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Research Article

Expression, Characterisation and Structural Homology Modelling of Recombinant Mercuric Reductase of *Streptomyces* sp. AS2

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ABSTRACT

Mercury pollution poses a significant environmental challenge worldwide, prompting extensive efforts over the past two decades to combat its detrimental effects. Cloning *merA* from *Streptomyces* sp. AS2 (Accession numbers LC026157) into the expression vector pET-28c (+) marks a critical advancement in this field, necessitating further investigation into the expression and structural analysis of the resulting recombinant mercuric reductase protein. This study aimed to optimise the expression and characterise the structural MerA protein. The study involved the expression of *merA* from AS2 isolate in the host *Escherichia coli* BL21 and the measurement of mercuric reductase using SDS-PAGE. Induction of *E. coli* BL21 was optimized by adding IPTG concentration and incubation time. Purification of mercuric reductase was attempted using ammonium sulphate precipitation, dialysis, and column chromatography. Protein structural characterisation was conducted using computational modelling tools Swiss-Model and Phyre2. Expression of *merA* from AS2 isolate was successfully performed *in E. coli* BL21, with SDS-PAGE showing a dominant band in the 55-70 kDa range using IPTG concentration 1 and 1,2 mM and 18-hour incubation time. The specific activity of mercuric reductase was obtained at an enzyme concentration of 294.07 Unit/mg. Protein structural characterisation revealed homology with *Lysinibacillus sphaericus* (Swiss-Model) and similar folding to c5c1Yc, a known mercuric reductase from the same species using Phyre2. The successful expression of recombinant pET-*merA* in *E. coli* BL21 offers new opportunities for bioremediation efforts targeting mercury contamination.

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 (Barkay et al. 2010; Bharagava et al. 2017). *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 (Cuozzo et al. 2018). Therefore, they are very suitable for the biological decomposition of environmental pollutants (Cuozzo et al. 2018). *Streptomyces* spp. can degrade the mercury in the environment because they harbour the *merA* (Putri et al. 2021). It could be utilized to address the issue of biological mercury exposure with its detoxification ability by *Streptomyces* spp. Detoxification primarily involves the conversion of toxic heavy metal ions into non-toxic ions (Mello et al. 2020; Rahayu et al. 2021).

Mercuric reductase (EC.1.16.1.1) is an oxidizing enzyme and *Flavin Adenine Dinucleotide* (FAD) is required for the conversion of mercury from Hg^{2+} to non-toxic Hg^{0} (Bafana et al. 2017). 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 (Bafana et al. 2017). 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 (Dash et al. 2017). Furthermore, the *merA* commonly present in bacteria and archaea is likely to survive at high mercury concentrations. Adenine Dinucleotide Phosphate (NADPH) and *merA*-FAD compounds are critical in mercury volatilization and subsequent binding (Singh & Kumar 2020).

The homology structure of mercuric reductase, elucidated through comparative modeling techniques, reveals conserved motifs and structural features across different bacterial species, highlighting its evolutionary significance and functional conservation (Bafana et al. 2017). Beyond its fundamental catalytic role, mercuric reductase finds diverse applications in environmental bioremediation, where engineered microbial systems utilise the enzyme to mitigate mercury contamination in soil, water, and industrial effluents (Bharagava et al. 2017). Additionally, the enzyme's ability to selectively bind and reduce mercury ions has garnered interest in biotechnological applications, including biosensors for mercury detection and novel approaches for mercury recovery from waste streams (Dash et al. 2017). These multifaceted applications underscore the importance of understanding both the catalytic mechanism and structural characteristics of mercuric reductase in addressing environmental and industrial challenges associated with mercury pollution.

