Cloning of an AA13 Polysaccharide Monooxygenase Gene from *Neurospora crassa* into the plasmid pEX2B

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Tóm tắt
Polysaccharide monooxygenases (PMOs) cleave glycoside linkages in polysaccharides, which has great potential in manufacturing biofuel from polysaccharide biomasses. Therefore, heterologous production of PMOs is required for the development of their application. We aim to clone PMO genes into the plasmid pEX2B to express PMOs in *Aspergillus oryzae*, a filamentous fungus widely used for industrial enzyme production. Here we cloned the *NCU08746* gene, an AA13 PMO from *Neurospora crassa*, into pEX2B. We successfully designed primers and amplified the *NCU08746* gene from the *N. crassa genome. NCU08746* was then cloned into the pEX2B vector. The recombinant plasmid pEX2B-NCU08746 was subsequently transferred into *A. tumefaciens AGL1* prior to being transferred into *A. oryzae*. This work provides the foundation for further study on PMOs expression in *A. oryzae.*

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Từ khóa
Polysaccharide monooxygenases, *NCU08746*, *Agrobacterium tumefaciens*, AA13, *Neurospora crassa*

1 Introduction

Biofuels are considered as a green and sustainable energy source that can replace fossil fuels in the current alarming situation of global warming caused by carbon dioxide emission [1]. Biofuels can be produced from various biomasses, including starch, one of the most abundant polysaccharides [1]. Regarding this, the classical biomass depolymerisation paradigm, in which exo-glycosidases operate in synergy with endo-glycoside hydrolases, was overturned by the discovery of a new group of enzymes only a few years ago. These new enzymes, called polysaccharide monooxygenases (PMOs), has been applied in the manufacturing of biofuel [2–5]. PMOs make use of an oxidative mechanism in cleaving glycoside linkages of polysaccharides, including cellulose [5,6], hemicellulose [6], chitine [7,8], xylan [5,9], and starch [6,10]. The starch-active PMOs, also known as AA13 PMOs, are among the seven families within the PMOs superfamily [4–14]. AA13 PMOs oxidatively degrade starch and can potentially be used with industrial amylases to convert starch into a fermentable carbohydrate [15]. The critical factor of PMOs's activity is that PMOs oxidizes the C–H links between the glycoside linkages in sugar units as well as polysaccharides [6,16,17]. The enhancement of starch degradation by AA13 PMOs on corn starch, amylose, and amylopectin was reported in 2010 in patent literature showing that the hydrolysis of these starches by canonical amyloglucosidases or a-amylases was observed to be boosted by "starch-active PMOs" derived from either *Neurospora crassa* or *Aspergillus nidulans* fungi [18].

Native PMOs undergo several co- and post-translational modifications in their native hosts, including the removal of the N-terminal signal peptide, methylation of the N-terminal histidine residue, as well as O - and N-glycosylation. Thus, heterologous PMOs expression has not been effective in the bacteria expression system [19]. Otherwise, PMOs can be expressed in filamentous fungi such as *Aspergillus oryzae*, allowing the mentioned post-translation modifications [12,19]. In this study, we cloned the AA13 PMO gene from *Neurospora crassa* (*NCU08746*) into a pEX2B vector and transformed *Agrobacterium tumefaciens* resulting in a recombinant vector, a necessary step for transferring the constructed expression into *Aspergillus oryzae* using a recently developed method [20].

2 Materials and Methods

2.1 Chemicals and microorganisms

Restriction Enzyme (RE) and necessary chemicals were purchased from New England Biolabs unless stated

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otherwise. Lysogeny broth (LB), Trisbase-Acid acetic-EDTA buffer (TAE buffer), Agarose M were purchased from Bio-Basic. Kanamycin sulfate ($C_{18}H_{36-37}N_{4-5}O_{10-11}.H_2SO_4$) from *Streptomyces kanamyceticus* was used as recommended by the manufacturer, Sigma-Aldrich. *Escherichia coli* strain *DH5α* (NEB) was used for subcloning. The gene coding for AA13 from *N. crassa* (GenBank: NCU08746) has been previously expressed and purified by Vu et al. [10]. Bacterium *A. tumefaciens* strain *AGL1* was used as the tool for gene transferring into *A.oryzae* cells [20].

