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Expression of Mycobacterium tuberculosis Protein Tyrosine Phosphatase B in Escherichia coli and Its Recovery from Inclusion Body

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Abstract

The present study aims at expressing and partially purifying PtpB in active form. To achieve this, Mtb PtpB gene has been cloned into pET30a vector and overexpressed in Escherichia coli BL 21(DE3) under IPTG induction in the form of an inclusion body. Following resolubilization by urea and dialysis, the resulted PtpB has been shown to be active against para-Nitrophenyl phosphate. It is concluded that the resulted PtpB has had been recovered from inclusion body to give the active form of the enzyme, and thus the success in overexpressing PtpB provides the required material to investigate the biochemical properties of the pathogen virulence factor further.

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INTRODUCTION

Tuberculosis still poses a significant threat to global health. The death toll caused by TB is estimated to be around 1.5 to 2 million casualties annually. The figure is worsened by HIV co-infection as well as by the development of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) strains of the bacteria (Palomino & Martin, 2016). Despite the unceasing global effort to eradicate TB, the decrease in global TB cases is not taking place equally throughout the world. In contrast to the significant reduction of global TB cases over past two decades (Chapman & Lauzardo, 2014), the figure in some regions is still beyond expectation. In Indonesia, for example, TB case is still high. A recent report suggested that over a million cases are found in the archipelagic country with mortality may as high as 45% and economic loss of about USD 6.9 billion (Collins *et al.,* 2017). Although vaccination against TB has long been implemented, its efficacy is a controversial matter. An array of new vaccines is currently on the horizon, but they will not be available in short time (Parida & Kaufmann, 2010).

M. tuberculosis, the causative agent of TB, are ingested by the host through the air, and, once entering the lungs, they are phagocytized by macrophages, the first line of defense against bacterial pathogens. This may result in either rapid elimination of the bacillus (Wahyuningsih *et al.,* 2016; Forrellad *et al.,* 2013) or triggering of an active tuberculosis infection (Forrellad *et al.,* 2013). However, the third scenario is also existing as the bacteria develop survival strategy to avoid macrophage degradation by secreting several modulator proteins, such as PtpA, PtpB, PknG, PknF, etc. (Sundaramurthy & Pieters, 2007; Ahmad, 2011). They can maintain viability in dormant phase in a different environment (Boshoff & Barry, 2005) whereas others restrict bacterial growth without necessarily sterilizing the infecting microorganisms. The physical and biochemical milieu in these lesions is poorly defined. None of the existing animal models for tuberculosis (except perhaps non-human primates). Reactivation of the so-called LTBI (latent tuberculosis infection) into active TB is an area that is not fully understood and many factors might be attributed to the development of active TB from LTBI. Moreover, development from active TB from dormancy may take place after the tenth of years (Lillebaek *et al.,* 2002) and make a comprehensive investigation even more constrained.

Increasing attention has recently been given to the latent *M. tuberculosis* infection and it belongs to an essential part of WHO End TB Strategy by 2050. A current model estimates one-fourth of the world population is infected by Mtb in latent form, as opposed to a long-standing prediction by WHO which predicted the figure could as high as one-third of world population (Bash, 2015).

The present study focuses on the Tyrosine phosphatase B (PtpB). It has been described that Mtb bacteria defect in PtpB are not capable of developing latent Mtb infection in Guinea pig ten weeks post inoculation (Chauhan *et al.,* 2013). This report underlines the important role of PtpB in the development of LBTI. Nevertheless, the detailed mechanism by which PtpB interfere with macrophage degradation is yet to be determined. Moreover, the human protein directly interacts with PtpB is also unknown to date. Apart from this, PtpB has been an interesting target for drug development. By inhibiting PtpB, it is expected that macrophage maintains its ability to degrade Mtb. Hence, biochemistry of PtpB, i.e., the pathway in human macrophage that it interferes and a possible way to inhibit the activity of PtpB to prevent the development of latent TB infection is an emerging study need to be solved.

As a prerequisite to investigating the biochemistry of PtpB, heterologous expression of PtpB to provide active PtpB in sufficient amount and purity is an important task. In this study, we have cloned Mtb PtpB gene in pET30a vector. We have also successfully overexpressed PtpB under T7 promoter and IPTG induction in *Escherichia coli* BL21(DE3) cells first in the form of inclusion body and further re-solubilize it into its active form. This result thus provides materials in order to investigate the biochemical property of PtpB, as well as assaying inhibitory potential of several PtpB inhibitor candidates resulted from computational analysis recently reported (Dhanjal *et al.,* 2014).

