Sub-Cloning of *ads* Gene Into pETDUET1_*cyp* For Co-Expression in *Escherichia coli*

Imam Adi Wicaksono[1], Tresna Lestari[2]*, Evi Umayah Ulfa[3], Catur Riyani[1], Elfahmi[1]

[1]Institut Teknologi Bandung, [2]STIKes Bakti Tunas Husada Tasikmalaya, [3]Universitas Jember

STIKes Bakti Tunas Husada Tasikmalaya

Jl. Cilolohan No 36 Tasikmalaya

*E-mail : beatsign@yahoo.com

***Abstract*— CYP71AV1 and ADS are two enzymes involved in artemisinin biosynthesis. In this research, sub-cloning of *cyp71av1* and *ads* in pETDUET1 (pETDUET1_*cyp/ads*) has been done. The result of transformation has been confirmed by migration, restriction and sequencing analysis. Overproduction of CYP71AV1 and ADS was done at temperature 37 °C using 0,5 mM IPTG induction. The protein produced mostly formed as inclusion bodies, therefore the optimization of overproduction condition is still needed.**

***Keywords*— CYP71AV; ADS; pETDUET1; Sub-cloning**

I. INTRODUCTION

Artemisinin is an antimalarial drug reported to have the high effectivity to overcome *Plasmodium falciparum* resistance against chloroquine and other formerly used antimalarial (Li, et al., 2006; Rathore, et al., 2005). World Health Organization (WHO) has recommended Artemisinin-Based Combination Therapies (ACTs) since 2001 as a malaria best treatment for overcoming resistance of *Plasmodium falciparum* (Duffy and Mutabingwa, 2006; White, 2008).

Artemisinin used in the treatment obtained through the isolation process from the *Artemisia annua* plant. The low levels of artemisinin in *A. Annua* (0,001 - 1 % of the simplicia) cause the treatment using artemisinin and their combinations become expensive and can't be reached by people who is on the endemic of malaria. The price of artemisinin itself in 2006 ranged about 900-1600 USD/kg (Mutabingwa T.K, 2005; Hale, et al., 2007).

Many experiments have been conducted to gain artemisinin in adequate amount. One of the methods being developed to improve levels of artemisinin is biosynthesis pathway engineering through genetic engineering against the enzymes that play role in the production of artemisinin using microbes, cell culture, tissue or organs of plants. One approach that potential to get artemisinin in large amount is by microbes biosynthesis engineering such as by inserting the artemisinic acid (precursor of artemisinin) biosynthesis pathway into *Escherichia coli* (*E. coli*). *E. coli* provides farnesyl pyrophosphate that need amorpha-4,11-diene synthase (*ADS*) and cytochrome p450 (*CYP71AV1*) to produce artemisinic acid. *ADS* and *CYP71AV1* are two enzymes involved in the final stages of artemisinic acid formation (Hale, et al., 2007).

Previous research, cloned of CYP71AV1 genes into pET_DUET1 (pETDUET1_*cyp*) as the co - expression vector has been done. In this research, cloning of ADS genes into pETDUET1_*cyp* and characterization of its co-expression resulted in *E. coli* BL21 using SDS-PAGE method will be done.

II. MATERIALS AND METHODS

A. Materials

Materials used in this research have the quality grade for the use of analysis (pro analysis, p.a) or pro molecular biology, including materials for Luria Bertani medium, materials for electrophoresis gel agar, DNA marker (Fermentas), *Polymerase Chain Reaction* (PCR) Kit reagents, PCR clean up (Geneaid), restriction enzymes *BamH*I, *Not*I (Roche), restriction buffer B and H (Roche), primers ADSFOR (5'–GAGGACG GATCCGATGTCACTTACAG-3'), ADSREV (5'-GGATTC GCGGCCGCTCATATACTCATA-3'), pET _UPSTREAM (5'-ATGCG TCCGGCGTAGA-3'), DUET_DOWN1 (5'-GATTATGCGGCCGTGTA CAA-3') and DUET_UP2 (5'-TTGTA CACGGC CGCAT AATC-3'), pGEM-T cloning vector, T4 ligase

enzyme and ligation buffer 10x (Promega), pETDUET1_*cyp* plasmid, materials for SDS-*Poliacrylamide Gel Electrophoresis* (SDS-PAGE), protein marker (Fermentas).

