Expression and Purification of Recombinant Malarial Antigen *Plasmodium falciparum* Lactate Dehydrogenase (*Pf*LDH) in *E.Coli* Expression System for Diagnostic Application

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Abstract

Malaria is still a fatal endemic disease caused by plasmodium parasites, most commonly transmitted by human bites transmitted by female Anopheles mosquitoes. It remains one of the biggest global threats to human health, especially in subtropical areas. The main stage of the parasite (Plasmodium falciparum) is responsible for most of those severe and lethal cases of malaria. The stage of the parasite's life cycle when it infects and replicates the phase occurring inside the host organism's red blood cells, is typically a human, called the erythrocytic stage. Plasmodium falciparum Lactate Dehydrogenase (PfLDH) is a glycolytic enzyme that catalyses the conversion of pyruvate and lactate into energy for the body's metabolism. It is used in antimalarial medications and diagnostic tests for finding malaria because the presence of PfLDH in blood samples suggests the presence of the parasite. The focus of this study is to make recombinant PfLDH protein in Escherichia coli BL21(DE3) strain.

The PfLDH gene was introduced into the expression vector pET27b(+)to construct recombinant plasmid, which was then transformed into E. Coli BL21(DE3). Next, recombinant DNA containing host cell was induced by IPTG at different concentrations, time intervals and temperature that can be grown in LB broth containing marker gene. Recombinant protein PfLDH was purified by IMAC, was analysed and was confirmed by SDS-PAGE and Western blot. It can be utilized as a biomarker for malarial Rapid Diagnostic Tests (RDTs).

Keywords: Malaria, *Plasmodium falciparum*, Lactate Dehydrogenase, BL21(DE3), RDT.

Introduction

Malaria still remains one of the deadly diseases caused by the plasmodium parasites exhibiting a complex life cycle, using infected female Anopheles mosquitoes as a vector for disease transmission which continues to be enormous crisis worldwide particularly in the tropical and subtropical areas[20]. As per the recent World malaria report, globally in 2023, about 263 million cases of Malaria, 597000 deaths worldwide were found. Approximately 11 million more cases and almost the same number of deaths were reported in 2023 as compared to 2022[21]. In addition, other animals such as birds, mammals and reptiles were also infected by these parasites. More than 200 species of plasmodium have been formally reported to date and each species affects a specific range of hosts.

There are five different plasmodium species that cause malaria in human: *P.falciparum*, *P.vivax*, *P.malariae, P.ovale* and *P.knowlesi*. *Plasmodium falciparum*, which is responsible for over 90% of deaths due to malaria worldwide, continues to be the single most significant threat to public health on a global basis[19]. Although severe cases of malaria are mostly caused by *P. falciparum,* it can also occur, albeit very rarely, from infections with *P. vivax* and *P. knowlesi*[22]. Patients initially have symptoms similar to a "viral syndrome" such as fever, chills, sweating, headaches, weakness and others. Subsequently, a severe illness with severe anemia, multisystem failure, altered consciousness and renal failure may appear[2].

World Health Organization (WHO) recommends that the patients who all are suspected of malaria should be diagnosed promptly to distinguish between malarial and non-malarial fevers to prevent the emergence of drug resistance and also to reduce the severity of the illness by appropriate treatments[7].

Malarial rapid diagnostic test (RDTs) can used to confirm the presence of malarial antigen which has significant prospect for enhancing the quality of Malaria infection management, simple to perform and interpret and can be used especially in the remote areas where access to high quality microscopy and other techniques are limited[6]. Immunochromatographic techniques known as rapid diagnostic tests (RDTs) used various biomarkers including lactate dehydrogenase (LDH)[11,10], histidine rich protein-2 (HRP-2)[14], glutamate dehydrogenase[12], aldolase[4] and merozoite surface protein[8]. Among them, histidine-rich protein 2 (PfHRP2) is the parasite biomarker which is used by the majority of RDTs used all over the world due to its higher reported sensitivity[15].