METHODS

Materials

Escherichia coli strain XL1-Blue was used for cloning and strain BL21(DE3) was used for expression. Template for PtpB amplification was pGS-21-PtpB, obtained from GeneScript USA and expression vector was pET30a. Bacterial growth media was standard LB which consists of yeast extract (BD), bacto tryptone (Bio-Basic), NaCl (Merck) and bacto agar (Difco). Colony selection was performed by addition of kanamycin (Bioworld) up to 25 μg/mL me-

dia. Inducer of gene expression was Isopropyl 1-Thio-β-D-galactopyranoside, IPTG, supplied by Thermoscientific whereas substrate for PtpB was *para*-Nitrophenyl phosphate (Sigma). Agarose for DNA electrophoresis provided by Thermoscientific, along with Tris base (Bio Basic), Sodium acetate (Merck) and Ethylene diamine tetraacetic acid (EDTA from Merck) for electrophoresis buffer, with a 1 kb DNA (Thermoscientific) as DNA marker. Materials for SDS PAGE were acrylamide and *bis*-acrylamide (Bio Basic, Canada), sodium dodecyl sulphate (Bio Basic), tetramethylene diamine (Bio Basic), ammonium persulphate (Bio Basic), glycine (Bio Basic), *beta*-mercaptoethanol (Sigma), brom phenol blue and coomassie brilliant blue (Sigma), dithiothreitol (Sigma) and protein ladder (Thermoscientific). Other substances for buffer and solution were provided by the major supplier of p.a. grade. Sample degradation by protease was prevented by addition of Phenylmethylsulfonyl fluoride (PMSF, Sigma).Isolation of plasmid DNA was undertaken by GeneJet plasmid DNA isolation kit (Thermo Scientific) and materials for PCR (Dream Taq DNA polymerase and dNTP mix) were provided by Thermoscientific.

Cloning

PtpB gene was amplified from a pGS-21-PtpB template by using forward primer 5'-CAT**GGTACC**GCTGTCCGTGAACTGCC-3' and reverse primer 5'- CAG**CTCGAG**TCCGAGCAGCACCCCG-3' which contain *Kpn*I and *Xho*I (Thermoscientific) restriction site in the forward and reverse primer, respectively.Cloning strategy is depicted in Figure 1. Design of cloning strategy and DNA sequence analysis/alignment were performed by SnapGene version 2.5 (GSL Biotech LLC, USA licensed to the authors). The resulted recombinant plasmid was maintained in *E. coli* XL1-blue from which the plasmid was further isolated and sequenced to ensure the absence of mutation.

Expression of PtpB in *Escherichia coli*

The resulted recombinant plasmid was isolated from XL1-but cell by using GeneJet mini plasmid (Thermoscientific) isolation kit according to manufacturer instruction and further introduced to competent *E. coli* BL21(DE) by using electroporator (Gene Pulser, Bio-Rad). Positive clones were tested by colony PCR. Production of PtpB was undertaken within LB medium containing 25 μg/mL kanamycin. To induce the expression of PtpB, transformed *E. coli* BL21 (DE3) was cultured in LB medium until OD_{600} of 0.3 is reached. At this point, IPTG was added to a final concentration of 0.5 mM and the culture was maintained at 37°C with vigorous shaking (250 rpm). Culture was harvested 4 hours after induction and cells were sedimented by cold centrifugation at 5000 rpm for 10 minutes. Cells were further resuspended in PBS (phosphate saline buffer) buffer containing 0.5 mM protease inhibitor PMSF and the cells were disrupted by sonication. The lysate was separated from debris by high-speed centrifugation at 13.000 rpm, 4°C for 20 minutes. Both soluble lysate and insoluble fraction were subjected to SDS-PAGE analysis.

PtpB recovery from inclusion body

Recovery of PtpB from inclusion body was undertaken by denaturating the protein with 8 M urea (Palmer & Wingfield, 2004). Initially, the so-called washed inclusion body (washed IB) was prepared by resuspending insoluble fraction in refolding buffer (50 mM Tris pH 8; 0.1 mM NaCl; 0.1 mM EDTA, 5% glycerol; 0.1 mM DTT). Following sonication, Triton X-100 was added to the lysate to a final concentration of 5% and let stand on ice bucket for 10 minutes before centrifugation at 14.000 rpm, 4°C for 10 minutes. The same treatment was undertaken twice with the exception that no Triton was added in the final step to give washed IB. Washed IB was further dissolved in the same buffer containing 8 M urea and shook with vigorous shaking (250 rpm) until clear lysate was obtained. Denatured protein was kept in a dialysis tube and dialysis was performed against refolding buffer containing 20 mM Tris pH 8; 5 mM EDTA; 5 mM DTT; 50 Mm NaCl, 20% glycerol, and 0.32 M urea. After 3 to 5 times washing, the presence of PtpB in soluble form was confirmed by SDS-PAGE.

