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Original Article

# Exploring of pyrazinamidase recombinant activity from PZA-sensitive and resistant *Mycobacterium tuberculosis* expressed in *Escherichia coli* BL21 (*DE3*)

## Exploração da atividade recombinante da pirazinamidase de *Mycobacterium tuberculosis* sensível e resistente à PZA expressa em *Escherichia coli* BL21 (DE3)

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**Abstract**

The mutations of *pnc*A gene encoding pyrazinamidase/PZase in *Mycobacterium tuberculosis* are often associated with pyrazinamide/PZA resistance. The H and R1 isolates showed significant phenotypic differences to PZA. The H isolate was PZA sensitive, but R1 was PZA resistant up to 100 ug/ml. The paper reports the *pnc*A profile for both isolates and the activity of their protein expressed in *Escherichia coli* BL21(*DE3*). The 0.6 kb of each *pnc*A genes have been subcloned successfully into the 5.4 kb pET30a vector and formed the pET30a-*pnc*A recombinant with a size of 6.0 kb. The *pnc*AR1 profile exhibited base mutations, but not for *pnc*AH against to *pnc*A from the PZA-sensitive *M. tuberculosis* H37RV published in Genbank ID: 888260. Three mutations were found in *pnc*AR1, ie T41C, G419A, and A535G that subsequently changed amino acids of Cys14Arg, Arg140His and Ser179Gly in its protein level. The mutant PZase R1 that expressed as a 21 kDa protein in *E. coli* Bl21(*DE3*) lost 32% of its performance in activating PZA drug to pyrazinoic acid/POA compared to the wild-type PZase H. The mutation in the *pnc*AR1 gene that followed by the decreasing of its PZase activity underlies the emergence of pyrazinamide resistance in the clinical isolate. Structural studies for the R1 mutant PZase protein should be further developed to reveal more precise drug resistance mechanisms and design more effective TB drugs.

**Keywords:** *pnc*A gene, *Mycobacterium tuberculosis*, pyrazinamide resistance, PZase.

**Resumo**

As mutações do gene *pnc*A que codifica a pirazinamidase (PZase) no *Mycobacterium tuberculosis* estão frequentemente associadas à resistência à pirazinamida (PZA). Os isolados H e R1 apresentaram diferenças fenotípicas significativas em relação à PZA. O isolado H foi sensível à PZA, mas o R1 foi resistente à PZA até 100 ug/ml. O artigo relata o perfil *pnc*A para ambos os isolados e a atividade de sua proteína expressa em *Escherichia coli* BL21 (DE3). Em cada gene *pnc*A, 0,6 kb foi subclonado com sucesso no vetor pET30a de 5,4 kb, formando o recombinante pET30a-*pnc*A com tamanho de 6,0 kb. O perfil *pnc*AR1 exibiu mutações de base, mas não para *pnc*AH contra *pnc*A do *M. tuberculosis* H37RV sensível à PZA publicado no Genbank ID: 888260. Três mutações foram encontradas em *pnc*AR1: T41C, G419A e A535G, que posteriormente alteraram aminoácidos de Cys14Arg, Arg140His e Ser179Gly em seu nível proteico. O mutante PZase R1, que se expressa como uma proteína de 21 kDa em *E. coli* Bl21 (DE3), perdeu 32% de seu desempenho na ativação do medicamento PZA para ácido pirazinoico (POA) em comparação com a PZase H de tipo selvagem. A mutação no gene *pnc*AR1, seguida pela diminuição de sua atividade PZase, está subjacente ao surgimento de resistência à pirazinamida no isolado clínico. Mais estudos estruturais para a proteína PZase mutante R1 devem ser desenvolvidos com o objetivo de revelar mecanismos de resistência aos medicamentos mais precisos e projetar medicamentos mais eficazes para a TB.

**Palavras-chave:** gene *pnc*A, *Mycobacterium tuberculosis*, resistência à pirazinamida, PZase

## 1. Introduction

Tuberculosis (TB) is a respiratory infectious disease caused by *Mycobacterium tuberculosis*. The main organs that *M. tuberculosis* bacteria attack are the lungs. In the mechanism of infection in the lungs, *M. tuberculosis*

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bacteria come into contact with macrophage cells, which involves the interaction between mycobacterial cell surface components such as lipoarabinomannan (LAM), porin protein OmpA and heme agglutin HbhA with macrophage receptors such as the mannose receptor, the surfactant protein SP-A and CD14 (Liu et al., 1997; Purkan et al., 2018a; Dabla et al., 2022). When contact occurs, *M. tuberculosis* bacteria secrete virulent compounds into macrophage cells. Through experimental studies, it was found that the proteins that functioned as intracellular pathogenic compounds in *M. tuberculosis* were ESAT-6, CFP-10 and PZase proteins (Purkan et al., 2018a; Khawbung et al., 2021; Kanabalan et al., 2021).

