



Genetic Stability Test of *Pichia pastoris* Carrying Integrated Pre-membrane Envelope Gene of DENV3 Indonesian Strain

Asri Sulfianti^{1,3}(✉), Angelina Gill^{2,3}, Astutiati Nurhasanah^{1,3}, Tika Widayanti^{1,3}, and Sabar Pambudi^{1,3}

¹ Research Center for Vaccine and Drugs Development, The National Research and Innovation Agency Republic of Indonesia (BRIN), Serpong, Tangerang Selatan 15310, Indonesia
asri.sulfianti@brin.go.id

² Indomabs Biosaintika Utama Ltd., Serpong, Tangerang Selatan 15310, Indonesia

³ Center for Biomedical Research, Research Organization for Health, National Research and Innovation Agency, Indonesia (BRIN), Cibinong, Bogor 16114, Indonesia

Abstract. In recent years, the methylotrophic *Pichia pastoris* has become an increasingly famous host for recombinant protein expressions. As a eukaryote, this yeast offers several advantages, including simplicity of genetic manipulation, stable expression, and low-cost scalable fermentation techniques. Previous study has confirmed the insertion of Dengue Virus Serotype 3 (DENV3) pre-Membrane Envelope (pr-M/E) gene in the recombinant *P. pastoris* X33 generated in our laboratory. The study has also confirmed the strain's ability to express the protein. In this study, we are reporting the genetic stability of the recombinant strain, confirming the steady expression of the heterologous protein in subsequent generations. The genetic stability test was performed by PCR and DNA sequencing on the recombinant *P. pastoris* cultured in non-baffled shake flasks. Generation time was estimated based on the yeast growth curve and calculated using a previously published formula. According to the growth curve, the generation time of this correlates recombinant yeast is four hours. It differs from the wild type, which took 4.3 h to complete. PCR of target gene performed at generation 1, 18, 39, 56, 81, and 100 revealed two DNA bands which indicating the presence of full AOX1 gene (2.2 kb) and AOX1 promoter plus pr-M/E gene (2 kb). In addition, sequencing of the PCR products show only minor variation, which might have been genuine or a result of PCR or sequencing errors. However, because the amino acid sequences of generation 100 differed from neither the RefSeq nor the original plasmid, we predict that our recombinant *P. pastoris* stably contains the gene of interest.

Keywords: dengue virus Serotype 3 · pre-Membrane Envelope · *Pichia pastoris* · genetic stability test · dengue vaccine

1 Introduction

Dengue fever, a viral disease spread by mosquitos, is endemic in over a hundred countries [1, 2]. As of May 5, 2022, 785. 736 cases and 588 deaths had been documented.

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The majority of cases have been reported from Brazil (607,546) whereas the majority of deaths have occurred in Indonesia (229) [3]. Since no antiviral medication is not available in Dengue treatment, prevention of infection by vaccination is most important. In addition, unexpected challenges in developing dengue vaccines, particularly in terms of safety, have rekindled interest in non-viral dengue vaccines [1]. Although there has been Dengue vaccine clinical trial conducted in Indonesia (Dengvaxia, Sanofi Pasteur), safety re-evaluation of the product by National Agency for Drug and Food Control, Republic of Indonesia, showed that the administration of the vaccine to immunologically naive individuals (seronegative) caused severe Dengue Hemorrhagic Fever (DHF) and increased the risk of hospitalization [4]. Therefore, development of Dengue vaccine is suitable for Indonesian population is crucial.

There are four Dengue virus subtypes DENV-1, DENV-2, DENV-3, and DENV-4, belonging to genus *Flavivirus* and *Flaviviridae* family. In our study, we focused on DENV-3 and make use of protein E (Envelope glycoprotein) and pr-M (non-glycosylated pre-Membrane protein) in our vaccine design. The pr-M/E proteins are two Dengue structural antigens that have a role in virus infection. Co-expression of these proteins are sufficient to produce recombinant VLPs, which have the shape and physicochemical properties of infectious virions [4, 5]. Membrane associated protein is an M glycoprotein of about 26 kDa which helps E protein to form a mature virus particle. During the maturation of virion, pre-Membrane (pr-M) protein generated the signal peptide, which was efficiently cut off when VLP mature. The E glycoprotein, on the other hand, is a surface protein that is responsible for host receptor recognition and host membrane fusion during infection. This protein is a major component of the viral particle, which is around 55 kDa in size, and has become the primary target of virus neutralizing antibodies generated during natural infection [1, 4, 6, 7].