Determination of optimum PtpB activity assay

The activity of PtpB recovered from inclusion body was assayed on a 96-well plate by measuring its ability to hydrolyze *para*-Nitrophenyl phosphate (pNPP). The released nitrophenol was measured at 410 nm by using UV-VIS spectrophotometer (SkanIT Multiskan Go, Thermoscientific). Optimum pH and temperature of PtpB were also determined by varying pH and temperature of the pNPP hydrolysis reaction.

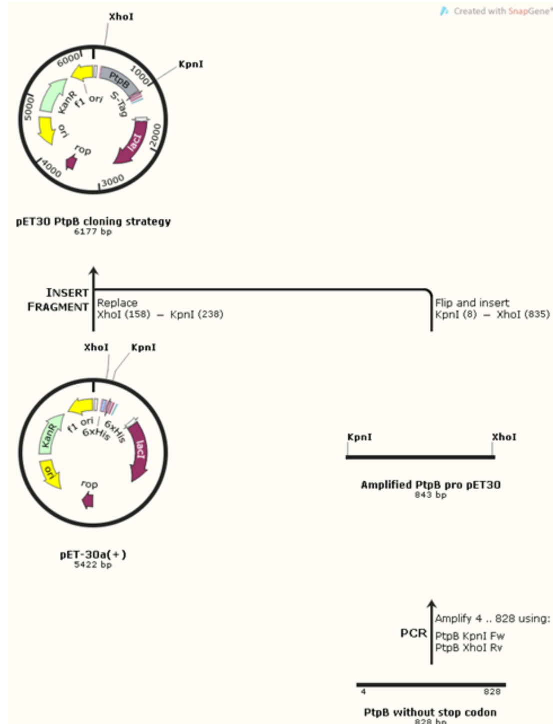

Figure 1. Cloning strategy of PtpB gene by using pET-30a as vector. Cloning strategy was designed by SnapGene software (GSL Biotech LLC, USA) licensed to the author.

RESULTS AND DISCUSSION

Cloning of PtpB gene

PtpB gene has successfully been inserted into pET30a vector via *Kpn*I and *Xho*I restriction site. The pET30a vector confers Kanamycin resistant gene that allows selection of positive clones on LB/kanamycin plate (Figure 2, left). Colony PCR on the growing colonies confirmed the present of the insert from selected colonies (Figure 2, right).

The pET30a vector allows overexpression of inserted gene under strong phage T7 promoter. BL21 (DE3) is a unique strain of *E. coli* designed to be an inducible host. By adding IPTG, the expression of T7 RNA polymerase fused in the chromosome of *E. coli* is increased. The resulted T7 RNA polymerase recognizes the T7 promoter at the upstream position of the target gene.

Expression of PtpB in *E. coli*

By using pET30a expression vector, we were able to overexpress Mtb *PtpB* gene. In *E. coli* BL21 (DE3), production of RNA polymerase of phage DE3 is induced by addition of lactose analog Isopropyl 1-Thio-β-D-galactopyranoside (IPTG). RNA polymerase of phage T7 strongly recognizes T7 promoter at the upstream position of PtpB gene that allows high transcription rate of the *PtpB* gene inserted in the pET30a vector. This expression system has a long time been established after pioneering work by Studier and Moffat (Studier & Moffat, 1986). As expected, PtpB appears at a molecular weight of around 31 kDa (Grundner, Ng, & Alber, 2005). However, PtpB is found in insoluble fraction (Figure 3, left). This indicates that PtpB is synthesized at both high rate and quantity that lead the synthesized proteins to aggregate and form an inclusion body. Formation of inclusion body is not a rare even in an overexpression system, especially when the synthesis of foreign protein takes place at a very high rate with the use of strong promoter such as T7.

