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Research Article

Keywords: expression, *E. coli* BL-21, mercuric reductase, SDS-PAGE

Posted Date: February 24th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1384627/v1>

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Expression and Structural Characterization of Recombinant Mercuric Reductase Protein Isolated from *Streptomyces*

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Abstract

Abstract: Efforts to control mercury pollution have been made for the last twenty years using various biological, physical, and chemical approaches. *Streptomyces* is able to degrade mercury because it possesses the mercuric reductase enzyme encoded by *merA*. The *merA* from *Streptomyces* isolate AS2 (*Accession numbers* LC026157) has been cloned into the expression vector pEt-28c(+). Therefore, it is critical to study recombinant mercuric reductase protein's expression and to analyse the protein structure. Expression of *merA* from an AS2 isolate was successfully performed in the host *Escherichia coli* BL21. Detection of mercuric reductase using SDS-PAGE showed a dominant band at 55–70 kDa. *E. coli* BL21 induction by IPTG was optimal at concentrations of 1 and 1.2 mM, and the optimal incubation times were 18 hours. The highest specific activity of mercuric reductase was 294.07 U/mg. Mercuric reductase protein in *Streptomyces* AS2 shares high homology with *Lysinibacillus sphaericus* (Swiss-Model), and similar folding to that of c5c1Yc refers to mercuric reductase from *L. sphaericus* using Phyre2. Based on amino acid sequences, the result revealed that *Streptomyces* AS2 strains were a group of *Streptomyces lividans*. These results give new insight to study further into a potential of *MerA* for bioremediation in the environment.

Keywords: expression, *E. coli* BL-21, mercuric reductase, SDS-PAGE

Introduction

Bioremediation is a biotechnological strategy that employs live organisms (plants, bacteria, fungi, or algae) or components of living organisms to eliminate pollutants and mitigate their environmental impact [1]. Actinomycetes are a stimulating group of microorganisms that are integral members of the microbial community in soil. These microorganisms exhibit excellent metabolic variety and specific growth features. Therefore, they are very suitable for the biological decomposition of environmental pollutants [2]. *Streptomyces* spp. can degrade the mercury in the environment because it harbors the *merA* gene [3]. It could be utilized to address the issue of biological mercury exposure caused by the detoxifying process of *Streptomyces* spp. Detoxification primarily involves the conversion of toxic heavy metal ions into non-toxic ions [4].

Mercuric reductase is an oxidizing enzyme and Flavin Adenine Dinucleotide (FAD) is required for the conversion of mercury from Hg^{2+} to non-toxic Hg^0 . As a result of elemental mercury's high vapor pressure, Hg^{2+} readily volatilizes and changes into Hg^0 , which causes the atmosphere to become mercury-free [5]. Mercury has a high vapor pressure and low solubility, allowing it to escape into the atmosphere freely. In addition, Hg^{2+} is a non-biodegradable element that can persist in the atmosphere for several years [6]. Furthermore, the *merA* gene commonly present in bacteria and archaea is likely to survive at high mercury concentrations. NADPH (Adenine Dinucleotide Phosphate) and MerA-FAD compounds are critical in mercury volatilization and subsequent binding [7].

Rahayu [4] successfully purified and characterized reductase from four strains of *Streptomyces* spp. isolated from *Cyperus rotundus* which located in a mercury-contaminated location within the traditional gold processing area of Selodong village, Buwun Mas village, West Lombok, Indonesia. They are *Streptomyces* spp. AS1, *Streptomyces* spp. AS2, *Streptomyces* spp. AS6, as well as *Streptomyces* spp. BR28. Resistance activity from highest

to lowest at a concentration of 1 mM HgCl₂ was *Streptomyces* BR28 (9.3 mm) *Streptomyces* AS2 (9.6 mm), *Streptomyces* AS1 (10.3 mm), and *Streptomyces* AS6 (20 mm), respectively. Mercuric reductase from *Lysinibacillus sphaericus* has a molecular weight of about 60 kDa; the K_m and V_{max} were found to be 32 μM of HgCl₂ and 18 units/mg respectively [5].

Cloning of the *merA* gene isolates *Streptomyces* AS2 was successfully performed [3]. The *merA* gene in AS2 isolates showed a length of 1456 bp. The AS2 isolate has similarities with *Streptomyces lividans*. The isolate AS2 has received an accession number from the DNA Data Bank of Japan/DDBJ with code LC026157 for AS2 clone 2. However, research on gene transformation of *merA* *Streptomyces* isolates AS2 into host *Escherichia coli* BL 21 to determine the results of protein expression, activity, and structural characterization was of critical importance.