Using recombinant DNA technology, infectious genetic material-free virus-like particles (VLPs) which was made exclusively of viral coat proteins are employed as vaccine because they can induce robust immunity without generating infection [5]. Previously, we successfully constructed a recombinant plasmid, from a shuttle vector pPICZ α -A, which contains pre-Membrane Envelope (pr-M/E) gene of DENV3 Indonesia strain. The plasmid is named pPICZ α -A-pr-M/E DENV3. Sequence analysis showed that the gene encoding the recombinant protein shared 100% similarity with the original protein sequence from the Indonesian wild type of dengue virus strain.

This recombinant plasmid was linearized and used to transform *Pichia pastoris* X33. Yeast *P. pastoris* has become one of the most prominent expression methods for the creation of commercially useful recombinant proteins. As a lower eukaryote, it possesses both prokaryotic (simple and economical handling) and eukaryotic (high cell density) characteristics (equipped for performing many post-translational modifications) [6, 7]. The utilization of a high yielding yeast system to produce these VLPs offers enormous promise for the creation of a dengue vaccine that is not only safe and effective, but also cheap, for use in resource-poor countries where dengue is widespread [1]. When used to transform *P. pastoris* X33, linearized pPICZ α -A will form single cross over and integrate into the yeast's genome. *P. pastoris* X33 possesses an AOX1 promoter gene in their chromosome, which allows for tight protein expression regulation [8, 9, 10]. This promoter gene is also the site of the plasmid integration (Fig. 1), thereby, two copies

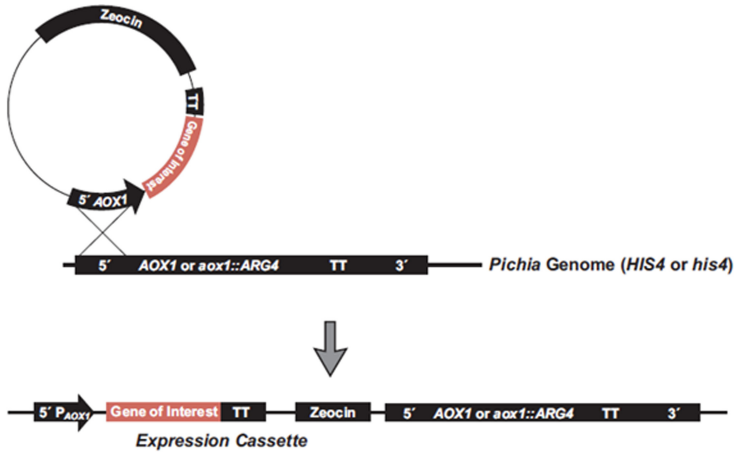


Fig. 1. The result of an insertion of the plasmid 5' to the intact AOX1 locus and the gain of PAOX1, the inserted gene, and the Zeocin™ resistance gene (figure copied from *P. pastoris* X33 Manuals, Invitrogen)

of the promoter gene will be in the yeast's modified genome. In this study, we assessed the genetic stability of the recombinant *P. pastoris* that were developed previously by our research group. This study proved that the recombinant *P. pastoris* stably carries the gene of interest for 100 generations.

2 Materials and Methods

2.1 *P. pastoris* Strain

P. pastoris Mut + strain X33 carrying an integrative plasmid pPICZ α -A with inserted DENV-3 pr-M/E gene were used in this study. The recombinant yeast was developed by the Dengue Research Group, Centre for Pharmaceutical and Medical Technology, BPPT, and currently is part of the collection at the Centre for Vaccine and Drug Research, BRIN.

2.2 Estimation of Generation Time

To test the stability of a genetic *P. pastoris* transformant over 100 generations, the yeast generation times must first be determined. Stock culture was spread on YPD agar media (Sigma Aldrich, US) containing 100 g/ml Zeocin (InvivoGen, US) and incubated for three days at 30 °C. A single colony was picked up and used to inoculate in 100 ml BMGY media (Sigma Aldrich, US) (1% yeast extract; 2% peptone; 100 mM potassium phosphate pH 6.0; 1.34% YNB; 4 × 10⁻⁵% biotin; 0.5% methanol) (10). For 24 h, the culture was incubated at 250 RPM in 30 °C, before being used as seed for the subsequent culture in 100 ml BMGY (initial OD₆₀₀ was set to 0.1). For 48 h, the cell was cultivated at 250 RPM in 30 °C. During this time, the yeast growth curve was observed. The culture