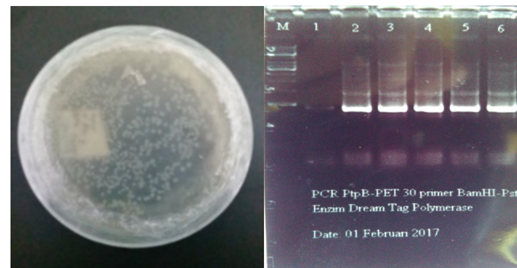

Figure 2. Transformed of *E. coli* XL1-Blue with pET30a-PtpB grown on kanamycin containing agar plate (left).The presence of PtpB insert was confirmed by PCR by using primer pair indicated in Method section. PtpB gene appears as 830 bps bands (right)

PtpB recovery from inclusion body

The high protein synthesis rate accompanying pET30a expression system in *E. coli* BL21(DE3) necessitates an additional step to obtain active PtpB. Recovery of PtpB was achieved by denaturing the inclusion body with 8 M urea. Upon dialysis to remove the excess or urea, the robust PtpB was found in the soluble form of protein with molecular weight of 31 kDa (Figure 3 right). Despite the fact that additional step is required as the target protein is produced in the form of inclusion body, it offers an advantage that the recovered protein presents in a sufficient purity (Li *et al.,* 2015) on SDS-PAGE electroforegram in Figure 3 (right).

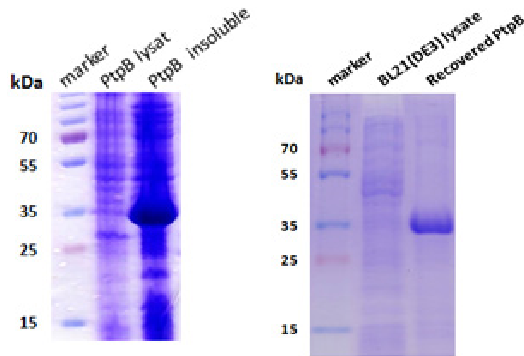

Figure 3. Expression of PtpB in *E. coli* BL21 (DE3) under T7 promoter and induction by 0.5 mM IPTG. Insoluble PtpB (left) and recovered PtpB (right) with a molecular weight of 31 kDa.

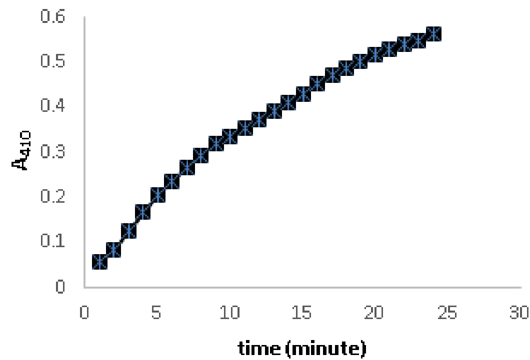

Figure 5. Activity of recovered PtpB. pNPP hydrolysis by recovered PtpB was measured over a period of time at 410 nm as the optimum absorption of *para*-Nitrophenol

Determination of optimum PtpB activity

The activity of PtpB was measured by its ability to hydrolyze substrate *para*-Nitrophenyl phosphate, pNPP, a colorless substance in its solution form which is commonly used to measure phosphatase activity (Lorenz, 2011). Upon hydrolysis by PtpB, the resulted *para*-Nitrophenol (Figure 4) appeared as yellow substance and was measured by spectrophotometer at 410 nm. *Para*-Nitrophenol absorption measured over a period of reaction time shows that the resulted PtpB is active against the pNPP substrate (Figure 5), thus indicating that the recovered PtpB is a functional enzyme.

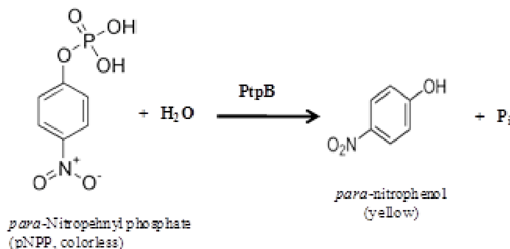

Figure 4. Hydrolysis of para-Nitrophenyl phosphate by PtpB which releases yellow color of *para*-Nitrophenol

