Optimization of culture conditions to express AA9 Polysaccharide monooxygenases AN3860 in *Escherichia coli*

Ngo Thi Cam Nhung[1], Le Quynh Loan[2], Vu Van Van[1]

[1] NTT Hi-Tech Institute, Nguyen Tat Thanh University
[2] Institute of Tropical Biology, Vietnam Academy of Science and Technology
ntcnhung@ntt.edu.vn

Abstract

Lignocellulose biomass is a copious source for second generation biomaterial production. The participant of Polysaccharide monooxygenases enzyme (PMO) in the reactions which convert lignocellulose biomass into monosaccharides enhances the activity and improve the efficiency of hydrolysis of hydrolase enzymes on lignocellulose substrate. Enzyme AN3860, obtained from *Aspergillus nidulans* strain belonging to AA9 PMO, is expected to catalyze flexibly at C1 and C4 carbon positions of β-glycosidic linkage. As an enzyme with high potential of improving cellulose crystals hydrolysis capacity, AN3860 was successfully cloned into the expression system of *E. coli* BL21 (DE3) strain. In this study, the culture process of recombinant strain with AN3680 gene is optimized to increase the target proteins yield, thus ensure the outcome of purification process, and save production cost. The results demonstrate that the *E. coli* recombinant strains grow sufficiently in TB (Terrific Broth) culture media and the highest yield of AN3680 protein achieved when the concentration of Isopropyl β-D-1-thiogalactopyranoside (IPTG) is 0.05 mM and the temperature of the reaction is 30 ^{0}C at 150 rpm. After 6 hours of induction, the biomass reaches 500 mg/L and the yield of AN3860 account for (7-10) % total protein generated. The recombinant AN3860 protein is later harvested on larger scale and purified by Ni-NTA column chromatography method for analysis of bioactivities on lignocellulose substrates in the future.

Received 22/09/2022
Accepted 27/10/2022
Published 02/11/2022

Keywords
AA9, AN3860, *E. coli*, optimization, polysaccharide monooxygenases

1 Introduction

Biofuel is a potential alternative for fossil fuel. The transition from using fossil fuels to using biofuel may reduce the negative impacts on the environment such as greenhouse effect, global warming, and pollution cause by energy industry. Nowadays, ethanol is the most popular fuel in the biofuel market of the world, and it is produced from renewable materials including corn, sugar, molasses or agricultural biowaste. Ethanol production is based on the fermentation of sugar extracted from starch and saccharose. Therefore, this process has caused many concerns related to food safety. Lignocellulose (LCB) exploitation is a promising solution which allow us to take advantage of abundant amount of food crops after harvest season, leftovers of logging process and other plants serve in sugar production and sugar fermentation and does not compete with food production. However, LCB has very complicated and stable molecular matrix structure which consists of two polymers: cellulose and hemicellulose, which strongly bond with lignin. To convert cellulose-rich biomass into monosaccharides, the biomaterial should be broken down and the bonds

in its molecular structure must be loosen by passing several steps of preprocessing including physical, chemical, and biological solutions combine with activities of different lyase enzymes on cellulose substrate. Previous studies have shown that the efficiency of conversion from polysaccharides to monosaccharides improves when cellobiohydrolase (CBH) and endoglucanases (EG) participate in the reactions together, compared with the case when each of those enzymes act individually. To optimize the biomass conversion process, a combination of broad-spectrum enzymes such as cellulase, hemicellulose and some types of oxidoreductases which act on different substrates is essential. Recently, polysaccharide monooxygenases (PMO) group of enzymes has shown promising potentials and has been commercialized into Cellic CTec2 and CTec3(Novozymes A/S) applied in industrial bioethanol production. To enhance the efficiency of the biomass conversion, studies focus on finding, recognizing, and analyzing new sequences of enzymes belong to PMO group have been established [1-3].

