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Research Article

OVERPRODUCTION OF MERCURIC REDUCTASE FROM MERCURY-RESISTANT BACTERIA *KLEBSIELLA PNEUMONIAE* ISOLATE A1.1.1

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ABSTRACT

Mercury is a highly toxic compound in human. It can, however, be detoxified by mercuric reductase (MerA) protein derived from mercury resistant bacteria. This study aims to obtaine MerA protein by transforming *merA* gene into *Escherichia coli BL21.* Nucleotide sequence of *merA* gene of mercury resistant bacteria *Klebsiella pneumoniae* isolates A111, optimized by using gene program designers (www.dna20/com) then commercially synthesized and cloned in pET32b expression plasmid vector. Plasmid was transformed into *Escherichia coli* BL21 to produce MerA protein recombinant, induced with isopropyl-β-D-thiogalactopyranoside (IPTG). MerA proteins were analyzed by 10% sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE). The result showed that MerA protein with 60kDa was detected on SDS PAGE. The obtained MerA protein can be used in further research for the enzymatic detoxification of inorganic mercury.

Key words: mercuric reductase, *mer*A gene, MerA protein, *Escherichia coli* BL21

INTRODUCTION

The release of heavy metals into the environment can cause serious harm to the ecosystem and human health. One of the most dangerous heavy metal is mercury. In any form, mercury is poisonous and its toxicity most commonly affects neurologic and renal organ systems (Al-Madani *et al*., 2010 ; Farina 2011). Minamata disease, a neurological syndrome, is one example of a disease caused by severe mercury poisoning. Currently it is predicted that more than 1500 tons of hazardous mercury wastes dumped into the environment each year in Asia and Africa. Therefore, there is a need for economical and efficient approach to eliminate or detoxify mercury (Barkay and Wagner-Döbler, 2005).

Efforts to mercury detoxification can be done using mercury-resistant microorganisms such as mercury resistant bacteria. Detoxification of mercury by mercury-resistant bacteria occurs becaus they harbor mer operon in their genome. The mer operon includes functional *mer*A and *mer*B genes along with promoter, regulator, and operator. The *mer*A gene codes for mercuric ion reductase (MerA) whereas *mer*B gene codes for organomercurial lyase (MerB) (Dash and Das, 2012). Those two enzymes transform methylmercury to elemental mercury. Carbon-mercury bond in methyl-mercury is cleaved by MerB protein to release methane. MerA protein reduces ionic mercury to volatile elemental mercury. MerA and MerB protein producing bacteria have been isolated from environment and exploited for bioremediation (Vetriani *et al*. 2005 ; Poulain *et al.,* 2007 ; Ni Chadchain *et al*., 2006; Barkay and Wagner-Dobler, 2005).

The use of mercuric ion reductase enzyme to overcome the problem of inorganic mercury contamination in the environment is potentially very high therefore the process of cloning and expression of MerA gene is very useful for obtaining MerA protein in significant amounts. Zeyaullah *et al*. (2010) have isolated *E. coli* bacteria that were resistant to mercury from mercury-contaminated environment in India. They have succeeded in cloning and expressing the gene in *E. coli* BL21 cells, and were able to obtain 66.2 kDA MerA protein.

Fatimawali *et al*. (2011) previously isolated 5 mercury-resistant bacterial isolates from an estuary in Manado, which can live in a medium with 20mg/L $HgCl_2$. One of these

isolates, A.1.1.1, had the ability to fast enough to reduce Hg to , 75% in 1h, 92% in 12h, and 99.4% in 24h. Identification using 16S-rRNA gene showed that this isolate had 99% identic to *Klebsiella pneumoniae* with 100% maximum coverage.

Amplification of partial *mer*A gene fragment of this isolate generated 285 bp fragment. Determination of the nucleotide sequence of the fragment showed 99% homology to several *mer*A genes of several strains of *K. pneumoniae* (strain I, M, N). Although there are some differences in the nucleotide, the alignment of the amino acid sequences showed only one difference in the amino acid at position 524 (Thr → Ser) (Fatimawali *et al*., 2014).

This study aims to obtain recombinant MerA protein from *mer*A gen of *Klebsiella pneumoniae* isolate A.1.1.1, cloned in expression vector and transformed into *Escherichia coli* Top-10 and BL21. The *mer*A gene was charactherized and overproduction of recombinant MerA protein was optimized.