was sampled every 4–6 h, and estimation of the generation time was determined using the following formula (11):

$$t_{gen} = \frac{t \log 2}{\ln X_t - \ln X_{t_0}}$$

t_{gene} : generation time

$x_{0,t}$: OD600 at times zero and t

t: the elapsed time during which growth is measured

2.3 Sampling of *P. pastoris* Culture to Assess the Genetic Stability

In a 500 ml non-baffled flask, a single colony of *P. pastoris* was cultured in 100 ml of BMGY media (Sigma Aldrich, US). The culture was grown in 30 °C for 20 days at 250 RPM. One ml culture was taken at each suitable time point and centrifuged at room temperature 12,000 rpm for 3 min to harvest the cells. The pelleted cells were kept at –80 °C until needed for genetic stability evaluation by PCR and sequencing. Cultivation was continued up to 100 generations, and harvests were performed at generation 5, 38, 69, and 100.

2.4 PCR Reaction and Gel Electrophoresis

Lysis of the *P. pastoris* cells was performed by heating. Twenty microliters cell was placed in a 1.5 ml tube containing 180 µl PBS 1X and heated to boil. The genomic DNA from the crude cell lysate was then utilized as the template for PCR. The PCR was performed using Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, US) with AOX1 universal primers (AOX1 5' primer: 5' GACTGGTTCCAATTGACAAGC 3' and AOX1 3' primer: 3' GCAAATGGCATTCTGACATCC 5'). The cycles of PCR were carried 30 times at the following condition steps: 98 °C, 30 s for pre-denaturation step; 98 °C, 5 s for denaturation step; 55 °C, 5 s for extension step; 72 °C, 1 min 30 s for polymerisation step; and 72 °C, 1 s for extension step. Visualization of the amplicon was performed by gel electrophoresis technique. Previously, 1% of agarose gel was put into Tris Acetic Acids (TAE 1X) buffer in electrophoresis chamber (Bio-Rad, US). PCR product that has been added by loading dye buffer (Thermo Fisher Scientific, US) was load on agarose gel. Gel was run at 100 V until the dye line is approximately 80% of the way down the gel. DNA fragments or referred to as bands will be seen after putting the gel into the EtBr solution and viewed under the UV light. The successfulness of PCR will be showed by two bands, which represented as full AOX1 gene at 2.2 kb and AOX1 promoter plus pr-M/E gene at 2 kb.

2.5 Sequencing of PCR Products

PCR products from generation 5 and 100 were Sanger sequenced using the universal AOX primer pair as used in the PCR reaction, and primers d3s3, d3s4, d3s5, d3a18, d3a19, and d3a20 as described in previous publication (12). The entire sequencing process was carried out using paid services from Genetika Science Ltd.

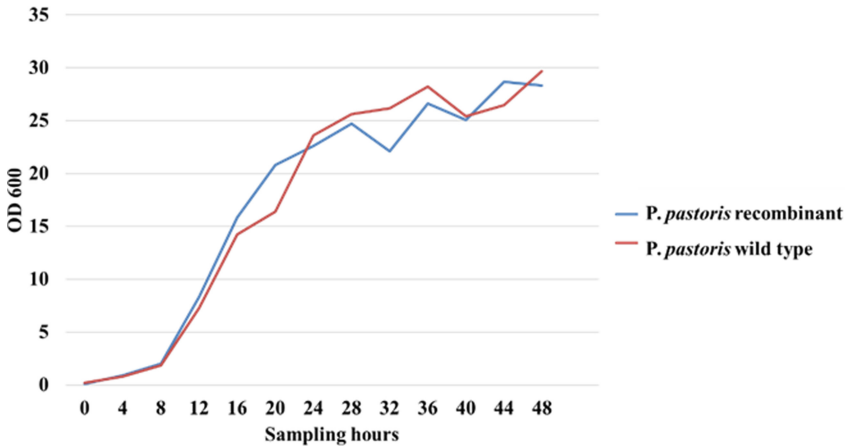


Fig. 2. Growth curve of *P. pastoris* X33 wildtype compared to the same strain carrying integrated DENV3 pr-M/E gene

2.6 Construction of Contigs and Bioinformatic Analyses

Sequencing results and contigs were visualized using BioEdit©. Sequences were edited when necessary before being used to construct contigs. Contigs were constructed from selected sequencing results using online tools CAP3 Sequence Assembly Program as provided by PRABI-Doua (Pôle Rhône-Alpes de Bioinformatique Site Doua) (<http://doua.prabi.fr/software/cap3>). Contigs were translated into amino acid sequences, which were then aligned to the translated reference sequence and original insert of plasmid pPICZ α -A-pr/ME, using BioEdit©, to determine the presence/absence of variations. DENV3 pr/ME sequence originated from DKI Jakarta, Indonesia was used as reference sequence (RefSeq) in this study.