Rahayu et al. (2021) purified and characterised mercuric reductase from four strains of *Streptomyces* spp. isolated from a mercury-contaminated site in Indonesia (Rahayu et al. 2021). These strains, namely *Streptomyces* spp. AS1, AS2, AS6, and BR28, exhibited varying resistance activities to $HgCl_2$, with *Streptomyces* sp. AS6 demonstrates the highest resistance. Mercuric reductase from *Lysinibacillus sphaericus* has been previously characterised , with a molecular weight of approximately 60 kDa, Km of 32 µM HgCl2, and Vmax of 18 units/mg (Bafana et al. 2017). Cloning of the *merA* from *Streptomyces* sp. AS2 was successfully achieved (Putri et al. 2021), revealing a gene length of 1456 bp and similarities with *Streptomyces lividans*. The AS2 has received an accession number from the DNA Data Bank of Japan (DDBJ) with code LC026157 for AS2 clone 2. However, research on *merA* transformation from *Streptomyces* sp. AS2 into *E. coli* BL 21 is important to assess protein expression, activity, and structural characterization. This study aims to

optimise recombinant protein expression and structural homology characterization of *Streptomyces* sp. AS2.

MATERIALS AND METHODS

Materials

A plasmid pET-28c (+) containing the *merA*, and competent *E. coli* BL21 cells used for transformation of the *merA* into *E. coli* BL21. Inoue buffer was used to transform *E. coli* using the pET-*merA* plasmid. Inoue buffer consists of 55 mM $MnCl_2.4H_2O$, and 15 mM $CaCl_2$. $2H_2O$, 250 Mm KCl, and 10 mM PIPES with pH 6.7 (Green & Sambrook 2020). Liquid Luria Bertani (LB) medium is used as a medium for the growth of *E. coli* BL21. The components of the Luria Bertani liquid medium are 10 g NaCl, 5 g yeast extract, 10 g tryptone, and 1 litre of distilled water. The pH of the solution is made to 7.0 (Sezonov et al. 2007). Iso Propyl Thio D-Galactoside (IPTG) is used to optimize the expression of mercuric reductase. The *Nicotinamide Adenine Dinucleotide Phosphate* (NADPH), Mercuric Reductase Assay (MRA) solution, PBS solution, $MgCl_2$, EDTA, β-mercaptoethanol, and $HgCl_2$ are used for determining the specific activity of mercuric reductase. Bradford reagent is used for quantification of protein concentration. Protease inhibitor, and DEAE Sepharose anion column chromatography are used for protein purification. A retained protein marker 180 kDa is used as a marker to determine protein molecular weight.

Methods

E. coli BL21 transformation using pET-*merA*

E. coli BL21 transformation using pET- *merA* into was carried out using the heat shock method (Sambrook & Russell 2001). A 3 µL volume of plasmid pET-28c(+) containing the *merA* from previous study (Putri et al. 2021) was added to 100 µL of competent *E. coli* BL21 cells. The mixture was then incubated on ice for 30 minutes, followed by a 52-second incubation at 42°C. After 52 seconds, the mixture was promptly transferred back to ice bath and further incubated for 3 minutes. Subsequently, 900 µL of SOC medium consists of 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose (Cold Spring Harbor Laboratory Press 2012) was added to the suspension and incubated in a 37°C shaker for 1 hour. The suspension was then centrifuged at 8000 rpm for 1 minute, and the supernatant (1100 µL) was discarded, leaving 100-200 µL for plating on LB medium supplemented with 20 µg/mL kanamycin. The plated cells were then incubated at 37°C for 24 hours.

Confirming and optimising mercuric reductase expression involved varying IPTG concentration and incubation time

The *E. coli* BL21 carrying pET-*merA* plasmid was inoculated in 5 mL of liquid Luria Bertani (LB) containing 20 µg/mL kanamycin and incubated for 3 hours. The study used IPTG as an inducer with concentrations of 1 and 1.2 mM and also 4 hours and 18 hours incubation time. The various treatments are IPTG 1 mM with an incubation time of 4 hours, IPTG 1 mM with an incubation time of 18 hours, IPTG 1,2 mM with an incubation time of 4 hours, and IPTG 1.2 mM with an incubation time of 18 hours. The cell culture was centrifuged at 6000 rpm for 30 minutes. The supernatant was discarded, and the pellet was washed twice with PBS, pH 7.2. The obtained pellets were broken down using sonication for 30 seconds, with 3 repetitions, each followed by a 10-second rest. The supernatant was directly analysed for expression of mercury reductase by SDS

PAGE or stored frozen at 80°C.

