HAYATI
Journal of Biosciences

Vol. 30 No. 3, May 2023 561-566
DOI:10.4308/hjb.30.3.561-566
ISSN: 1978-3019
EISSN: 2086-4094

Enhancing Solubility of Recombinant Plasmodium Lactate Dehydrogenase (pLDH) Using Combination of Cold-Inducible Expression System and Cold-Stirred Bioreactor

Alimuddin[1], Muhamad Ali[1], Sahrul Alim[2], Muhamad Amin[3]*

[1]*Laboratory of Microbiology and Biotechnology, Faculty of Animal Sciences, University of Mataram, Mataram 83115, Indonesia*
[2]*Aquaculture Study Program, Faculty of Agriculture, University of Matram, Mataram 83115, Indonesia*
[3]*Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Campus C, Surabaya 60115, Indonesia*

ARTICLE INFO

Article history:
Received March 15, 2022
Received in revised form February 6, 2023
Accepted February 14, 2023

KEYWORDS:
Cold-inducible expression system,
Escherichia coli BL21 (DE3) RIPL Codon Plus Strain,
pLDH, solubility

ABSTRACT

A major drawback associated with an expression of a high-level Plasmodium Lactate Dehydrogenase (pLDH) using *Escherichia coli* is the low solubility due to the formation of an inclusion body (IB). This study aimed to develop a suitable protocol for enhancing the solubility of pLDH expressed in *E. coli*. Firstly, a pLDH-encoding gene was amplified from the blood sample of malaria-infected patients and ligated into pBlueScript II KS+ for sequencing. Afterward, the pLDH gene was digested and cloned into pColdTF for expression. The recombinant plasmid was transformed into the *E. coli* BL21 (DE3) RIPL Codon Plus Strain. Then, the bacterial host was initially cultured at 37°C until reaching optical density (OD) at 600 nm: 0.5. Thereafter, the growth temperature was lowered to 15°C, followed by the addition of 0.1 mM IPTG into the culture medium for inducing pLDH expression. Thereafter, the bacterial hosts were cultured in a cold-stirred bioreactor (15°C). The result showed that a combination of the low culture conditions (15°C) and a low amount of IPTG increased the solubility of pLDH. This result suggests that this protocol can be a convenient method for generating high-quality recombinant protein using the *E. coli* system.

1. Introduction

Producing a highly soluble form of plasmodium lactate dehydrogenase (pLDH) is very important to provide high-quality materials for immunochromatographic rapid diagnostic tests (RDTs), immunization studies, and inhibition kinetic assays (Rosano and Ceccarelli 2014). In addition, large quantities of protein production nowadays can be performed in *Escherichia coli* (Berwal *et al.* 2008). However, a major drawback in the high-level expression of proteins in *E. coli*, is the formation of an inclusion body (IB), which leads to the inactivity of the generated proteins (Bzik *et al.* 1993; Piper *et al.* 1999; Rosano and Ceccarelli 2014).

Kleber-Janke and Becker (2000) reported that the overexpression of protein in *E. coli* BL21 (DE3) RIPL Codon Plus strain could enhance the solubility of protein recombinant. This might be because the bacterial cells have extra copies of tRNA genes including ileY, argU, proL, and leuW. Therefore, the expression of targeted proteins using a host with 11% rare tRNA gene content could be efficiently obtained. In addition, the use of cold temperature (15°C) with a low amount of IPTG (0.1 mM) has been described to reduce host cell growth, slow down the transcription and translation rates, as well as reduce the strength of hydrophobic interactions contributing to protein misfolding (Berwal *et al.* 2008). It was hypothesized that the combination of these two approaches could be used to produce a high solubility of protein recombinants.

Thus, this study aimed at investigating the expression of pLDH recombinant in *E. coli* BL21 (DE3) RIPL Codon Plus strain cultured under a cold-inducible expression system and a low amount of IPTG.