However, it has been proposed that *Pf*HRP2-detecting RDTs have low clinical specificity to detect current malaria

infection in areas of high transmission, because the protein remains in the blood stream even after parasite clearance and also recent years have seen the reports of deletion of HRP-2 gene in the parasites and were not expressed in the suspected patients' blood which shows in false-negative results[12,13].

Lactate dehydrogenase is an oxidoreductase enzyme which functions the conversion of lactate to pyruvate in reversible manner. The malarial parasite *Plasmodium falciparum* expresses the LDH isoform, which is an essential enzyme for the parasite's energy production to obtain their energy from anaerobic glycolysis[5]. All other four species (*P.vivax, P.malariae, P.ovale and P.knowlesi*) of plasmodium express LDH exhibit 90% similar to *Pf*LDH, which can be used as a biomarker in the RDT for the diagnosis of malaria. The structural and kinetic characteristics of PLDH can be examined in order to explore for the potential as antimalarial drugs[16].

Thus, the aim of the present study is to express recombinant *Pf*LDH in the *E.Coli* expression host BL21(DE3) by cloning the target gene that encodes for the *Pf*LDH into the expression vector pET-27b(+) and optimization of protein expression. Sodium Dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed to confirm the presence of expressed recombinant *Pf*LDH protein and were used for the diagnostic application.

Material and Methods

Construction of Recombinant plasmid: The gene sequence which encodes for the *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH) was identified and the target sequence was synthesized. By the Polymerase chain reaction, 951bp of *Pf*LDH gene based on the sequence of *Plasmodium falciparum 3D7 L-lactate dehydrogenase* was amplified using specific forward primer and reverse primer. PCR reaction was performed under starting denaturation at 95◦C for 5 mins, followed by 35 cycles of 95◦C for 1 min, 55◦C for 1 min and 72◦C for 1 min and the final extension at 72◦C for 10 mins. The tubes were collected from the thermal cycler, analysed by loading the PCR product into the wells of 1% agarose gel and observed using UV transilluminator.

The amplified *Pf*LDH gene was cloned into expression vector pET-27b(+) by digesting the *Pf*LDH gene with restriction enzymes *Nde*I and *Eco*RI reaction mixture. Using T4-DNA Ligase reaction mixture, the digested *Pf*LDH fragment was ligated to the digested pET-27b(+) to construct a recombinant plasmid and incubated at 16◦C for 16 hours.

Transformation into host cell: The fresh competent *E.Coli* BL21(DE3) cells were made by chemical transformation of treating with $CaCl_2$. Competent cells of 100µl were taken in the sterile microfuge, added with 10µl of cloned DNA and incubated on ice for 30 mins. After transforming the recombinant plasmid into the *E. coli* BL21(DE3) strain, the cells underwent heat shock method. Keep the tubes for 90 seconds at 42°C and then immediately in ice for 10 minutes to stabilize them. 400µl of Luria-Bertani (LB) medium was added and incubated in the shaker at 37◦C for 45 min. Centrifuge the cells at 7000rpm for 10 minutes. 400µl of supernatant was removed and the pellet with remaining supernatant was suspended. 100µl of cells were spread plated onto kanamycin (100µg/ml) supplemented Luria-Bertani (LB) agar plate using L-rod and incubated at 37◦C for 12-14 hours. The growth of the transformants was observed on the antibiotic plate.

Optimization of *Pf*LDH protein expression: In order to optimize *Pf*LDH expression in the transformed *E. coli* BL21(DE3) strain with pET-27b(+)-*Pf*LDH plasmid, cells were grown in 20ml of LB broth that included 100µg/ml of kanamycin. The culture was maintained at 37◦C and incubated at 175rpm for 16 hours in the shaking incubator. After the culture reaches 0.6 OD, the Induction of isopropyl-1-thio-D-galactopyranoside (IPTG) was performed at low-temperature 28◦C for 3 hours at concentration of 0.25mM, 0.75mM and at high-temperature 37◦C for 6 hours at concentration of 0mM, 0.5mM, 1mM. The *E coli* BL21 (DE3) strain was centrifuged at 6000rpm for 15 min. After resuspending with PBS (phosphate Buffer Saline) and SSB (Solid staining Buffer), the pellets were boiled at 100◦C for 10 mins. The 12% polyacrylamide gel was performed to verify the *Pf*LDH expression pattern in accordance with different induction conditions.