Materials and Methods

Transformation of the *merA* gene into the host *E. coli* BL21

The *merA* gene from *Streptomyces* AS2 has been successfully ligated into the plasmid pET-28c [3]. Competent cells of *E. coli* BL21 were made by using the method of Inoue [8]. Transformation of the *merA* gene into *E. coli* BL21 was performed by the heat shock method.

Optimization of mercuric reductase expression with a variation of IPTG concentration and incubation time

The colony of *E. coli* BL21 carrying the pET-28c (transformed) vector was inoculated into 5 mL liquid LB containing 20 μg/mL kanamycin and incubated for 3 hours. IPTG induction was then conducted with concentrations of 1 and 1.2 mM and incubation times of 4 and 18 hours. Centrifuged the cell culture at 6000 rpm for 30 minutes. The supernatant was

discarded, and the pellet was washed with PBS, pH 7.2 twice. The result was directly analyzed for expression of mercury reductase by SDS PAGE or stored frozen at -80°C .

Resistance test of *E. coli* BL21

The resistance test was conducted by the paper disk method. A 100- μl volume of the *E. coli* BL21 (which has been overexpressed) was inoculated onto the surface of a petri plate containing LB medium. A paper disk measuring 6 mm was placed on the medium, and then an 8- μl drop of 1 mM HgCl_2 solution was placed on it. The medium was incubated at 37°C for 24 hours, and the growth inhibition zone formed was measured.

Protein Extraction and Purification

The results of IPTG induction and incubation time optimization were further used as standard cell culture conditions to obtain optimal mercuric reductase production. Pellets from *E. coli* BL21 were sonicated to disrupt the cells. Following the addition of a protease inhibitor (1 tablet to 50 ml cell culture), sonication was performed for 30 seconds, in three replications, with a rest between each one of 10 seconds. A supernatant and a pellet solution were obtained. The supernatant was removed for analysis because mercuric reductase is a soluble enzyme. The supernatant acquired was called the cell-free extract of crude enzyme. Crude enzymes can be analyzed directly with SDS PAGE or stored at -20°C . Purification was performed in three stages, i.e., precipitation with ammonium sulfate using a saturation level of 20%–70%, dialysis, and column chromatography (DEAE *Sepharose anion column chromatography*).

Measurement of Molecular Weights

Protein was analyzed by SDS PAGE. The marker used was the Prestained Protein Marker 180 kDa. The enzyme sample (10 μ L) was mixed with ten μ L of sample buffer. The samples were heated at 100°C for 2 minutes, placed in ice water, and allowed to stand for 10 minutes. Samples and protein markers were applied to the gel formed. Electrophoresis was performed in running buffer for 1.5 hours with a 100 Volt electric current. The gel was then stained with Coomassie brilliant blue, and the background color was removed by destaining.

Protein Modeling, Prediction, and Analysis

Tertiary protein structure prediction techniques can be divided into homology modeling, fold recognition, and *ab initio* prediction. Homology modeling was predicted using the Swiss model [9] and modeling based on fold recognition using phyre2 [10].

Phylogenetic Tree of Amino Acid Sequences

Reconstruction and analysis of phylogenetic trees based on mercuric reductase protein sequences were performed using MEGA11 [11]

Data Analysis

Protein concentration was determined using the raw protein curve's regression line $y = ax + b$. The activity of mercuric reductase was measured by determining the concentration of oxidized NADPH₂ per milligram of protein per minute (M NADPH/mg protein/min) using the equation of the regression line $y = ax + b$ of the NADPH standard curve.

Result

Transformation of the *merA* gene into the host *E. coli* BL21

Figure 1 shows that the isolates of *Streptomyces* AS2 were successfully transformed.

Fig 1. Transformation of plasmid pET-28c which has been inserted the *merA* gene isolate *Streptomyces* AS2 into *E. coli* BL21 is showing a positive result

This is demonstrated by the ability of the isolates to grow in selective LB + kanamycin 20 µg/ml. After incubation for 24 h at 37°C, there were approximately 40 colonies. This positive result represents the host transcription capability of a pET plasmid with a strong promoter to produce target proteins.

Optimization of Mercuric Reductase Expression and Measurement of Molecular Weight

Optimization of Mercuric Reductase Expression *E.coli* BL21 using IPTG variation and incubation time. Confirmation of heterologous mercuric reductase expression can be seen from the specific protein band profiles formed at 55–70 kDa using SDS-PAGE. The IPTG concentrations used in this study were 1 and 1.2 mM. The incubation time was optimized using 18 hours. From the results. Mercuric reductase AS2 was expressed with IPTG induction. The molecular weight of mercuric reductase was determined using SDS-PAGE (Acrilamide 30%).

