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A Systematic Approach for Optimization of Up-scaling the Expression and Kinetic Characterization of Recombinant *Plasmodium falciparum* Lactate Dehydrogenase (*Pf*LDH) in *E.coli*

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Plasmodial lactate dehydrogenase enzyme is known to be a potential immunodiagnostic marker as well as a novel target for chemotherapy and hence its economical bulk production is required. Although *Escherichia coli* offers a mean for rapid, high yield, and economical production of recombinant proteins, high-level production of functional recombinant *Plasmodium falciparum* lactate dehydrogenase enzyme in *E. coli* is quite challenging. To explore a new approach for high level soluble expression of *Pf*LDH in *E. coli,* the recombinant clone carrying the transgene was subjected to optimizations of expression conditions at shake flask level. The comparative analysis of different culture parameters revealed that modified-TB medium at a specific pH has the highest capacity to produce the high level of expression of *Pf*LDH. There was an increase of 23% pure protein from the culture grown under optimised conditions in comparison to normal medium. The purified r*Pf*LDH was found to be biologically active with specific activity of 485.3 μmol/min/mg. We believe that this strategy could be of special interest due to its capacity to improve the expression level of *Pf*LDH and the procedure described in this study may provide a reliable and simple method for production of large quantities of soluble and biologically active *Pf*LDH.

Key words: Malaria, *Pf*LDH, optimized expression, affinity purification, enzyme kinetics.

Malaria, a vector-borne disease caused by the *Plasmodium* species, continues to be a major global health problem in tropical and subtropical regions of the world. It has been estimated that malaria kills over 2 million people each year globally and approximately 90% of child mortality in Africa is due to this disease[1]. The development of a malaria diagnostics and an anti-malarial compound could be the most effective means of controlling the impact of the disease due to the emergence of resistance against common anti-malarial drugs. *Plasmodium* requires a high level of energy production to maintain its rapid multiplication rates during asexual, intraerythrocytic cycle in the human host. Lactate dehydrogenase (LDH), the terminal enzyme of anaerobic glycolysis is very essential for parasite survival as it generates NAD from NADH for continuation of glycolysis. Hence *Pf*LDH is a good target for drug design as its inhibition results in parasite death within cultured red blood cells[2].The plasmodial LDH is known to be a potential target for chemotherapy[3-6] and the *P. falciparum* LDH has been recently used for anti-

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malarial screening by docking studies[7]. The efficacy of conventional antimalarial drugs and insecticides in controlling falciparum malaria outbreaks is declining with increasing resistance of parasites and their vectors[8, 9]. This has alarmed the immediate requirement to develop novel and affordable anti-malarial drugs to overcome the detrimental effects of drug resistance, particularly in developing countries. So as to monitor the success of anti-malarial drug therapy particularly in the presence of fever, it is crucial to accurately differentiate between malaria and another cause. (http://www.malariajournal.com/content/8/1/211) Due to the increase in drug-resistant infections, it has been suggested that LDH-based tests are more useful for this purpose, since they become negative soon after parasite clearance from the blood[10, 11, 12]. The role of plasmodial LDH in malaria diagnosis has been well established[13, 14-16, 17, 18, 19]. Advances have been also made in developing rapid malaria diagnostic tests based on detection of plasmodial LDH[20-24]. Hence, large scale production of pure recombinant LDH is required either for developing rapid diagnostic assays or *in vitro* screening of anti malarial compounds. As LDH enzyme of *P. vivax*, *P. malariae* and *P. ovale* exhibit ~90% identity to *Pf*LDH, it would be desirable to have new anti-pLDH drugs, particularly ones that are effective against *P. falciparum*, the most virulent species of human malaria[18]. We have previously reported the expression of *Pf*LDH enzyme in *E.coli*[25] and more recently *Plasmodium knowlesi* lactate dehydrogenase is also expressed in *E.coli*[26]. Although *Escherichia coli* offers a mean for rapid, high yield, and economical production of recombinant proteins, high-level production of functional recombinant *Plasmodium falciparum* lactate dehydrogenase enzyme in *E. coli* is quite challenging. Obtaining large amount of *Pf*LDH has frequently proven to be difficult in *E. coli* typically resulting in the expression of most of the protein as insoluble inclusion bodies[27, 28]. No reports exist till date on systematic evaluation of several culture conditions for expression of *pf*LDH enzyme. To address all these needs, the present study was focussed to optimize the heterologous expression conditions to get high yield of recombinant *Pf*LDH protein in soluble form at shake flask level.