Separation and purification of *Pf*LDH protein: The *E. coli* BL21(DE3) bacterial cells were lysed by ultrasonication at 40% amplitude for 15 seconds, followed by a 10s rest interval. Bacterial cells which were lysed by ultrasonication were centrifuged at 9000rpm, 4◦C for 30min. The prepared sample was allowed to run through the column immobilized with Ni-NTA resin. The purification of the expressed recombinant *Pf*LDH protein was performed by Immobilized Metal Affinity Chromatography. Protein containing 6x His-tag binds to nickel ions after it is passed over the column.

For the removal of the unbounded protein, wash buffer (10mM Imidazole, 50mM-Tris HCL, 300mM NaCl) was added and to elute the target protein, elution buffer (250mM Imidazole, 50mM-Tris HCL, 300mM NaCl) was added. After 10mins, the fractions containing target recombinant *Pf*LDH protein sample were collected and run on 12% polyacrylamide gel electrophoresis. The purified sample was loaded in the dialysis bag in order to achieve further purification, to ensure that remaining impurities were removed.

Western Blot analysis: The gel was incubated in transfer buffer for 10 to 15 minutes after being washed with distilled water. A 100mA, 20V electric charge was applied for 1 hour and 30 minutes to transfer the protein from the gel to a nitrocellulose membrane. After a minute of immersion in Ponceau stain, the membrane was thoroughly washed with PBS. The molecular weight marker lane was visualized with amido black staining for 2-3 minutes and destained until the

background is eliminated. Rest of NCP membrane was blocked overnight at 4◦C with 3-5% BSA milk in PBS and then washed 5 times with PBST. The primary mouse anti-PLDH antibody was diluted 1:500 in 1% BSA in PBS and incubated on the membrane for 2 hours.

After 5 times wash with PBST, secondary antibody goat anti-mouse PLDH/alkaline phosphatase was diluted 1:1000. BSA in PBS was added to the membrane. The nitrocellulose membrane was washed 5 times with PBST and then the blots were developed in 10ml of substrate buffer containing 33µl of 5-Bromo-4-Chloro-3-indolyl phosphate (BCIP) and 66 µl of Nitro Blue Tetrazolium (NBT).

Results

Construction of recombinant plasmid and Its Transformation into bacteria: *Pf*LDH gene was introduced with NdeI and EcoRI restriction sites to construct recombinant plasmid. Using certain forward and reverse primers, 951bp *Pf*LDH gene was amplified by PCR for cloning. Figure 1(A) represents the sequence of *Pf*LDH gene and fig. 1(C) shows the organizational map of pET-27b(+) vector. Amplified genes were studied by agarose gel electrophoresis shown in figure 1(B). The amplified *Pf*LDH gene was inserted into pET-27b(+) vector approximately in the multiple cloning site. pET-27b(+)-*Pf*LDH recombinant plasmid was constructed by the digesting of both the PCR amplified *Pf*LDH gene and the pET-27b(+) plasmid with NdeI and EcoRI, followed by ligation using T4 DNA ligase.

The pET27b(+) vector includes a T7 promoter to enhance protein expression in the *E. coli* BL21(DE3) host strain, which is engineered with a λDE3 lysogen to express the T7 RNA polymerase gene under the control of the lac promoter. This recombinant plasmid pET-27b(+)-*Pf*LDH was transformed into *E.coli* BL21(DE3) grown in kanamycin added agar plate, the transformed bacterial cells which contain recombinant plasmid survived in the medium by the formation of colonies showed in the figure 2(B). No colonies were fond in the figure 2(A) as a negative control.