PMO is a group of enzymes catalyze the hydroxylation of polymer carbohydrate chains. PMO belongs to Auxiliary Activities family classification, which demonstrates their origins and substrates. Until now, there have been 7 main groups of AA: AA9, AA11, AA13 and AA14, which originate from fungi and act on hemicellulose, chitin, starch, and xylan, respectively. AA10 was found in bacteria, performing the breakdown on cellulose or/and chitin substrates, AA15 and AA16 most recently discovered in viruses and some animals, they act on cellulose or chitin [4-6]. AN3860 is a theoretical PMO gene sequence found in *Aspergillus nidulans*. According to a study on gene expression regulation in *A. nidulans* in straw-containing media, there are only five of the total nine PMO induced genes in which AN3860 accounts for more than 93% of total gene expressed in FPKM (Fragments Per Kilobase Million) unit [7]. Simultaneously, phylogenetic tree shows that AN3860 belongs to group 3 AA9 PMO. Enzymes in this group may catalyze flexibly at C1 or C4 carbon position of β-glycosidic bond, thus increase the activity of cellulase effectively, compared with group 1 AA9 (oxidate at C1 position) and group 2 AA9 (oxidate at C4 position) [4,7]. Additionally, till now, AN3860 has not been studied thoroughly, therefore recombinant gene expression method is applied to actively massed produce AN3860 enzyme, serving in further studies on its structure, activities, and functions.

Some of the most popular and successful expression systems of PMO are fungi, yeasts, and *E. coli*. Each of them has their own advantages, disadvantages and different compatibility with target expressed protein sequences. In previous study, AN3860 was successfully cloned into *A. oryzae* expression system [8]. However, the complicated biophysical characteristics of fungi lead to low quantity of proteins secreted to extracellular environment, caused difficulties in target protein extraction. Hence, AN3860 was cloned and successfully expressed in *E. coli* BL21 (DE3) expression system with exceed yield of AN3860 protein. To obtain large amount of target protein and reduce production cost, research on optimization of culture conditions to enhance the AN3860 protein expression efficiency is essential.

In this study, *E. coli* BL21 (DE3) stain with recombinant AN3860 gene is cultured in different media and different culture conditions to optimize the recombinant AN3860 protein production.

2 Materials and Methodology

2.1 Materials

Escherichia coli BL21 (DE3)) [F– ompT gal dcm lon hsdSB(rB –mB –) λ(DE3) was used as recombinant strain carrying pET22b/AN3860 expression vector encode for target AN3860 protein. This strain was obtained from NEB and pET22b-AN3860 was ordered from Biobasic. The synthesised vector pET22b-AN3860 was tranfered to *E. coli* cells following the introduction of NEB protocol for competent cells. A tube containing competent cells was added 20 ng of plasmid DNA. The mixture was placed on ice for 30 minutes. Then, the cells were heat shocked at 42 ^{0}C for exactly 60 seconds. After placing on ice for 5 minutes, 950 µL of LB was pipetted into the mixture at room temperature and incubated for 60 minutes. The mixture was spread onto LB-Ampicillin-Agar (LB-Amp-Agar) plate. The grown colonies on LB-Amp-Agar were checked by colony PCR method. The DNA sequences (Fw 5'- ATA CGT GAC GAA GAT GAC G -3'; Rv 5'- ACC ACT GTA CAG CTC AGG- 3') were used as the primer pair for verifying the transformed colonies

using PCR method. The PCR was done with 30 cycles of denaturation step in 30 seconds at 95 ^{0}C, annealing steps in 30 seconds at 51 ^{0}C and extension step in 60 seconds at 72 ^{0}C.

All the chemicals used in this project were purchased from Sigma Aldrich company, USA.

2.2 Methodology

Bacteria culture process

Single colonies of *E. coli* BL21 (DE3) with pET22b-AN3860 vector were cultured in 20 mL LB medium containing 100 µg/mL ampicillin, at 37 ^{0}C, shaking speed at 150 rpm over night then subculture 5 % to five 100 mL − erlenmeyer flasks, each contains 50 mL of fermentation culture. The culture conditions were the same in every flasks for approximately (3-4) hours. When OD_{600} value reached 0.4-0.8, IPTG induction was performed so that final concentration was 0.5 mM and continue shaking at 30 ^{0}C in 6 hours. After induced culturing, samples were collected and mixed with loading dye (4X) then heat-treated at 100 ^{0}C in 10 minutes. SDS-PAGE (12 % of SDS-polyacrylamide gel electrophoresis) was used for sample analysis. Collected cell biomass was dissolved in NPI-10 (NaH_2PO_4 50 mM pH = 8.0, NaCl 0.5 M, imidazole 10 mM), then cells were disrupted by ultrasonic sonicator. Total protein amount was assessed by Bradford Assay (Biobasic kit) at 595 nm-wavelength (Genway, USA).