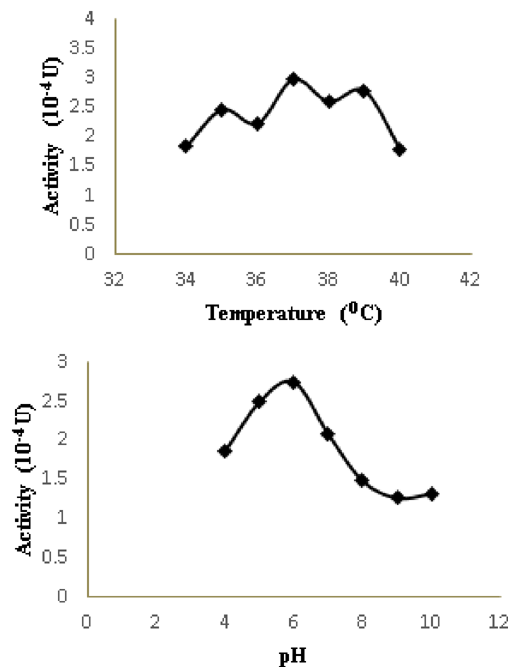

Figure 6. Optimum temperature and pH for hydrolysis of pNPP with PtpB

Further biochemical characterization of recovered PtpB was done to determine the optimum temperature and pH of pNPP hydrolysis. Figure 6 shows that PtpB activity is optimum at 37°C and pH of 6.0. These findings are in agreement to the previous reports (Mascarello *et al.,* 2013; Chiaradia *et al.,* 2008). Thus the recovered PtpB produced by this approach is ready to be utilized in the further study, such as screening of novel inhibitors for PtpB.

Whereas some countries have successfully dealt with the disease, many countries,-especially and unfortunately low and middle-income countries- still have to struggle to go along with the Global TB Eradication program. Novel vaccine research, drugs development against Mtb, and accurate and rapid TB diagnostic kit development have so far been of focal points and hence become major direction of research funding in the war against TB, whereas much of latent TB problems are yet to reveal and increasing demands are lauded to invest more effort to deal with LTBI. The importance of the study of virulence factors or Mtb has only currently been widely recognized to combat this life-threatening disease.

The present study shows an example of such effort at the initial stage in an attempt to understand the potential TB development. Since PtpB increasingly becomes an interesting target to prevent LBTI (Zhou *et al.,* 2010), where it mediates mycobacterial survival in the host. Consequently, there is considerable interest in understanding the mechanism by which mPTPB evades the host immune responses, and in developing potent and selective mPTPB inhibitors as unique antituberculosis (antiTB). By using this result to produce functional PtpB, it is tempting in the future to test the potencies of several chemical agents resulted from *in silico* study (Dhanjal *et al.,* 2014) to inhibit PtpB both *in vitro* and *in vivo.* Of equally important, screening of inhibitory effect of new compounds from a chemical library or new sources might also pave a way to find drug leads (Chen *et al.,* 2016; He *et al.,* 2015).

CONCLUSIONS

We have successfully cloned *M. tuberculosis* PtpB gene within pET30a vector and expressed it in *E.coli* as inclusion body following IPTG induction. We have subsequently been able to resolubilize the inclusion body into active PtpB form. The resulted PtpB has an optimum reaction condition at 37°C and pH of 6.0 against substrate *para*-Nitrophenyl phosphate. It is concluded that the resulted PtpB is a functional enzyme which is in the future can be used to study some chemicals that might inhibit PtpB in an attempt to seek drug candidates against latent *M. tuberculosis* infection.

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REFERENCES

Ahmad, S. (2011). Pathogenesis, immunology, and diagnosis of latent Mycobacterium tuberculosis infection. *Clinical and Developmental Immunology*, *2011*.

Bash, E. (2015). Latent tuberculosis infection. *Ph.D. Proposal*, *1*(5), 62–66.

Boshoff, H. I. M., & Barry, C. E. (2005). Tuberculosis — metabolism and respiration in the absence of growth. *Nature Reviews Microbiology*, *3*(1), 70–80.

Chapman, H. J., & Lauzardo, M. (2014). Advances in diagnosis and treatment of latent tuberculosis infection. *Journal of the American Board of Family Medicine*, *27*(5), 704–12.

Chauhan, P., Reddy, P. V., Singh, R., Jaisinghani, N., Gandotra, S., & Tyagi, A. K. (2013). Secretory Phosphatases Deficient Mutant of Mycobacterium tuberculosis Imparts Protection at the Primary Site of Infection in Guinea Pigs. *PLoS ONE*, *8*(10),

Chen, D., Chen, H., She, Z., & Lu, Y. (2016). Identification of Bostrycin Derivatives as Potential Inhibitors of Mycobacterium tuberculosis Protein Tyrosine Phosphatase (MptpB). *Medicinal Chemistry*, *12*(3), 296–302.