The Sustainable Development Goals (SDG's) have put the elimination of tuberculosis (TB) as one of its goals. As many as 1.6 million people have died from TB in 2021 and 187 thousand of them with HIV co-infection (Hadi et al., 2023; Soeroto et al., 2021; WHO, 2022). TB is the 13th cause of death in the world, and is classified as the 2nd leading infectious disease cause of death after COVID-19. Indonesia ranks second in the world after India for TB cases in 2022, with a total of 969 thousand cases and 93 thousand deaths per year or the equivalent of 11 deaths per hour (WHO, 2022; Republic of Indonesia, 2022).

The emergence of drug-resistant TB has made it more difficult to cure the disease. As many as 450 thousand of the world's population suffered the TB resistant in 2021 and accompanied by a death rate of 191 thousand people. As 78% of these resistant cases were recorded as multidrug resistant-TB (MDR-TB). MDR-TB cases enhanced at an average rate of 3.6% per year, while XDR-TB increased 8.5% per year from MDR cases. Indonesia has ranked fourth in MDR-TB cases with a total of 24,000 cases in 2021. Studies of drug resistance mechanisms in *M. tuberculosis* clinical isolates are needed for the development of new, more potent TB drugs (Liu et al., 1997; WHO, 2022; Republic of Indonesia, 2022).

Pyrazinamide (PZA) is a TB drug with a high bacteriocidal effect, because it can kill semidormant of *M. tuberculosis* (Shi et al., 2022; Shrestha et al., 2022).The efficacy of PZA as a TB drug is highly dependent on the action of the pyrazinamidase (PZase) enzyme encoded by the *pnc*A gene of *M. tuberculosis*. As many as 80% of clinical isolates of PZA-resistant *M. tuberculosis* were reported to have *pnc*A mutations (Khawbung et al., 2021; Lamont et al., 2020). The types of mutations in the *pnc*A gene were reported to be very diverse, and showed specificity in each geographic area where these clinical isolates were found (Hadi et al., 2023; Shi et al., 2022; Shrestha et al., 2022; Lamont et al., 2020; Purkan et al., 2016). *M. tuberculosis* strains that are defective in pncA thereby eliminating its PZase activity are reported to be resistant to PZA. (Khan et al., 2021; McHenry et al., 2020; Konno et al., 1967), then the transformation of the PZA–resistant bacilli of *Mycobacterium bovis* and *M. tuberculosis* that had no PZase activity with a functional *pnc*A gene restored the enzyme activity and PZA susceptibility (Scorpio and Zhang, 1996). The relationship between PZase activity and pyrazinamide resistance still raises many questions because the ultimate deficiency of *pnc*A in clinical isolates of *M. tuberculosis* has never been found.

The important role of PZase-encoding *pnc*A in PZA action is underscored by the fact that most clinical isolates of PZA-resistant *M. tuberculosis* harbor mutations in this gene. The mode of action described by a number of papers shows that PZA is a prodrug which is then activated by the PZase to become POA, then in turn it can further kill mycobacteria (Figure 1) (Hadi et al., 2023; Zhang et al., 2013).

The initial action of POA takes place from the entry of PZA into mycobacterial cells through passive diffusion, and then PZase/nicotinamidase converts it into POA. The resulting POA compounds can kill mycobacterial cells through various mechanisms. POA binds explicitly to the RpsA protein and inhibits the trans-translation process in *M. tuberculosis* (Tunstall et al., 2021; Vallejos-Sánchez et al., 2020). POA also inhibits the biosynthesis of pantothenate or CoA compounds, two essential compounds in cell metabolism processes (Karmakar et al., 2020; Mehmood et al., 2019). Another mode suggests that POA triggers cytoplasmic aciditation due to its ionization to [POA-] and H+. The acidity increases due to the accumulation of H+ produced by the transformation of POA under acidic conditions into HPOA, the passive diffusion mechanism, and the efflux of the two intermediates on the mycobacterial cell surface. Excessive cytoplasmic acidification causes mycobacterial cells to break down (Figure 2) (Dookie et al., 2022; Vallejos-Sánchez et al., 2020; Zhang et al., 2013).

