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Construction and Expression of Synthetic Gene Encoding MPT64 as Extracellular Protein in *Escherichia coli* BL21 (DE3) Expression System

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Abstract

Aim: This study was purposed to produce MPT64 protein extracellularly by contructing pelB signal peptide into expression vector containing synthetic gene of mpt64 from *Mycobacterium tuberculosis* H37Rv

Methods: The mpt64 synthetic gene was derived from gene sequence of *M. tuberculosis* H37Rv. Therefore, the mpt64 gene sequence need codon optimization using a graphical codon usage analyzer (GCUA) to improve gene expression in *E. coli*. The optimized codon then design and construct into an *E. coli* expression vector (pD861-SR) containing such component that high replication, strong RBS, induction system with rhamnose and supplemented with kanamycin selection markers. The pelB gene, a gene coding for extracellular proteins was inserted into the vector. The transformation of pD861-SR plasmid into *E. coli* BL21 (DE3) was conducted using electroporation method and the isolated plasmid was verified by sequencing method. The overproduction of MPT64 protein was induced by 4 mM rhamnose in different time of induction. Followed by characterization of MPT64 protein using sodium dodecyl sulfate polyacrilamide electrophoresis (SDS-PAGE).

Results: The gene sequencing results showed there was no nucleotide changes in the sequences of mpt64 and pelB genes. Based on SDS PAGE analysis, the pelB signal peptide could translocate the MPT64 protein into the medium as an extracellular protein with a molecular weight of 24 kDa.

Conclusion: The expression of mpt64 gene combined with pelB as signal peptide could be a choice design in plasmid recombinant construction to yield MPT64 as extracellular protein.

Keywords: MPT64, *Mycobacterium tuberculosis* H37Rv, pelB, extracellular, *Escherichia coli*

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. TB has been existed for thousands of years and is still a major global health problem. This disease causes health problems in millions of people every year. In 2015, TB is one of the 10 causes of death worldwide. It is estimated that there are 10.4 million new TB cases worldwide, of which 5.9 million (56%) are male, while 3.5 million (34%) are female and 1.0 million (10%) among them are children [1]. In 2015, there were 330.729 tuberculosis patients in Indonesia. The increasing of tuberculosis patients in the overall age has induced the need for rapid and accurate detection of *M. tuberculosis.*

The diagnosis is an important effort to treat TB patient promptly and to control TB spread among the population. There are several established method to detect TB, such as: sputum microscopy, chest X-ray, culture in growth media, and nucleic acid amplification using polymerase chain reaction (PCR) method [2]. Among those of TB diagnostic method, sputum observation using microscopy is a simple, rapid, and inexpensive tool to diagnose the pulmonary TB, but it has low sensitivity [1]. Moreover, the common TB diagnosing methods are conducted by isolating the pathogen, which is difficult to obtain the active cells of *M. tuberculosis* in high concentration or to ascertain the site of infection. These methods may take long process, an infected patient may spread the TB pathogen to others. Early detection of TB patients can help prevent TB transmission. Beside that, some of the methods require expensive chemical, tools, and trained personnel so that they cannot be applied practically in all health services, especially in developing countries. Because of these, TB patients are usually treated using empiric anti-TB treatment, based on clinical symptoms diagnosing and without microbiological examination. The most severity problems are that the similar symptoms might produce by different kind of mycobacterium, not only *M. tuberculosis* complex (MTBC) but also mycobacteria other than tuberculosis (MOTT). The differentiation of both mycobacterium is important to decide the choice of treatment. Thus, it is very essential to use the diagnostic component that can differentiate both Mycobacterium groups.

The *M. tuberculosis* protein 64 (MPT64) is a specific protein (24 kDa) of *M. tuberculosis* (MTB) and encoded by the RD2 region genes which secreted during the bacterial growth [3-5]. This protein is a specific antigen that can differentiate the MTBC from MOTT [6,7]. In addition, the sequence of mpt64 encoding gene is sustainable, thus it can be used for diagnostic purposes [8]. Therefore, MPT64 protein can be used as a diagnosis tool for tuberculosis. In the process, large amounts of MPT64 protein are needed to produce antibodies against the secreted MPT64 protein. The interactions of anti-MPT64 with MPT64 can be developed through a more simple and economical immunochromatography test.