MATERIAL AND METHODS

Codon optimization, synthesis of merA gene of *K. pneumoniae* isolate A.1.1.1, and *mer*A gen cloning

The size of full length of *mer*A gen is 1686 bp while the size of previous isolated *mer*A gene was only 285 bp. Based on the obtained *mer*A gene sequence, codon optimization was conducted to adapt codon usage of *mer*A gene of *K. pneumoniae* isolate A.1.1.1 towards codons of *E. coli* BL21 (DE3). This is because *mer*A gene was aimed to be expressed in *E. coli*. Codon optimization was done using online computer programs namely JCat (http://www.jcat.de/) and Gene Designer (www.dna20/com). Optimized *mer*A gene sequence was synthesized commercially and cloned in pET32b expression vector and transformed into *E. coli* BL21 (DE3). The MerA protein was expressed as fusion protein with thioredoxin and histidine residues (6x His-tagged).

Transformation of plasmid into *E. coli* BL21 (DE3)

A 5µL of pET32b expression vector harboring *mer*A gene from isolate A1.1.1.1 was pipetted into 200µL of *E. coli* BL21 and incubated in thermo block at 42°C for 90s, followed by 10min in ice. Liquid LB medium of 800µL was added and the tube was incubated in shaker incubator for 60min at 250rpm. The cells were grown until the absorbance at 600nm (OD_{600}) reached approximately 0.4. Cells were centrifuged down at 3.670rpm for 10min. The supernatant was decanted leaving only 200µL of pellet with a small quantity of supernatant. Pellet was stirred gently and 50µL of suspension was transferred onto solid LB medium containing ampicillin, spread evenly and incubated at 37°C for 17h. Colony was picked using oose and streaked onto solid medium containing ampicillin and incubated at 37°C for 17h. A single colony of recombinant *E. coli* BL21 was picked using oose and lysed quickly using EDTA 10mM pH 8 containing 50µL of fresh solution which consisted of 2µL NaOH 5N, 50µL SDS 10%, 400µL sucrose 50% and 548µL aquadest. The suspension was vortexed gently for 30s, incubated on ice for 5min then centrifuged down at 12.000rpm for 3min. Supernatant was then electrophoresed on 1% agarose gel for 45min. Gel was submerged in buffer solution containing ethidium bromide for 5min, subsequently immersed in aquadest for 20min then visualized on a UV illuminator. The presence of 1696kb DNA fragment on the gel indicated that transformation was successful.

Overproduction of MerA protein of *K. pneumoniae* isolate A.1.1.1

Recombinant *E. coli* BL21 was cultured in LB liquid medium containing 100µg/mL ampicillin at 25°C and 37°C until log phase was reached (OD_{600}~0.7). The cells were then induced with IPTG with final concentration of 1.0mM for 3h. Cells were centrifuged down and pellet was lysed. Protein was analysed using SDS-PAGE. Overproduction was successful if thick band of 60kDa MerA protein present.

Overproduction optimization of MerA protein of *K. pneumoniae* isolate A.1.1.1

Overproduction optimization was conducted under the optimum induction temperature with 3h induction time. After the optimum induction temperature was obtained, several final concentration of IPTG (0.1; 1.0

and 5.0mM) was used for optimization. Pellet produced during optimization step was analyzed using SDS-PAGE. The optimum IPTG concentration was obtained when thick band of 60kDa merA protein was observed.

Purification of MerA protein

The cells of *E. coli* BL21 bearing pET32b-merA were grown in liquid LB media, incubated for 2h at 37°C, or until OD_{600}~0.7 was reached. The cells were subsequently induced with 0.1mM IPTG and incubated for further 3h at 37°C. Cells were centrifuged and the pellet was lysed. Supernatant was poured into polypropylen column containing nickel-nitro-acetic acid (NTA) resin and eluted with imidazole containing buffer with concentration of 15, 50, 60, 70, 80, 90, 100, 200, and 250mM. The fraction obtained was centrifuged at 1000rpm for 2min and the supernatant was analyzed using 10% SDS-PAGE.

RESULTS AND DISCUSSION

Plasmid transformation into *E. coli* BL21

Before overproduction was conducted, the presence of plasmid containing *mer*A gene inside *E. coli* BL21 was confirmed. Confirmation was carried out in 1% agarose gel. The result is shown in Figure 1. Fragments of *mer*A gene with the size of 1695 bp isolated from recombinant *E. coli* BL21 were observed. This shows that plasmid transformation was successfully done in recombinant *E. coli* BL21.