3 Results

3.1 Growth Evaluation

Based on our observation, the growth started with 4 h lag phases, followed with logarithmic phase between 4–24 h. Afterwards, the culture entered stationary phase (Fig. 2). Generation time was calculated using t_0 : 4, t_t : 24. Estimation of generation of the recombinant culture was around 4.0 h compared to the wild type at 4.3 h. Thus, it took 17 days to study 100 generation.

3.2 PCR Evaluation of Integrated pr-ME Gene

Based on sampling at generations 5, 38, 69, and 100, it was discovered that all generations studied had pr-M/E gene integrated into their genomes. It was demonstrated by two distinct bands on an agarose gel, one for full AOX1 gene (2.2 kb) and the other for AOX1 promoter plus pr-M/E gene (2 kb) (Fig. 3). Thus, the gene was present in generation 5, 38, 69, and 100. The amplicons of generation 5 and 100 were later sequenced to confirm the stability.

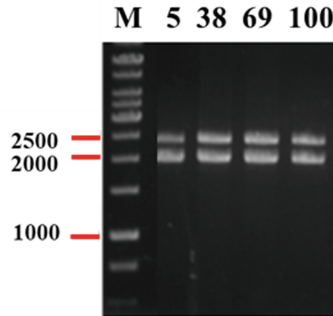


Fig. 3. PCR with AOX1 primers showed that the pr-M/E gene was integrated in the genome of generation 5, 38, 69, and 100.

3.3 Alignment of Generation 5 and 100 pr-M/E Gene to the DENV3 pr-M/E Reference Sequence

pr-M/E amino acid sequence of generations 5 and 100 were aligned to the translated RefSeq and original insert. The alignment was generated with the ClustalW Multiple Alignment in Bioedit® Software. Residues are colored coded according to their conservancy. Our finding shows one hundred percent conservancy of generation 100 558-amino acids in comparison to RefSeq and the original plasmid. On the contrary, five amino acid substitutions and one deletion were observed in generation 5, hence 89.60% similarity (Fig. 4).

4 Discussion

So far, the dengue vaccine remains an unmet need. Many known live attenuated vaccines face significant challenges, especially in the difficulties of eliciting a balanced immune response against all four DENV serotypes. This served as the foundation for subsequent findings using non-replicating dengue vaccinations. In this regard, genome-free dengue VLPs could be a promising alternative [4]. It is because VLP is structurally and physiochemically like native viruses but lacks infectious genetic content. In addition, it also made up of envelope structural proteins which are easily recognized by the immune system and can elicit both B-cell and T-cell immune responses [2, 13, 14].

Previously, we successfully integrated the recombinant plasmid which contains the pr-M/E gene of DENV3 strain Indonesia on *P. pastoris* X33 strain. We established the *P. pastoris* to produce homologous protein because yeast cultures are easy to maintain, which makes them faster and cheaper to use than other eukaryotic expression system [7, 13]. Moreover, this yeast offers a very strong methanol-inducible alcohol oxidase (AOX1) promoter which regulate the productivity of its gene interest [15]. The integration of the gene interest to the AOX1 *P. pastoris* chromosome via homologous recombination generates the stable clones which can be used in high scale fermentation process over generations [16].