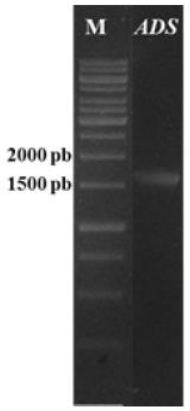
B. Methods

ADS gene construction was done by PCR methods using primers with *Bam*HI restriction site addition at 5'-end and *Not*I restriction site addition at 3'-end. PCR result was then cloned into intermediate cloning vector, pGEM-T, to give recombinant pGEM-T_*ads*. This recombinant was then transformed into *E. coli* TOP10. The recombinant DNA was characterized by migration and restriction analysis. For sub-cloning the *ADS* gene into pETDUET1_*cyp*, pGEM-T_*ads* and pETDUET1_*cyp* were restricted using *Bam*HI and *Not*I and cloned to give recombinant pETDUET1_*cyp/ads.* The recombinant plasmid was then transformed into *E. coli* TOP10. The recombinant pETDUET1_*cyp/ads* were characterized by migration, restriction and sequencing analyses. For overproduction purposes, pETDUET1_*cyp*/*ads* were transformed into *E. coli* BL21 (DE3). The protein produced was then characterized using SDS-PAGE analysis.

III. RESULT AND DISCUSSION

ADS gene from pET15b_*ads* was constructed for sub-cloning into pETDUET1_*cyp* using primers ADSFOR and ADSREV by PCR method. ADSFOR and ADSREV primers resulting *ADS* gene with *Bam*HI restriction site addition at 5' end and *Not*I restriction site addition at 3' end. The result of 1% agarose gel electrophoresis against PCR product showed a DNA band located between 1500 bp and 2000 bp of DNA marker (picture 1). Experimentally calculation showed that PCR product was 1684 bp with theoretical size is 1641 bp, so the PCR product was suspected as *ADS* gene.

The purified *ADS* gene was then cloned into pGEM-T vector for copying the gene. *ADS* gene was amplified using Taq DNA polymerase during the PCR process which preferentially adds an adenine to the 3' end of the product, while pGEM-T is a cloning vector that has complementary 3' thymine overhangs (T-overhangs). The ability of adenine (A) and thymine (T) (complementary basepairs) on different DNA fragments to hybridize and, in the presence of ligase, make the DNA become ligated together to give pGEMT_*ads*. The ligation product was then transformed into competent cells of *E. coli* TOP 10 using heat shock method (Chung dkk, 1989). Transformant cells selection carried out in Luria Bertani (LB) medium containing 100 µg/mL of ampicillin, 100 mM of IPTG and 10 µL of X-gal 2,5%.

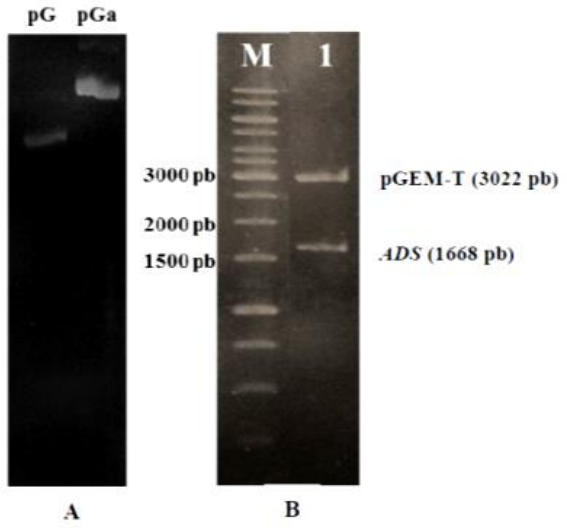


Picture 1. Electrophoregram of PCR result. (*ADS*) : Amorpha-1,4-diene Synthase Gene (M) : 1 kb DNA marker

Successful cloning of an insert into the pGEM-T vector interrupts the coding sequence of β-galactosidase because the insertion site lies on *lacZ* gene that is coding for β-galactosidase. Recombinant clones can be identified by color screening on indicator plates, the X-gal that was added into LB medium has role as the indicator. Clones containing a DNA insert produce white colonies because the cells could not express β-galactosidase so the X-gal as the analog of lactose could not be degraded, and blue colonies that show the result of X-gal degradation can be resulted from DNA fragments that are cloned in-frame with the *lacZ* gene.