MATERIALS AND METHODS

Bacterial clone, molecular reagents and chemicals

The *E.coli* clone (SG13009) known to express r*Pf*LDH protein harbouring recombinant plasmid pQE-30Xa-*Pf*LDH was obtained from Vector Management Division, DRDE, Gwalior. Stock cultures of the micro-organisms were maintained in 30% (v/v) glycerol at -80ºC. Pre stained protein marker were from MBI Fermantas Inc., USA. The chemicals like Ampicillin (Amp), Isopropyl â-D- thiogalactoside (IPTG), Diaminobenzidine (DAB), hydrogen peroxide, TMB/H_2O_2 Liquid substrate solution, HRP conjugated anti-HIS monoclonal antibodies, HRP-conjugated anti-human IgG antibodies were from Sigma, USA. The nitrocellulose membrane for western blotting, syringe filters (0.22µm and 0.44µm) were procured from Millipore Corporation, USA. Ni-NTA resin and pre packed disposable Ni-NTA affinity columns were obtained from Qiagen, Germany.

Instruments

Centrifuge (Sorvall Evolution RC, USA), ultrasonic disintegrator VibraCell VCX 750 (Sonics, USA), ELISA reader (Bio-Tek, USA), electrophoresis apparatus Mini-Protean III and Semi Dry blotting system (Bio-Rad, , USA), Shaker with incubator (ISF-1-W, Kuhner, Switzerland), Spectrophotometer (Biomate 3, Thermo Electron Corporation , USA), AKTA explorer (GE Healthcare, Sweden) were used in the present study.

Selection of Medium

We have used the following standard media (Hi-Media, Mumbai, India) with/without modifications for bacterial culture *viz.*, Luria Bertani (LB; 25.0g/L) broth, Super broth (SB; 60.0g/L), SOB broth (28.0g/L), Terrific broth (TB; TB-47.6g/L, Glycerol-4.0ml/L), Terrific broth (with different concentrations of glycerol), Semi Defined (SD with glucose; Glucose-20.0g, Yeast extract-25.0g, Trace metals-1.00g, $MgSO_4$-1.20g, M9 salts-1X), Semi Defined (SD with glycerol; Glycerol-20.0g, Yeast extract-25.0g, Trace metals-1.00g, $MgSO_4$-1.20g, M9 salts-1X). Several modifications in the media composition were made such as glycerol concentration in TB, M9 minimal salts, $MgSO_4$ and trace metal concentration (Modified TB; Terrific Broth-47.6g, Glycerol-8.00ml, M9 minimal salts-1X,

Yeast Extract-48.0g, Trace metals-1.00g, $MgSO_4$-1.20g). All the above media were individually tested for expression of the r*Pf*LDH protein at shake flask level.

Optimization of Medium at shake flask level

The seed culture was prepared by harbouring the glycerol stock of *E.coli* (SG13009) pQE30Xa*Pf*LDH (Fig.1) was inoculated into freshly prepared sterile LB broth containing 100µg/ml ampicillin and incubated for overnight at 37ºC. Overnight grown seed culture was inoculated into 100 ml each of the media as above (containing100µg/ml ampicillin) in 500ml conical flask. The flasks were kept under incubation at 37ºC shaker incubator set at 200rpm. The absorbance of the culture was measured at A_{600} using spectrophotometer at every 2h time interval. The culture was induced with 1mM IPTG when the absorbance reached 0.9 -1.1 and incubation was continued at 22ºC, 200rpm for 4hrs. For biomass analysis, 2ml aliquots of the induced culture were harvested into pre-weighed centrifuge tubes in triplicates. The wet weight and dry weight of the pellets were analysed after removing the spent medium *via* centrifugation. The pellets were washed with PBS and resuspended in solubilisation buffer (50mM NaH_2PO_4, 50mM NaCl, 20mM Imidazole, pH 8.0) and the cells were lyzed and soluble supernatant containing protein of interest was obtained as described earlier[25]. The obtained supernatants were further analysed on SDS-PAGE for protein expression analysis as described earlier[29].

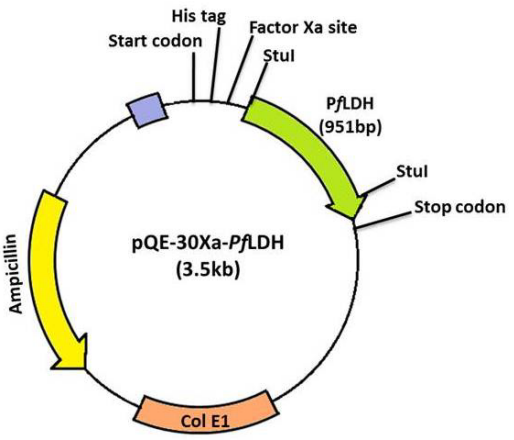

Fig. 1. pQE-30Xa-*Pf*LDH vector map: The recombinant plasmid pQE-30Xa-*Pf*LDH carrying *Pf*LDH gene cloned at *Stu I* sites under T5 promoter with N-terminus 6X-His fusion tag. The construct has ampicillin resistance gene for selection of transformants.