A)

Sequence 951 BP; 341 A; 127 C; 187 G; 296 T; 0 other;

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atggcaccaa aagcaaaaat cgttttagtt ggctcaggta tgattggagg agtaatggct      60
accttaattg ttcagaaaaa tttaggagat gtagttttgt tcgatattgt aaagaacatg     120
ccacatggaa aagctttaga tacatctcat actaatgtta tggcatattc aaattgcaaa     180
gtaagtggtt caaacactta tgacgatttg gctggagcag atgtagtaat agtaacagct     240
ggatttacca aggccccagg aaagagtgac aaagaatgga atagagatga tttattacca     300
ttaaacaaca agattatgat tgaaattggt ggtcatatta agaagaattg tccaaatgct     360
tttattattg ttgtaacaaa cccagtagat gttatggtac aattattaca tcaacattca     420
ggtgttccta aaaacaagat tattggttta ggtggtgtat tagatacatc aagattgaag     480
tattacatat ctcagaaatt aaatgtatgc ccaagagatg taaatgcaca cattgtaggt     540
gctcatggaa ataaaatggt tcttttaaaa agatacatta ctgtaggtgg tatcccttta     600
caagaattta ttaataacaa gttaatttct gatgctgaat tagaagctat atttgataga     660
actgttaata ctgcattaga aattgtaaac ttacatgcat caccatatgt tgcaccagct     720
gctgctatta tcgaaatggc tgaatcctac ttaaaagatt tgaaaaaagt attaatttgc     780
tcaaccttgt tagaaggaca atatggacac tccgatatat tcggtggtac acctgttgtt     840
ttaggtgcta atggtgttga acaagttatc gaattacaat taaatagtga ggaaaaagct     900
aaatttgatg aagccatagc tgaaactaag agaatgaagg cattagctta a              951
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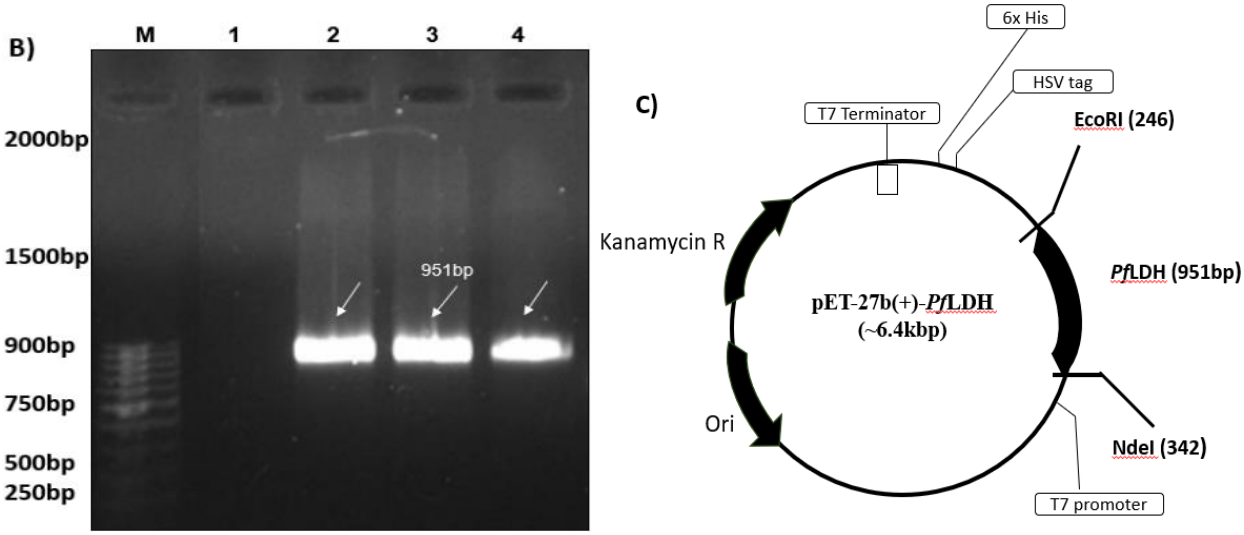

Figure 1: (A) Nucleotide sequence of *Pf*LDH gene (B) Agarose gel electrophoresis analysis of *Pf*LDH coding gene amplified by PCR using respective primers was shown in the lane 2, 3 and 4 as DNA fragment length (951bp). (C) Schematic representation of recombinant plasmid pET27b(+)-*Pf*LDH containing *Pf*LDH gene.