Mercuric Reductase Enzyme Activity Assay

Mercuric reductase enzyme activity was determined by measuring $NADPH_2$ oxidation at λ 340 nm. The method involved adding 17 µL of the enzyme extract to 83 µL of Mercuric Reductase Assay (MRA) solution, which comprised 50 mM PBS solution (pH 7), 100 µM $NADPH_2$, 0.2 mM $MgCl_2$, 0.5 mM EDTA, 0.1% (vol/vol) β-mercaptoethanol, and 200 µM $HgCl_2$. Incubation occurred for 60 minutes at 37°C in darkness. Spectrophotometric measurements at 340 nm λ were conducted before and after incubation to determine initial and final $NADPH_2$ concentrations, enabling calculation of oxidized $NADPH_2$ quantity using the $NADPH_2$ standard curve equation y = ax + b. Activity was expressed as units of oxidised $NADPH_2$ per mg of protein per minute (µM $NADPH_2$/mg protein/min). 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 Nicotinamide Adenine Dinucleotide Phosphate ($NADPH_2$) 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.

Quantification of Protein Concentrations Using Bradford Assay

The standard curve was initially generated. Protein concentration testing was conducted using the Coomassie Blue (Bradford Assay) method. Crude enzyme samples (8 µL) were combined with 200 µL of Bradford reagent and incubated for 2 minutes at room temperature. Absorbance was then measured at a wavelength of λ 595 nm. Protein concentration (mg/mL) was determined using the regression equation of the standard protein curve.

Resistance test of *E. coli* BL21 to $HgCl_2$

The resistance tests were carried out using the paper disk method. A 100 µL volume of *E. coli* BL21 pET-*merA* suspension which has been added IPTG was grown on the surface of a petri dish containing LB medium. A 6 mm paper disk was placed on top of the medium, and a drop of 8 µL of 1 mM $HgCl_2$ solution was added. The media was incubated at 37°C for 24 hours, and the diameter of the inhibition zone was measured.

Production and Purification of MerA Recombinant

The optimized IPTG induction and incubation time results were utilised as standard cell culture conditions for optimal mercuric reductase production. *E. coli* BL21 pellets were added with protease inhibitor then sonicated to disrupt the cells. Sonication, performed in three 30-second cycles with a 10-second rest between each, was supplemented with a protease inhibitor (1 tablet per 50 mL cell culture). A supernatant and pellet solution were obtained, with the supernatant collected for analysis due to the solubility of mercuric reductase. This supernatant constituted the crude enzyme extract, which could be directly analysed with SDS-PAGE or stored at -20°C. The purification process involved three stages: precipitation with ammonium sulphate at a saturation level of 20-70%, dialysis, and DEAE Sepharose anion column chromatography.

Characterisation of MerA Recombinant using SDS-PAGE

Protein analysis was conducted using SDS-PAGE with the Pre-stained

Protein Marker 180 kDa. 4% stacking gel and 12% resolving gel were used for SDS PAGE. Enzyme samples (10 µL) were mixed with sample buffer and heated at 100°C for 2 minutes, followed by a 10-minute incubation in an ice bath. Samples and protein markers were loaded into the wells of the electrophoresis gel, and electrophoresis was run for 1.5 hours at 100 volts using a running buffer. The gel was stained with Coomassie Brilliant Blue and destained to remove the background colour.

Protein Modelling, Prediction, and Structural Analysis

Tertiary protein structure prediction techniques can be divided into homology modelling, fold recognition, and *ab initio* prediction. Homology modelling was predicted using the Swiss model (Biasini et al. 2014) whereas modeling based on fold recognition using Phyre2 (Kelley et al. 2015). The protein prediction template was sourced from the Protein Data Bank (PDB) dataset.

Phylogenetic Tree of Amino Acid Sequences

The reference protein was downloaded from the PDB ID (Protein Data Bank). Reconstruction and analysis of phylogenetic trees based on mercuric reductase protein sequences were performed using MEGA11 (Zeyaullah et al. 2010).