In this study, *E. coli* acts as a host cell that will produce recombinant MPT64 protein. Considering that *E. coli* also produces other proteins and the plasmid construction did not use protein coding genes for purification using the affinity method, so that a strategy is needed to facilitate the acquisition of the target MPT64 protein. However, it is not uncommon for recombinant proteins, do not produce their conformations correctly. Furthermore, those proteins can undergo proteolytic degradation or interact with each other to produce insoluble aggregates or nonnative proteins known as inclusion bodies [9]. To overcome these problems, the secretion of protein was targeted to the periplasm or culture medium, is called extracellular expression. The extracellular production of recombinant protein secretion has several advantages than intracellular production, such as: overcoming the inclusion body because it increases protein folding, reduces proteolytic degradation and facilitates better purification [10]. For these reasons, the peptide signal coding gene is inserted into the recombinant plasmid. This signal peptide

plays a role in translating protein into the medium as an extracellular protein. Some signal peptides that are widely used are PelB, OmpA, PhoA, PhoE, Lpp, OmpF, OmpC, TorA, Tap, endosilanase, sfmC, and StII. Among these signal peptides, PelB is one of the best signal peptides that can be used for extracellular protein secretion in *E. coli* [11].

MATERIALS AND METHODS

Materials

The materials used were *E. coli* BL21 (DE3) empty, pD861-pelB-MPT64 recombinant plasmid (synthesized by ATUM, California, USA), bacto, agarose, kanamycin antibiotics, 10% APS, hydrochloric acid , distilled water, bis-acrylamide 40% (Biorad), SDS-PAGE electrophoresis buffer pH 8.3, lysis buffer pH 8.6, SDS sample buffer, TAE buffer, TSE buffer pH 8.0, buffer tris-HCl pH 7.5 10 mM, tris-HCl buffer pH 6.8, Tris-HCl buffer pH 8.8, Commassie Briliant Blue R-250 staining solution, destaining solution, yeast extract, absolute ethanol, 70% ethanol, GelRed (Biotium), 85% glycerol, glucose, potassium chloride, plasmid isolation kit (TianGen Miniprep Kit), loading dye (Thermo), L-ramnosa, 1 kb Ladder (Thermo) DNA markers, unstained protein markers (PeqLab), sodium chloride, nuclease free water, PMSF, SDS, TEMED, tripton, and urea 8M.

Gen mpt64 Design and Optimization

In this study, MPT64 gene design was prepared using synthetic genes. The nucleotide sequence of the mpt64 (687 bp) gene used was derived from the *M. tuberculosis* H37Rv with the access code in Genbank: AY208674 (www.ncbi.nlm.nih.gov). The nucleotide sequence of the mpt64 gene was expressed in *E. coli* BL21 (DE3). Therefore, the adjustment of the mpt64 gene amino acid coding codon from *M. tuberculosis* H37Rv was adapted to the codon reading system in *E. coli* using a graphical codon usage analyzer (GCUA). The codon optimization results were then translated by Blastx analysis to obtain an amino acid sequence. The selection of vectors used in this study was carried out using ATUM (DNA 2.0) application with the specification of expression vectors that had high replication ability, strong RBS, induction with rhamnose and supplemented with kanamycin selection markers. In this research, synthetic peptide signal pectate lyase B (pelB) was used. This addition was made because the target MPT64 protein would be secreted into the medium, so that protein acquisition was easier than making it as an intracellular protein. From these optimizations, the type of expression vector was obtained and completely contain of the mpt64 target gene, pD861-SR: 319895-mpt64-pelB (hereinafter referred to as pD861-SR).

Preparation of pD861-pelB-MPT64

Paper containing genes was placed into a sterile petri dish using sterile tweezers. A total of 100 µL sterile pH 7.5 Tris-HCl buffer with a concentration of 10mM was added right in the middle of the paper containing the gene. After that, the paper containing the genes was rolled using sterile tweezers and put into a sterile 0.6 mL micro tube that had been perforated on the bottom using a sterile needle, then the micro tube was inserted into a 1.5 mL sterile microtube and centrifuged for 1 min at 12.000 rpm. The supernatant collected in a 1.5 mL sterile microtube was taken. The a volume of 90µL supernatant containing 20 ng/µL of the vector gene was diluted to a concentration of 2 ng/µL.

Preparation of Competent *E. coli* Cells

One Ose of *E. coli* colonies from slant agar was inoculated into 5 mL of Luria Bertani (LB) broth media and incubated for 20 h in a shaker incubator at 37 °C, 180 rpm. A volume of 1 mL bacterial suspension was taken and inoculated with 100 mL of LB broth. The media then incubated for 3.5 h in the shaker incubator at 37 °C, 180 rpm. Each of 25 mL cell suspension was poured into the falcon tube and centrifuged for 5 min at a speed of 5.000 rpm, until pellet cells and supernatants separated. The supernatant obtained was discarded, then the bacterial cell suspension was re-poured and centrifuged. This process was repeated, until the entire cell suspension was exhausted. The cell pellets obtained were then resuspended using 5 mL sterile distilled water, then centrifuged at 5,000 rpm for 5 min and the supernatant was discarded. The pellets obtained were then resuspended with 10% glycerol sterile and stored at -20^{o}C as stock culture.