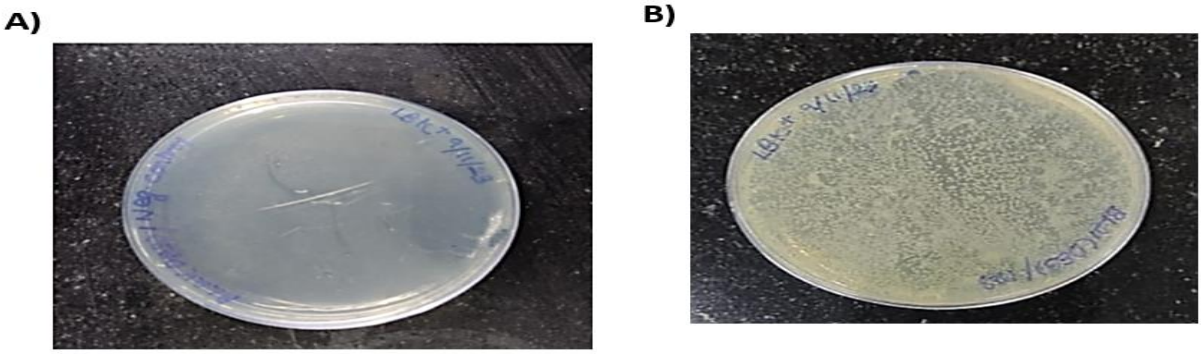

Figure 2: (A) Negative Control (B) Positive control which represents the pET27b(+)-*Pf*LDH transformed *E.coli* BL21(DE3) cells formed with the colonies on kanamycin added LB agar after incubating at 37◦C for 12-14 hours.