2.2 Growth and maintenance of cultures

A. tumefaciens and *E. coli* bacteria were cultured in LB medium with Kanamycin supplement (to maintain bacterial cells carrying pEX2B plasmid) at 37^0C with shaking. Vogel's medium was used to culture *N. crassa* [19].

2.3 DNA isolation

pEX2B vector was isolated from *E. coli DH5α* by Monarch® Plasmid Miniprep Kit. The Phenol/Chloroform method developed by Sacchi et al. was used to extract DNA [21]. DNA concentration is estimated by measuring the absorbance at 260 nm and 320 nm. The concentration was calculated using the following formula:

DNA concentration (µg/mL) = $(A_{260} - A_{320})$ × dilution factor × 50 µg/mL

(The DNA Purification Chapter of the Promega Protocols and Applications Guide)

2.4 Primers design

Base on the pEX2B map (Fig. 1), we chose *BamHI* and *AflII* sequences to design primers to amplify the *NCU08746* gene. The *DsRed* gene of pEX2B was replaced with the *NCU08746* gene using the RE (*AflII* and *BamHI).* The designed PCR product includes the *NCU08746* gene and both *BamHI* and *AflII* sequences.

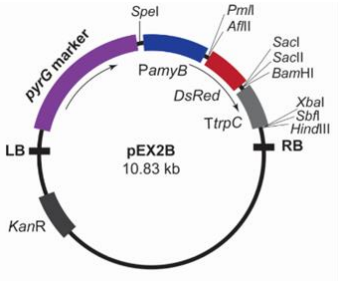

Figure 1 Map of the binary vectors pEX2B. The vector carries KanR and pyrG markers.

2.5 Vector construction

Restriction endonucleases and T4 DNA ligase (New England Biolabs) were used according to the manufacturer's recommendations. *E. coli* strain *DH5α* was transformed with plasmid DNA through heat shock method (42^0C for 45 seconds). *E. coli* transformants were selected via LB agar containing Kanamycin (100 µg/mL) to be incubated at 37^0C for 24 h. *A. tumefaciens AGL1* was transformed using electroporation. *A tumefaciens* transformants were selected via LB agar containing Kanamycin (100 µg/mL).

2.6 *NCU08746* gene detection and sequencing

Bacteria colonies were checked for the presence of *NCU08746* using the Colony PCR assay. Each PCR assay was performed in a total volume of 50 µL, containing five units of Taq DNA polymerase, 200 µM of each type of dNTPs, a few cells from each colony, and 10 pmol of each primer. Amplification was performed by the following cycle: initial denaturation at 95^0C for 5 min, followed by 35 cycles of denaturation at 95^0C for the 30 s, annealing for 1 min at 53^0C, and primer extension for 1min at 72^0C with a final extension period at 72^0C for 10 min. Then, samples were cooled to 4^0C. Aliquots of 5.0 µL of the amplified products were subjected to electrophoresis in 1.5% w/v agarose gels in TAE buffer.

Positive colonies were cultured in LB agar containing Kanamycin (100 µg/mL) to extract the target plasmid. These extracted plasmid samples were sequenced. In this study, we used the sequencing service of First BASE Laboratories (http://base-asia.com/).

3 Results and Discussion

3.1 PCR amplify *NCU08746* gene

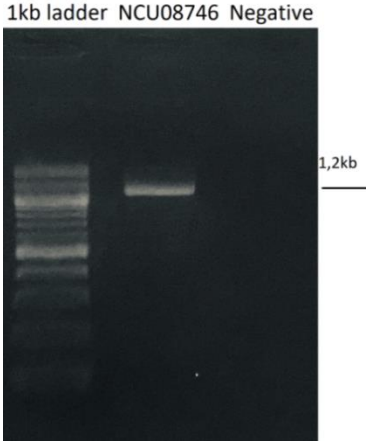

Figure 2 Agarose gel electrophoresis of *NCU08746* PCR products. Lanes: 1, 1kb ladder; 2, NCU08746; 3, Negative control without template DNA.