* Corresponding Author
E-mail Address: muhamad.amin@fpk.unair.ac.id

2. Materials and Methods

2.1. Collecting of Blood Samples

Samples were collected according to a protocol of Ali *et al.* (2013) with slight modifications. In brief, blood samples were drawn from patients with fever by a fingerpick at Gunung Sari, Lombok Island, West Nusa Tenggara, Indonesia. Thick and thin peripheral blood smear from each blood sample was made on a slide and subsequently stained with Giemsa. The slide was then examined for the presence of malaria parasites by a light microscope. Afterward, 1-5 ml of blood with positive malaria was redrawn from the venous blood of infected patients and washed with an RPMI medium to get rid of white-blood cells present on the buffy coat layer.

2.2. Isolation of *Plasmodium falciparum* DNA

DNA was extracted from blood samples with positive *Plasmodium falciparum*. While the buffy coat of leucocytes and plasma was discarded through centrifugation at 200 g for 10 min. Thereafter, the genomic DNA of *P. falciparum* was extracted from the Red Blood Cells (RBCs) using a QIAamp® DNA Blood mini kit (QIAGEN) as the manufacturer's protocol. Then, the quality of the extracted DNA samples was assessed by agarose gel electrophoresis.

2.3. Amplification of pLDH-encoding Gene

Oligonucleotide primers pLDH S Kpn: 5'-AGAGAGGGTACCGCACCAAAAGCAA AAATC'-3 and pLDH AS Eco: 5'-CACACAGATTCTTAAGCTAATGCCTTCATTCTC'-3 which were pLDH open reading frame (ORF) were constructed based on pLDH gene sequence (K1 strain). Thereafter, PCR was performed according to the protocol of Berwal *et al.* (2008).

2.4. Cloning of pLDH-encoding Gene Into pBluescript II KS+ and pColdTF Vector

The PCR products were purified, phosphorylated, and then ligated to pBlueScript II KS+ (Invitrogen). Then, the recombinant vector was introduced into a competent *Escherichia coli* Top10. The recombinant vector was sequenced to confirm the correct sequence. Then, the pLDH gene was extracted from the pBlue-pLDH plasmid using KpnI and EcoR1 restriction enzymes and subsequently ligated to the pColdTF expression vector to generate a recombinant pCold-pLDH plasmid. Thereafter, the recombinant plasmid was transformed into *E. coli* BL21 (DE3) RIPL Codon Plus strain (Invitrogen, Carlsbad, CA). Colony PCR was conducted to confirm *E. coli* BL21 (DE3) RIPL Codon Plus strain which bore the pCold-pLDH recombinant plasmid according to a protocol of Amin *et al.* (2017).

2.5. Expression of Recombinant pLDH

Ten µl *E. coli* BL21 (DE3) RIPL Codon Plus strain stock which contained the recombinant plasmid was initially cultured in 10 ml LB broth supplemented with ampicillin and chloramphenicol. After reaching the optical density (OD) value of 0.5 at 600 nm wavelength, the bacterial culture was incubated overnight at 15°C, IPTG 0.1 µg/L, and shaken at 200 rpm for inducing protein expression to optimize protein expression (Ali *et al.* 2015). Afterward, bacterial cells were centrifugated at 6,000 g at 4°C to harvest the bacterial cells, which were later stored at -20°C until further assay.

2.6. Measuring pLDH Solubility

The pellet of bacterial cells was diluted in 160 ml lysis buffer consisting of 300 mM NaCl, 20 mM phosphate buffer, 10 mM imidazole, 1 mM PMSF, and pH of 8.0 and sonicated to lyse the bacterial cells (10 × 30 s pulse with 45 s interval). Thereafter, the lysate was collected by centrifugation at 12,000 g for 15 min. The solubility of recombinant proteins was analyzed by SDS–PAGE using 12.5 % mini-gels as described by Ali *et al.* (2005). In brief, 5 µl of samples were boiled with an equal volume of sample buffer for non-reducing conditions and loaded into an SDS-PAGE gel for electrophoresis. RainbowTM protein (MW 1,000-8,000 Da; Amersham Pharmacia Biotech., Tokyo) was used as a marker. High solubility was indicated by a thick band on the gel.