Plasmid Transformation

The pD861-SR transformation into the *E. coli* was conducted using electric field-mediated transformation (electroporation) method. A total of 2 µL pD861-SR was suspended into a microtube containing 50 µL of competent cells. Before electroporation, the media was pre-warmed to room temperature, then agar plates containing antibiotic were incubated to 37°C, and electroporation cuvettes were cooled on ice. Bacterial cells mixed with pD861-SR were loaded into the electroporation cuvette and run for a few milliseconds. The mixture then resuspended into to a test tube containing 1 mL LB broth and incubated at 37^{o}C for 4 h. After that, 100 µL of cell suspension containing pD861-SR was spread onto a petri dish containing LB-kanamycin medium (25 µg/ mL) and incubated at 37 °C overnight.

Selection and Characterization of Recombinant Clones

Bacteria transformed with the plasmid (transformant) should form colonies on the media containing kanamycin. The transformant colonies were re-inoculated on new agar plates using the streak plate method to isolate a single colony. Then pD861-SR plasmids were isolated using the TIANprep Mini Plasmid Kit to confirm that the transformed plasmid. A total of 1 transformant colony was taken from agar plates, then inoculated into 5 mL LB medium containing with kanamycin antibiotics 25 µg / mL. The culture was incubated for 12-16 h at 37ºC ,180 rpm. The process of isolating plasmid DNA should use 1-5 mL of bacterial culture. A total of 1 mL of bacterial suspension was put in a 1.5 mL microtube and centrifuged at a speed of 12,000 rpm for 1 min at 25^{o}C, then the supernatant was discarded. The centrifugation process was repeated until the bacterial suspension run out. Bacterial pellets were added with 250 µL of P1 buffer, resuspended until late, then distorted. Then 250 µL of P2 buffer was added and inverted 6-8 times. After that, 350 µL of P3 buffer was added and inverted 6-8 times. The mixture was then centrifuged at a speed of 12,000 rpm for 10 min. The supernatant was taken and put into a balanced CP3 column. Then the CP3 column was centrifuged at a speed of 12,000 rpm for 1 min, then the supernatant was discarded. Followed by the addition of 500 µL of PD buffer and then centrifuged at 12,000 rpm for 1 min. The obtained supernatant was discarded. Then 600 µL of PW buffer was added, centrifuged at a speed of 12,000 rpm for 1 minute, then the supernatant was discarded (done 2 times). The empty CP3 column was centrifuged at a speed of 12,000 rpm for 2 min. Then the CP3 column was transferred to a micro tube and added 60 µL of EB buffer to the center of the CP3 column, allowed to stand for 2 min, then centrifuged at 12,000 rpm for 2 min. Then 40 µL of EB buffer was added to the center of the CP3 column, allowed to stand for 2 min and centrifuged at a rate of 12,000 rpm for 2 min. Then the supernatant obtained has contained a plasmid isolates. The characterization of isolated plasmids was carried out using agarose gel electrophoresis method. The first analysis was a plasmid migration analysis. Each sample contained 5 µL plasmid, 1 µL loading dye, and 1 µL of gelRed was put into well of 1% (b / v) agarose gel. The electrophoresis was run at a voltage of 80 V for 40 min. After that the gel was observed under UV light. Then, the second analysis was the restriction analysis with two restriction enzymes (EcoRI

and SapI) then characterized by agarose gel electrophoresis. The third analysis was the confirmation of nucleotide sequences using sequencing method with forward primer and reverse primer. The primers were designed using PrimerSelect (DNASTAR) software.

Bacterial Growth Curve

From the glycerol stock, 1 Ose of transformant colony was inoculated into 5 mL of LB broth media containing 30 µL of kanamycin (25 µg / mL) and incubated overnight at 37 °C. Then, a volume of 1 mL cell suspension was re-inoculated into 99 mL LB broth media containing 494 µL kanamycin (25 µg/mL). The medium was then incubated at 37 °C. Every hour, the turbidity of the bacterial suspension was measured using a spectrophotometer on OD600.

Protein Overproduction

One Ose of *E. coli* BL21 (DE3) [pD861-SR: 319895] was inoculated in 5 mL LB broth medium containing 30 µL kanamycin (25 µg/mL) and incubated for 16-18 h at 37 °C, 180 rpm. Furthermore, a volume of 1 mL bacterial suspension was transferred and inoculated to Erlenmeyer flasks containing 99 mL of LB broth and kanamycin. Then, the culture was incubated at 37 °C with a shaking rate of 180 rpm, for 3 h, until the absorbance of bacterial suspension reached 0.6-1 at OD600. A volume of 1 mL culture was taken and L-Rhamnosa was added to the culture until the final concentration of rhamnose reached 4 mM. The culture was then re-incubated at 37 ° C, 180 rpm, 24 h. The protein harvest in the supernatant /medium was carried out after incubation for 18, 19, 20, 21, 22, 23, and 24 h. The isolation of extracellular proteins was carried out using centrifugation at 6,000 g, 4 ° C, for 20 min to separate pellets cell and its supernatant. The supernatant was taken and transferred to a new microtube, then PMSF was added to the final concentration of 1 mM culture.