Optimization of culture conditions for improved expression of *Pf*LDH in *E.coli*

After selecting the optimum growth media from the above experiment, other parameters *viz*., pH, IPTG concentration, glycerol concentration, M9 minimal salt concentration, Yeast extract concentration, $MgSO_4$ concentration, trace metal concentration and percentage of inoculum were optimised. Optimised medium with different pH ranging from 6.5-7.8 were tested. Similarly, different concentrations of IPTG ranging from 0.25mM to 1.25mM were tried for finding the optimum concentration required for better expression of the recombinant protein. Several concentrations of glycerol (4-15ml/L), M9 salts (1X/2X) and varied Yeast extract concentrations (1X/2X) in TB medium were evaluated. The obtained recombinant protein yields in each case were estimated using Pierce BCA kit (Pierce, USA) following the manufacturer's instructions. Bio mass obtained in terms of wet cell weight and dry cell weight were analysed in triplicates as described earlier[30].

Expression of r*Pf*LDH under optimized conditions for purification

Finally, we expressed *Pf*LDH in one litre optimized media (modified Terrific broth) under optimised expression conditions (pH-7.0, 1mM IPTG concentration,8ml/L Glycerol concentration, 2X-M9 salts, 2X-Yeast extract concentration, 1.2ml/L $MgCl_2$, 1ml/L of trace metal solution and 5% of inoculum). The cell lysate was analyzed by SDS-PAGE for consistent expression further proceeding to protein purification.

Purification of expressed r*Pf*LDH protein

In this study, r*Pf*LDH protein was purified using NI-NTA affinity columns using AKTA explorer as per the standard protocol of the manufacturer. The sonicated cell lysate was centrifuged to get the supernatant containing our protein of interest as described earlier [25]. The soluble cell lysate (5ml) was loaded onto pre-equilibrated gravity flow disposable affinity column. The column was then washed with a step gradient of 20-150mM imidazole (in buffer containing 50mM NaH_2PO_4, 300mM NaCl, pH 8.0). This was followed by elution of r*Pf*LDH with

350mM imidazole containing elution buffer (50mM NaH_2PO_4, 300mM NaCl, pH 8.0). The eluted protein was collected as one milli litre fractions and individually analyzed on SDS-PAGE.The fractions containing the highest amount of recombinant protein were pooled and concentrated using centrifugal ultra filtration devices (10KDa MWCO). The purified r*Pf*LDH protein was than analysed *via* SDS-PAGE and protein concentration was determined as mentioned earlier.

Confirmation of expressed r*Pf*LDH protein *via* western blotting and Indirect ELISA

To confirm the authenticity of the purified rP*f*LDH protein, we performed the western blot analysis using HRP-conjugated anti-His monoclonal antibody as described earlier[30]. For Indirect ELISA, the purified r*Pf*LDH protein was coated onto MaxiSorp Immuno plates (Nunc, Denmark) at a concentration of 100ng/well and blocked with 3%BSA overnight at 4ºC. The wells were reacted in triplicates with nine different human clinical sera samples (obtained from ISPAT general hospital, Rourkela-769 005) at 1:500 dilution [The samples used in the present study were previously tested and found positive for *Plasmodium falciparum* infection through microscopic examination and BinaxNOW malaria ICT strip test (Binax, Inc, USA)] for 60 min at 37°C along with healthy sample as negative control. The wells were washed thrice for 3 minutes each with Phosphate buffered saline containing 0.025%Tween-20 (PBST) and incubated with anti-human IgG-HRP conjugate (1:10,000 in 5%BSA) for 1hr at 37p C. The plate was washed as above and enzymatic colour was development was done using 100µl of liquid TMB/ H_2O_2 solution as chromogenic substrate. The reaction was stopped by adding 50µl of 1M sulfuric acid and the absorbance were recorded at A_{490nm} using microplate reader. The samples with double the OD values of the negative control plus 2 standard deviation (SD) value were considered to be positive.

Enzyme Kinetics

Kinetic characterization of the purified r*Pf*LDH has also being done to establish the activity of the r*Pf*LDH. The kinetic study of r*Pf*LDH, i.e., the reduction of pyruvate to l-lactate was performed in 0.1 M sodium phosphate buffer, pH 7.5, containing 5 mM pyruvate and 1 mM NADH and the change in molar absorbance of NADH monitored at 340 nm. And the activity of r*Pf*LDH in oxidation of lactate to pyruvate, was performed in 0.1 M Tris buffer, pH 9.2 with 0.2% Triton X-100, 50 mM lactate and 1 mM NAD^+ by following changes in absorbance at 340 nm, å = 6.2 mM^{-1} cm^{-1}. The analysis was performed in a 250µl volume using an enzyme concentration of 3.17 nM. Michaelis constants for substrates (pyruvate, lactate) and cofactors (NADH, NAD^+, $APAD^+$) were determined from double reciprocal Lineweaver–Burk plots of initial rate measurements at varied concentrations. The K_M for pyruvate was determined with a range of pyruvate concentration from 20-800µM and for lactate range of substrate concentration from 2.5–60mM. K_M for NADH was determined with a range of substrate concentration 3.5–20µM. For APAD/ NAD^+, concentration was 50-800µM. K_M determinations were based upon collection of 8 experimental points. Data were used to determine the Catalytic rate constants (k_{cat}) and catalytic efficiency values (k_{cat}/K_M).