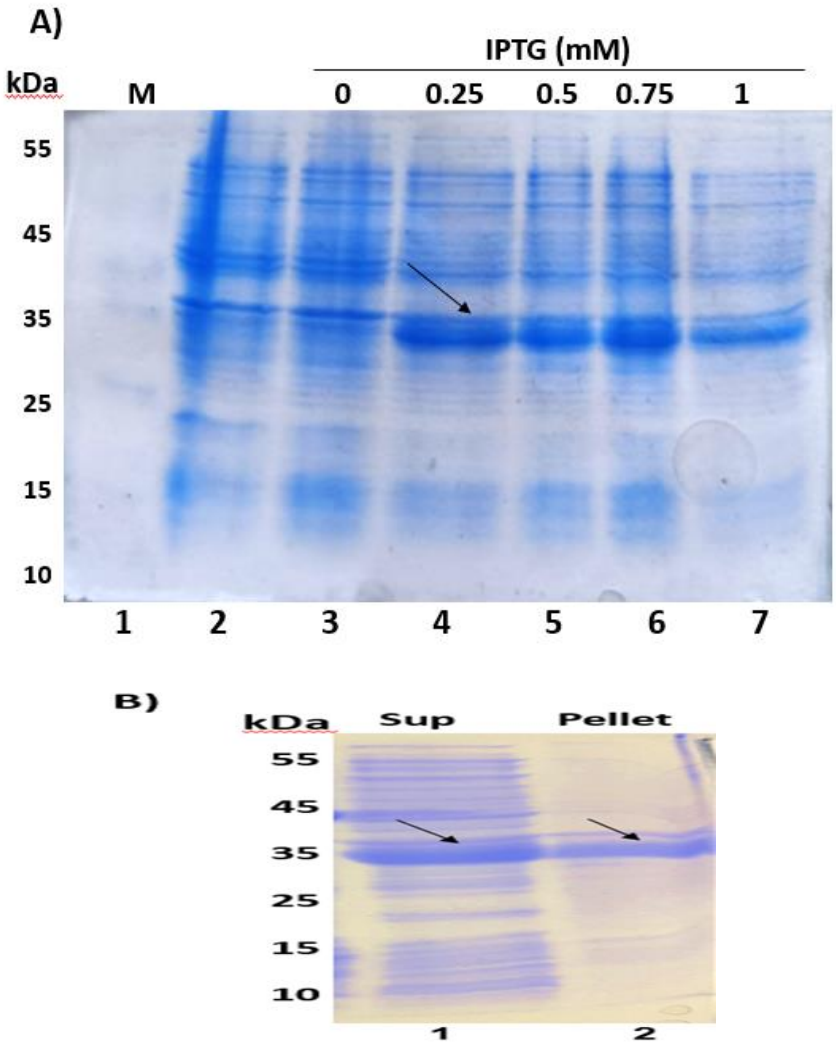

Figure 3: A) SDS-PAGE analysis of recombinant *Pf*LDH expressed in *E.Coli* BL21(DE3) which is subjected to induction of protein with IPTG at low and high temperatures, different concentrations and time intervals. B) SDS-PAGE image showing the thick band in lane 1 observed to be high amount of protein present in the supernatant as soluble form.

Finding of optimal condition for *Pf*LDH expression: The best competence for the overexpression of recombinant *Pf*LDH for pET27b(+)-*Pf*LDH was established. The transformed *E. coli* BL21(DE3) strain containing 4.5284523 predicted plasmid *Pf*LDH expression pattern was experimentally validated by the appearance of band of approx. 34kDa. Low *Pf*LDH protein expression was induced at both temperatures 28^0C and 37^0C, 3hrs, 0.25mM IPTG treatment. Low temp 28^0C was found as the best expression level for purification of *Pf*LDH. In the figure 3(A), lane 1 represents protein ladder, lane 2 represents Host-vector control which was loaded with sample

containing only BL21(DE3)-pET27b(+) without *Pf*LDH gene to compare the protein profile between host-vector control and the sample containing *Pf*LDH gene. After the protein expression, the bacterial cells were centrifuged and pellet was collected and suspended with buffer. It was lysed by ultrasonication and it was found that recombinant protein showed that the high amount of recombinant *Pf*LDH was found in the supernatant as a soluble form. PvLDH expression was confirmed by the formation of a ~35 kDa band that got thicker with increasing IPTG concentration.

After induction with 0.1 mM IPTG, PvLDH was sufficiently overexpressed[9]. Figure 3(B) represents the thick band in lane 1 observed to be high amount of protein present in the supernatant as soluble form. By decreasing the cell growth at 15^0C and the IPTG concentration to 0.25 mM, around 82% of the produced protein could be obtained in soluble form[1].

Analysis of purified *Pf*LDH: To collect the soluble lysate, a Ni-NTA resin infiltration column was set up. Eluted fractions of the purified *Pf*LDH protein were collected and analyzed on 12% polyacrylamide gel electrophoresis. The purified protein was confirmed using anti-HIS monoclonal antibodies by Western blot at approximately ~36 kDa, the expected amount, representing the r*Pf*LDH protein[18].

Figure 4 (A) showed the result of formation of prominent band at the ~34kDa molecular weight confirming the purification of the recombinant *Pf*LDH in the lane 2 and lane 3. Lane 1 shows the result of soluble fraction. After the proteins were transferred from nitrocellulose membrane, the membranes were treated successively with Mouse anti PLDH antibodies and Goat anti-mouse PLDH. The minor band was formed at ~34kDa molecular weight and the colour change confirmed the presence of recombinant *Plasmodium falciparum* lactate dehydrogenase (*Pf*LDH) by western Blot analysis. Figure 4(B) shows Western blot analysis result image that confirms the presence of plasmodium falciparum lactate dehydrogenase (*Pf*LDH).