Most of the mutations found in the pncA gene from clinical isolates of M. tuberculosis are point mutations such as insertion, substitution, and deletion of nitrogen bases in the gene. Identification of pncA gene mutations

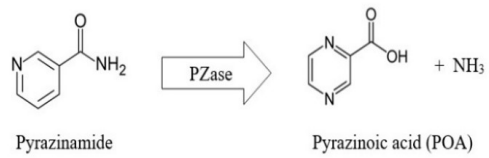


**Figure 1.** Conversion of Pyrazinamide to POA by PZase (Zhang et al., 2013; Hadi et al., 2023).

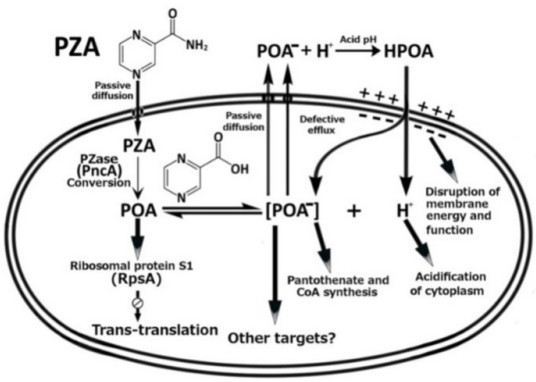


**Figure 2.** The action mode of PZA on tubercle bacilli (Zhang et al., 2013).

from clinical isolates of PZA-resistant M. tuberculosis is needed to reveal the basis of drug resistance at the genotypic level, and then in turn at the enzymatic level, it requires elucidating the function of the accompanying PZase mutants.

*M. tuberculosis* isolates, H and R1 had significant phenotypic differences to PZA drug. The H isolate is PZA sensitive, while the R1 isolate is PZA resistant up to 100 ug/ml. The basis of PZA resistance in clinical isolate R1 has been unrevealed in term of genotype aspect and its intermediary protein function. The paper reported the difference of *pnc*A gene profile from the R1 isolate compared to the H, equipped with the activity of the PZase protein encoded by each gene, in order to support the explanation of PZA resistance from the protein's role aspect. The research work was carried out using cloning technique and *pnc*A gene expression in *Escherichia coli* BL21 (*DE3*) host cells.

## 2. Materials and Methods

### *2.1. Materials*

Plasmid samples used in the research consisting of pGemT-*pnc*A and pET30a. The recombinant plasmid, pGemT-*pncA* obtained from previously research, containing the *pnc*A gene from H37RV and R1 isolate respectively. The another was the pET30a plasmid that used as expression vector. The chemicals used were yeast extract, NaCl, tryptone, bacto agar, $CaCl_2$, EDTA, NaOH, glycerol, lysozyme, isopropanol, ethanol, agarose, ethidium bromide, Wizard Genomic DNA Purification Kit (Promega), QIAprep Miniprep Kit (Qiagen), the enzyme of *Nde*I, *Bgl*II and T4 DNA ligase, PMSF, acrylamide, bis-acrylamide, Glycine, commassie brilliant blue R-250.

### *2.2. Methods*

#### *2.2.1. Subcloning of pncA gene to pET30a expression vector*

The plasmid pGemT-*pnc*A was digested simultaneously by *Nde*I and *Bgl*II restriction enzymes, likewise for the pET30a plasmid. The fragment of *pnc*A and linier plasmid pET30a was isolated and purified with DNA purication kit (Promega), then both joined each other by T4-DNA ligase enzyme. The product of joining reaction then introduced to the *Escherichia coli* BL21 (*DE3*) by transformation method with cold $CaCl_2$ reagent. The positive clone was selected in Luria Bertani Medium with additional the antibiotic of kanamycin. Characterization to the clone was performed by restriction analysis of recombinant plasmid and nucleotides sequencing for *pnc*A gene.

#### *2.2.2. Digestion of DNA sample with restriction enzyme*

The cutting reaction was carried out in a volume of 50 µL of the reaction mixture containing 1 µL of restriction enzyme *Nde*I and 1 µL of restriction enzyme *Bgl* II; 1 µL DNA sample; 5 µL NE 10x enzyme buffer; and 42 µL sterile ddH2O (Purkan et al., 2018b; Green and Sambrook, 2012). Incubation for cutting restriction enzymes was carried out at 37 °C for 15 minutes, so that plasmids were cut with restriction enzymes *Nde*I and *Bgl*II. Then the result was being analyzed using agarose gel electrophoresis.