RESULTS AND DISCUSSION

Medium Optimization

Medium optimization was the major parameter of our study. Medium plays a vital role in producing the highest yield in shake flask culture and batch cultivation. The final cell density obtained in the present study varied with the type of medium used as well as the composition of medium. As summarised in Table 1, among all the media tested, Terrific broth recorded the highest optical density (2.462) at the time of harvest (11 h) and appears to be more suitable basic medium for the growth of our recombinant *E.coli*. From Table 1, it is also evident that TB medium had produced the highest biomass (2.22g/L) than any other medium tested. The total protein content obtained was the highest (702.25mg/ L) in the case of terrific broth. The induced cell lysates obtained from different media were analysed on SDS-PAGE (Fig.2). As expected, a thick protein band of size ~36 kDa was observed in the case TB medium. Interestingly the expression level of the recombinant protein was very poor in case of SD-gly-28, Semi-defined glucose (SD-glu)-12, SD-glu-20 and SD-glu-28 media. TB medium had rich nutrient contents. Its osmolarity was close to optimal for *E.coli* cell growth at early log phase. *E.coli* strains often grow reasonably fast in TB at early log phase. As optical

density, dry mass weight, protein concentration and r*Pf*LDH expression level were the highest in the case of TB, this appears to be optimal medium for *rPf*LDH expression. All though it is difficult to point out the reason, the present findings may be attributed to the presence of some co-factors, or ligands in TB medium that are often critical for protein folding, solubility and stability. As seen in Table 2, the net protein content was high in culture grown in TB (702.25mg/L) medium from which a total of 30.5mg/L of pure *Pf*LDH protein was obtained from the soluble lysate.

Optimization of culture conditions for improved expression of *Pf*LDH in *E.coli*

After identifying the medium for optimum growth, the following culture conditions that play an equally important role in the expression of the recombinant protein were studied.

Optimization of pH range

The pH has major effect on the growth of micro-organisms. Any wide change in pH can affect adversely to the growth of the organism and eventually reduce the final protein yield. Therefore, the pH of the media must be optimized and regulated so as to achieve the maximum biomass or protein yield. The pH of the media was optimized over a range (6.5; 6.8; 7; 7.2 & 7.8) as described under materials and method section. In the present study, pH 7.2 was found to be optimum. However, no significant OD change was noticed in case of other pH tested except for high pH (7.8) that showed decreased OD as seen in Figure 3.

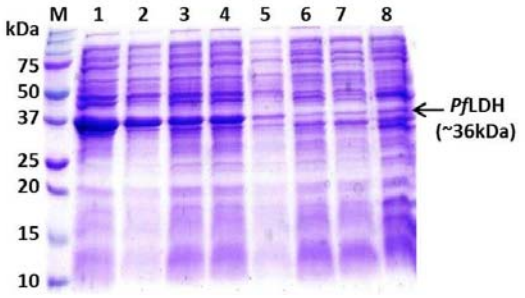

Fig. 2. SDS-PAGE analysis of proteins derived from induced cell lysates grown in different media
Lane M: Pre-stained protein marker; **Lane 1, 2, 3, 4, 5, 6, 7 and 8:** Recombinant pQE-30Xa-*Pf*LDH transformed into *E.coli*(SG13009) culture induced with 1mM IPTG in a different media *viz.* Terrific broth, Super broth, Luria Bertani, Semi-Defined glycerol (SD-gly)-12, SD-gly-20, SD-gly-28, Semi-defined glucose (SD-glu)-12, SD-glu-20, SD-glu-28 respectively.

Optimization of IPTG concentration

In most of the bacterial systems, IPTG is used as an inducer of protein expression. In the present study, we optimized the concentration IPTG as an inducer for expression of r*Pf*LDH. Several concentrations of IPTG (0.25, 0.5, 0.75, 1.0 and 1.25mM) were tested in the present study. An IPTG concentration of 1mM showed slightly better OD i.e., 2.420; compared to the 0.25mM, 0.50mM, 0.75mM and 1.25mM i.e., 1.038, 1.737, 1.565 and 2.229 respectively.