Discussion

In this study, we constructed recombinant plasmid pET27b(+)-*Pf*LDH by inserting the *Pf*LDH gene which encodes for *Plasmodium falciparum* lactate dehydrogenase protein into pET27b(+) vector. The heat shock method was used to transfer the recombinant into host cells. *Pf*LDH protein expression was induced by addition of IPTG at various concentrations, time intervals and temperature. The optimal condition for the overexpression of *Pf*LDH protein was observed to be 0.25mM at low temperature 28◦C for 3 hrs. Low-temperature expression promotes stable protein synthesis and protein solubility. Then, the bacterial cells were isolated, lysed and recombinant protein was released. Bacterial vector pET27b(+) is used to induce the production of T7-tagged proteins containing histidine at their C-terminus.

His-tag is a commonly used expression method because of its low molecular weight, which prevents it from altering the structure and activities of proteins. The observed molecular mass is around 34kDa which is similar to the molecular weight of previously documented plasmodium LDH[3,16]. Western blot result also supports this prediction by showing its reactivity with anti-PLDH antibodies with protein at molecular weight of ~34kDa.

Conclusion

The optimised expression work and purification protocol described in this work give a simple and quick way to get plentiful *Pf*LDH usage in many applications ranging from biochemical assays, diagnosis and screening assays. The process outlined is the systematic way being followed in the present production of LDH. This study shows novel changes of other plasmodial species and of other recombinant proteins. In this study, *Pf*LDH was successfully cloned, expressed and purified from bacterial cells and confirmed by SDS-PAGE analysis and western blot analysis.

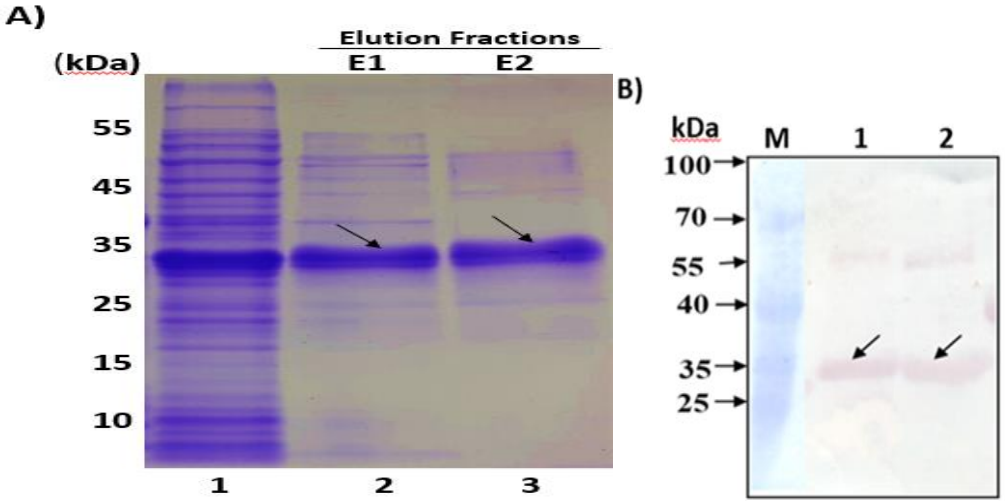

Figure 4: (A) SDS-PAGE analysis shows the presence of recombinant *Pf*LDH protein, E1 and E2 represent the elution fractions separated from IMAC. (B) Western blot analysis result image shows the minor band formed approximately at 34kDa confirms the presence of (*Pf*LDH).

This result will provide a platform for large scale production and purification of *plasmodium falciparum* lactate dehydrogenase which can be used as biomarker in rapid diagnostic test kit for detection of malaria. This will also provide an avenue for conducting further research on parasite metabolism development into potential antimalarial drugs.

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