornstarch [2]. However, the expansion of quantity ethanol product has been challenged by the concerns over food security. On the other hand, lignocellulosic biomass (LCB) consists of cellulose, hemicellulose, and lignin that can be used for ethanol production. LCB from food crop residue, or forest residue, and plant biomass is much more abundant than starch and could serve as the source for fermentable sugars that does not compete with food [3]. However, the hydrolysis process of LCB into soluble sugar still faces great barriers due to its complicated structure. The poor efficiency of LCB conversion threatens the commercial viability of biofuels [4]. For these reasons, recently many research projects have been devoted to discovering new enzymes, among which polysaccharide monooxygenases stand out thanks to their novel mechanism and ability to boost LCB conversion [5,6].

Polysaccharide monooxygenases (PMOs) are copper dependent redox enzymes that degrade polysacharides. PMO-encoding genes were found in a wide range of microorganism genomes such as fungi, bacterial and viruses for decades [7-9]. In 1974, Erikson discovered the cellulose hydrolytic enzyme in the presence of O_2, which plays a role in the redox reaction [10]. However, only until recent years this group of enzymes was described to have catalytic activity on chitin in study of Vaaje-Kolstad G *et al.*, 2010[5]. Subsequently, the PMOs have been investigated to clarify the structure, mechanism of action and synergistic activity for biomass hydrolysis applications on industrial scale [11-13].

PMOs are currently categorised into seven auxiliary activity (AA) families including AA9 – AA11 and AA13 – AA16 in the CAZy database (http://www.cazy.org/) based on similarities in sequence. Each group has a different origin and/or a substrate specificity. The AA9, AA11, AA13, and AA14 families are found primarily in fungi with cellulose, chitin, starch, and xylan, respectively. Members of AA10, which exhibit activity on chitin and cellulose, are found across several kingdoms, including viruses, bacteria, and archaea [14]. AA15 PMOs are found in viruses and invertebrates, some of which act on cellulose while others act on chitin [15]. Recently, the new fungal AA16 family was found to degrade cellulose [16]. Among PMO families, AA9 PMOs are important components in commercial cellulase cocktails (Cellic Ctec3 by Novozymes A/S) [17]. These enzymes share the same conserved structure including a Cu(I/II) ion at the active site coordinated by two Histidines in the flat protein surface. To have its catalytic activity, AA9 PMOs need four external electron donors to activate one O_2 molecule for carrying on oxidative cleavage of β-1,4-glucosidic linkage in cellulose chain. AA9 PMOs exhibit different regioselectivities. Some AA9 PMOs specifically hydroxylate C1 or C4 of the glycosidic linkages, which are classified as Type 1 and Type 2 AA9 PMOs, respectively. Some other AA9 PMOs classified as Type 3 AA9 PMOs oxidize both C1 and C4 positions.

The filamentous fungus *Aspergillus nidulans*, also known as a fungal saprotroph, has significant biomass degradation. Its genome possesses 9 PMO sequences, but only AN3860 was dominantly expressed when the fungus was grown in the presence of wheat straw as the sole carbon source [18]. Phylogenic analysis indicated that AN3860 belongs to type 3 AA9 PMO subfamily which is capable of hydroxylating C1 and C4 of the glycosidic linkages. In the Harris *et al.*, (2010) study, TaGH61A (a Type 3 AA9 PMO) showed the highest ability to enhance the hydrolytic activity of cellulase comparing to the TaGH61E (type 1) and cellulase alone treatment [19]. So, AN3860 may be a promised enzyme for improving catalysis degradation. Nonetheless, the AN3860 properties are currently not fully researched. Heterologous expression of AN3860 is needed for further characterization of this enzyme. The native PMO requires post-translation processing including cleavage to produce N-terminal histidine residues, methylation of this residues, and addition of O- or N- glycosidic linkages. Because the bacterial or *Pichia pastoris* expressive systems have not shown the methylation of N histidine residues, some AA9 PMO sequences from fungi were not ensured successful expression. To improve that, the filamentous fungi system, such as *Aspergillus oryzae*, *Neurospora crassa*, can theoretically perform all post-translational modifications [20].

In this study, we carried out the first step of AN3860 protein production by heterologous expression in *Aspergillus oryzae*. AN3860 sequence was fused with a CBM20 tag to its C-terminal to facilitate purification. The recombinant vector was transferred to *A. oryzae* through *Agrobacterium tumefaciens*-mediated transformation (ATMT) method [21].