#### *2.2.3. Joining of the DNA fragments with ligase enzyme*

Ligation of the *pncA* gene to the pET30a vector was carried out by mixing 1 µL of T4 Ligation Buffer, 1 µL of T4 DNA ligase, 2 µL of restricted and purified *pncA* gene, 2 µL of restricted and purified pET30a plasmid, and 4 µL of sterile ddH2O into a new and sterile microtube. Then it was incubated overnight at 4 °C. After that, the *E. coli* BL21(*DE3*) transformation was carried out on the ligated recombinant DNA plasmid (Green and Sambrook, 2012; Purkan et al., 2017).

#### *2.2.4. Sequencing of pncA gene*

Sequencing or determination of the nucleotide sequence was determined by the dideoxy-Sanger method using an automatic sequencer (ABI PRISM) at Macrogen, Malaysia. The nucleotides of *pnc*A gene in pET30a-*pnc*A was sequenced by using T7 promoter and terminator. The nucleotides sequence was analyzed in silico by using SeqManTMII and MegAlignTM DNASTAR program (Lasergene, 1997).

#### *2.2.5. Plasmid isolation*

Plasmid extraction was performed by QIAprep Spin Miniprep Kit according to the manual from QIAGEN. For 5 mL of the transformed culture filled into a microtube tube, then centrifuged at 8.000 rpm at 20 °C for 3 minutes. The pellets from transformant cells were lysed with reagents provided by the QIAprep Kit to release the plasmid DNA. The plasmid resulted from this step was stored at -20°C and analyzed by agarose gel electrophoresis (Ahmad et al., 2023).

#### *2.2.6. Agarose gel electrophoresis*

The DNA was detected by agarose gel electrophoresis method using 1% (w/v) of gel and 1x of TAE buffer. The DNA sample was firstly mixed with loading dye, then inserted to the well of agarose gel. The electrophoresis process was run at 70 V for 45 minutes. The gel immersed in the ethidium bromide solution, then was visualized by UV transluminator (Purkan et al., 2012, 2017).

#### *2.2.7. Expression of pyrazinamidase*

Single colony of recombinant bacteria *E. coli* BL21(*DE3*) [pET30a-*pncA*] was cultured in LB-kanamycin liquid medium at 37°C and shaking at 150 rpm. When the optical density at λ600 nm reaches 0.4-0.6, it was added by 0,05 mM IPTG. The cultures were incubated again with 150 rpm stirring overnight but at room temperature 16 °C. The same treatment was also carried out on *E. coli* BL21(*DE3*) [pET-30a] culture as a control (Purkan et al., 2018b, 2020).

The cell pellet was separated from the culture by centrifugation, then lysed using an ultra sonicator at a power rate 1% for 15 minutes on a pulse *ON/OFF* in 30 seconds. The lysed cells were centrifuged at 12.000 rpm, on 4°C for

20 mins. The supernatant obtained was added with 1 mM phenylmethylsulfonyl fluoride (PMSF), then analyzed by SDS-PAGE and enzyme activity test (Purkan et al., 2020).

*2.2.8. Enzyme activity test*

As much as 25 µL of the enzyme extract was mixed with 500 µL of 2 mM PZA solution in sodium phosphate buffer pH 7.0, then incubated at 37 °C for 15 minutes. The reaction was then stopped with 25 µL of 25 mM ferrous ammonium sulfate followed by the addition of 450 µL of cold glycine-HCl buffer (pH 3.4). The absorbance of the mixture was measured at λ 460 nm. The product of this enzymatic reaction is calculated using the constant $\Delta\varepsilon_{460}$ POA, $6 \times 10^{-4}\ \mu M^{-1} cm^{-1}$. One unit (U) of enzyme activity is expressed by the amount of PZase enzyme needed to produce 1 µmol pyrazinoic acid (POA) per minute per mL of enzyme under experimental conditions (37 °C) (Rueda et al., 2018; Zhang et al., 2008; Sheen et al., 2012).

## 3. Results and Discussions

*3.1. Construction of recombinant DNA of pET30a-pncA*

The formation of the pET30a-pncA recombinant was prepared to express the pncA gene into PZase protein, because the pET30a plasmid is a type of expression vector. The source of the pncA gene for recombinant formation was taken from the pGemT-pncA plasmid obtained from previous studies and has a size of ~3.6 kb. This size accuracy was also confirmed by cutting the pGemT-pncA plasmid with both NdeI and BglII restriction enzymes, and the results correctly yielded ~3.6 kb DNA fragments (Figure 3).