Results And Discussion

Results of Gen mpt64 Design and Optimization

Mpt64 coding gene expression occurs in *E. coli* BL21 (DE3) as host cells. The *E. coli* is one of the microorganisms commonly used to produce recombinant proteins. It has been known as a protein-producing cells and has become the most common expression platform. For this reason, there are many molecular tools and protocols for the production of heterologous proteins in high levels, such as a large catalog of expression plasmids, a large number of engineering strains and many cultivation strategies. But due to the difference in the origin of the source mpt64 gene from the host cell used, so the mpt64 coding gene sequence must be optimized. The purpose of this optimization was to increase the percent adaptive gene reading in the process of protein synthesis in *E. coli*. The optimization process was carried out using the Graphical Codon Usage Analyzer (GCUA) web server. The first thing to do is to check the adaptive percent of the mpt64 gene sequence by analyzing it through the web so that it got a percent view of amino acid adaptive before the optimization process was carried out. The result of GCUA analysis was performed at figure 1.

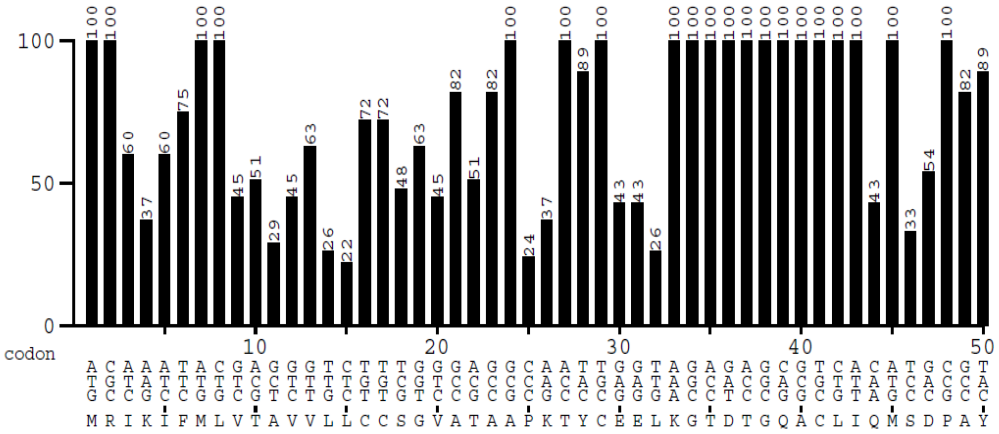

Figure 1: Graph of codon frequency percentage before the optimization process using GCUA

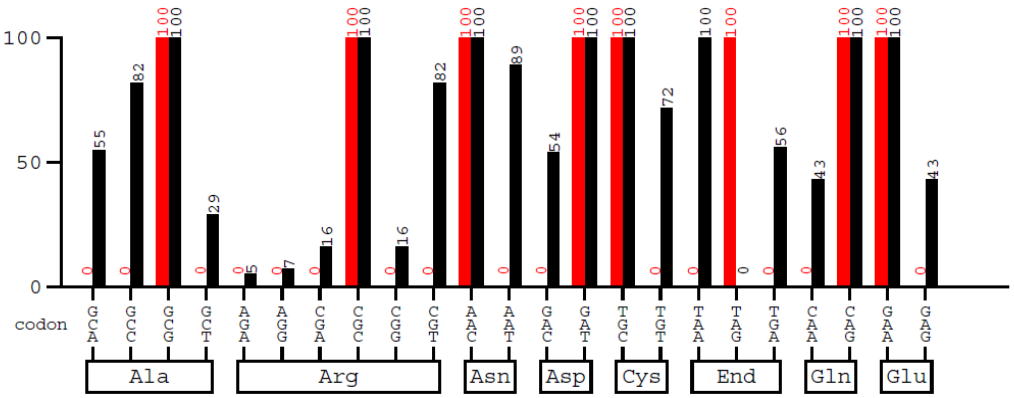

Figure 2: Percent adaptive gene sequence (the red bar diagram showed the percent adaptive gene sequence derived from *M. tuberculosis* H37Rv. The black bar diagram showed the percent adaptive gene sequence in *E. coli*)