2 Materials and methods

2.1 Strains and plasmid

Escherichia coli DH5α strain [F^- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1] (NEB) was used as the host for plasmid cloning. *Agrobacterium tumefaciens AGL1* [*C58, recA::bla,* pTiBo542ΔT-DNA, *Mop*+, Cb^R] was used to transfer DNAs into the expression strain *Aspergillus oryzae* AUT1-PlD (*niaD*$^-$ *sC*$^-$ *adeA*$^-$ Δ*argB::adeA*$^-$ Δ*ppA::argB* Δ*pepE::adeA aut1*$^-$ Δ*ligD* Δ*pyrG)*. The strain was obtained from Genomic Lab, Ha Noi University of Science. The vector pEX2B has the size of 10.83kb and contains an *amyB* promoter for protein expression through maltose inducing.

2.2 Vector construction

AN3860 DNA sequence (AN3860.2, Uniprot: A0A1U8QLQ8) was designed from *Aspergillus nidulans* added the restriction site sequences with *Pml*I at 3' end and *Sac*II at 5' end and synthesized in pBHA-AN plasmid. For gene amplification, THE/A plasmid containing AN3860 was transformed into *Escherichia coli* DH5α and selected on LB agar (peptonE 1%, yeast extract 0.5%, NaCl 0.5%) containing ampicillin (100 µg/ml). pBHA-AN plasmid was extracted and *Pml*I/*Sac*II restriction enzymes were used to release AN3860 from this plasmid. This sequence was then ligated into the binary vector pEX2B at *Pml*I/*Sac*II sites and transferred into *E. coli* DH5α. Then, the mixture was spread on LB agar containing 100 µg/ml kanamycin. The recombinants were tested for the presence of the targerted gene by colony PCR method using the specific primer pair F/R of pEX2B vector.

2.3 *Agrobacterium tumfaciens*-mediated transformation (ATMT)

The recombinant vector was introduced to *A. tumfaciens* AGL1 following the procedure of Talhinhas *et al*., (2008) with some modifications [22]. To prepare the competent cells, the bacterial strain was cultured in liquid LB medium until OD_{600} of 0.8 - 0.9 was reached. Then, the bacterial cells were harvested by centrifugation at 4,000 rpm, 4^0C for 10 min. The cell pellet was washed three times with 100 mM HEPES and once with 10% glycerol before being resuspended to 1ml of 10% glycerol. Competent cells (50 µl) were mixed with 200 ng vector and transferred to 1-mm cuvette. The mixture was put on

ice and performed electroporation at 1.8 kV, 25 µF (BTX Electro Cell Manipulator 600). Following electroporation, 1ml LB medium was added to the cuvette. The electroporated cells were allowed to regenerate through mixture shaking for 2 hrs at 28^{0}C and 200 rpm. The bacterial cells were then collected with configuration at 8000 rpm for 30s and subsequently spread on kanamycin (100 µg/ml) LB agar plates. To acquire bacterial colonies, the plates were incubated at 28^{0}C for 60 - 72 hrs. Colony PCR was used to screen for positive colonies using a specific primer pair.

2.4 TranSformation of *A. oryzae*

ATMT method was carried out for transformation of the auxotrophic AUT1 strain according to the optimized report by Nguyen *et al.*, (2016) [21]. Briefly, spores of *A. oryzae* were collected by suspension of sterile water on the surface of PDA plates containing cultivated fungi (supplement 0.1% uracine and 0.1% uridine). The obtained solution was filtered and washed twice with sterile distilled water before adjusting volume to gain 10^6 spores/ml. The positive AGL1 transformant was inoculated in liquid LB contain kanamycin (100 µg/ml) in shaken condition overnight at 28^{0}C. Subsequently, the bacterial culture (1ml) was transferred to 9 ml IM liquid containing 200 µM acetosyringone (AS) to gain an OD_{600} value of apProximately 0.25, then incubated at 28^{0}C in the dark for 6 - 8 hrs to reach an OD_{600} value from 0.6 to 0.8. Then, 100 µl of the induced AGL1 was mixed with 100µl fungal spore suspension, distributed over the filter paper on an IM agar plate containing 200 µM AS, uracin (0.05%) and uridine (0.05%).