Overproduction optimization of MerA protein

Recombinant *E. coli* BL21 cells were cultured in liquid LB medium containing 100μg/mL of ampicillin. Overproduction was initiated by induction of cells with 0.1mM IPTG, and incubated at 25°C and 37°C. OD_{600}~0.7 was reached when incuated for 3h at 37°C. Incubation at 25°C for 3h only reached OD_{600}~0.3. Therefore incubation temperature of 37°C was subsequently used in this study. The IPTG with concentration of 0.1mM, 1,0mM and 5.0mM, respectively, was added into respective culture and the cells were incubated at 37°C to obtain OD_{600}~0.7. Figure 2 shows SDS-PAGE result of different IPTG concentration. Induction with 0.1mM IPTG produced thicker MerA protein band with molecular weight of 60kDa.

Purification of affinity-tagged MerA protein

Recombinant 6x His-tagged MerA protein expressed during overproduction was purified on Ni-NTA beads and eluted with washing buffers containing 15, 50, 60, 70, 80, 90, 100, 200, and 250mM imidazole, respectively. Approximately 60kDa merA protein was detected after resolving on 10% SDS PAGE (Figure 3). The figure shows that MerA recombinant protein is relatively pure although there are bands of approximately 57kDa, which presumably are fragmented MerA proteins.

The *mer*A gene encodes for mercuric reductase enzyme, an enzyme that plays a role in the reduction of highly toxic ionic Hg^{2+} into nontoxic volatile metallic Hg^{0} species (Nascimento and Chartone-Souza 2003). This gene presents in operon system called *mer operon* contained on bacterial plasmid (Ravel *et al.* 2000; Nascimento and Chartone-Souza 2003) and on bacterial chromosomal DNA (Osborn *et al.* 1997).

Nowadays there have been many techniques developed for mercury bioremediation, so as encouraging in-depth study on associated genes. The development of modern biotechnology allows *mer*A gene transformation into competent bacterial cells to produce large amounts of MerA protein that can be used in enzymatic detoxification of mercury. Even though further research is still needed for commercialization of the MerA product, this preliminary finding will serve as a stepping-stone into enzymatic mercury bioremediation. Competent bacterial cell used in this study was *E. coli* BL21. This bacterium was designed genetically to harbor bacteriophage T7 RNA polymerase gene under control of *lac operon*. Synthesis of T7 RNA polymerase can be induced by lactose analog IPGT. Induction of *E. coli* cell at different temperature was targeted to obtain the optimum temperature for overproduction of recombinant MerA protein.

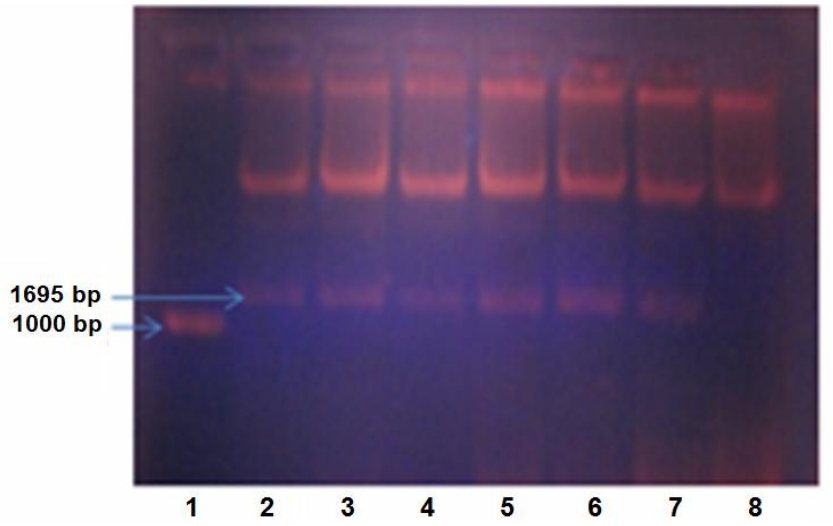

Figure 1. Electroforegram of *mer*A gene isolated from recombinant *E. coli* B21. Lane 1: 1000bp DNA Ladder; Lane 1-7: *mer*A gene fragments; Lane 8: non-recombinant *E. coli* BL21