Optimization of glycerol concentration in modified terrific broth medium

Glycerol is the main carbohydrate source of the composed medium. Different concentrations of glycerol (4, 8, 12 and 15ml/L) were used in terrific broth medium in order to find out the optimum glycerol concentration that would produce the highest yield of the protein of interest. Our findings indicate that the medium containing ⁻8ml/L of glycerol concentration had shown better expression of *Pf*LDH protein with a total protein yield of 31.5mg/L (Table 2). An addition of glycerol has no visual change in the expression profile on SDS-PAGE gel. But glycerol may have a role in giving enhanced stability to the expressed recombinant protein.

Optimization of M9 minimal salt concentration in modified terrific broth medium

The effect of M9 minimal salt concentration was optimized by examining 1X and 2X concentrations along with other optimized parameters like TB medium containing 8ml glycerol.

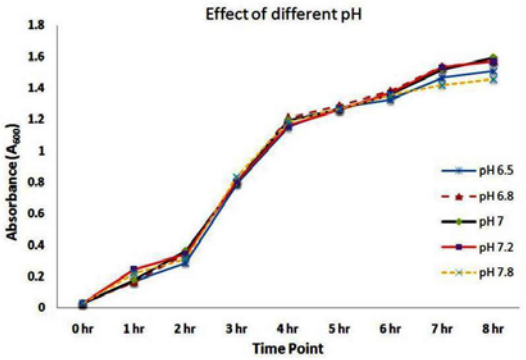

Fig. 3. Effect of pH on bacterial growth

Table 1. Concise summary of Growth and protein yield of E.coli cultured in different media

S. No.	Type of media Time Point	OD (A_{600}) 0hr	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr	9hr	10hr	11hr	Dry Cell wt. (mg/L) At induction	At harvest	Total Protein content (mg/L)	Purified Protein content (mg/L)
1	LB Broth	0.023	0.071	0.263	0.563	0.97	1.196	1.353	1.522	1.662	1.834	1.923	2.023	0.52	1.15	575.25	18
2	SB Broth	0.042	0.079	0.209	0.664	1.069	1.377	1.51	1.71	1.844	1.967	2.04	2.121	0.65	1.65	639	25.75
3	Terrific) Broth (TB	0.021	0.034	0.162	0.646	1.162	1.534	1.756	1.973	2.116	2.254	2.374	2.462	0.8	2.22	702.25	30.5
4	SD (glu 12mL/L)	0.023	0.032	0.15	0.677	1.349	1.522	1.644	1.685	1.748	1.774	1.776	1.804	1.45	1.95	550	2.5
5	SD (glu 20mL/L)	0.015	0.027	0.126	0.691	1.394	1.523	1.634	1.664	1.706	1.717	1.719	1.747	1.01	1.71	425.5	5
6	SD (glu 28mL/L)	0.001	0.023	0.118	0.648	1.317	1.463	1.595	1.627	1.696	1.717	1.726	1.784	1.26	1.59	575	5.25
7	SD (gly 12mL/L)	0.016	0.031	0.118	0.526	1.097	1.333	1.599	1.822	2.019	2.108	2.198	2.27	0.65	1.76	602.5	16
8	SD (gly 20mL/L)	0.01	0.023	0.114	0.487	1.043	1.269	1.578	1.804	2.002	2.045	2.169	2.243	0.68	1.72	625.25	16.75
9	SD (gly 28mL/L)	0.007	0.018	0.108	0.475	1.028	1.249	1.529	1.76	1.972	2.081	2.135	2.207	0.46	1.68	375.25	6.23

Table 2. Effect of the different concentrations of glycerol in TB medium on growth kinetics and protein yield

S. No.	Glycerol concentration in TB Medium	OD (A_{600})							Dry Cell wt. (mg/L)		Total Protein content (mg/L)	Purified Protein content (mg/L)
Time Point		0hr	2hr	4hr	6hr	8hr	10hr	12hr	At induction	At harvest		
1	TB (Glycerol 4ml/L)	0.021	0.162	1.162	1.756	2.116	2.374	2.421	1.26	2.22	720.5	30.5
2	TB (Glycerol 8ml/L)	0.113	0.467	1.107	1.672	2.048	2.395	2.533	1.24	2.5	730	31.5
3	TB (Glycerol 12ml/L)	0.115	0.337	1.047	1.651	2.074	2.319	2.398	1.2	2.21	715	30
4	TB (Glycerol 15ml/L)	0.123	0.282	0.997	1.505	1.889	2.187	2.247	1	2.1	712.5	29.75

We observed the better OD and biomass in 2X concentration of M9 minimal salt solution i.e., 2.40 and 3.92, in comparison to the 1X concentration i.e., 2.31 and 3.20 respectively.

Optimization of Yeast extract concentration

Yeast extract contributes as a major nitrogen source in media. The concentration of yeast extracts along with 2X-M9 were optimized by examining two different concentrations of yeast extract viz. 24mg/L and 48mg/L as 1X and 2X respectively. Our study suggests that the use of 2X concentration of yeast extract gives better cell growth as well as protein yield (761mg/L of total protein and 34.7mg/L of pure protein) in comparison to that of 2X concentration (756mg/L of total protein and 33.25mg/L of pure protein).