After incubating for 60 hours at 22^{0}C in the dark, the filter paper was moved to a M+met plate containing cefotaxime (300 µg/ml) to eliminate the AGL1. Finally, the plate was kept at 30^{0}C for 3 - 5 days until the fungal transformants appear.

2.5. Analysis of the fungal recombinant putative

Single spore of fungal transformants was isolated and cultivated continuously FOR at least three generations for examining mitotic stability in the liquid M+met medium at 30^{0}C, 150 rpm. The collected mycelia were used for genomic DNA extraction following the Khumallambam *et al.*, study 2013 [23]. The integration of the target gene into *A. oryzae* genome was confirmed by PCR using the F/R specific vector primers.

3 Result

3.1 Sequence analysis and vector construction

The sequence analysis shows that *AN3860* does not carry *Pml*I and *Sac*II restriction sites present in the multiple cloning sites (MCS) of the vector pEX2B. Therefore, the *Pml*I and *Sac*II restriction sites were added to 5'- and 3'-ends of the *AN3860* sequence for cloning into pEX2B with two sticky ends (Fig. 1A).

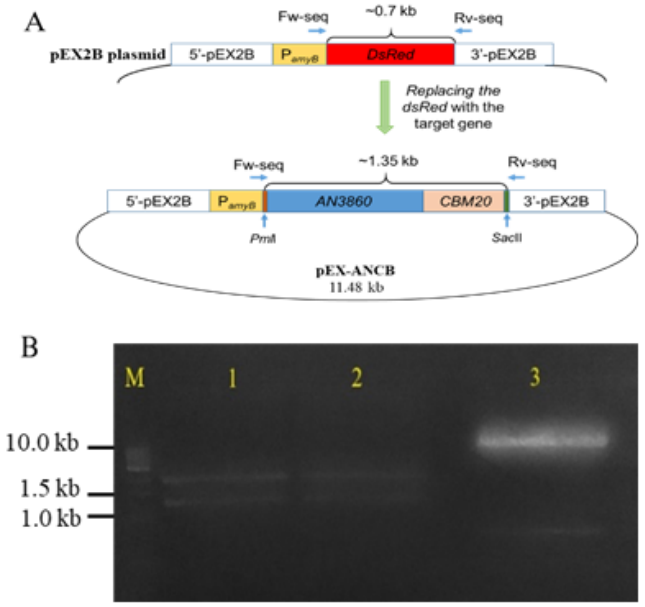

Fig. 1 AN3860-CBM20 vector construction
A. The structure of target sequence expression vector.
B. The results of restriction enzymes treated by *Pml*I and *Sac*II; 1, 2: pBHA-AN digestion; 3: pEX2B digestion; M: 1kb Ladder

The modified *AN3860* sequence and pEX2B plasmid was digested with *Pml*I and *Sac*II, which released the expected fragments of 1.3 kb and 10.0 kb, respectively (Fig. 1B). These indication fragments were purified for ligation. The ligation mixture was transferred and screened into *E. coli* DH5α on LB medium containing kanamycin (50 µg/ml). Only *E. coli* bacteria carrying kanamycin resistance gene can survive in the selected medium (data not shows).

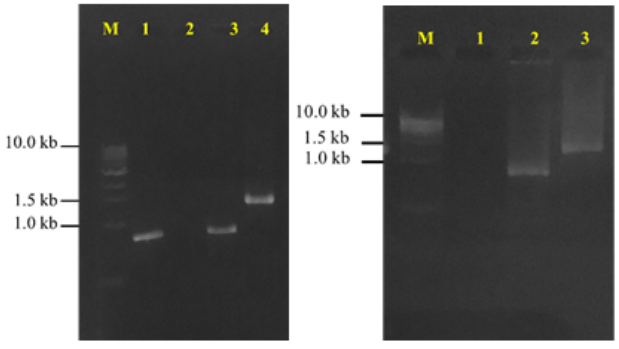

Fig. 2 Confirmation of the pEX-ANCB vector contruction
A. Colony PCR for screening the recombinant *E. coli* DH5α/pEX-ANCB. 1: Negative control, pEX2B plasmid; 2: Negative control, distilled water; 3,4: Tested clones
B. Results of the recombinant vector selection by PCR with plasmid template. 1: Negative control, distilled water; 2: Negative control, pEX2B plasmid; 3: The plasmid of the selective clone sample. M: 1kb Ladder

The recombinant was confirmed by colony PCR with the forward (Fw) and reverse (Rv) sequence specific primers (Fig. 2A). Agarose gel analysis of colony PCR exhibits a DNA band of 1.3 kb (lane 4) as expected.