RESULTS AND DISCUSSION

Transformation of *E. coli* BL21 using pET-*merA*

The successful expression and characterisation of recombinant MerA from *Streptomyces* sp. AS2 underscores its potential for mercury detoxification applications, confirming its functionality as a mercuric reductase enzyme involved in bacterial mercury resistance mechanisms. *Streptomyces* sp. AS2 isolates demonstrated successful transformation, as evidenced by their growth in selective medium. Approximately 40 colonies were observed, indicating the effective transcriptional activity of the pET-28c (+) plasmid with a strong promoter for target protein production (Figure 1).

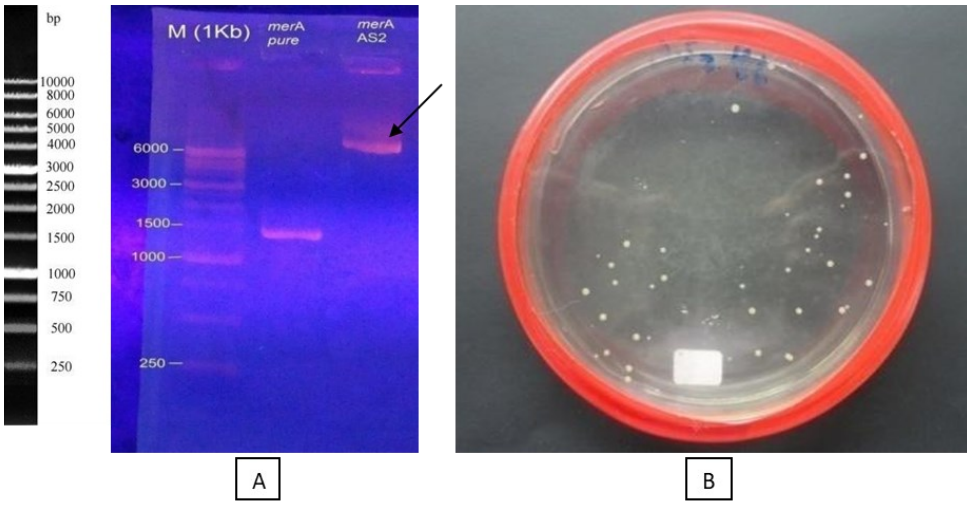

Figure 1. (A) Confirmation of *merA* after transformation; (B) *E. coli* BL21 recombinant inserted pET-*merA*.

Optimisation of Mercuric Reductase Expression and Measurement of Molecular Weight

Heterologous expression of mercuric reductase was confirmed by specific protein bands appearing at 55–70 kDa on SDS-PAGE (Figure 2). The

study used IPTG concentrations of 1 and 1.2 mM, with an optimal incubation time of 18 hours. Our findings demonstrate successful expression of mercuric reductase from *Streptomyces* sp. AS2 upon IPTG induction in *E. coli* BL21. The results of this study are in line with of Zeyaullah et al. (2010), who obtained a plasmid containing *merA* (1695 bp) from *E. coli* isolated from a mercury-contaminated site and expressed it in the pQE-30U/A vector, resulting in the expression of a 66.2 kDa mercuric reductase protein (Zeyaullah et al. 2010).