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ATGCGCATTAAAATTTTTATGCTGGTGACCGCGGTGGTGCTGCTGTGCTGCAG
CGGCGTGGCGACCGCGGCGCCGAAAACCTATTGCGAAGAACTGAAAGGCACCG
ATACCGGCCAGGCGTGCCTGATTCAGATGAGCGATCCGGCGTATAACACCAAC
ATTAGCCTGCCGAGCTATTATCCGGATCAGAAAAGCCTGGAAAACTATATTGC
GCAGACCCGCGATAAATTTCTGAGCGCGGCGACCAGCAGCACCCCGCGCGAAG
CGCCGTATGAACTGAACATTACCAGCGCGACCTATCAGAGCGCGATTCCGCCG
CGCGGCACCCAGGCGGTGGTGCTGAAAGTGTATCAGAACGCGGGCGGCACCCA
TCCGACCACCACCTATAAAGCGTTTGATTGGGATCAGGCGTATCGCAAACCGA
TTACCTATGATACCCTGTGGCAGGCGGATACCGATCCGCTGCCGGTGGTGTTT
CCGATTGTGCAGGGCGAACTGAGCAAACAGACCGGCCAGCAGGTGAGCATTGC
GCCGAACGCGGGCCTGGATCCGGTGAACTATCAGAACTTTGCGGTGACCAACG
ATGGCGTGATTTTTTTTTTTAACCCGGGCGAACTGCTGCCGGAAGCGGCGGGC
CCGACCCAGGTGCTGGTGCATCGCAGCGCGATTGATAGCATGCTGGCGTAG
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Figure 3: The optimized mpt64 gene sequence

A B C

Figure 4: Transformant selection (A) *E. coli cells* BL21 (DE3) without kanamycin addition into the medium (positive control) (B) *E. coli* BL21 (DE3) with the addition of kanamycin in the media (negative control) (C) Transformant of *E. coli* BL21 (DE3) [pD861-SR] with the addition of antibiotic kanamycin in the media.

More than one codon encodes some amino acids and cognate tRNAs determines the codon usage. Therefore, codon usage of each species can be quite different. Based on the percentage of codon frequency in figure 1, it could be seen that there were differences in the reading of the original bacterial codon (*M. tuberculosis* H37rv) which were not optimal reading in *E. coli*, because it showed a value of less than 100%. Thus, the codon optimization needs to be done by replacing certain nitrogen bases at the low percentage codon with codons that were 100% recognized by *E. coli* K12 to produce the same amino acids. Comparison of the initial mpt64 gene codon with optimal codon preference in *E. coli*, could be seen in figure 2. The codon sequence of mpt64 gene that has been optimized can be seen in figure 3. The mpt64 gene sequence with the optimal codon was then inserted in the expression vector.

The results of the mpt64 gene design inserted into the expression vector pD861-SR: 319895 resulted in a total plasmid size of 2990 pb. The plasmid pD861-SR used in this study is a vector of expression so that it can be used directly by the host cell expression. tThis plasmid consists of he coding sequences of kanamycin antibiotic-resistant genes, rhaBAD promoters, strong RBS, Ori, and recombinant gene insertion sites. The constructed plasmids were selected by kanamycin antibiotic resistance as a selection marker to distinguish *E. coli* host cells that were successfully inserted with the target plasmid and did not carry the target plasmid. The rhaBAD promoter was used because it has a high level of expression control and prevent leaky expression. In addition, in the plasmid also has a promoter that was regulated by L-rhamnose inducer, and the promoter regulated by L-rhamnose could improve the expression of mpt64 gene to get the optimal yield of MPT64 protein. This purposed also supported by the use of strong RBS (Ribosomal Binding Site) in the plasmid construction. The strong RBS has high expression specifications so that it could accelerate the process of translational initiation and MPT64 protein expression can be done quickly and optimally. The Ori area was the point of the area to carry out the replication process, and MCS was the area to insert the optimized mpt64 recombinant gene.

Results of Selection and Characterization of Transformant

In this study, the definition of transformant is a host cell that has received a recombinant plasmid. The *E. coli* strain used in this study is susceptible to all antibiotics. The transformant was transformed by plasmid recombinant containing an inserted kanamycin resistance gene. This character made transformant easier to select, because only the transformant of *E. coli* could survive in media containing kanamycin. So, if the *E. coli* lack of resistance gene, then the kanamycin will inhibit the *E. coli*'s growth. The growth of transformant colonies was performed in figure 4.