2.7. Purification of Recombinant pLDH

The supernatant of sonicated host cells was subsequently purified using BD TalonTM metal affinity resin (Clontech Laboratories, Paolo Alto, CA, USA) according to the manufacturer protocol.

3. Results

3.1. Amplified pLDH

pLDH-encoding gene was amplified from the blood of malaria-infected patients with gene size ~951 bp (Figure 1A). The gene was subsequently ligated into a linearized pBlueScript II KS vector after phosphorylation using T4 Polynucleotide kinase. The

gene size of the vector was 3,000 bp, therefore the size of the recombinant plasmid (pBlueScript II KS vector + pLDH gene) was ~4,000 bp (Figure 1B).

pLDH gene which was ligated to pBluescript II KS+ vector (pBlue-pLDH) and successfully transformed into *E. coli* BL21 (DE3) RIPL Codon Plus strain appeared to be white colonies. While *E. coli* BL21 (DE3) RIPL Codon Plus strain which contains the original plasmid (pBluescript II KS+ vector) appeared to be blue since the vector has a β-lactamase gene which could degrade X-gal in the LB medium. Afterward, pLDH gene was extracted from the pBlue-pLDH plasmid, ligated into the pColdTF vector, and subsequently transformed into *E. coli* BL21 (DE3) RIPL codon plus for protein expression.

3.2. Expressed Recombinant TF-pLDH Protein

E. coli BL21 (DE3) RIPL codon plus strain harboring pLDH-pCold TF plasmid was initially cultured at 37°C for 48h to get OD_{600} nm: 0.5. Then, the growth temperature was lowered to 15°C for 36 h and addition of 0.1 mM IPTG to induce the protein expression. SDS-PAGE assay showed that ~48 kDa protein band appeared in the supernatant of sonicated cells, corresponding to the TF chaperon, Figure 2 (line 1). Since the molecular weight of pLDH was ~32 kDa, the recombinant protein expressed by the pCold-pLDH plasmid became ~80 kDa (Figure 2, lane 5).

As shown in Figure 2, a band of sonicated supernatant on lane 5 was thicker than a band of sonicated pellet on lane 4, indicating that the soluble expressed protein was higher than the insoluble fraction or inclusion body. This result showed that the expression of the target protein in cold temperatures improves the solubility of the generated protein.

3.3. Purified pLDH

Cell lysates which had the soluble fraction of TF-pLDH recombinant protein were purified using a Ni-NTA spin kit (Qiagen, USA). As presented in the SDS-PAGE results (Figure 3), the amount of purified pLDH appeared to be high since the pLDH protein band (Lane 5) was much thicker compared to the flowthrough fraction (Lane 3) and washing fraction (Lane 4). Since protease inhibitors were not used during cell lysis and purification, a smaller protein band corresponding to degraded recombinant protein also appeared as a result of endogenous protease degradation.