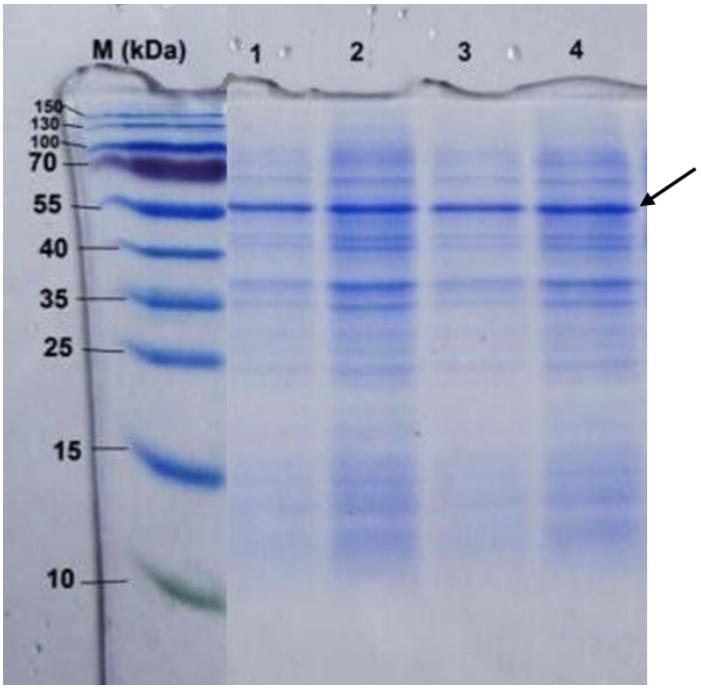

Figure 2. Expression of mercuric reductase *Streptomyces* sp. AS2 after induction IPTG 1 mM (1,2) & 1.2 mM (3,4) with 18 hours incubation (two replicates).

The observed size of the mercuric reductase aligns with findings from prior research. Gene expression analysis using *E. coli* BL21 (DE3) as the host revealed a mercuric reductase size of 66.2 kDa, consistent with reports on mercuric reductase expression from *Zymomonas mobilis*, which had a size of 65 kDa (Jones et al. 1992). These findings are attributed to the presence of a pET-28c (+) plasmid containing a robust T7 promoter, facilitating more efficient transcription of mercuric reductase compared to other proteins. Activation of T7 RNA polymerase occurs upon induction with IPTG, a lac operon inducer (Ogunseitan 1998).

Resistance test of *E. coli* BL21 against $HgCl_2$

The resistance test conducted in *E. coli* BL21 aims to evaluate its resistance level to 1 mM $HgCl_2$. Figure 3 depicts a 5.65 mm inhibitory diameter in *E. coli* BL21 carrying the *merA* isolate AS2 against 1 mM $HgCl_2$ (3 replications). The inhibitory diameter of *E. coli* BL21 against 1 mM HgCl2 was measured at 5.65 mm. Rahayu et al. (2021) conducted a similar test with *Streptomyces* sp. The inhibitory diameter produced by *Streptomyces* sp. AS2 is 9.6 mm (Rahayu et al. 2021). Comparison before and after transformation revealed that the resistant activity of *E. coli* BL21 surpassed the endogenous inhibitory activity of *Streptomyces* sp. AS2. The increase in mercuric reductase resistance is characterised by a decrease in the diameter of the resistance. The greater resistance activity may be due to the high quantity of mercuric reductase in the cell, resulting from the strong T7 promoter, compared with the quantity of mercuric reductase from endogenous *Streptomyces*.

Protein Extraction and Purification

In this study, the specific activity of recombinant mercuric reductase crude enzyme was higher than that of precipitation with ammonium sulphate, dialysis, and DEAE sepharose anion column chromatography (294.07 Unit / mg protein), and the lowest specific activity was found in dialysis results (29.22 Units / mg protein) (Table 1). Recovery of purified enzymes is also quite low. This less optimal result can be caused by several factors, including inclusion body, incubation time after less optimal induction of IPTG, ineffective lysis method, and purification method not specific for recombinant protein (Paul et al. 2020). So, another purification method for recombinant protein still needs to be performed for further study (Zeroual et al. 2003).

Protein Modelling, Prediction, and Analysis

To understand the structural properties of mercuric reductase, protein modelling, and prediction techniques were employed. Swiss-Model and Phyre2 were utilised for homology modelling and fold recognition, respectively. The resulting three-dimensional structure provided insights into the protein's architecture and spatial arrangement of amino acid residues. 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* sp. AS2 shares a 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. Figure 4 illustrates the three-dimensional structure of mercuric reductase generated by the Swiss -Model (Figure 4A), the results of a reference structure search (Figure 4B), and the phyre2 prediction results (Figure 4C). These computational modelling approaches enhance our understanding of AS2 mercuric reductase structural features and facilitate further biochemical investigations.