Optimization of $MgSO_4$ and trace metals

Presence or absence (+/-) of $MgSO_4$ and trace metal solution in media (TB containing 8ml Glycerol, 2X-M9, 2X-yeast extract) showed positive effect on biomass and yield of *Pf*LDH protein. Presence of 1.2g/L $MgSO_4$ along with trace Metals (1ml/L) increased the protein level (765mg/L of total protein and 36.5mg/L of pure protein) while in the only presence of $MgSO_4$ (755.5mg/L of total protein and 35.5mg/L of pure protein) and the trace metal (725.75mg/L of total protein and 35.0mg/L of pure protein) the protein level was comparatively low.

Optimization of Inoculum volume

The final volume of inoculum may affect the growth of culture. Low volume of the inoculum may not grow properly, whereas more volume of inoculum may inhibit the growth of the culture. In our study, we observed that 5% (v/v) inoculum used had shown better OD than 1% and 2% inoculum size tested (Fig. 4).

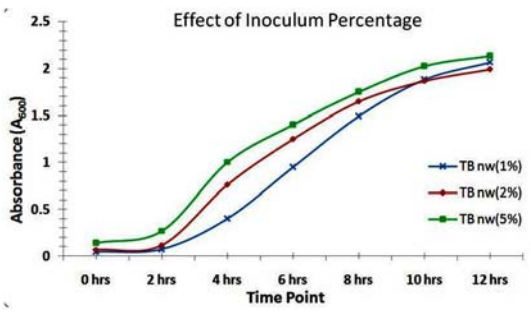

Fig. 4. Effect of different volumes of inoculum percentage on the growth kinetics and protein expression

Table 3. Substrate and Cofactor kinetic parameters of rPfLDH

S.No.	Substrate/ Cofactor	K_M (µM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} M^{-1})
1	Pyruvate	63.38 ± 1.36	(4.0 ± 0.04) × 10^4	6.3 × 10^8
2	Lactate	5289.6 ± 131.8	(3.3 ± 0.01) × 10^4	6.2 × 10^6
3	NADH	15.31 ± 0.95	(3.8 ± 0.03) × 10^4	2.5 × 10^9
4	NAD^+	54.92 ± 3.23	(3.3 ± 0.01) × 10^4	6.0 × 10^8
5	APAD	177.33 ± 2.20	(13.7 ± 0.01) × 10^4	7.7 × 10^8

Comparison of protein yield in terrific broth and modified terrific broth medium

After optimizing all the parameters, we compared the terrific broth with modified terrific broth and noticed a clear difference in biomass and protein yield between both the media as shown in Fig.5. An increase of wet cell weight from 24g/L to 31g/L (Fig.5A) and dry weight from3.7g/L to 4.5g/L (Fig.5B) was noticed when modified terrific broth was used in place of terrific broth. There was also an elevation in the total protein yield (from 680mg/L to 770mg/L- Fig. 5C) and pure protein yield (from 30mg/L to 37mg/L- Fig.5D) when modified terrific broth was used. This accounts for nearly 23% increase in the purified protein derived from the culture grown in modified terrific broth. We conclude that modified TB is the optimum medium for scaling up the expression of r*Pf*LDH.

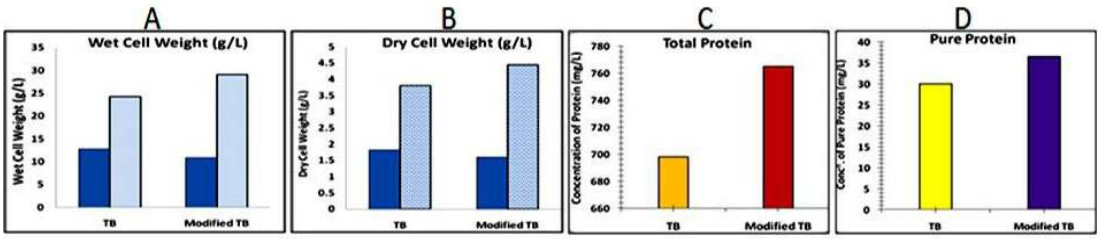

Fig. 5. Comparative analysis of biomass and protein yield obtained from TB and modified TB medium
Panel A: wet weight from pre-induced and induced culture
Panel B: Dry weight from pre-induced and induced culture
Panel C: Total Protein estimation Panel D: Pure protein estimation

Expression analysis of r*Pf*LDH protein from optimized culture conditions

After careful consideration of the several factors that influences the growth of the culture, biomass and protein yield, we were able to arrive at a conclusion that Modified Terrific broth media at optimized parameters *viz*., pH-7.2, 1mM IPTG concentration, 8ml/L glycerol, 2X- M9 salts, 2X-Yeast extract, 1.2ml/L of $MgSO_4$, 1ml/L of trace metal solution and 5% (v/v) of inoculum gives better expression of soluble *Pf*LDH upon induction with 1mM IPTG. A consistent expression of 36kDa was observed with the above culture conditions as seen in Figure 6.