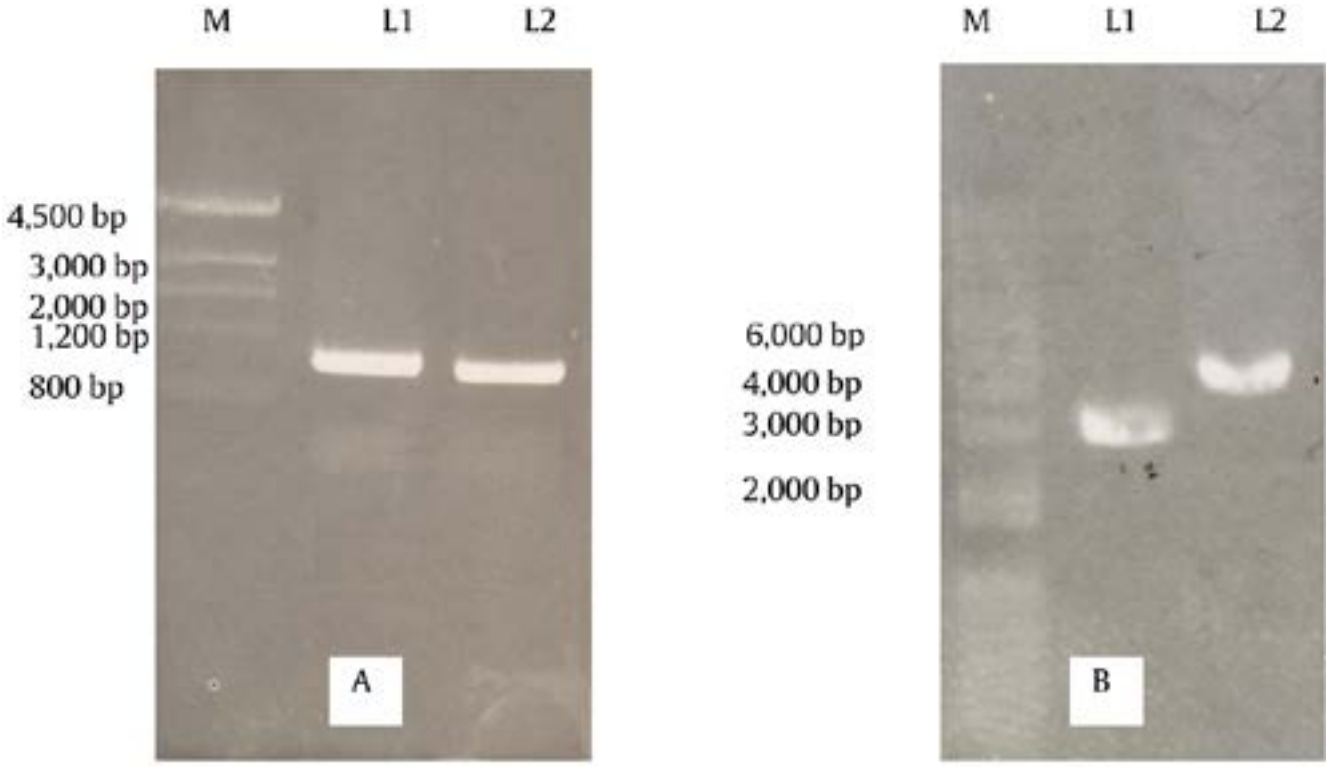

Figure 1. (A) DNA bands of pLDH-encoding genes. M = marker; L1-L2 = amplified pLDH-encoding gene, (B) pBlueScript II KS+ vector and recombinant pLDH-pBlueScript II KS plasmid. M: marker; L1: pBlueScript II KS+ vector; and L2: recombinant plasmid (pBlueScript II KS + pLDH gene)