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 modelled 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 (Tamura et al. 2021). The *Streptomyces* sp. AS2 mercuric reductase protein has a sequence length of 466 amino acids and a globular protein. Characterisation and modelling of proteins using the Swiss model for homology view and Phyre2 for folding view.

Protein modelling using the Swiss model showed that the mercuric reductase protein in *Streptomyces* sp. AS2 shares high homology with

Table 1. Purification of Mercury Reductase of *E. coli* BL21 as the result of transformation.

Stage	Volume (mL)	Protein		Mercury Activity			Purification	
		Concentration of Protein (mg/mL)	Total Protein (mg)	Activity of Enzyme (Unit)	Total Activity	Specific Activity (Unit/mg protein)	Purification Factor	Results (%)
Crude Enzyme	80	0.2518	20.1405	74.05	5923.69	294.07	1	100
Precipitation Am. Sulphate	18	0.3052	5.4941	68.94	1240.96	228.01	0.7754	20.95
Dialysis	6	1.306	7.8361	38.15	228.92	29.22	0.1282	3.86
DEAE sepharose anion column chromatography	4	0,3454	1,3871	40.91	118.32	163.65	5.6006	2.00

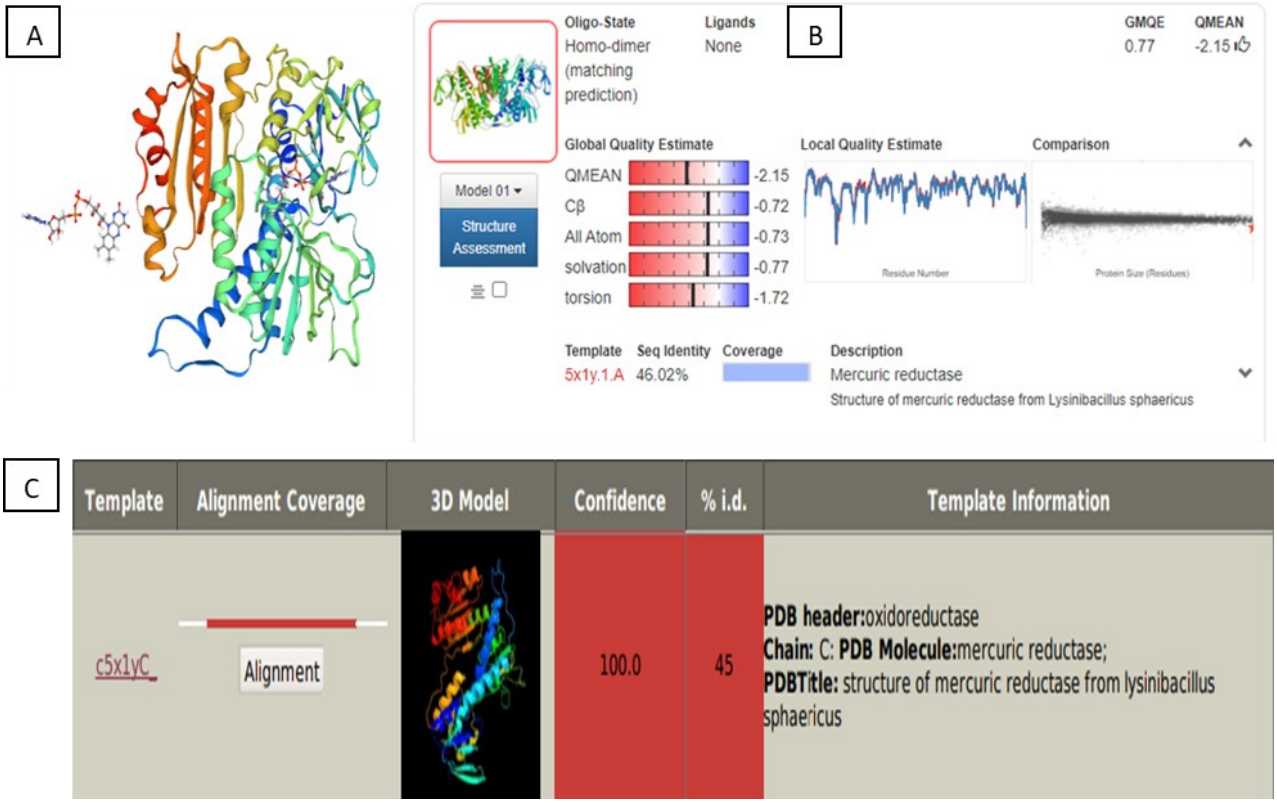

Figure 4. Image of the three-dimensional structure of the mercuric reductase protein; (A) Modelling the 3D structure result using Swiss-Model; (B) Results of a reference structure search using mercuric reductase *Streptomyces* AS2 protein sequence query; (C) Display of phyre2 prediction results with the best reference structure.