Result of Plasmid Recombinant Analysis

The plasmid isolation was carried out using TIANprep Mini Plasmid Kit based on modification of the alkaline lysis method on bacterial cells followed by DNA adsorption into silica using high salt concentrations. There are three basic steps of this method, as follows: the bacterial lysis process, DNA adsorption on the membrane, washing and elution of plasmid DNA. The reagents used in this isolation process were P1 buffer, P2 buffer, P3 buffer, PW buffer, and EB buffer. The P1 buffer functions were to

suspend pellets and the addition of RNase serves to degrade RNA as an unwanted component in plasmid isolation. The P2 buffer functions were as an alkaline lysis which damages the walls of *E. coli* cells and denatures chromosomal DNA and other components apart from plasmids. P3 buffer consists of sodium hydroxide and sodium deodoxyl sulfate. Sodium deodoxyl sulfate served to break down lipids in the bacterial cell wall, while sodium hydroxide provided an alkaline atmosphere that can denaturated *E. coli* chromosome DNA. The inversion procedure in the plasmid isolation process was aimed to prevent the plasmid damage. The release of plasmid DNA without chromosomal DNA release was influenced by the optimum lysis time. P3 buffer that contains potassium acetate and acetic acid, have functions to precipitate components that have been damaged and denatured. Potassium acetate plays a role to return the pH to neutral, so that DNA renaturated and bound to other cell debris such as lipids and proteins form insoluble deposits, while small plasmid DNA remain in solution. The plasmid DNA in the dissolved fraction was placed in the column and adsorbed by the silica membrane and the remaining macromolecules eluted out of the matrix and removed. PW buffers that have been added to ethanol has function to remove salt in the matrix. Plasmid DNA in the silica matrix was eluted with EB buffer. The collected plasmid DNA was then characterized using agarose gel electrophoresis 1% (b / v). The plasmid characterization was also carried out with restriction enzyme analysis using ecoRI and SapI enzymes. The plasmid characterization was carried out by 1% agarose gel electrophoresis. The visualization of gene bands was shown in Figure 5.

Based on the band pattern in figure 5A, the uncut plasmid DNA was still circular with different conformations such as supercoiled monomers, supercoiled dimers and nicked circular (nc). In contrast with the resulting fragment of pD861-SR mpt64 plasmid which was successfully cut with two restriction enzymes produced two bands in gel with each different size of 755 pb and 2235 pb. The different of DNA conformation will produce different migration rates. Supercoiled plasmid DNA has a compact conformation, so it migrated faster in the gel. Next, followed by a DNA fragment in the form of a nicked circular migrate [12].

Results of Sequencing Analysis

The primer was designed using the primer select application (DNAStar) by choosing 20 bases of the plasmid. The quality of primer was analyzed based on secondary structures which may be formed from the selected primary sequences, such as: hairpin (loop structure) and dimer (self dimer and cross dimer). Both secondary structures were avoided because they can cause the primer not to stick with the DNA template. The sequencing results were then analyzed using the SeqMan (DNAStar) application. In this application, it can be seen whether there were mutations in the sequencing results compared to the origin of mpt64-pelB nucleotide sequence. The results could be seen in figure 6-7.

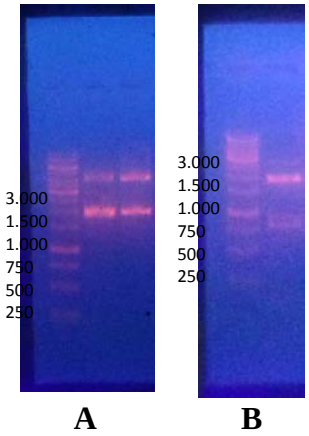

Figure 5: Plasmid electrophoregram, (M) 1 kb gene Marker (A) Plasmid pD861-SR mpt64 (uncut) (B) Plasmid pD861-SR mpt64 / EcoRI-SapI.

Based on the electrogram pattern using forward primer (Fig. 5.10), in the pelB base sequence there was a difference in C base at position 43. But the difference was found at the beginning of the sequence reading and the band was wide, thus it can be concluded that there was no change in the pelB base sequence. Likewise, the results of the mpt64 gene sequencing did not show any change in the nucleotide sequence using both primers. Confirmation of the sequencing results can be seen based on a homology analysis of the mpt64 gene nucleotide sequence compared with the gene source in the bank gene using a blast system. The results of the blast showed that the mpt64 gene sequence used had a 100% similarity with the mpt64 nucleotide sequence. The blast result of mpt64 gene was performed in figure 8.