Optimization of expression conditions for recombinant protein

Culture parameters, inducer (IPTG) concentration and induction temperature were varied respectively to evaluate the ability of AN3860 synthesis and biomass synthesis based on total amount of protein obtained from culture process.

Five popular culture media containing glucose, yeast extracts were used to study the effects of environment on AN3860 protein synthesis, including M9 (Na_2HPO_4 12.8 g; KH_2PO_4 3.0 g; NaCl 0.5 g; NH_4Cl 1.0 g; and micronutrients) + 2 % glucose, $M9^+$ 2 % glucose + 2 % yeast extract, LB (yeast extract 0.5 %, peptone 1.0 %, NaCl 1.0 %), TB (yeast extract 2.4 %, peptone 1.2 %, K_2HPO_4 72 mM, KH_2PO_4 17 mM, glycerol 0.4 %), SB (3.0 % tryptone, 2.0 % yeast extract, 1.0 % MOPS free acid, 2.0 % glucose, 1.0 % NaCl) [9]. Other parameters of culture media were maintained the same between treatments during culture.

In the experiment to determine the optimal concentration of inducer IPTG, this value was evaluated in the range of (0.05-1) mM. Optimal culture medium is utilized and expression rate was assessed when final concentration of IPTG were (0.05, 0.1, 0.25, 0.5, 0.75 and 1) mM respectively. Biomass samples were collected after 6 hours of induction at 30 ^{0}C, with shaking speed at 150 rpm.

Temperature parameter of culture process was evaluated by shake culturing the recombinant strain at 150 rpm in optimal conditions, induced by optimal concentration of IPTG mention in previous treatment and the induction temperature was varied in range of (20-40) ^{0}C. After 6 hours, samples were collected to quantify the expression rate and protein yield.

Data Analysis

The experiments were biologically repeated 3 times. Expression rate of target protein in SDS-PAGE assay was calculated by ImageJ software. Total protein amount was the average value of 3 replicates and was analyzed by one-way ANOVA, Turkey test ($p < 0.05$).

3 Results and Discussion

3.1 Cloning the recombinant *E. coli* BL21 (DE3) carrying vector pET22b-AN3860

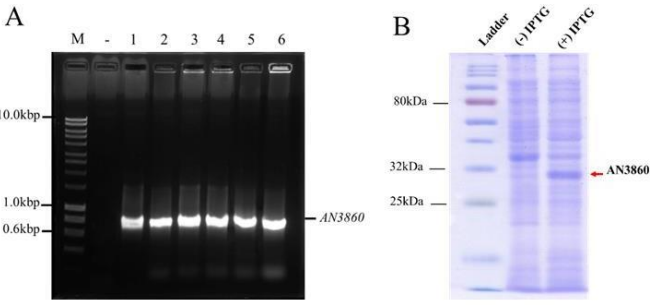

Figure 1 Examination of the recombinant *E. coli* by PCR reaction and SDS-PAGE method.

A. PCR reaction to screening the *E. coli* BL21 (DE3) recombinants. M- Marker hyperLadder 1kbp; 1- Negative control; 2, 3, 4, 5, 6- The tested colony

B. Result of the protein components of the recombinant cells induced with IPTG and without IPTG for AN3860 expression.

The synthesized vector was introduced into the host cells *E. coli* BL21 (DE3) by the chemical transformation method. After the process, the colonies were selected on LB containing Ampicillin (100 µg/mL). The single colony growth in LB-Amp-Agar was checked by PCR method with the specific primer. The PCR band of the target gene expected fragments of

0.76 kbp. The PCR results were shown on agarose gel (Figure 1). The size of all target bands (from well 1 to well 6) from the test colonies was approximately 0.8 kbp, matching the AN3860 theoretical length. Therefore, the *E. coli* BL21 (DE3) recombinants were cloned successfully.