The purified plasmid DNA derived from the positive colony (lane 4) was verified by PCR using the Fw/Rv-seq primer pair, which results in a band of 1.3kb on agarose gel (Fig. 2B, lane 3). Thus, the target DNA was inserted successfully into pEX2B and replaced *DsRed* reporter gene (0.7 kb). Finally, the sequencing of recombinant plasmid pEX-AN gave 100% identification with the initial design and the *AN3860* sequence was fused in-frame into pEX2B vector. Therefore, we can infer that the vector for expressing AN3860 in *A. oryzae* (pEX-ANCB) was constructed successfully.

3.2 *Agrobacterium tumfaciens*-mediated transformation method

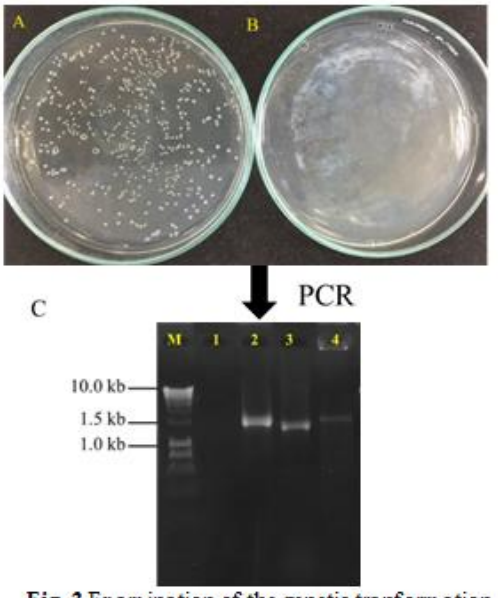

Fig. 3 Examination of the genetic tranformation into *A. tumefaciens*.

A. Result of *A. tumefaciens* AGL1 tranformation by electroporation on LB (50 µg/ml kanamycin)
B. Control plate without pEX-ANCB
C. Confirmation of the AGL1/pEX-ANCB transformants by PCR reaction.
M: 1 kb Ladder; 1: Negative control with distilled water; 2: Positive control with pEX-ANCB; 3,4: The selective clones.

The ATMT approach has recently been shown to be A USEFUL tool for the genetic transformation in fungal auxotrophy [22]. *A. tumefaciens* AGL1 is a soil phytopathogen that causes tumors in plant via transferring T-DNA region coding for the virulence (*vir*) genes into the host cells. To make the recombinant *A. tumefaciens*, we introduced the vector pEX-AN into competent cells by electroporation. The bacterial cells received the recombinant vector can grow on LB medium added with kanamycin (50 µg/ml) (Fig. 3A) while negative control did not have any colonies (Fig. 3B). The screening of recombinant AGL1 bacterium was performed by PCR using Fw-seq and Rv-seq primers. All transformants show a band of 1.3 kb similar to the band of positive control (Fig. 3C, lane 3,4).

The *A. tumefaciens* AGL1 strain carrying the expression vector (called AGL1/pEX-ANCB strain) was then prepared for the co-cultivation process.

3.3 Transformation of *A. oryzae* AUT1-PlD

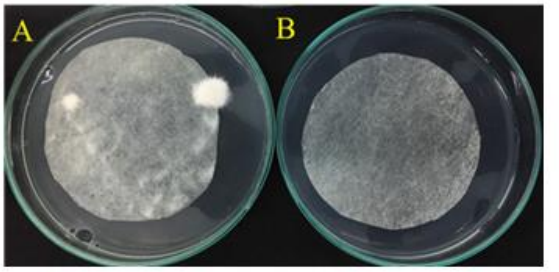

Fig. 4 Results of the co-cultivation process for the auxotrophic *A. oryzae AUT1 strain.*

A. The spores of AUT1 was cultived with AGL1/pEX-ANCB after 5 days incubated on M+met medium
B. Negative plate with distilled water

The genus *Aspergillus* has a long history in food applicationS such as secondary metabolite production. Nowadays, they are being employed in protein manufacturing with high levels of secretion. Hence, *Aspergillus* strains were modified genetically for easy selection [24]. Among them, *A. oryzae* AUT1-PlD was created the *pyrG* mutation leading to auxotrophic for uridine/uracine. This strain has enabled the ability to grow in the minimal medium when the *pyrG* cassette on pEX2B plasmid randomly integrated into the fungus genome by *A. tumefaciens*. The genetic transformation occurs in the co-cultivation process between AGL1/pEX-ANCB and *A. oryzae* AUT1-PlD spores. The result shows that we are collected several fungal colonies on the selected medium M+met (Fig. 4).