The pncA gene is released from pGemT-pncA by simultaneously double cleaving with NdeI and BglII restriction enzymes. The double cutting resulted in two DNA fragments at ~3.0 kb and ~0.6 kb respectively (Figure 4). The 3.0 kb DNA fragment constituted to the size of pGemT plasmid while the 0.6 kb for the pncA gene size as reported in Genbank (ID: 888260).

The 0.6 kb fragment of pncA was isolated and then inserted into the pET30a plasmid. Before inserting the pncA gene, the pET30a plasmid was also cut with the same two restriction enzymes, NdeI and BglII. The cutting of pET30a plasmid resulted in a single fragment DNA at 5.4 kb on the agarose gel electrophorogram (Figure 5).

A recombinant plasmid of pET30a-*pnc*A was created by joining a linear pET30a vector with a *pnc*A fragment with T4 DNA ligase. The ligation reaction product was then introduced into *E. coli* BL21 (*DE3*) cells to produce positive *E. coli* clones carrying pET30a-*pnc*A recombinant DNA. The growth of transformed *E. coli* showed different profiles in LB-Kanamycin media (Figure 6) according to the type of plasmid DNA used in the transformation.

Based on Figure 6, it could be seen that *E. coli* BL21 (*DE3*) containing the plasmid pET30a DNA grew on the solid LB medium with kanamycin. The countless colonies were obtained in the product (Figure 6C). However, some colonies around 30 colonies were obtained when using pET30a-*pnc*AH wild type and pET30a-*pnc*AR1 mutant (Figure 6A and 6B). The colonies were not grown when

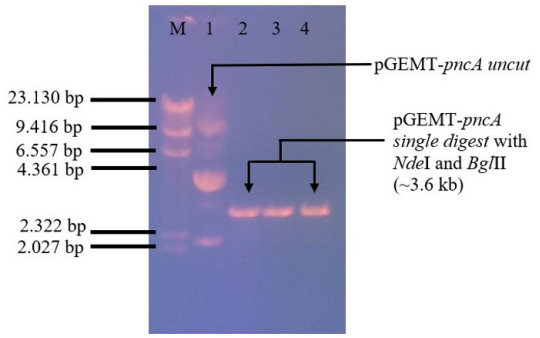


**Figure 3.** Electrophoregram of digest of pGEMT-*pncA* recombinant plasmid with restriction enzymes. (M) Marker DNA λ/*Hind*III, (1) pGEMT-*pncA uncut*; (2 and 3) pGEMT-*pncA*/*NdeI*; (4) pGEMT-*pncA*/*Bgl*II.

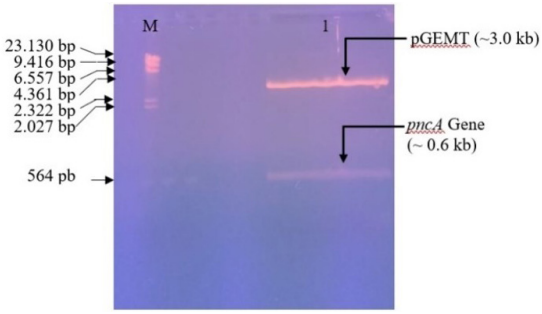


**Figure 4.** Electrophoregram of double digest pGEMT-*pncA*. (M) Marker DNA λ/*Hind*III; (1) Double digest pGEMT-*pncA*/*Nde*I + *Bgl*II.

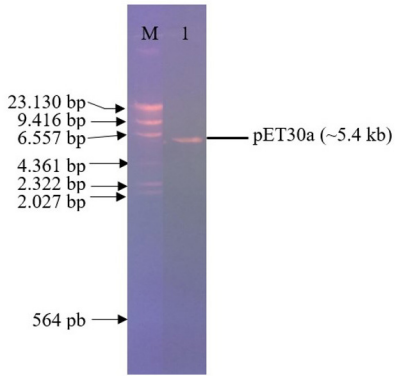


**Figure 5.** Electrophoregram of double digest pET30a plasmid. (M) Marker DNA λ/*Hind*III; (1) pET30a/ *Nde*I and *Bgl*II.

linear pET30 was cut with *Nde*I and *Bgl*II restriction enzymes used in the transformation (Figure 6D). Settlements resulting from modification with a mixture of ligation results (Figure 6A and 6B) were identified as positive clones as a source for gene identification. Next, the plasmids pET30a-*pnc*AH and pET30a-*pnc*AR1 were isolated from these positive clones for further characterization.