The target protein expression in *E. coli* BL21 (DE3) was shown on gel SDS-PAGE 12 % acrylamide (Figure 1. B). The predicted molecular weight of overexpressed protein - AN3860 was approximate 30 kDa in lane (+) IPTG whereas the negative control (without IPTG inducement) had no band at the same position. Thus, AN3860 was expressed successfully in *E. coli* BL21 (DE3).

3.2. Investigation of expression media of *E. coli* BL21 recombinant strain carrying vector pET22b-AN3860

Culture media provide the microorganisms with essential nutrients for their growth and recombinant protein production. The differences in culture media contents lead to different expression rates of target protein and different states of them. Hence, determining the optimal culture conditions is necessary to assure the best outcome of host cell development as well as highest yield of recombinant proteins.

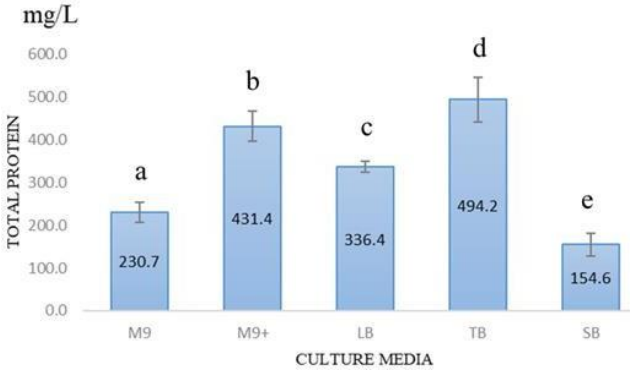

Figure 2 Quantification result of total protein produced in different culture media assessed by Bradford Assay. The presented result is the mean of three replicates and standard deviation, analyzed by one-way ANOVA, Turkey test. a, b, c, d, and e letter represented for statistically significant difference.

The augmentation of total protein concentation demonstrated the growth of culture biomass. The amount of biomass is proportional to the total protein amount. The results after 6 hours of induction (Figure 2) showed that TB culture medium achieved superior amount of total protein produced: 490 mg/L, followed by $M9^+$ medium supplemented with 1 % peptone reached 433 mg/L , on the other hand, the LB, M9, SB medium recorded low biomass growth. At the same time, electrophoresis results of the total protein solution obtained after culture showed that the ratio of the target protein (AN3860) to the total protein induced in different culture media did not have a significant difference, about (6.7-7.2) %, Figure 3. Although expression rate of target protein did not have statistically significant difference between culture media, in this experiment, TB media culture produced the highest amount of target protein AN3860. On the other hand, SB medium containing high concentration of tryptone (3 %) and yeast extract (2 %) was considered not appropriate for supporting the growth of recombinant strains.

The result demonstrated that TB media provided an adequate source of carbon and amino acids. In detail, SB medium consists of high concentration of amino acids source (tryptone, yeast extract) but this medium reduced the cell growth of the recombinant strain. The basic media, M9 and LB, contain the less quantity of carbon and amino acids sources, leading to the less biomass growth. In addition, TB medium contains a stable buffer system (K_2HPO_4, KH_2PO_4) for the growth of host cell *E. coli* BL21 and the production of AN3860 protein, therefore, it was chosen as culture media for subsequent experiments

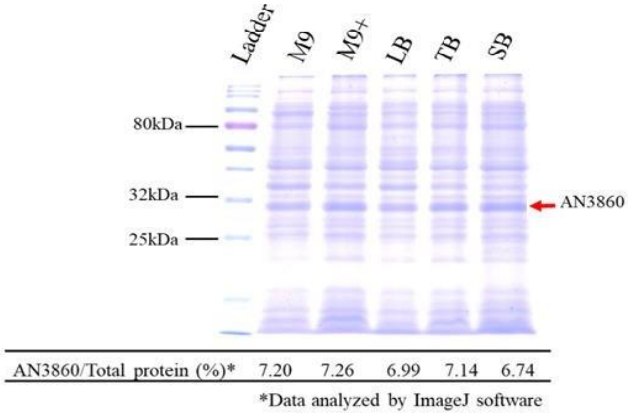

Figure 3 Investigation of AN3680 protein expression rate in different culture media assessed by SDS-PAGE method. Data was analyzed by ImageJ software.