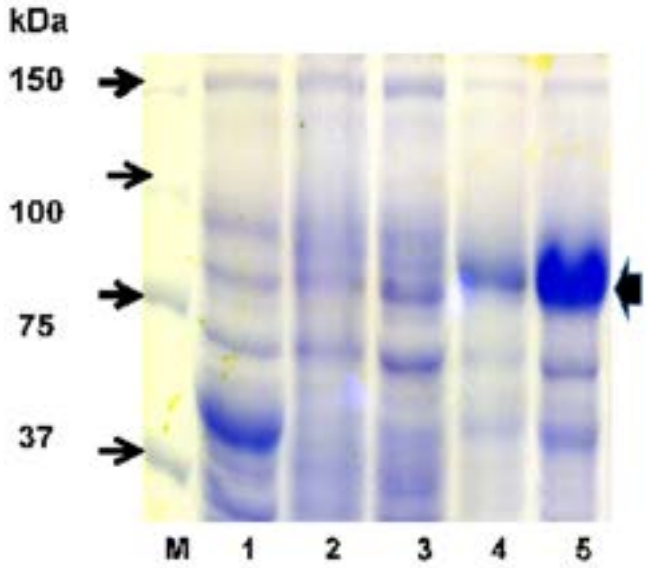

Figure 2. The expression and solubility analysis of the recombinant protein expression in *E. coli* BL21 (DE3) RIPL codon plus strain. Lane M was protein standards; Lane 1 was sonicated supernatant of TF protein; Lane 2 was sonicated pellet of uninduced cells; Lane 3 was sonicated supernatants of uninduced cells; Lane 4 was sonicated pellet of induced cells; and Lane 5 was sonicated supernatants of induced cells

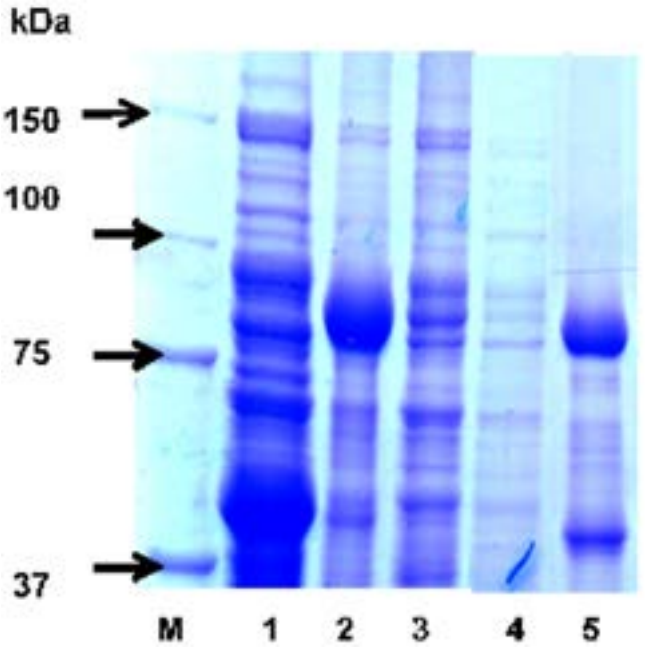

Figure 3. SDS-PAGE analysis of purified recombinant pLDH. M was the protein standard. Lane 1 was TF chaperon expressed by pColdTF vector, Lane 2 was unpurified TF-pLDH, Lane 3 was flowthrough fraction, Lane 4 was washing fraction, and Lane 5 was elution fraction (purified TF-pLDH).

4. Discussion

Plasmodium Lactate Dehydrogenase (pLDH) is a well-known protein for malaria diagnostic targets as its comparable structure to the human lactate dehydrogenase isoform (Baker *et al.* 2005; Howard *et al.* 1986; Piper *et al.* 1999; Tjitra *et al.* 2001). Thus, the protein has been used as material tests to detect multiple species of malaria agents and is an important material for immunochromatographic rapid diagnostic tests (RDTs).

Up to now, pLDH was expressed in *E. coli* BL21 (DE3) RIPL Codon Plus strain as a cell factory. However, the major drawback of the produced pLDH was the formation of an inclusion body (IB), which caused low solubility and protein inactivity (Bzik *et al.* 1993, Piper *et al.* 1999, Rosano and Ceccarelli 2014). A previous study by Bzik *et al.* (1993) reported that pLDH which was expressed in the pKK223-3 vector was insoluble and attached to the pellet fraction after sonication. The same result was documented by Turgut-Balik *et al.* (2001) using the same vector, in which 95% of expressed protein was in an inactive form (pellet fraction) and only 5% was inactive soluble form. Thus, various strategies have been tried to enhance the soluble expression of pLDH.