Chiaradia, L. D., Mascarello, A., Purificação, M., Vernal, J., Cordeiro, M. N. S., Zenteno, M. E., … Terenzi, H. (2008). Synthetic chalcones as efficient inhibitors of Mycobacterium tuberculosis protein tyrosine phosphatase PtpA. *Bioorganic and Medicinal Chemistry Letters*, *18*(23), 6227–6230.

Collins, D., Hafidz, F., & Mustikawati, D. (2017). The economic burden of tuberculosis in Indonesia. *The International Journal of Tuberculosis and Lung Disease*, *21*(9), 2017.

Dhanjal, J. K., Grover, S., Sharma, S., Singh, A., & Grover, A. (2014). Structural insights into mode of actions of novel natural Mycobacterium protein tyrosine phosphatase B inhibitors. *BMC Genomics*, *15 Suppl 1*(Suppl 1), S3.

Forrellad, M. A., Klepp, L. I., Gioffré, A., Sabio y García, J., Morbidoni, H. R., de la Paz Santangelo, M., … Bigi, F. (2013). Virulence factors of the Mycobacterium tuberculosis complex. *Virulence*, *4*(1), 3–66.

Grundner, C., Ng, H. L., & Alber, T. (2005). Mycobacterium tuberculosis protein tyrosine phosphatase PtpB structure reveals a diverged fold and a buried active site. *Structure*, *13*(11), 1625–1634.

He, R., Yu, Z. H., Zhang, R. Y., Wu, L., Gunawan, A. M., & Zhang, Z. Y. (2015). Cefsulodin Inspired Potent and Selective Inhibitors of mPTPB, a Virulent Phosphatase from Mycobacterium tuberculosis. *ACS Medicinal Chemistry Letters*, *6*(12), 1231–1235.

Li, Y., Wang, B., Zhang, H., Wang, Z., Zhu, S., & Ma, H. (2015). High-level expression of angiotensin converting enzyme inhibitory peptide Tuna AI as tandem multimer in Escherichia coli BL21 (DE3). *Process Biochemistry*, *50*(4), 545–552.

Lillebaek, T., Dirksen, A., Baess, I., Strunge, B., & Thomsen, V. Ø. (2002). Molecular Evidence of Endogenous Reactivation of *Mycobacterium tuberculosis* after 33 Years of Latent Infection. *The Journal of Infectious Diseases*, 185(3), 401-404.

Lorenz, U. (2011). Protein Tyrosine Phosphatase Assays. *Curr Protoc Immunol.*, (434), 1–14.

Mascarello, A., Mori, M., Chiaradia-Delatorre, L. D., Menegatti, A. C. O., de Monache, F., Ferrari, F., … Botta, M. (2013). Discovery of Mycobacterium tuberculosis Protein Tyrosine Phosphatase B (PtpB) Inhibitors from Natural Products. *PLoS ONE*, *8*(10), 1–12.

Palmer, I., & Wingfield, P. T. (2004). Preparation and extraction of insoluble (Inclusion body) proteins from Escherichia coli. *Current Protocols in Protein Science*, (8 M), 1–25.

Palomino, J. C., & Martin, A. (2016). The potential role of trimethop sulfamethoxazole in the treatment of drug-resistant tuberculosis. *Future microbiology*, 11(4), 539-547.

Parida, S. K., & Kaufmann, S. H. E. (2010). Novel tuberculosis vaccines on the horizon. *Current Opinion in Immunology*, *22*(3), 374–384.

Studier, F. W., & Moffat, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of molecular biology,* 189(1), 113-130.

Sundaramurthy, V., & Pieters, J. (2007). Interactions of pathogenic mycobacteria with host macrophages. *Microbes and Infection*, *9*(14–15), 1671–1679.

Wahyuningsih, S. P. A., Pramudya, M., & Sugiharto, S. (2016). Influence of Polysaccharide Krestin from Coriolus versicolor Extract on Nitrite and Malondialdehyde Concencentrations of Mus musculus Serum Exposed by Mycobacterium tuberculosis. *Biosaintifika: Journal of Biology & Biology Education*, 8(1), 12-17.

Zhou, B., He, Y., Zhang, X., Xu, J., Luo, Y., Wang, Y., … Zhang, Z.-Y. (2010). Targeting mycobacterium protein tyrosine phosphatase B for antituberculosis agents. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(10), 4573–8.