3.3 Investigation of IPTG concentration during the induction of AN3860 protein expression

Isopropyl-β-d-thiogalactopyranoside (IPTG) is a compound which has similar structure with lactose but does not participate in metabolism and it is widely utilized in *E. coli* expression system. IPTG acts efficiently in induction of operon lac, however, inappropriate concentration of IPTG leads to cytotoxicity and high production cost [10]. Effects of different concentration of IPTG in induction process on

final concentration of the solution reached (0.05, 0.1, 0.2, 0.5, 0.75 and 1) mM, represented by the SDS-PAGE assay results of total synthesized proteins and target protein expression.

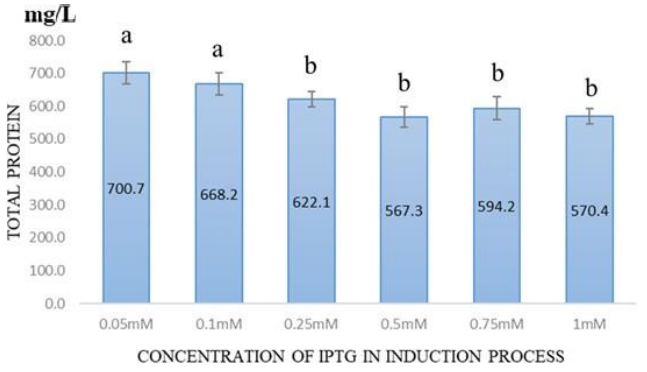

Figure 4 Effects of IPTG concentration in induction process on total protein synthesis. The presented result is the mean of three replicates and standard deviation, analyzed by one-way ANOVA, Turkey test. a, b letter represented for statistically significant difference.

The amount of total protein produced proved that recombinant gene expression strain tended to reduce the biomass created when IPTG concentration inceased. When IPTG concentration was in the range of (0.05-0.1) mM, the highest amount of total protein created was approximately 700 mg/L and slightly decreased at subsequent concentrations then levelled off in the range of (0.5-1) mM with values in the range of (567-594) mg/L, Figure 4. Besides, electrophoresis results (Figure 5) demonstrated expression rate of target protein did not have significant difference between treatments with difference concentration of IPTG (approximately 10 %). Hence, the IPTG concentration of 0.05 mM was chosen to induce the expression process, to ensure the target protein and biomass production and to cut down the cost of producing AN3860 protein at larger scale.

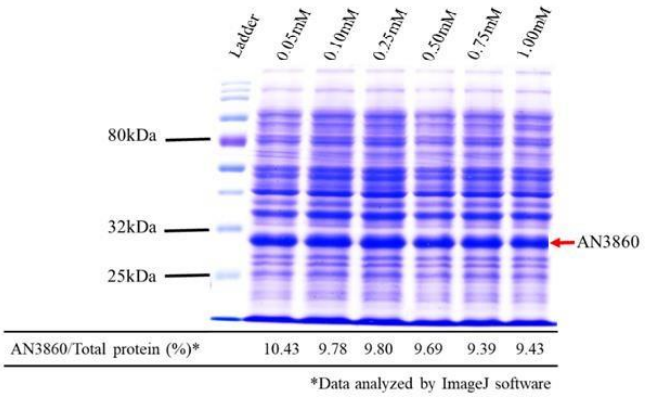

Figure 5 SDS-PAGE result presented the amount of target protein in *E. coli* BL21/AN3860 strain at different IPTG concentration after 6 hours of induction. AN3860/Total protein ratio analyzed by ImageJ software.

3.4 Studying on optimal temperature for AN3860 protein expression

Temperature is a crucial factor in efficiency of target protein expression and growth rate of biomass of the host strain. For *E. coli*, the temperature from 37 ^{0}C to 39 ^{0}C is optimal for cell proliferation as well as for the optimal activity of *lac* and *tac* promoter. However, at high levels of metabolism, it might lead to undesirable metabolic reactions for the synthesis of foreign proteins, increasing the activity of proteolytic enzymes, leading to a decrease in the efficiency of producing the target protein [11,12]. To determine the appropriate induction temperature, we investigate temperature for protein expression in the range of (20-40) ^{0}C, specifically at 5 main temperature marks of (20, 25, 30, 37 and 40) ^{0}C.

