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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Fermentation and downstream process for high yield production of *Plasmodium falciparum* recombinant HRP II protein and its application in diagnosis

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Abstract Malaria represents the world's greatest public health problem in terms of number of people affected, levels of morbidity and mortality in tropical and subtropical countries. Malaria parasites are members of the *Apicomplexa*, family of *Plasmodiidae*. Histidine-rich protein-II secreted by *Plasmodium falciparum* is known to be a compelling marker in malaria diagnosis and follow-up. In our present study, we have optimized the batch fermentation and downstream process for large scale production of recombinant *P. falciparum* HRP-II 62 kDa protein for diagnostic application. The culture broth was effectively induced with IPTG twice at different time intervals to sustain induction for a long period. Batch fermentation resulted in a wet weight of 61.34 g/L and dry cell biomass 12.81 g/L. With the improved downstream process, purified recombinant protein had a yield of 304.60 mg/L. The authenticity of the purified recombinant protein was confirmed via western blotting using indigenously developed HRP-II specific monoclonal antibodies and known positive human clinical sera samples. Further, the reactivity of recombinant HRP-II protein was validated using commercially available immuno chromatographic strips. Indirect ELISA using recombinant purified protein recognized the *P. falciparum* specific antibodies in suspected human sera samples. Our results clearly suggest that the recombinant HRP-II protein produced via batch fermentation has immense potential for routine diagnostic application.

Keywords Batch fermentation · *P. falciparum* · Histidine-rich protein II · Malaria diagnosis

Abbreviations

HRP II	Histidine-rich protein II
RDTs	Rapid diagnostic tests
MAbs	Monoclonal antibodies
DAB	Diaminobenzidine
OPD	Ortho phenylenediamine
ELISA	Enzyme linked immunosorbent assay
ICT	Immuno chromatographic strip test

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Introduction

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. *Plasmodium falciparum* infection has become the leading cause of morbidity and mortality in tropical areas [1]. It has been estimated that malaria kills over 2 million people each year globally and approximately 90 % of mortality in children occur in Africa. Accurate diagnosis of malaria is necessary to prevent morbidity and mortality while avoiding the unnecessary use of anti-malarial agents [2]. Microscopic diagnosis of blood specimens is sensitive and specific, but

difficult for field application as it requires specific equipment and technical expertise for accurate identification of the pathogen [3]. By contrast, rapid diagnostic tests (RDTs) offer many advantages, including ease of use, applicability to a broad range of medical conditions, rapid development time with relatively low cost to the patient [4]. Protein biomarkers are important targets for RDTs [5, 6]. Most of the currently available RDTs for malaria are based on detection of the *P. falciparum* histidine-rich protein II (*Pf*HRP-II) by monoclonal antibodies (mAbs) [7, 8]. The HRP-II is a water-soluble protein produced young gametocytes of *P. falciparum* at asexual stage of multiplication [9]. The *Pf*HRP-II protein contains central repeats, rich in alanine and histidine residues, the number of which varies between parasite clones. This protein is abundantly produced specifically by *P. falciparum* and not by any other malaria parasites infecting humans [10]. The HRP-II is known to be a soluble, heat stable protein secreted from *P. falciparum* infected erythrocytes and, hence, is an interesting and sensitive target antigen for detecting falciparum species of malaria in biological fluids [11]. The performance of *Pf* HRP II based RDTs developed by several companies has been recentlyassessed and compared [12–21]. Considering the practical applications of HRP II for diagnosis, it is important to produce the HRP-II protein in bulk using recombinant DNA technique. To the best of our knowledge, no studies have been reported on the fermentation process for large scale production of HRP-II protein. Hence, the present study was undertaken to develop a fermentation and downstream process for large scale production of rec. HRP-II protein.

Materials and methods

Small scale expression analysis of HRPII gene

The *Escherichia coli* M15 cells harboring pQE-UAHRP II recombinant plasmid, known to express *P. falciparum* HRP II gene were used in the present study. The HRP II protein expression was initially tested in small scale using shake flask culture that has been previously reported by us [22]. Three different media viz, Lauria Bertani broth, Terrific broth and modified Terrific broth were tested to analyze the expression level recombinant HRP II protein at optimized expression conditions (pH-7.0, 1 % (v/v) inoculum, 1 mM IPTG concentration 25 µg/mL Kanamycin, Ampicillin 100 µg/mL at 37 °C). Protein expression was induced with final concentration 1 mM IPTG at two different points during the exponential phase (OD_{600} ~0.7 and ~2.5) [23]. After 7.5 h of induction with IPTG, cells were harvested by centrifugation at 7,000*g* at 4 °C for 15 min and the expression was checked on SDS-PAGE [24].