Lysinibacillus sphaericus. The query mercuric reductase protein *Streptomyces* sp. 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 from *Lysinibacillus sphaericus* is the best reference structure among other predictive reference structures for mercuric reductase *Streptomyces* sp. 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, a total of 461 or 97% residues of the *Streptomyces* sp. AS2 mercuric reductase protein are estimated to have 100% structural similarity to the reference protein. The prediction of secondary structure mercuric reductase *Streptomyces* sp. AS2 using the phyre2 program showed that mercuric reductase *Streptomyces* sp. AS2 has 32% α-helix bonds, 30% β-strands, and 4% transmembrane helices.

Phylogenetic Tree of Amino Acid Sequences

The phylogenetic tree based on mercuric reductase amino acid sequences, depicted in Figure 5, illustrates the evolutionary relationships among various organisms. Our study identified a 100% similarity between *Streptomyces* sp. AS2 and *Streptomyces lividans* in terms of the mercuric reductase sequence. Mercuric reductase sequences were obtained from the alpha fold protein database, comprising 11 organisms, primarily from the

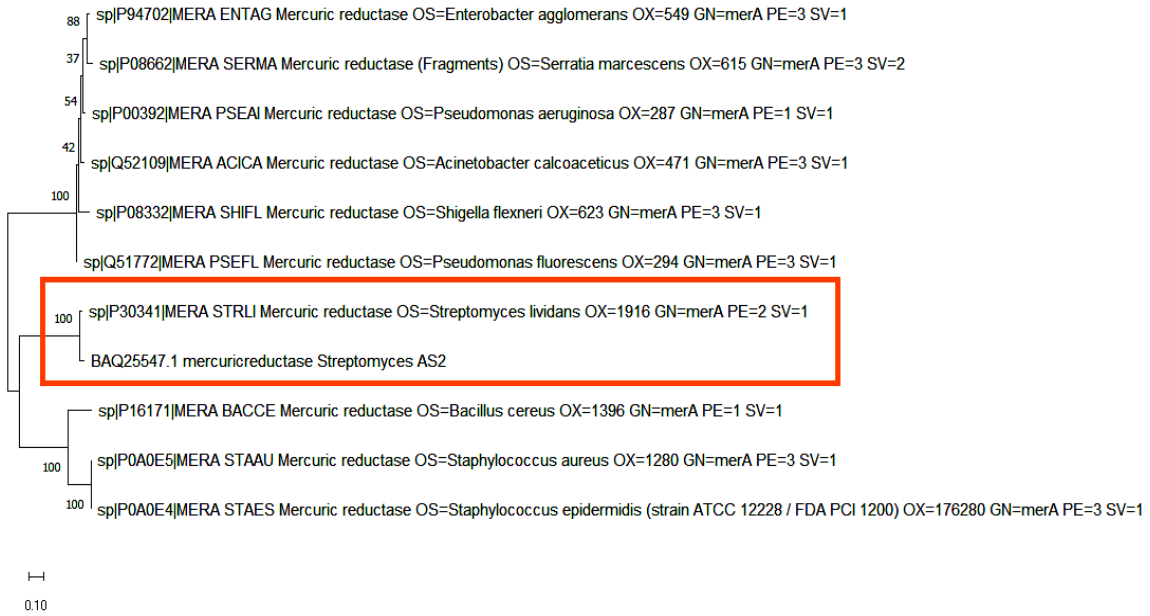

Figure 5. The results of the reconstruction of the phylogenetic tree using a MEGA11 program based on the mercuric reductase protein.