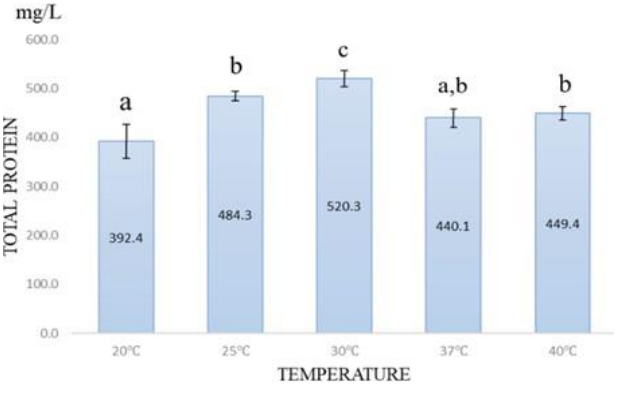

Figure 6 Effects of temperature on total protein synthesis of E. coli BL21 recombinant strain. The presented result is the mean of three replicates and standard deviation, analyzed by one-way ANOVA, Turkey test. a, b, and c letter represented for statistically significant difference.

Figure 6 shows that the growth of recombinant *E. coli* cells was affected by temperature. At 30 ^{0}C, the most produced protein reached the amount of 520 mg/L, there was a difference between therest treatments by ANOVA analysis of variance at the significance level of 0.05 and the highest rate of recombinant protein AN3860 expression accounting for 7.78 % of the total protein produced (Figure 7). The 25 ^{0}C mark had a total protein value of 484 mg/L. At a low temperature of 20 ^{0}C, the metabolism was reduced, thus the amount of biomass formed reached the lowest value of about 392 mg/L with low amount of target protein synthesis accounting for 6.43 % of total protein. Although 37 ^{0}C and 40 ^{0}C are the optimal temperatures for *E. coli* cultures, the total protein produced tended to be lower than those of 25 ^{0}C and 30 ^{0}C marks. This result is similar to the study of author Le Ngoc Giang and colleagues, which optimized α-glucuronidase expression when examining the culture temperature above 30 ^{0}C with a decrease in the measured biomass [9]. Some causes of this phenomena may be that high temperature accelerates the expression of target proteins, forms unfavorable structures which interact with cell membranes, leading to rapid cell degradation and lysis [10]. Therefore, the temperature investigation results proved that maintaining the protein expression process at 30 ^{0}C, which is close to laboratory temperature, is very benificial in many aspects, such as in terms of energy, in the stability and efficiency of the growth of *E.coli* and the expression rate of proteins. Hence, the expression temperature at 30 ^{0}C was chosen as the optimal parameter for the expression of AN3860 protein in recombinant strain *E. coli* BL21.

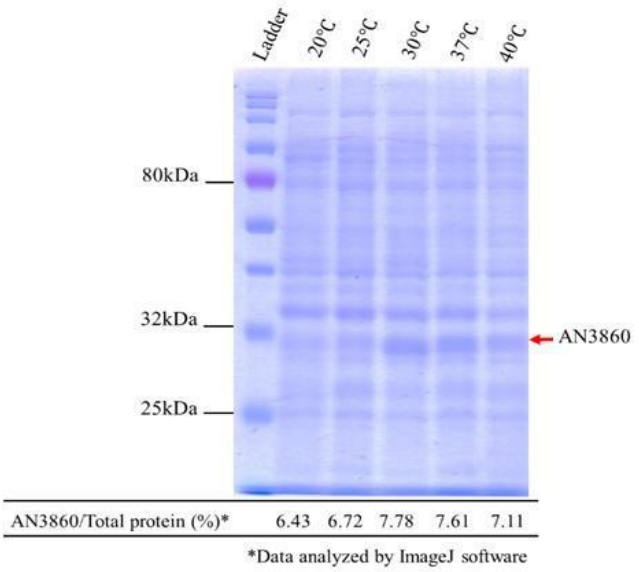

Figure 7 Different temperatures of induction process effects on target protein expression rate assessed by SDS-PAGE method. Data analyzed by ImageJ software.