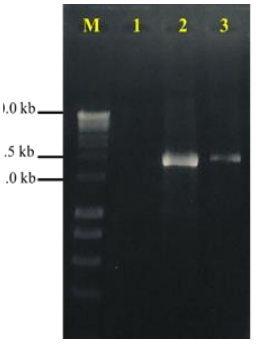

Fig. 5 Examination of the AN3860-CBM20 presence in the recombinant *A. oryzae* chromosome using PCR. 1: Negative control, distilled water; 2: Positive control, pEX-ANCB; 3: Genome template of selective clone. M: 1 kb Ladder.

These clones were continuously used to examine the presence of the T-DNA region in the fungal chromosome. The funga recombinant'S genome was extracted and used to as the template for the PCR reaction using the Fw- and Rv-seq primers (Fig. 5, lane 4). The amplified DNA appears as a band of 1.3 kb on AN agarose gel as expected. Thus, we have successfully generated *A. oryzae* carrying the recombinant vector for the heterologous expression of AN3860.

4 Conclusion

Identifying and characterizing of new potential PMOs is necessary to improve the performance of current hydrolytic enzymes. AN3860, a putative AA9 PMO active on cellulose substrates, is of great interest as it belongs to the Type 3 AA9 subgroup capable of oxidizing both C1 and C4 position of glycosidic bond in cellulose. In this study, we used a new strategy for AN3860 heterologous expression in the auxotrophic *A. oryzae* fungi. The results showed that the recombinant vector was designed and constructed successfully. Simultaneously, we have obtained the clone of *A. oryzae* carrying the target gene by the ATMT method.

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Conflict of Interest The authors declare that there is no conflict of interest.

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Tạo dòng gen mã hóa enzyme AA9 Polysaccharide Monooxygenase AN3860 trên vector pEX2B hướng tới biểu hiện trên *Aspergillus oryzae*

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Tóm tắt Polysaccharide monooxygenases (PMO) là enzyme xúc tác phản ứng oxi hóa thủy giải các chuỗi carbohydrate có cấu trúc khó phá vỡ như cellulose, tinh bột và chitin. Trong lĩnh vực năng lượng sinh học, sự chuyển đổi nguồn sinh khối lignocellulose dồi dào thành đường đơn cho quá trình lên men bằng enzyme li giải cellulase có thể tăng hiệu suất đáng kể khi bổ sung enzyme nhóm AA9 PMO. Những enzyme này được tìm thấy với một số lượng khá lớn trong các loại nấm sinh trưởng sử dụng nguồn sinh khối làm cơ chất. *Aspergillus nidulans* thuộc nhóm nấm sợi sở hữu một số gen mã hóa cho PMO, tuy nhiên chỉ có AN3860 được biểu hiện ở mức cao khi nuôi trong môi trường bổ sung sợi rơm lúa mì làm nguồn cacbon duy nhất. Phân tích tin học trình tự gen cho thấy AN3860 thuộc loại 3 AA9 PMO có thể oxi hóa linh động cả hai vị trí C1 và C4 của liên kết glycosidic. Do đó, AN3860 có tiềm năng cải thiện được hoạt tính li giải cellulose nhưng cho đến nay vẫn chưa được mô tả. Trong nghiên cứu này, trình bày cách tiếp cận AN3860 bằng phương pháp tạo dòng biểu hiện tái tổ hợp trong *Aspergillus oryzae* AUT1-PlD. Để thuận lợi cho quá trình tinh sạch AN3860, đuôi CBM20 được gắn vào đầu C của gen mục tiêu. Quá trình tối ưu biểu hiện và nghiên cứu thử nghiệm hoạt tính sẽ được thực hiện tiếp theo.

Từ khóa AA9, AN3860, *Aspergillus oryzae*, cloning, Polysaccharide Monooxygenase.