In this research, we developed a protocol for producing pLDH antigen with a low formation of IB and a high-level solubility to support the production of local RDTs kits. A pLDH-encoding gene derived from the blood of malaria-infected patients from Lombok island Indonesia was cloned into a pBlueScript II KS+ and fused into a competent *E. coli* Top10 competent cells. A colony carrying the recombinant plasmid (indicated by a white colony) was isolated and digested using KpnI and EcoR1 restriction enzymes. pLDH gene was extracted and ligated into the pColdTF plasmid to express the Trigger Factor chaperone as a soluble fusion tag (Li *et al.* 2018). The chaperone is a 48 kDa chaperone protein which is associated with the prokaryotic ribosome and facilitates the co-translational folding of emerging polypeptides. Due to its *E. coli* BL21 (DE3) RIPL Codon Plus strain origin, the trigger factor is highly expressed in *E. coli* BL21 (DE3) RIPL Codon Plus strain expression system. The pCold TF vector contains the cspA promoter with additional downstream sequences such as a 5'-UTR (5'-untranslated region), a TEE (translational enhancing element), a multi-cloning site (MCS), and a His-Tag sequence. By using the plasmid presented in Figure 4, most *E. coli* strains can serve as expression hosts.

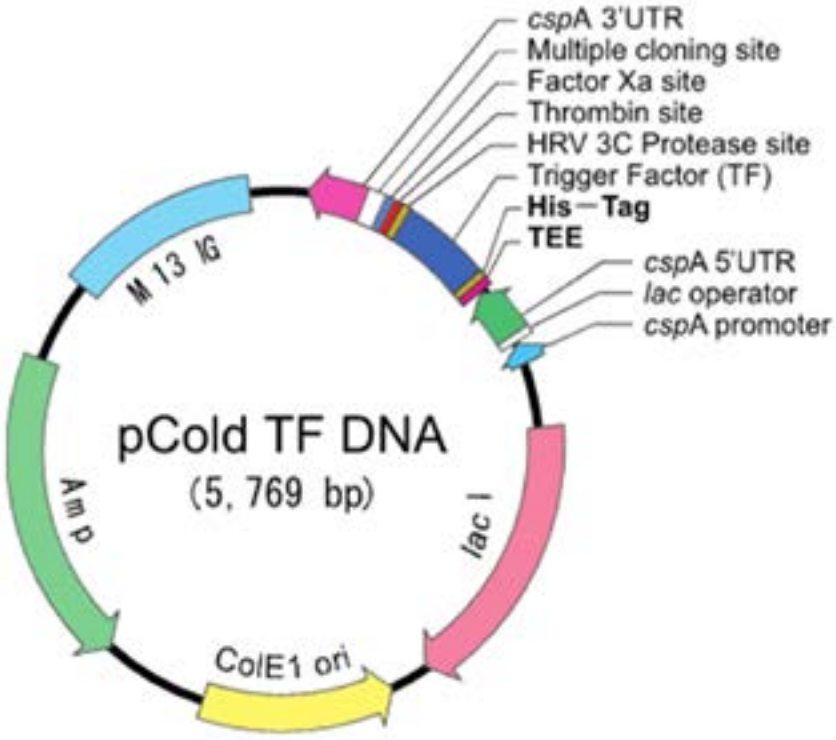

Figure 4. Map of pCold TF vector

The pCold-pLDH plasmid was then transformed into *E. coli* BL21 (DE3) RIPL Codon Plus strain as factory cells. The bacterium was used as the host due to containing extra copies of argU, ileU, leuW, and proL tRNA. The tRNA genes recognize some rare codon, which frequently restricts the recombinant protein translation in *E. coli*, including AGA, AGG (arginine), AUA (isoleucine), and CCC (proline) (Wu *et al.* 2004). Thus, those bottlenecks of recombinant protein expression in *E. coli* BL21 (DE3) RIPL Codon Plus strain can be solved. The host cells harboring pLDH-pCold TF plasmid were cultured at 37°C until OD_{600} nm: 0.5 (~48h after inoculation). Then, the growth temperature was decreased to 15°C, and addition of 0.1 mM IPTG to induce the protein expression. A low amount of IPTG was used to avoid an incorrect folding effect of the chemicals on the synthesized protein. The bacterial host was cultured in a stirred bioreactor during the induction process.