Purification of expressed r*Pf*LDH protein

Purification was done by Ni-NTA affinity chromatography using AKTA explorer system as described under materials and methods. The chromatogram of affinity purification is as shown in Figure 7A. The peak corresponding to r*Pf*LDH protein was obtained between 56-62 min during elution step. After affinity purification, a single band corresponding to ~36kDa was observed on the SDS-PAGE gel (Fig.7B) indicating the homogeneity of the purified protein.

Confirmation of expressed purified r*Pf*LDH protein

Western blot analysis

For the confirmation of the purified protein, western blot was performed using anti-HIS monoclonal antibodies and the protein was detected at about ~36 kDa as expected (Fig.8) that represents the r*Pf*LDH protein.

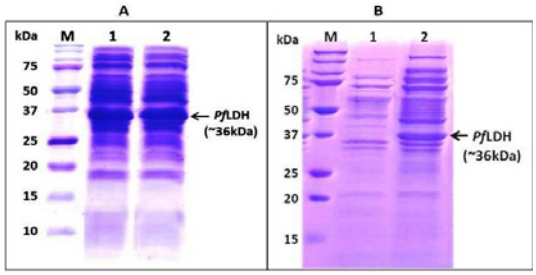

Fig. 6. Panel 6A: SDS-PAGE analysis of induced cell lysate grown in modified Terrific broth and Terrific broth medium

Lane M: Pre-stained protein marker; **Lane 1 and 2:** Expression of recombinant pQE-30Xa-*Pf*LDH transformed *E.coli* (SG13009) culture into the modified terrific broth and terrific broth medium.

Panel 6B: SDS-PAGE analysis of expression of r*Pf*LDH from optimized media conditions in modified Terrific broth

Lane M: Pre-stained protein marker; **Lane 1 and 2:** Uninduced rPfLDH and the Expression of recombinant pQE-30Xa-*Pf*LDH transformed *E.coli* (SG13009) culture after induction into the modified terrific broth respectively

The ELISA result have confirmed the immuno reactivity of the expressed recombinant protein as it selectively reacts only with positive sera samples and not with negative ones. However, the present study on diagnosis is preliminary and requires extensive validation with more number of established positive and negative samples for determining the specificity and sensitivity of the ELISA assay. As our main focus was to optimise the r*Pf*LDH protein expression, further evaluation of the diagnostic potential of r*Pf*LDH protein was beyond the scope of this study.

Enzyme Kinetics

Preliminary steady state kinetic parameters obtained for r*Pf*LDH with substrates (pyruvate, lactate) and cofactors (NADH, APAD$^+$ and NAD$^+$) are summarized in Table 3. From Table 3 (Fig.10), it can be observed that for both the substrates (pyruvate and lactate) and cofactors (NADH, APAD$^+$ and NAD$^+$) catalytic efficiencies (k_{cat}/K_M) as well as Catalytic rate constant (k_{cat}) and K_M values of r*Pf*LDH are comparable to those of previous reports [4, 31]. The k_{cat} value for APAD$^+$

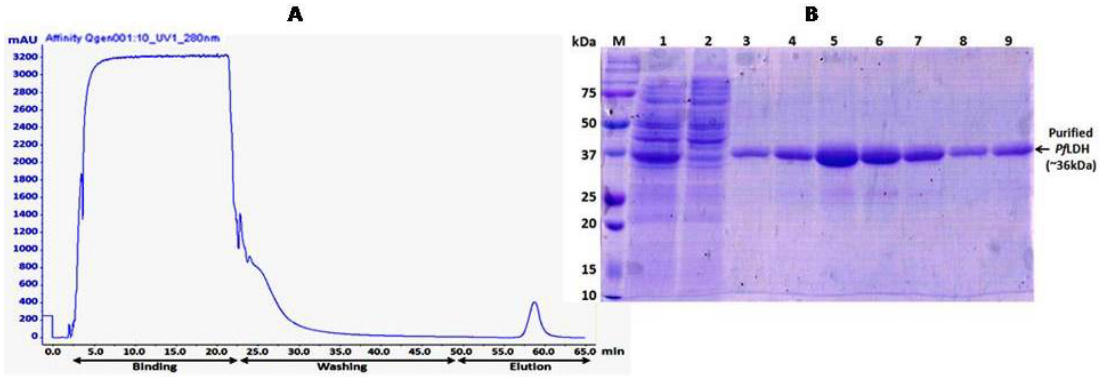

Fig.7. Panel 7A: Affinity chromatogram showing the r*Pf*LDH elution profile

Panel 7B: SDS-PAGE analysis of Ni-NTA affinity column fractions of Purified r*Pf*LDH protein