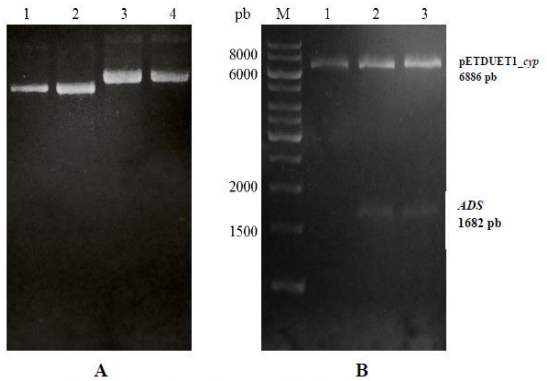
The cloning result examined by analysis of migration and restriction. The migration analysis on gel agarose 1 % (b / v) showed that pGEMT_*ads* (4656 bp) migrate slower than pGEM-T (3015 pb) because its greater size (Picture 2 A). While based on the results of restriction analysis with *Bam*HI and *Not*I restriction enzymes obtained two DNA bands with size 3000 bp and 1500-2000 bp. Theoretically *ADS* gene has size 1641 bp and pGEM-T 3015 bp (Picture 2 B). This indicates that the *ADS* genes has successfully pasted on pGEM-T vector.

For sub-cloning the ADS gene into pETDUET1_cyp, pGEMT_ads and pETDUET1_cyp were restricted by BamHI and NotI enzymes to give ADS gene and linear pETDUET1_cyp with proper ends. Ligation process was done in 1:4 molar ratio between vector and DNA insert. Ligation product was then transformed into E. coli TOP10 competent cells and grown in solid LB medium containing ampicillin. For overproduction purposes, pETDUET1_cyp/ads was transformed into E. coli BL21(DE3). Both of transformant cells were isolated for pETDUET1_cyp/ads and characterized by migration, restriction and sequencing analysis.



Picture 2. Electrophoregram of pGEMT_*ads* characterization result. A. Migration analysis pf pGEMT_*ads*, (pG) pGEM-T, (pGa) pGEM-T_*ads*; B. Restriction analysis using enzymes *Bam*HI dan *Not*I, (M) 1 kb DNA marker, (1) pGEM-T_*ads*.

The result of migration analysis showed that pETDUET1_*cyp/ads* migrate slower than pETDUET1_*cyp* because its greater size. Theoretically pETDUET1_*cyp/ads* size is 8522 bp while pETDUET1_*cyp* is 6892 bp (Picture 3A). The next characterization was restriction analysis, pETDUET1_*cyp/ads* were cut using *Bam*HI and *Not*I restriction enzyme to give *ADS* and pETDUET1_*cyp*. The result of 1% agarose gel electrophoresis showed two DNA band with size between 6000-8000 bp and 1500-2000 bp. Theoretically, the *ADS* gene size is 1641 bp and pETDUET1_*cyp* is 6892 bp. While experimentally the size of *ADS* gene is 1682 bp and pETDUET1_*cyp* is 6886 bp (Picture 3B).



Picture 3. Electrophoregram ofpETDUET1_*cyp/ads* characterization. A. Migration analysis, (1) pETDUET1_*cyp* from *E. coli* TOP10, (2) pETDUET1_*cyp* from *E. coli* BL21(DE3), (3) pETDUET1_*cyp/ads* from *E. coli* TOP10, (4) pETDUET1_*cyp* from *E. coli* BL21(DE3); **B.** Restriction analysis, (M) 1 kb DNA Marker. (1) pETDUET1_*cyp*, (2) pETDUET1_*cyp/ads* from *E. coli* TOP10, (3)pETDUET1_*cyp/ads* from *E. coli* BL21(DE3)