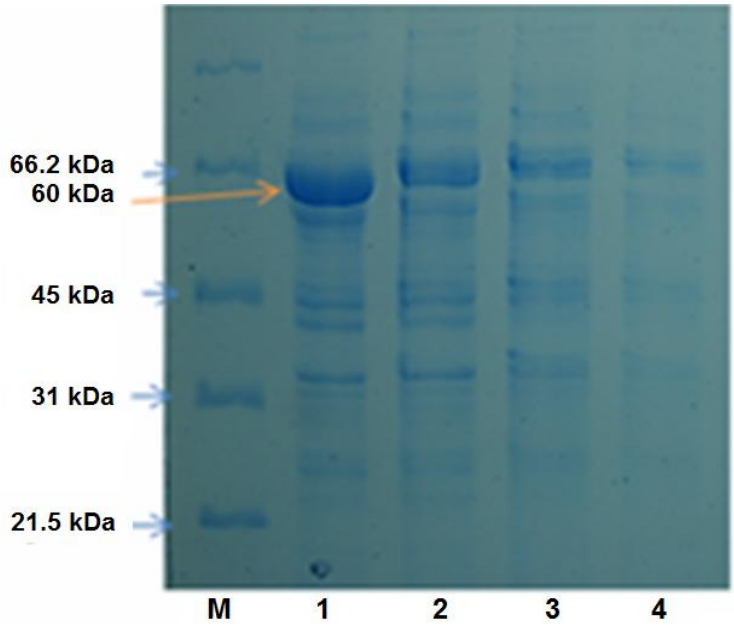

Figure 2. SDS-PAGE results of recombinant MerA protein. Lane M: Protein marker; Lane 1: MerA protein induced with 0.1 mM IPTG; Lane 2: MerA protein induced with 1.0 mM IPTG; Lane 3: MerA protein induced with 5.0 mM IPTG; Lane 4: negative control

This study proves that recombinant MerA protein can be produced by *E coli* BL21. Protein expression was high enough and resulted in efficient protein isolation and purification, thus allowing production of mercuric reductase in large scale. This enzyme can be used in mercury detoxification. Zeyaullah *et al.* (2010) explained that expression of bacterial *mer*A gene had been expressed in plants but none of them have been already applied in the field and remains debatable. Although MerA alone can detoxify mercury enzimatically, MerB is also required to achieve higher level of mercury detoxification.

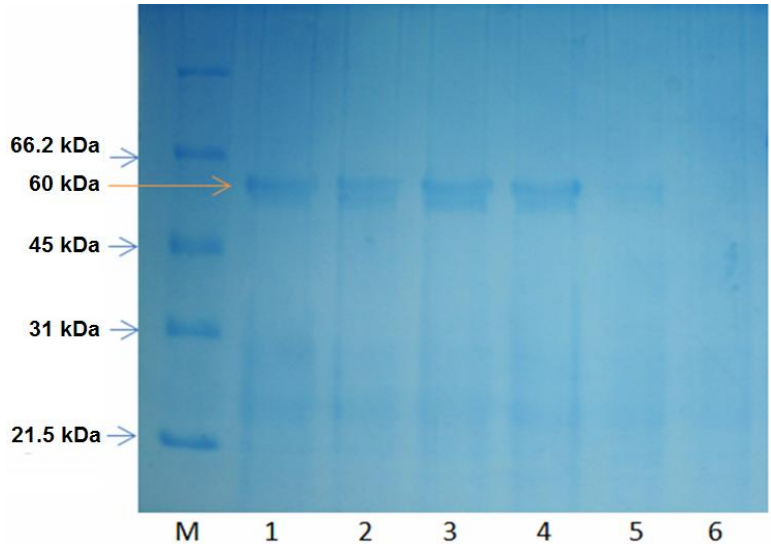

Figure 3. SDS-PAGE result of purified recombinant MerA protein. Lane M : Protein Marker Lane 1 : recombinant MerA protein eluted with 100 mM imidazole; Lane 2-3: recombinant MerA protein eluted with 200 mM imidazole; Lane 4-5: Recombinant MerA protein eluted with 250 mM imidazole; Lane 6: Negative control.

Therefore MerA protein is limited to enzymatic detoxification of inorganic mercury compounds therefore MerB protein is needed to complement the detoxification process since it transforms both organic and inorganic mercury into volatile forms (Mathema *et al.* 2011).

CONCLUSION

Gene cloning and over production of MerA protein was successfully performed in *E. coli* BL21 mediated by plasmid pET32b, resulting MerA protein with a molecular weight of 60 kDa, with the optimum at 37°C incubation temperature, incubation time of 3 hours and 0.1 mM IPTG induction. MerA protein obtained can be used in further research on the enzymatic detoxification of inorganic mercury.

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