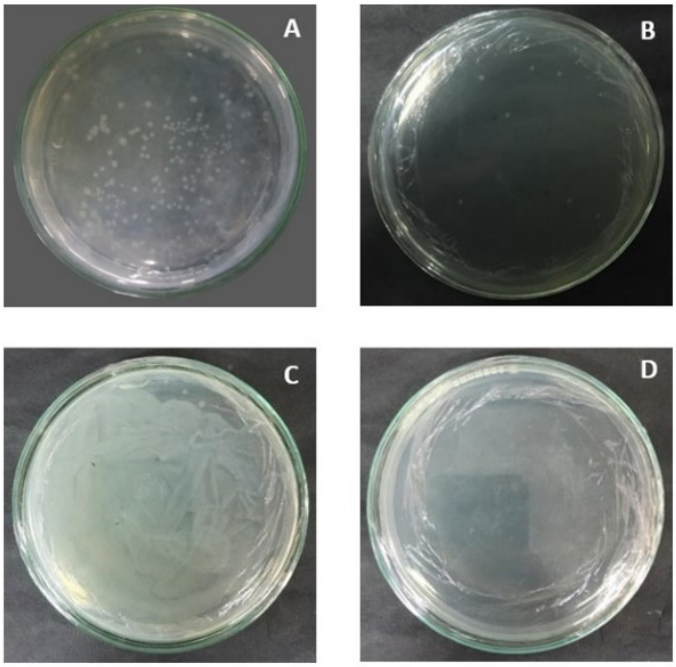


**Figure 6.** The growth of *E. coli* BL21 (*DE3*) transformant cells in LB medium-kanamycin. Some colonies grow for the transformation with pET30a-*pnc*AH and pET30a-*pnc*AR1 (A, B). Countless colonies were obtained with the addition of uncut pET30a (C), and no colonies were obtained in transformation with linear pET30 after cutting by *Nde*I and *Bgl*II restriction enzymes (D).

### *3.2. Profile of pET30a-pncA Recombinant*

The restriction map of pET30a-*pnc*A R1 and H7 plasmids after cut by *Bgl*II and *Nde*I enzyme showed single fragment with 6.0 kb in electrophoregram agarose gel electrophoresis (Figure 7), resulted from joining of pET30a (5.4 kb) and *pnc*A gene (0.6 kb). Confirmation of double digest for the pET30a-*pnc*A R1 and H7 with enzyme *Nde*I and *Bgl*II simultaneously resulted a suitable fragment, at 5.4 and 0.6 kb (Figure 7 lane 4 and 7).

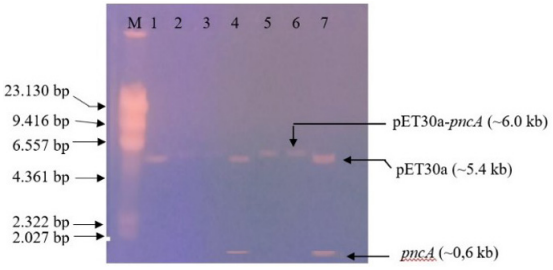


**Figure 7.** Electrophoregram of pET30a-*pncA* restriction analysis. (M) Marker DNAλ/*Hind*II; (1) pET30a/*Nde*I+*Bgl*II; (2) pET30a-*pncA* R1/*Nde*I; (3) pET30a-*pncA* R1/*Bgl*II; (4) pET30a-*pncA* R1/*Nde*I+*Bgl*II; (5) pET30a-*pncA* H/*Nde*I; (6) pET30a-*pncA* H/*Bgl*II; (7) pET30a-*pncA* R1/*Nde*I+*Bgl*II.

#### *3.2.1. Nucleotide sequence of the pncA gene*

The sequencing of the *pnc*A gene in the pET30a-*pnc*A recombinant plasmid was determined by T7 promoter and terminator primers. The binding site for the two primers is located in the upstream and downstream of *pnc*A gene. The entire nucleotide sequence of the *pnc*A gene consisting of 561 nucleotides was all sequenced with additional 18 nucleotides for His codon in upstream of plasmid pET30a. The nucleotide alignment of the genes that compared to *pnc*A from *M. tuberculosis* H37RV that published in the Genbank (Gene ID: 888260) showed mutation for *pnc*AR1 but no for *pnc*AH (Figure 8, and Table 1). Three mutations was found in *pnc*AR1, ie T41C, G419A, and A535G that change the amino acids Cys14Arg, Arg140His and Ser179Gly for its protein (Table 1). Multiple mutation in *pnc*A gene was also found in some PZA-resistant *M. tuberculosis* strains. Approximately 10-15% of the total *pnc*A mutations in PZA-resistant *M. tuberculosis* strains are reported to be multiple mutations. The variety and position of mutations in these genes are unique to each geographic area where resistant strains are obtained (Shi et al., 2020; Kahbazi et al., 2018; Shi et al., 2022; Shrestha et al., 2022).