Our designed primers are named BamHI_NCU08746_ rv (5'- ATA TAT GGA TCC CTA CTT CCA CGA CGA CTC AAC -3') and AflII_NCU08746_fw (5'- ATA TAT CTT AAG ATG AAG TTC TCC ATC ATC TCG GTT G -3') containing BamHI (TAT GGA) and AflII (CTT AAG). PCR products were analyzed with electrophoresis on a 1% agarose gel (Fig. 2). Only a single band at ~ 1.2 kb is observed on the gel. The PCR product was purified for further experiments.

3.2 Cloning of *NCU08746* gene

The plasmid pEX2B was digested with *BamHI* and *AflII*. The linearized vector (50 ng) was mixed with the purified PCR product described above (20 ng) for ligation reaction following the T4 DNA Ligation protocol (M0202). The reaction mixture was incubated overnight at 16^0C. Subsequently, the REs were inactivated by incubation at 65^0C for 10min. 2 µl of the ligation reaction was then used to transform *E. coli DH5α*. LB agar with Kanamycin (100 mg/L) was used for selecting transformed strains, and the results are shown in Figure 3.

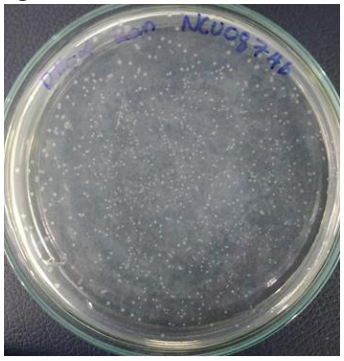

Figure 3 *E. coli DH5α* colonies on LB agar with Kanamycin after transformation

The control plate incubated with unmodified *E. coli* DH5α strain contained no colony. However, the transformed *E. coli* DH5α plate had many colonies suggesting bacterial strains resistant to Kanamycin on the sample. To have a specific conclusion, some colonies were tested by the Colony PCR assay.

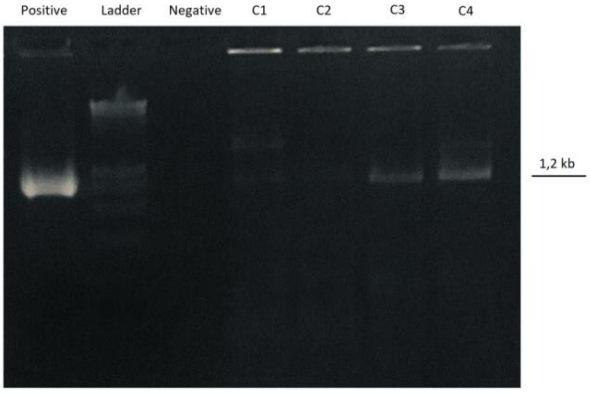

Figure 4 Electrophoresis analysis of the Colony PCR assay of transformed DH5α colonies. Lanes C1–C4 corresponds to colony samples. Lane 1: Positive control; Lane 2:1kb ladder; Lane 3: Negative control.

The result of the Colony PCR assay showed specific bands in all four colony samples. Colony No. 3 and No. 4's samples had the clearest bands while No. 2 Colony's sample showed a fainter band, and Colony No. 1's sample had another non-specific band. The result suggested that we had successfully produced *NCU08746* on a pEX2B vector. To ensure that the *NCU08746* sequence had been successfully inserted on pEX2B, the purified plasmid from colony No. 3 was sequenced (Fig. 5).