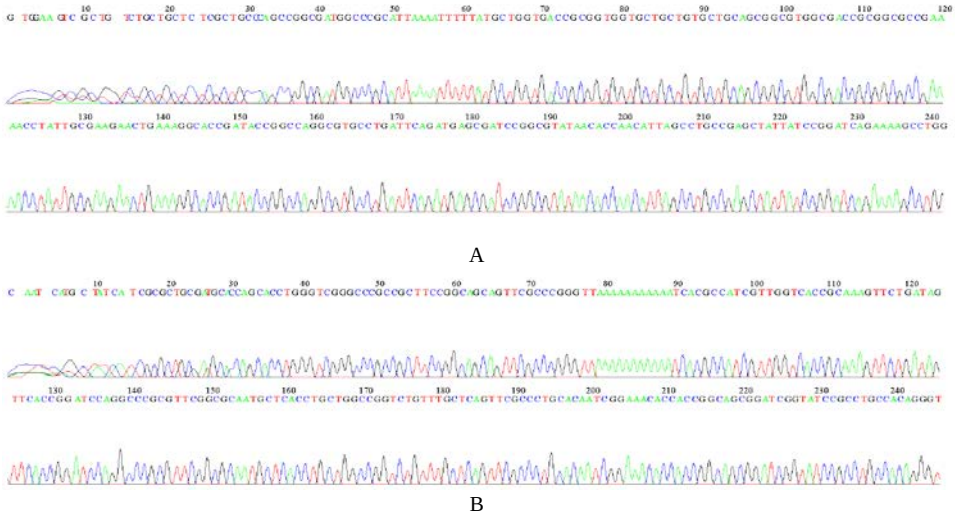

Figure 6: The results of pD861-SR plasmid sequencing (A) sequencing results with forward primer; (B) sequencing results with reverse primer

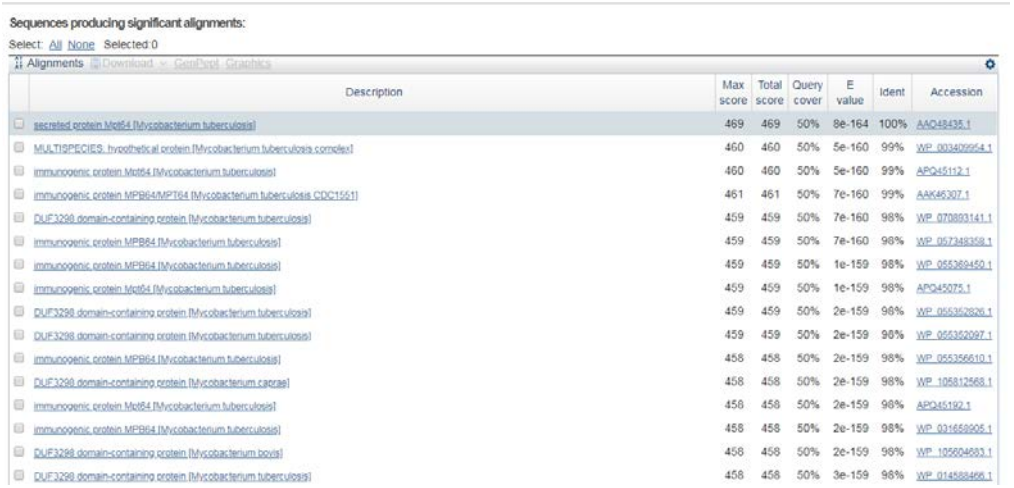

Figure 7: The results of pelB sequencing

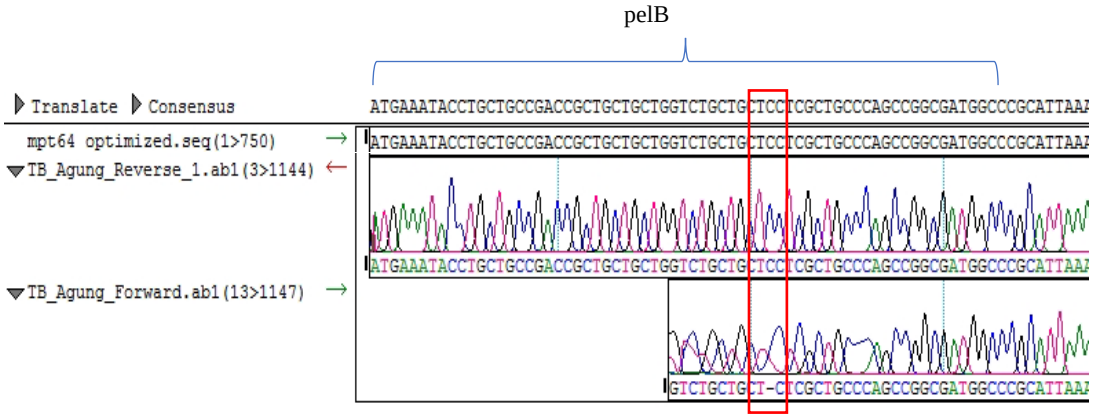

Figure 8: The blast results of the mpt64 nucleotide sequence

Bacterial Growth Curve

Before working on a gene expression procedure, the bacterial growth curve was conducted to determine the most active time of bacterial cell dividing. The result correlated with the determination of the induction time in the gene overexpression process. The observation of bacterial optical density was done every hour until 24 h. The induction time could be determined from bacterial growth curve by observing the optical density. The optical density has a linier value with the number of bacterial cells in the suspension. The growth curve of *E. coli* BL21 (DE3) [pD861-SR mpt64] was displayed in figure 9.