The type mutation in *pnc*AR1 gene was be checked to compare with other mutant of *pnc*A from PZA-resistant *M. tuberculosis* strains. A mutation at position 535 base that resided in the *pnc*AR1 gene was also found in the *pnc*A of the PZA-resistant strain from Hunan Province, China, but differed in the type of base mutated. The A535T mutation that changes the Ser179Cys amino acid was found in the Hunan strain *pnc*A (Shi et al., 2020), while the A535G mutation changes the Ser179Gly amino acid in the *pnc*AR1 gene. Multiple mutations in *pnc*AR1 represented new mutations that have not been found in various references.

**Table 1.** Profile of *pncA* R1 and H against *pnc*A Genbank (ID: 888260) belonging to PZA-sensitive *Mycobacterium tuberculosis* H37RV.

| 3.1. Sample | Mutation compared to *pnc*A CenBank (ID: 888260) | | Fenotipe |
|---|---|---|---|
| | Nucleotides | Amino Acids | |
| pncA H7 | No mutation | No mutation | PZA Sensitive |
| pncA R1 | T41C | Cys14Arg | PZA Resistant |
| | G419A | Arg140His | |
| | A535G | Ser179Gly | |

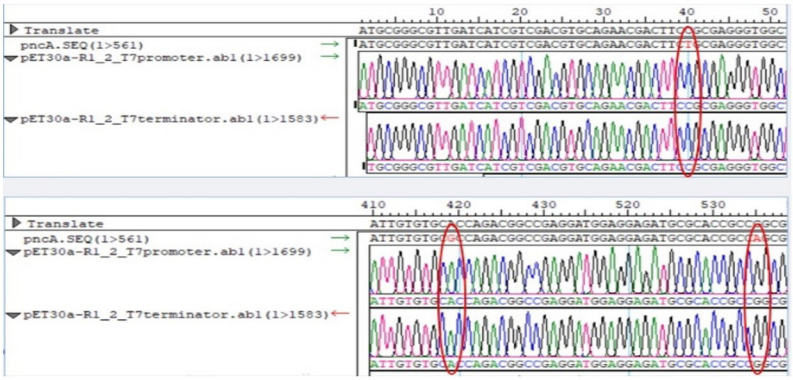


**Figure 8.** The alignment result of the *pncA* R1 against *pncA* Genbank (ID: 888260) belongs to PZA-sensitive *M. tuberculosis* H37RV. The *pnc*AR1 had three mutations, i.e., T41C, G419A, and A535G, corresponding to amino acid change for Cys14Arg, Arg140His and Ser179Gly respectively.

### *3.3. The product of PZase expression in Escherichia coli BL21(DE3)*

The PZase protein expressed from pET30a-*pnc*AH and pET30a-*pnc*AR1 recombinants in *Escherichia coli* BL21 (*DE3*) have appeared as a band ~21 kDa in electrophorogram SDS PAGE (Lane 2 and 3, Figure 7). The band was not found in control of *E. coli* BL21 (*DE3*) containing vector pET30a (Lane 2, Figure 9).

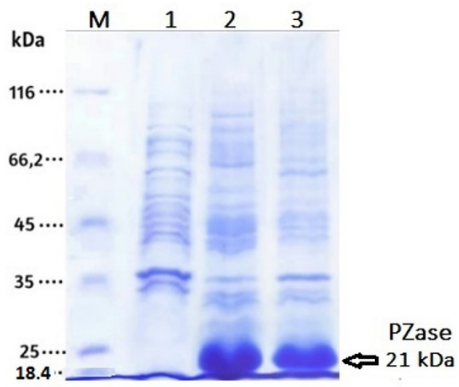


**Figure 9.** SDS-PAGE electrophoregram of PZase Protein Expressed in *E. coli* BL21 (*DE3*). (M) protein marker; (1) *E. coli* BL21(*DE3*) [pET30a]; (2) *E. coli* BL21(*DE3*) [pET30a-*pnc*AH]; (3) *E. coli* BL21(*DE3*) [pET30a-*pnc*AR1].