SDS-PAGE results showed mercuric reductase with an apparent molecular size of 55–70 kDa (Figure 2).

Fig 2. Expression of mercuric reductase *Streptomyces* AS2 after induction IPTG 1 mM (1,2) & 1.2 mM (3,4) with 18 hours incubation (two replicates)

Resistance test of *E. coli* BL21

The purpose of the resistance test in *E. coli* BL21 is to determine the resistance level of *E. coli* BL21 to 1 mM HgCl₂.

Fig 3. Resistance test of *E. coli* BL21 as the results of transformation to HgCl₂ 1 mM

Figure 3 shows the 5.65 mm inhibitory diameter in *E. coli* BL21 that carries the *merA* gene isolate AS2 against one mM HgCl₂ (6 replications).

Protein Extraction and Purification

Cells were disrupted using the method of sonication. Then, cell cultures were centrifuged to separate the pellets from the supernatants. The supernatant was removed for

analysis because mercuric reductase is a soluble enzyme. The supernatant formed was termed the cell-free extract of crude enzyme. As the result of the transformation, Mercuric reductase was purified in three stages: precipitation with ammonium sulfate, dialysis, and column chromatography (*DEAE sepharose anion column chromatography*).

Protein Modeling, Prediction, and Analysis

The Swiss model is an automated approach for deriving a protein's three-dimensional structure from its amino acid sequence using homology modeling techniques. Predicting the tertiary structure of proteins based on the fold approach can be performed using phyre2.

Fig 4. Modeling the 3D structure result using Swiss-Model

- (a) Results of reference structure search using mercuric reductase *Streptomyces* AS2 protein sequence query (b) Display of phyre2 prediction results with the best reference structure (c)

The search result shows several proteins similar to the query sequence based on the alignment of sequence pairs (Figure 4). The mercuric reductase protein in *Streptomyces* AS2 shares high homology with *Lysinibacillus sphaericus*. The query of mercuric reductase was submitted in phyre2 using standard mode. The summary showed that a single template could model 97% of the query sequences.

Phylogenetic Tree of Amino Acid Sequences

The results of the reconstruction of the phylogenetic tree based on the mercuric reductase amino acid sequence are shown in Figure 5.

Fig 5. The results of the reconstruction of the phylogenetic tree using a MEGA11 program based on the mercuric reductase protein

Our study reported that *Streptomyces* AS2 has a similarity 100% of mercuric reductase sequence with *Streptomyces lividans*.

Discussion

MerA gene was successfully expressed to the host *E. coli* BL 21. A similar action was taken by Zeyaulah [12], who obtained a plasmid containing *merA* with 1695 bp from *E. coli* isolated in a mercury-contaminated area and then expressed it in an expression vector (pQE-30U/A), obtaining expression of mercuric reductase protein 66.2 kDa.

Figure 2 shows the expression of mercuric reductase with a size of 55–70 kDa. This size of mercuric reductase refers to some previous studies. The results of gene expression of *merA* using *E. coli* BL21 (DE3) Plys as the host showed a mercuric reductase with a size of 66.2 kDa. The expression of mercuric reductase from *Zymomonas mobilis* has a size of 65 kDa [13]. These results are associated with a pET-28c plasmid that has a strong T7 promoter and can transcribe more mercuric reductase than other proteins. The resulting target protein reached more than 50% abundance compared with other proteins. T7 RNA polymerase is activated in the presence of the lac inducer in the form of IPTG.

The isolate of *E. coli* BL21 had an inhibitory diameter of 1 mM HgCl₂ of 5.65 mm. Rahayu [4] conducted a similar test with an inhibitory diameter result of *Streptomyces* AS2 of 9.6 mm. When viewed from before and after transformation, the resistant activity of *E. coli* BL21 is greater than the endogenous inhibitory activity of *Streptomyces* AS2. The clear zone indicated the most significant resistance activity, which was getting smaller (5.65 mm from the previous 9.6 mm compared with the quantity of endogenous mercuric reductase in *Streptomyces*). This means that *E. coli* transformed by the *merA* gene can produce higher amounts of mercuric reductase due to robust transcription by the T7 promoter. The most increased specific activity of mercuric reductase was obtained with a concentration of 294.07 U/mg.

The structure of a protein is more evolutionarily conserved than its amino acid sequence, and the sequence of the desired protein (target) can be pretty accurately modeled

using a very distant sequence of known structure (substrate), as long as the relationship between the target and the model can be distinguished by the association of sequences [14] The *Streptomyces* AS2 mercuric reductase protein has a sequence length of 466 amino acids and a globular protein. Characterization and modeling of proteins using the Swiss model for homology view and phyre2 for folding view.