Streptomyces genus. Maximum likelihood estimation using the JTT matrix model was employed to infer the evolutionary history (Aprilyanto & Sembiring 2017), resulting in a tree with the highest logarithmic probability (3361.13). Branches on the tree indicate the percentage of trees supporting the clustering of related taxa. The tree, drawn to scale, reflects branch lengths denoted by the number of substitutions at each site. The analysis of the amino acid sequences of the 53 TNLs in roses and other Rosaceous species, with a bootstrap consensus tree derived from 500 iterations deemed representative of their evolutionary history (Terefe-Ayana et al. 2012). These analyses were conducted using MEGA11 (Tamura et al. 2021). Interestingly, a previous study based on the 16S rRNA gene suggested that *Streptomyces* sp. AS2 closely related *Streptomyces ardesiacus* NRRL B-1773. Our protein database (Alpha fold) analysis revealed *Streptomyces* sp. AS2 mercuric reductase its similarity to *Streptomyces lividans*. This investigation demonstrated of the tertiary structure of mercuric reductase protein in *Streptomyces*.

Reconstruction of the phylogenetic tree using the mercuric reductase protein database in several bacteria was intended to determine the similarity of mercuric reductase in *Streptomycetes* to other bacterial strains. Reconstruction of the tree using the phylogeny.fr program. The phylogenetic reconstruction in this program is arranged in the form of a pipeline, starting from the juxtaposition of multiple sequences, calculating the evolutionary distance, to the reconstruction of the tree itself. Tree reconstruction is done through a one-click menu (Dereeper et al. 2008). The mercuric reductase data base on various bacteria was obtained from the NCBI protein database. The phylogenetic tree shows the mercuric reductase protein *Streptomyces* sp. AS2 used in this study, has 60% similarity to *Lysinibacillus sphaericus*. These results are following the results of the reconstruction of the tertiary structure of 3D proteins using the Swiss-model and Phyre, both of which use mercuric reductase in *Lysinibacillus sphaericus* as a query and reference template. *Elizabethkingia anophelis* in the phylogenetic tree reconstruction was used as an outgroup due to the type of protein oxidoreductase but not mercuric reductase. *Elizabethkingia anophelis* is one of the species from

Elizabethkingia, a bacterial genus commonly found in soil and water. *Elizabethkingia anophelis* is a gram-negative bacteria and has been isolated from *Anopheles* mosquitoes (Centers for Disease Control and Prevention 2024). This bacteria was used as a comparison in the phylogenetic tree reconstruction.

CONCLUSION

In conclusion, our study represents a significant step forward in mercury bioremediation research, paving the way for innovative strategies to address environmental mercury contamination. The *mer*A gene isolated from *Streptomyces* sp. AS2 can be expressed in *E. coli* BL21 and was confirmed by specific protein bands appearing at 55–70 kDa. IPTG concentration 1 and 1.2 mM IPTG as inducer and 18-hour incubation time are optimal variations to determine the optimal expression of mercuric reductase. The specific activity of purified mercuric reductase on crude enzyme with a concentration of 294.07 units/mg Structural characterization of the *Streptomyces* sp. AS2 MerA protein has homology to *Lysinibacillus sphaericus* and has a similar fold to c5c1Yc. By elucidating MerA expression, enzymatic activity, and structural characteristics, we contribute to the development of advanced biotechnological interventions aimed at preserving ecological health and safeguarding human well-being.

AUTHORS CONTRIBUTION

A.U.K. designed and performed the experiments and wrote the manuscript. W.A.P. performed the experiments and edited the manuscript. H.M.R. performed the experiments and edited the manuscript. L.S. designed the experiments. Y.A.P. designed the experiments and edited the manuscript.

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CONFLICT OF 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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