### *3.4. Activity of PZase wild type and mutant*

PZase recombinant from PZA-sensitive *M. tuberculosis* (H) exhibited a performance activity higher than PZase R1 mutant in converting PZA to POA. The activities of PZase H and R1 mutant were 0.167 and 0.114 U/m respectively. There was a 32% decrease in PZase activity in the R1 mutant compared to wild type H (Figure 10). The decreasing factor of PZase activity in the R1 mutant against wild type H is thought to trigger the emergence of PZA resistance in clinical isolate R1.

The decreasing level in the activity of PZase mutants due to gene mutations tends to vary, depending on the amino acid type that changed. Petrella et al. (2011) reported the effect of mutations on critical residues of PZase, especially in the catalytic triad region (Cys138, Asp8, Lys96), in the PZA binding sites (Trp68, Phe13 and His137), and in the iron-binding site (Asp49, His51, His57, His71). All the mutants were affected in the PZase activity, with no detectable PZA hydrolysis for Cys138Ala, Lys96Gln and Asp49Gly, and deficient residual activity for Ala134Val, His51Ala, His57Asp, Trp68Leu, and Phe13Leu. The PZAse mutants on TSA analysis displayed atypical unfolding more than their wild type.

Regarding the effect of multiple mutations in the pncA R1 gene on reducing the activity of the PZase enzyme, it is possible that the mutations disrupt the structural stability of the PZase protein, leading to the formation

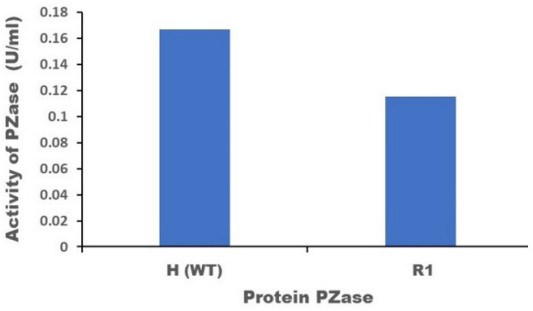


**Figure 10.** The activity of PZase recombinant of wild type H and mutant R1.

of a less compact structure than the wild type. Another possibility is that these mutations change the substrate binding space of the mutant PZase R1 enzyme, making the space less compatible with the PZA substrate. This temporary answer needs to be proven in the future through structural studies of the mutant Pzase R1 protein, such as docking techniques and molecular dynamics simulations.

*Mycobacterium tuberculosis* clinical isolate R1 with a pyrazinamide resistant phenotype had the *pnc*A gene with 3 types of mutations, namely T41C, G419A, and A535G, which change the amino acids Cys14Arg, Arg140His and Ser179Gly in its PZase protein. Characterization of the recombinant PZase protein as a result of its expression in *E. coli* showed that PZase mutant R1 had a lower ability to activate PZA than PZase wild type H which was derived from a PZA-sensitive strain of *M. tuberculosis*. It can be postulated that the *pnc*A mutation of *M. tuberculosis* clinical isolate R1 accompanied by a decrease in PZase activity in activating pyrazinamide underlies the occurrence of pyrazinamide resistance in isolate R1. Multiple mutations in PZase R1 might all contribute to the reduced activity. Which amino acid residue dominantly reduced this activity needs to be investigated further. It is essential to carry out molecular dynamics simulation studies of PZase mutant R1 and wild type in the future to observe the relationship between the structure and function of the protein in order to complete the explanation of the mechanism for PZA resistance in the isolates studied.

## 4. Conclusion

The *M. tuberculosis* isolates, H and R1 had significant phenotypic differences to pyrazinamide. The H is PZA sensitive isolate, but R1 is PZA resistant isolate up to 100 ug/ml. Profiling of the *pnc*A gene from both isolates showed the *pnc*AR1 carrying out base mutations, but not for *pnc*AH against to the *pnc*A (ID: 888260) from PZA-sensitive *M. tuberculosis* H37RV. The *pnc*AR1 exhibited three mutations, ie T41C, G419A, and *A535G* that subsequently changed amino acids of Cys14Arg, Arg140His and Ser179Gly in its protein level. The mutant PZase R1 that expressed as a 21 kDa protein in *E. coli* Bl21(*DE3*) lost 32% of its performance in activating PZA drug to pyrazinoic acid/POA compared to the wild-type PZase H. The mutation in the *pnc*AR1 gene that followed by the decreasing of its PZase activity underlies the emergence of pyrazinamide resistance in the clinical isolate. The structural dynamics of the PZase mutant R1 and wild type molecules need to be studied in the future to complete the explanation of this PZA resistance postulate.

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