4 Conclusion

Results collected from erlenmeyer flask culture of *E. coli* BL21 strain with recombinant protein AN3860 showed that the recombinant strain grew well in TB medium in collaboration with optimal final concentration of IPTG at 0.05 mM at 30 ^{0}C and produced highest amount of target protein AN3860. After 6 hours of induction, the biomass created may reach a value of more than 500 mg/L and the amount of AN3860 protein synthesized accounted for (7-10) % of total protein. With these results, the optimal parameters can be applied into the cultivation of *E. coli* BL21/AN3860 in fermentors with larger volume to harvest larger biomass and to save the production cost. AN3860 protein will then be purified by Ni-NTA column chromatography, collected in large quantities and process through various analysis methods, including activity analysis, mass spectrometry (MS) on Lignocellulose substrate in the future.

Foundation Acknowledgement

This research is funded by NTTU for Science and Technology Development under grant number 2021.01.11/HĐ-KHCN

Loan Le Quynh was funded by Vingroup Joint Stock Company and supported by the Domestic Master/ PhD Scholarship Programmed of Vingroup Innovation Foundation (VINIF), Vingroup Big Data Institute (VINBIGDATA), code VINIF.2020.TS.62

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

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Tối ưu điều kiện biểu hiện AA9 polysaccharide monooxygenases tái tổ hợp trong hệ thống *Escherichia coli*

Ngô Thị Cẩm Nhung[1], Lê Quỳnh Loan[2], Vũ Văn Vân[1]
[1]Viện Kĩ thuật Công nghệ cao NTT, Đại học Nguyễn Tất Thành
[2]Viện Sinh học Nhiệt đới, Viện Hàn lâm Khoa học và Công nghệ Việt Nam
ntcnhung@ntt.edu.vn

Tóm tắt Sinh khối lignocellulose là nguồn nguyên liệu dồi dào cho sản xuất nhiên liệu sinh học thế hệ thứ hai. Sự tham gia của enzyme Polysaccharide monooxygenases (PMO) trong phản ứng chuyển hóa sinh khối lignocellulose thành đường đơn đóng vai trò tăng cường hoạt động và nâng cao hiệu suất thủy phân của các enzyme hydrolase trên cơ chất lignocellulose. Enzyme AN3860 thu nhận từ chủng nấm *Aspergillus nidulans* thuộc nhóm AA9 PMO được dự đoán có thể xúc tác linh hoạt ở vị trí carbon C1 và C4 của liên kết β-glycosidic. Với tiềm năng là một enzyme có thể cải thiện khả năng thủy phân tinh thể cellulose, AN3860 đã được dòng hóa thành công trong hệ thống biểu hiện *E. coli* BL21 (DE3). Trong nghiên cứu này, chủng tái tổ hợp mang gen AN3860 được tối ưu quy trình nuôi cấy nhằm thu nhận lượng lớn protein mục tiêu đảm bảo thuận lợi cho quá trình tinh sạch và tiết kiệm chi phí sản xuất. Các kết quả tối ưu quy trình tạo dòng cho thấy trong tế bào *E. coli* tái tổ hợp tăng trưởng tốt trong môi trường TB (Terrific Broth) và lượng protein AN3860 thu được cao nhất với nồng độ chất cảm ứng IPTG là 0,05 mM kết hợp nhiệt độ cảm ứng ở 30 ^{0}C. Sinh khối tạo thành sau khi cảm ứng 6 giờ có thể đạt giá trị hơn 500 mg/L và lượng protein AN3860 được tạo ra chiếm khoảng (7-10) % lượng protein tổng số. Protein AN3860 tái tổ hợp tiếp tục được thu nhận ở quy mô lớn hơn và tiến hành tinh sạch qua cột sắc kí Ni-NTA cho các thí nghiệm phân tích hoạt tính, khối phổ trên các cơ chất lignocellulose trong tương lai.

Từ khóa AA9, AN3860, *E. coli*, tối ưu hóa, polysaccharide monooxygenases