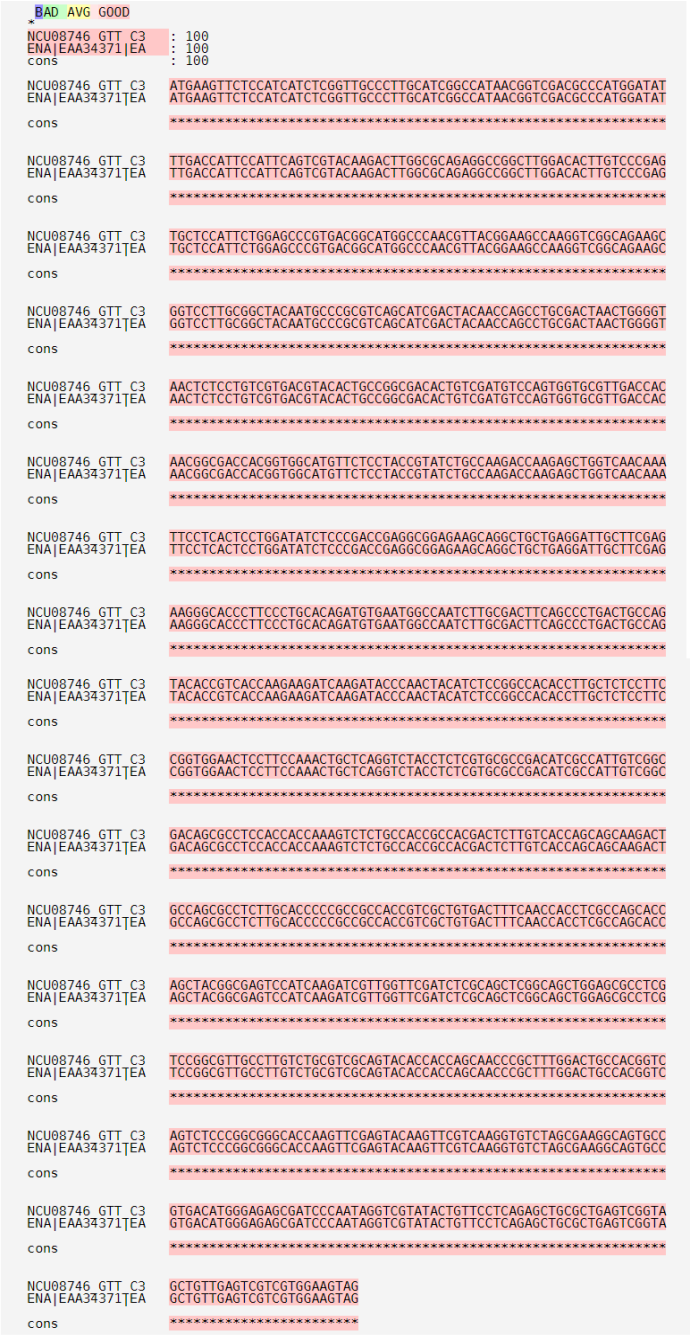

Figure 5 Pair-wise alignment of DNA sequencing result and NCU08746 sequence previously reported.

The No. 3 colony DNA sequence was aligned to the *NCU08746* sequence (accession number EAA34371) from NCBI Genbank. The result shows that the No. 3 colony DNA sequence is 100% identical to the *NCU08746* sequence (Fig. 5). It can be confirmed that the *NCU08746* gene was successfully cloned into the pEX2B vector.

3.3 Preparing *Agrobacterium* strain carries pEX2B-NCU08746

The pEX2B-NCU08746 vector was transferred into *A. tumefaciens AGL1* using electroporation as previously described by Nguyen *et al*. [20]. The electroporation was performed in 2 mm cuvettes. The instrument parameters were set at 2500 V, 25 µF, and 400 Ω [20]. Transformants were then incubated on LB agar plates containing Kanamycin (100 µg/mL) at 28^0C for 24 hrs. The transformed colonies growing on LB plate (Figure 6) were also examined by the colony PCR assay.