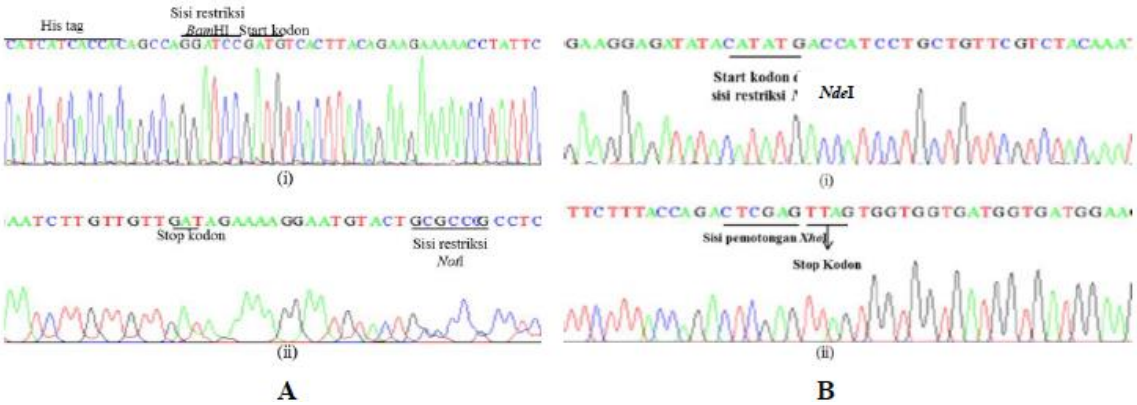
Sequencing analysis of nucleotides were done for CYP71AV1 and ADS. Based on homology analysis by BLAST program using pET_UPSTREAM and DUET_DOWN1 primers, the result of ADS gene sequence obtained homology value 99% with the gene of ADS coding enzyme from A. annua with access code FJ432667.1 (www.ncbi.nlm.nih.gov). The result was not 100% homolog because of the change of nucleotide sequence at base 27 that change the amino acid sequence. The change of nucleotide sequence may occur during the PCR process because the Taq polymerase that was used has a low proofreading. The presence of nucleotide sequence changes cause the change of amino acid sequence, but did not make the formation of a codon stop in the middle of ADS sequence and did not make the change in active site residue of ADS enzyme, so it is predicted would not disrupt the activity of ADS enzyme (Julsing, 2006).

Primers DUET_UP2 and T7 terminator used for *CYP71AV1* gene sequencing. Alignment result of *CYP71AV1* gene from pETDUET1_*cyp/ads* with *CYP71AV1* synthetic gene showed that many changes occur in nucleotide base sequence. This is unacceptable because *CYP71AV1* gene was prepared by cut and paste method from pJexpress401_*cyp*, different form *ADS* gene that was prepared by PCR method that may cause nucleotide sequence changes. The mistake of proofreading in *CYP71AV1* sequencing may occur due to the concentration of DNA used as the sample was overly diluted, so the sequencing analysis of *CYP71AV1* gene still needs to be done with higher concentration of the DNA.

Based on sequencing analysis result (Picture 4), *ADS* and *CYP71AV1* genes were successfully cloned into pETDUET1 vector. Sequencing analysis result showed the designed components of *ADS* and *CYP71AV1* gene such as codon start, codon stop, and restriction sites.

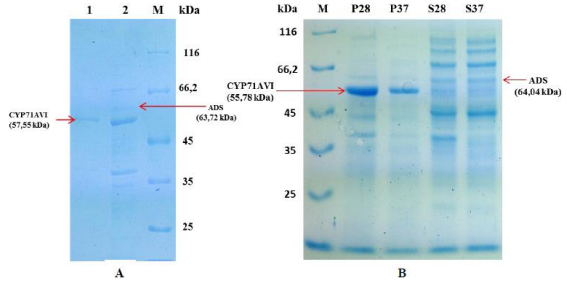
A. Abbreviations and Acronyms

CYP71AV1 and ADS overproduction was done in *E. coli* BL21(DE3) that carries pETDUET1_*cyp/ads*. For optimization of overproduction condition, cell cultures were differentiated with and without addition of IPTG 0,5 mM as the inductor. The result of overproduction showed two protein bands on SDS-PAGE analysis indicated as CYP71AV1 aand ADS (Picture 5A). Based on experimental calculation obtained molecular weight of CYP71AV1 band was 57.55 kDa that is similar with theoretical size of CYP71AV1(53.57 kDa), and the molecular weight of ADS band was 63.72 kDa based on experimental calculation that is similar with theoretical size of ADS (65.55 kDa). Cell culture with IPTG induction produce thicker protein band compared to cell culture without IPTG induction.