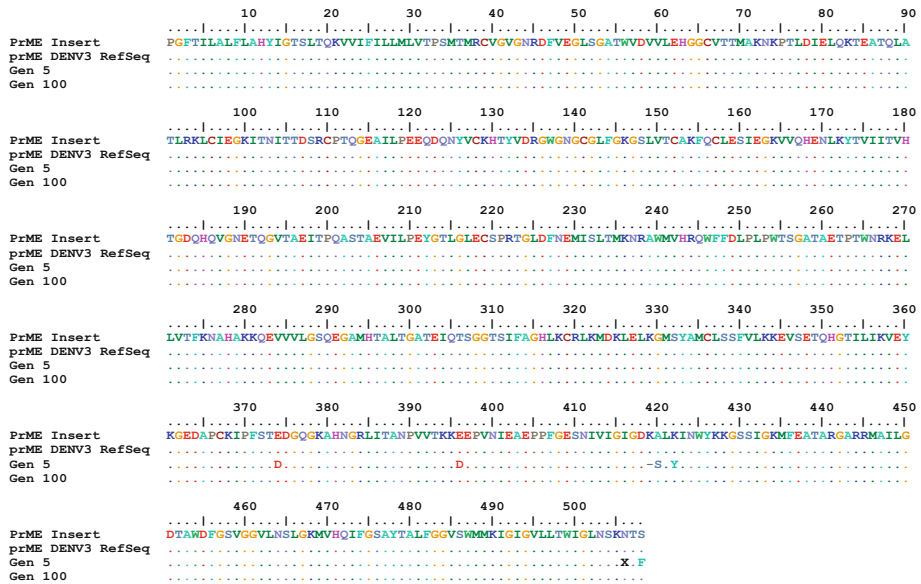


Fig. 4. Amino acid sequence alignment of predicted pr-M/E sequence from generations 5 and 100, compared to the pr-M/E protein in recombinant plasmid and the DENV3 DKI Indonesian strain as the reference sequence.

Electron microscopy revealed that the pr-ME recombinant protein was seen as VLP as we predicted (data not shown). However, to scale up the production, we must confirm the seed stability to produce the required protein over generations, thus, in this study we tested the genetic stability of the recombinant *P. pastoris* over 100 generations. To start with, we investigated the generation time of the recombinant *P. pastoris*. The generation or doubling time is the amount of time that it takes for a cell to double in number during a specified period [23]. An optical density at 600 nm (OD600 nm), which correlates to cell population in liquid suspension, is generally used to calculate yeast doubling time at different time intervals during log phase development at 30 °C with vigorous shaking [17, 18].

The growth curve shows that the generation time of this correlates recombinant yeast is four hours. It differs from the wild type, which took 4.3 h. However, this finding is consistent with the theory which described that every yeast double in 4–6 h [10]. In accordance with this finding, the recombinant strain was cultivated for 17 days to reach 100 generations. Sampling of cultures were performed at certain time points, estimated based on the generation time. Thus, sampling of generations 5, 38, 69 and 100 were performed at 20, 152, 276, and 400 h, respectively, after inoculation.

The single cross over event during plasmid integration to the genome produce two copies of AOX1 gene [10] (Fig. 1). Since the AOX1 primers used in PCR bind to these genes, two bands were observed, indicating the presence of full AOX1 gene (2.2 kb) and AOX1 promoter plus pr-M/E gene (2 kb) [19, 20]. PCR is the most sensitive and specific molecular assay, with results that are extremely accurate and can be evaluated

quickly [21]. However, because we used crude cell lysates as PCR templates without further purification in this study, there was high chance that there were impurities that might affect the reaction and hence the DNA sequences obtained. One possible impurity is proteinase, which is known to interfere with PCR [22]. Yet the presence of endogenous protease in *P. pastoris* X33 had been reported before [23]. If this was the case in our investigation, it is possible that the endogenous enzyme influenced the polymerase enzyme in the reaction mixture, and hence the sequence alterations reported in generation 5 were not genuine but an artifact. However, since the amino acid sequences of generation 100 showed no variation from either the RefSeq or the original plasmid, we predict that our recombinant *P. pastoris* stably carries the gene of interest. To demonstrate the stability at protein level, the expression analysis of each generation must yet be carried out.

5 Conclusion

Previous research has confirmed the insertion of the Dengue Virus Serotype 3 (DENV3) pre-Membrane Envelope (pr-M/E) gene into the recombinant *P. pastoris* X33 produced in our lab. The study also confirmed that the strain can express the protein. We report the genetic stability of the recombinant strain in this study, confirming the steady expression of the heterologous protein in subsequent generations. The genetic stability of recombinant *P. pastoris* cultured in non-baffled shake flasks was tested using PCR and DNA sequencing. The generation time was estimated using a previously published formula based on the yeast growth curve. The generation time of this correlates recombinant yeast is four hours, according to the growth curve. It is not the same as the wild type, which took 4.3 h to complete. PCR of the target gene at generation 1, 18, 39, 56, 81, and 100 revealed two DNA bands indicating the presence of the full AOX1 gene (2.2 kb) and the AOX1 promoter plus pr-M/E gene (2 kb). Furthermore, sequencing of the PCR products reveals only minor variation, which could be genuine or the result of PCR or sequencing errors. However, because neither the RefSeq nor the original plasmid amino acid sequences differed from generation 100, we predict that our recombinant *P. pastoris* stably contains the gene of interest.

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