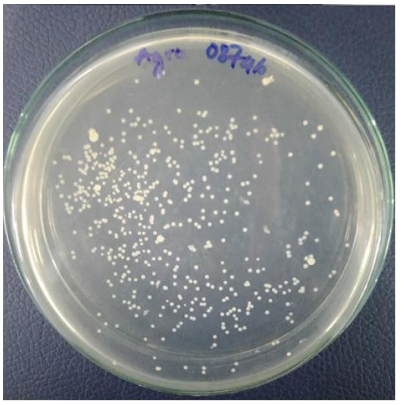

Figure 6 Transformed *A. tumefaciens* colonies growing on LB agar containing Kanamycin

We chose three random colonies to examine the presence of *NCU08746*. All Colony PCR samples had a band at about 1.2 kb (Figure 7) – the same size ascorresponds to the plasmid obtained from DH5α used as a positive control. Thus, we have successfully generated an *A. tumefaciens* strain containing the recombinant plasmid pEX2B-NCU08746.

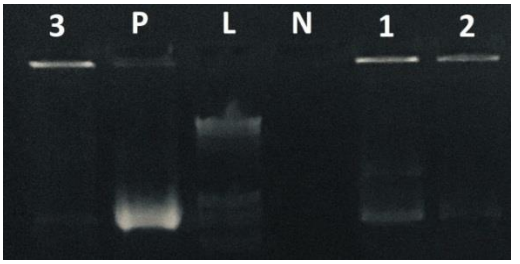

Figure 7 Electrophoresis analysis of PCR assays of *A.tumefaciens* colonies. Lanes 1, 2, and 3 correspond to colony samples. P: Positive control; L: 1kb ladder; N: Negative control

3.4 Conclusion

Cloning and gene transfer are the key procedures for protein activity and structure research. In Vietnam, no cloning or expression research has been carried out on *NCU08746* – a gene encoding AA13 PMOs or starch-active PMOs. Currently, there are three commonly used methods for fungal transformation, including electroporation, PEG-mediated protoplast transformation, and *A.tumefaciens*-mediated transformation [20]. The electroporation procedure for filamentous fungi requires a high-cost electroporator with specific parameters, while the PEG-mediated protoplast transformation method is laborious and requires more complicated procedures. Among the three transformation methods, *A. tumefaciens*-mediated transformation is more comfortable to implement due to the direct manipulation of fungal spores [20]. In this study, we have successfully cloned the AA13 PMO gene (*NCU08746*) into the plasmid *pEX2B* and generated an *A. tumefaciens* strain containing the recombinant plasmid pEX2B-NCU08746, which serve as the foundation for further study on the heterologous expression of PMOs in *A. oryzae.*

Acknowledgment

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Tạo dòng gen mã hóa Polysaccharide Monooxygenase AA13 từ *Neurospora crassa* lên plasmid pEX2B

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Tóm tắt Polysaccharide monooxygenases (PMOs) - nhóm enzyme có hoạt tính cắt liên kết glycoside trong các phân tử polysaccharide – được xem như một nhóm enzyme có tiềm năng ứng dụng cao trong công nghệ sản xuất biofuel từ nguồn sinh khối polysaccharide. Nghiên cứu này hướng đến tạo dòng gen PMO vào plasmid pEX2B nhằm biểu hiện enzyme PMO trên loài nấm *Aspergillus oryzae*, một loài nấm sợi được sử dụng rộng rãi trong công nghiệp sản xuất enzyme. Gen *NCU08746* từ nấm *Neurospora crassa* thuộc nhóm PMOs AA13 được tạo dòng lên plasmid pEX2B. Nghiên cứu đã thành công trong việc thiết kế bộ mồi và nhân bản gen NCU08746 từ bộ gen của *N. crassa*. Gen NCU08746 đã được tạo dòng lên plasmid pEX2B. Plasmid tái tổ hợp pEX2B-NCU08746 được chuyển vào *A. oryzae* thông qua vi khuẩn trung gian *Agrobacterium tumefaciens* AGL1. Nghiên cứu này góp phần đặt nền tảng cho các nghiên cứu tiếp theo về biểu hiện PMOs trên nấm *A. oryzae*.

Từ khóa Polysaccharide monooxygenases, *NCU08746, Agrobacterium tumefaciens,* AA13, *Neurospora crassa*.