The result showed that the molecular weight of pLDH was ~32 kDa and the pCold TF vector bearing the TF chaperone was~48 kDa. Thus, the recombinant protein which was expressed by the pCold-pLDH plasmid was approximately 80 kDa. Figure 3 shows that about 42 kDa protein bands appeared in the expression of pCold TF plasmid corresponding to the TF chaperon. The size of the band was increased to approximately 80 kDa after the fusion of the protein with pLDH protein. One way to increase protein solubility is by suppressing the synthesis of the target gene product which can be achieved by lowering the growth temperature of a bacterial host (Sørensen and Mortensen 2005). The application of low temperatures not only slowed down transcription and translation rates but also reduced the strength of hydrophobic interactions, an important factor which contributes to protein misfolding (Baneyx and Mujacic 2004). In addition, the reduction of cell growth with low temperature (15°C) increased the protein expression in soluble form.

Furthermore, the expression of the recombinant plasmid in cold temperatures improves the solubility and stability of the recombinant protein (Qing *et al.* 2004). The protein expression at the low temperature increased the solubility of recombinant protein and reduced their degradation by heat shock protein generated in the culture. Additionally, most of the TF-pLDH recombinant protein which was expressed in this research was primarily in soluble form. These results may suggest that a combination of pCold TF expression vector, *E. coli* BL21 (DE3) RIPL codon plus strain, and cold temperature are suitable

strategies to produce a high quantity and quality of pLDH recombinant. Thus, this protocol can be a good solution to address some issues regarding the production of insoluble pLDH with low activity.

Acknowledgements

This research was granted by SINAS Research Incentive of the Indonesian Ministry of Research and Higher Education. The authors would like to thank Prof. Masaharu ISOBE and Dr. Nobuyuki KUROSAWA (Laboratory of Molecular Biology, Toyama University) for providing research materials and Prof. Hideo NAKANO (Laboratory of Molecular Biotechnology, Nagoya University) for helping with some devices required in the part of this research.

References

Ali, M., Suzuki, H., Fukuba, T., Jiang, X., Nakano, H., Yamane, T., 2005. Improvements in the cell-free production of functional antibodies using cell extract from protease-deficient *Escherichia coli* mutant. *J. Biosci. Bioeng.* 99, 181-186. https://doi.org/10.1263/jbb.99.181

Ali, M., Hidayatullah, T.A., Alimuddin, Z., Sabrina, Y.. 2013. Sequence diversity of pfmdr1 and sequence conserve of pldh in *Plasmodium falciparum* from Indonesia: Its implications on designing a novel antimalarial drug with less prone to resistance. *Iranian journal of parasitology*. 8, 522.

Ali, M., Depamede, S.N., Setyono, B.D., Mukhlis, A., Amin, M., Ashari, M., 2015. Stirred bioreactor for the robustness production of recombinant gst. Vp28 in fed-batch cultivation of *Escherichia coli*. *Scientific Study and Research Chemistry and Chemical Engineering, Biotechnology, Food Industry*. 16, 245.

Amin, M., Bolch, C.J.S., Adams, M.B., Burke, C.M., 2017. Isolation of alginate lyase-producing bacteria and screening for their potential characteristics as abalone probionts. *Aquac. Res.* 48, 5614-5623. https://doi.org/10.1111/are.13383

Baker, J., McCarthy, J., Gatton, M., Kyle, D.E., Belizario, V., Luchavez, J., Bell, D., Cheng, Q., 2005. Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. *The Journal of Infectious Diseases.* 192, 870-877. https://doi.org/10.1086/432010

Baneyx, F., Mujacic, M., 2004. Recombinant protein folding and misfolding in *Escherichia coli*. *Nat Biotechnol.* 22, 1399-1408. https://doi.org/10.1038/nbt1029