Protein modeling using the Swiss model showed that the mercuric reductase protein in *Streptomyces* AS2 shares high homology with *Lysinibacillus sphaericus*. The query mercuric reductase protein *Streptomyces* AS2 has the best GMQE and QMEAN scores among the three structures (Mercuric reductase Crystal Structure of TN501 *MerA*, Mercuric reductase Crystal Structure of the C136, and Mercuric reductase Crystal Structure of C558). The thumb symbol pointing up to the right of the QMEAN score indicates that the reference structure is sufficient to represent the query protein structure.

Protein modeling using Phyre2 reported that a protein with code c5c1Yc is the best reference structure among other predictive reference structures for mercuric reductase *Streptomyces* AS2. The code is a phyre2 accession code adopted from the SCOP database. The letter “c” at the beginning indicates that the entire protein chain is used as the reference structure if “d” shows only certain domains. The code of c5c1Yc refers to mercuric reductase from *Lysinibacillus sphaericus*. Based on the confidence level of the predicted results, 97% or 461 residues of the mercuric reductase protein *Streptomyces* AS2 were expected to have structural similarities to the reference protein with a 100% confidence level. The prediction of secondary structure mercuric reductase *Streptomyces* AS2 using the phyre2 program showed that mercuric reductase *Streptomyces* AS2 has 32% α -helix bonds, 30% β -strands, and 4% transmembrane helices.

Reconstruction of the phylogenetic tree using mercuric reductase amino acid sequences. Mercuric reductase sequences were retrieved from the alpha fold protein database.

Based on the alpha fold database, it is known that only one in the genus *Streptomyces* and the other are different genera. The total number of amino acid sequences compared was 11 organisms. Maximum likelihood estimation and the JTT matrix model were used to infer the evolutionary history. [15]. The tree with the highest logarithmic probability (3361.13) is displayed. Under the branches, the percentage of trees for which related taxa have clustered is indicated. The starting trees for the heuristic search were generated automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances determined using the JTT model and then adopting a useful topology. The tree is drawn to scale, with branch lengths denoted by the number of substitutions at each site. This investigation entailed the examination of 11 amino acid sequences. The final dataset contains 355 locations in total. The bootstrap consensus tree is inferred from 1000 iterations [16] considered representative of the evolutionary history of the analyzed units. Evolutionary analyzes were performed in MEGA11 [11].

Previous study reported that the reconstruction of the phylogenetic tree using the 16S rRNA gene, *Streptomyces* AS2 belong to *Streptomyces ardesiacus* NRRL B-1773 [3]. However based on protein database (Alpha fold) *Streptomyces* AS2 similar to *Streptomyces lividans*. This study revealed the characterization of tertiary structure of the mercuric reductase protein in *Streptomyces* for the first time.

Conclusions

The *merA* gene isolated from *Streptomyces* AS2 can be expressed in the host *Escherichia coli* BL21. The optimum specific activity of mercuric reductase was found 292.47 U/mg protein. Mercuric reductase protein in *Streptomyces* AS2 shares high homology with *Lysinibacillus sphaericus* (Swiss-Model), and similar folding to that of c5c1 Yc refers to mercuric reductase from *Lysinibacillus sphaericus* using Phyre2. Based on alpha fold

database mercuric reductase sequences, this study reported that *Streptomyces* AS2 has a similarity of 100% with *Streptomyces lividans*. Characterization of the tertiary structure of the mercuric reductase protein in *Streptomyces* was carried out for the first time.

Abbreviations

merA: Mercury-resistance operon gene A

IPTG: Isopropyl β -D-1-thiogalactopyranoside

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

SDS-PAGE: Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Acknowledgment

We thank Professor Masashi Kawaichi for an excellent chance to perform some experiments in his laboratory by Special Research Student Program, NAIST, and Gadjah Mada University collaboration.

Author contributions

Anis Uswatun Khasanah designed and performed the experiments and wrote the manuscript.

Wahyu Aristyaning Putri performed the experiments and edited the manuscript. Hanum

Mukti Rahayu performed the experiments and edited the manuscript. Langkah Sembiring designed the experiments. Yekti Asih Purwestri designed the experiments.

Funding

This research was funded by BPPDN Dikti (Beasiswa Pendidikan Pascasarjana Dalam Negeri) Ministry of Research and Technology Indonesia.

Availability of data and materials

Not Applicable

Ethics approval and consent to participate

Not Applicable

Consent for Publication

Not Applicable

Conflict of interest / Competing Interest

The authors have no conflict of interest in participating in this research. The funder had no role in the study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

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Figures

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Figure 5

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