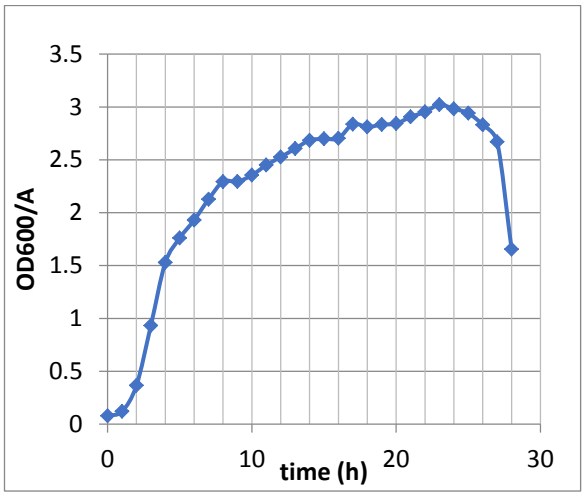

Figure 9: Growth curve of *E. coli* BL21 (DE3) [pD861-SR mpt64]

Based on the growth curve, the optimum induction time could be determined in range of exponential phase before entering the stationary phase. Because during the stationary phase, the cell growth stops and the number of living cells is the same as the number of dying cells so that if the induction time was selected in that range, then production of recombinant protein will be low. Meanwhile, the lag phase is a phase where the bacteria adapt to their environment, so that the induction in this phase will produce recombinant proteins in low amounts. In this study, the lag phase of *E. coli* BL21 (DE3) [pD861-SR mpt64] was shown at 0-1 h, while in the range of 2-16 h was an exponential phase and after that was the stationary phase. Based on the growth curve, it could be determined that a good exponential phase to be induced was in the exponential initial phase, which was in the 3rd hour after incubation, with the absorbance of 0.933. In this condition, the cells were in the active cell divides.

MPT64 Characterization

The mpt64 gene expression was regulated by an inducible system using L-rhamnose as an inducer. Therefore the expression process was controlled by promotor. The promoter of L-rhamnose induction or rhaBAD promoter (PrhaBAD) has been used extensively to control recombinant protein in an expression system of *E. coli* and other bacteria. PrhaBAD is a member of the AraC group XylS from the expression system which are regulated positively. The promoter has several advantages compared to other promoter systems such as systems Ptet induction

(tetracycline), Plac (lactose) or ParaBAD (arabinose), and their derivatives. PrhaBAD can be used for expression in highest level [13]. rhaBAD as the regulon allows *E. coli* to carry out L-Rhamnose metabolism. The promoter consists of genes rhamnosa transporter (rhaT), the gene for rhamnose catabolism (rhaBAD) which coded proteins RhaB, RhaA, and RhaD, as well as the coding rhaSR regulator gene protein rhaR and rhaS. Of the three promoters that are induced by rhamnose in the operon Rhamnosa, a promoter of RHABAD which is generally used for protein production [14].

The secretion of the MPT64 as an extracellular protein was demonstrated by protein recovery in the medium. The pelB as signal peptide play a role in this secretion process. In *E. coli*, proteins are directly secreted by peptide signals to periplasm and the outer membrane [15]. Characterization of recombinant MPT64 protein was carried out with SDS PAGE. The MPT64 recombinant showed as a protein with a molecular weight of 24 kDa, illustrated in figure 10. Optimal production of MPT64 protein was obtained after 24 h induced by rhamnose.

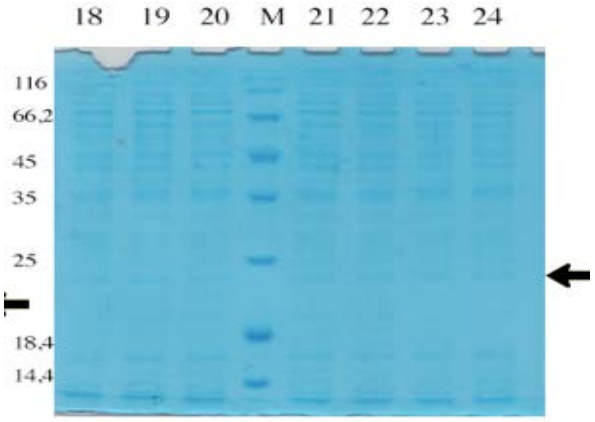

Figure 10: Characterization of secreted MPT64 protein in medium

CONCLUSION

Our results demonstrated that the construction of mpt64 gene fused with pelB gene in the used expression vector has achieved the target expression to produce MPT64 as extracellular protein.

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