Berwal, R., Gopalan, N., Chandel, K., Prasad, G., Prakash, S., 2008. *Plasmodium falciparum*: enhanced soluble expression, purification and biochemical characterization of lactate dehydrogenase. *Exp Parasitol.* 120, 135-141. https://doi.org/10.1016/j.exppara.2008.06.006

Bzik, D.J., Fox, B.A., Gonyer, K., 1993. Expression of *Plasmodium falciparum* lactate dehydrogenase in *Escherichia coli*. *Mol. Biochem. Parasitol.* 59, 155-166. https://doi.org/10.1016/0166-6851(93)90016-Q

Howard, R.J., Uni, S., Aikawa, M., Aley, S.B., Leech, J.H., Lew, A.M., Wellems, T.E., Rener, J., Taylor, D.W., 1986. Secretion of a malarial histidine-rich protein (Pf HRP II) from *Plasmodium falciparum*-infected erythrocytes. *The Journal of Cell Biology*. 103, 1269-1277. https://doi.org/10.1083/jcb.103.4.1269

Li, J., Han, Q., Zhang, T., Du, J., Sun, Q., Pang, Y., 2018. Expression of soluble native protein in *Escherichia coli* using a cold-shock SUMO tag-fused expression vector. *Biotechnology Reports*. 19, e00261. https://doi.org/10.1016/j.btre.2018.e00261

Kleber-Janke, T., Becker, W.M., 2000. Use of modified BL21(DE3) *Escherichia coli* cells for high-level expression of recombinant peanut allergens affected by poor codon usage. *Protein Expr Purif*. 19, 419-424. https://doi.org/10.1006/prep.2000.1265

Piper, R., Lebras, J., Wentworth, L., Hunt-Cooke, A., Houze, S., Chiodini, P., Makler, M., 1999. Immunocapture diagnostic assays for malaria using *Plasmodium lactate* dehydrogenase (pLDH). *Am. J. Trop. Med. Hyg.* 60, 109-118. https://doi.org/10.4269/ajtmh.1999.60.109

Qing, G., Ma, L.C., Khorchid, A., Swapna, G., Mal, T.K., Takayama, M.M., Xia, B., Phadtare, S., Ke, H., Acton, T., Montelione, G.T., 2004. Cold-shock induced high-yield protein production in *Escherichia coli*. *Nat. Biotechnol.* 22, 877. https://doi.org/10.1038/nbt984

Rosano, G.L., Ceccarelli, E.A., 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol.* 5, 172. https://doi.org/10.3389/fmicb.2014.00172

Sørensen, H.P., Mortensen, K.K., 2005. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microbial Cell Factories.* 4, 1-8. https://doi.org/10.1186/1475-2859-4-1

Tjitra, E., Suprianto, S., McBroom, J., Currie, B.J., Anstey, N.M., 2001. Persistent ICT malaria Pf/Pv panmalarial and HRP2 antigen reactivity after treatment of *Plasmodium falciparum* malaria is associated with gametocytemia and results in false-positive diagnoses of *Plasmodium vivax* in convalescence. *J. Clin. Microbiol.* 39, 1025-1031. https://doi.org/10.1128/JCM.39.3.1025-1031.2001

Turgut-Balik, D., Shoemark, D.K., Moreton, K.M., Sessions, R.B., Holbrook, J.J., 2001. Over-production of lactate dehydrogenase from *Plasmodium falciparum* opens a route to new antimalarials. *Biotechnol Lett*. 23, 917-921. https://doi.org/10.1023/A:1010555803606

Wu, X., Jörnvall, H., Berndt, K.D., Oppermann, U., 2004. Codon optimization reveals critical factors for high level expression of two rare codon genes in *Escherichia coli*: RNA stability and secondary structure but not tRNA abundance. *Biochem Biophys Res Commun.* 313, 89-96. https://doi.org/10.1016/j.bbrc.2003.11.091