Lane M: Pre-stained protein marker; **Lane 1:** Induced Cell lysate, **Lane 2:** Flow through,

Lane 3, 4, 5, 6 and 7: Eluted fractions of purified r*Pf*LDH protein

Indirect ELISA

r*Pf*LDH protein was used in indirect ELISA for detection of anti-*Pf*LDH antibodies in nine known positive human sera samples (samples A - I). All nine positive human clinical sera samples tested were recognised by r*Pf*LDH protein in indirect ELISA (Fig.9). The mean absorbance value for the healthy subject studied was 0.18 ± 0.06.

[$(13.7 \pm 0.01) \times 10^4$] was found to be greater than NAD$^+$ [$(3.3 \pm 0.01) \times 10^4$] either in this study or elsewhere [25, 4], including the ability of *Pf*LDH to use APAD$^+$ more efficiently than NAD$^+$. Whereas, NAD$^+$ is a strong cofactor as compared to APAD$^+$ for human LDH[4], this property is being used previously to develop an enzyme based diagnostic assay for malaria[10, 17].

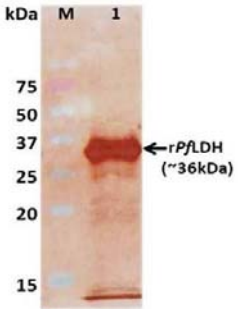

Fig.8. Western blot analysis of purified r*Pf*LDH using anti-HIS monoclonal antibodies

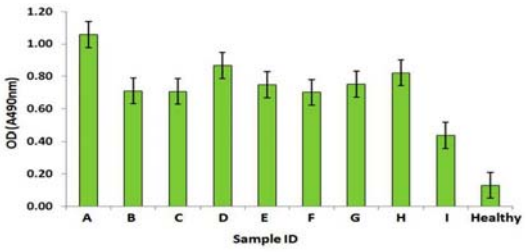

Fig.9. Indirect ELISA for *Pf*LDH specific IgG antibody detection from human clinical sera samples

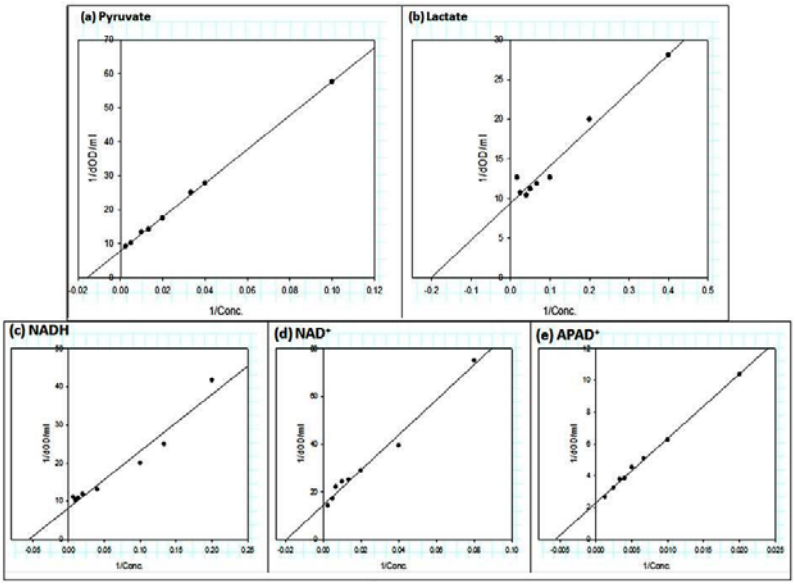

Fig. 10. Double reciprocal Lineweaver-Burk plots of initial rate measurements of r*Pf*LDH at varied concentrations of Pyruvate (a), Lactate (b), NADH (c), NAD^+ (d), $APAD^+$(e). Calculated kinetic constants corresponding to the plots are given in table 3.

CONCLUSIONS

To conclude, the optimised expression and purification procedure described in this study provide a simple and efficient method to obtain pure *Pf*LDH in large quantities for wide range of applications that include biochemical assays, antibody screening assays, diagnosis and high throughput screening of anti-malarial compounds. The systematic approach followed in the present study could be of interest for production of LDH from other plasmodial species as well as other recombinant proteins. We have successfully improved the expression level of r*Pf*LDH enzyme by careful analysis of different culture parameters that influences the biomass, protein yield and having better specific activity (485.3 µmol/min/mg) by a more simple protocol. Comparative study carried out over different culture parameters with the different media revealed that the modified-TB with the specified culture conditions have the highest potential to produce and express r*Pf*LDH enzyme. There was an increase of nearly 23% pure protein recovery from the culture grown under optimised conditions in comparison to the normal medium.

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