Picture 4. Sequencing analysis result of pETDUET_*cyp/ads*. A. Sequencing analysis result of *ADS* gene, (i) forward primer pET_UPSTREAM, (ii) reverse primer DUET_DOWN1; B. Sequencing analysis result of *CYP71AV1* gene, (i) forward primer DUET_UP2, (ii) reverse primer T7 Terminator

The next optimization was against the temperature of overproduction condition. Cell cultures was grown with IPTG 0.5 mM induction and incubated at temperature 28 °C and 37 °C. Based on SDS-PAGE analysis, protein bands resulted from cell culture incubated at 28 °C was thicker compared to protein bands resulted from cell culture incubated at 37 °C, either on supernatant and pellet cells. But protein bands resulted from pellet cells was thicker than in supernatant either from cell culture incubated at 28 °C nor 37 °C (Picture 5B).



Picture 5. Electrophoregram of SDS-PAGE. A. Cell lysis extraction, (1) Non IPTG induction, (2) With IPTG 0.5 mM induction, (M) Unstained protein marker; B. CYP71AV1 and ADS production, (M) Unstained protein marker, (P28) Pellet cells at 28 °C, (P37) Pellet cells at 37 °C, (S28) Supernatant at 28 °C, (S37) Supernatant at 37 °C.

This result showed that CYP71AV1 and ADS protein produced in this research still found as inclusion bodies form. Therefore, solubilization and renaturation against those proteins is needed to give protein in soluble form.

Co-expression of two genes in pETDUET1 probably will produce protein in inclusion bodies form. Other research using pETDUET1 vector for co-expression of *staphain A* gene and *staphostatin A* gene from *Staphylococcus aureus* also produced protein in inclusion bodies form, so protein refolding was done in order to get soluble protein (Wladyka, 2005).

Acknowledgment

Thank you to Indonesian Ministry of Health for funding this research through RISBIN IPTEKDOK 2013.

References

[1] Duffy PE, Mutabingwa TK, Artemisinin combination therapies, **Lancet,** 367, 2037–2039, 2006.

[2] Hale, V., Keasling, JD., Renninger, N and Diagana, N.N, Microbially Derived Artemisinin: A Biotechnology Solution to the Global Problem of Access to Affordable Antimalarial Drugs, **Am. J. Trop. Med. Hyg**., 77(Suppl 6), 198–202, 2007.

[3] Julsing, M. K., Pouderoyen G. V., Veen J. V., Woerdenbag H. J., Bioconversion and combinatorial biosynthesis of selected terpenoids and lignans, **Thesis**, Department of Pharmaceutical Biology of The University of Groningen, Netherlands, 4, 47-56, 2006.

[4] Li Y, Huang H, Wu YL, Qinghaosu (artemisinin)—a fantastic antimalarial drug from a traditional Chinese medicine, In: Liang XT, Fang WS (eds) **Medicinal Chemistry of Bioactive Natural Products**, Wiley, New York, pp 183–256, 2006.

[5] Mutabingwa TK. Artemisinin-based combination therapies (ACTs): best hope for malaria treatment but inaccessible to the needy! **Acta Trop**. 95:305–315. doi: 10.1016/j.actatropica.2005.06.009.

[6] Rathore, D., McCutchan, T.F., Sullivan, M., and Kumar, S., Antimalarial drugs: current status and new developments. Expert Opin. **Investig. Drugs**, 14, 871–883. doi:10.1517/13543784.14.7. 871. PMID:16022576, 2005.

[7] White NJ, Qinghaosu (artemisinin): the price of success, **Science,** 320, 330–334, 2008.

[8] Wladyka, B., Puzia, K., and Dubin, A., Efficient Co-expression of a Recombinant Staphopain A and Its Inhibitor Staphostatin A, **Biochem. J.**, 385, 181-187, 2005.