Batch fermentation

The modified Terrific broth with optimized formulation [Terrific broth, (Himedia, Mumbai, India) containing 8 mL/L glycerol, 2X-M9 salts (Difco, USA), 2X-Yeast extract, 1.2 mL/L of $MgCl_2$, 1 mL/L of trace metal solution) that was found to give better expression of the recombinant protein at shake flask level was finally chosen for culturing the cells in batch fermentation. The primary seed culture was prepared by the inoculating of frozen glycerol stock of the clone expressing recombinant HRP-II of *P. falciparum* protein into 10 mL modified Terrific broth containing Kanamycin (25 µg/mL) and Ampicillin (100 µg/mL). The culture was incubated for 8 h at 37 °C at 200 rpm in a shaker-incubator. The secondary seed culture was prepared by inoculating the primary seed culture into 450 mL of terrific broth medium and grown for overnight as above. The secondary seed culture was further inoculated into the bioreactor containing pre sterilized 4.5 L modified terrific broth. Fermentation was carried out in a fully automated NBS fermenter (Bioflow-3000, New Brunswick scientific USA) with 5 L working volume. Biocommand plus software was used for operation of the fermenter as per the instructions from the manufacturer. Calibrations of dissolved oxygen (DO) and pH probes were done before sterilizing the medium as previously described by us [25]. The initial batch conditions of the bioreactor were as follows: culture medium volume 4.5 L, 10 % inoculum, temperature 37 °C, airflow rate 3.0 LPM and agitation speed 200 rpm. Kanamycin and ampicillin were added into the fermentation vessel to final concentrations of 25 and 50 µg/mL respectively via the inoculation port. Temperature was monitored by a temperature probe and maintained at 37 °C throughout the fermentation process. The pH was maintained at 7.0 using 10 % (v/v) liquid ammonia and 1 N hydrochloric acid as required. The DO was maintained at 30–75 % of air saturation either through agitation (200–500 rpm) or aeration (3–5 LPM). At higher turbidity, the DO level was maintained by injecting 5–10 % pure oxygen blended into the spurge air. Active Silicone polymer antifoam-A solution (Sigma, USA) was added to avoid foaming. The culture was induced twice with 0.50 mM IPTG at OD's 0.793 and 2.652. The cells were cultivated for a total period of 7.5 h after IPTG induction and were harvested by centrifugation (Sorvell-RC5C) at 7,000 *g* for 15 min at 4 °C. The obtained cell pellet was washed with sterile PBS and then stored at −80 °C until used further for downstream processing.

Biomass analysis

The Biomass was analyzed by measuring the dry cell weight (DCW), wet cell weight (WCW), and optical

density (OD) in triplicate at every 30 min of the fermentation (pre- and post-induction stage). For determining the dry weight, 2 mL aliquots of fermentation broth collected in pre-weighed centrifuge tubes were spun at 10,000 *g* for 5 min. Supernatant was completely removed and the cell pellet, along with the tubes, was weighed. For dry cell weights, the above cell pellets were dried overnight in an oven at 100 °C. The OD was measured by measuring optical density at 600 nm using a spectrophotometer (Thermo, Electron corp., USA).

Purification of recombinant HRPII protein using immobilized metal affinity chromatography

Purification of recombinant HRPII protein was carried out by affinity chromatography in native conditions using commercially available Ni–NTA columns (Qiagen, Germany) using the AKTA-explorer FPLC system (GE healthcare, Sweden) as per the manufacturer's instructions. Briefly, the harvested pellet was lysed using ultra sonication as described earlier [26] and the soluble recombinant protein was bound to pre- equilibrated (in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0) Ni–NTA column at a flow rate of 1 mL/min. The unbound proteins were washed with 50 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM Imidazole, pH 8.0). The desired protein was eluted in 1 mL fractions with 20 mL of elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 350 mM Imidazole pH 8.0. The eluted protein fractions were analyzed on SDS-PAGE. The fractions containing the highest amount of recombinant protein were pooled, concentrated using Amicon ultra filtration device (10 kDa MWCO), and dialysed in phosphate buffered saline (PBS). The protein concentration was estimated through the Bradford assay [27].

Western blot analysis using monoclonal antibodies and positive human clinical serum sample

The purified rHRP-II protein was confirmed via western blotting using monoclonal antibodies (MAb) developed against rHRP-II protein previously by us [22] and also against a known positive human clinical serum sample. The expressed protein after affinity purification and desalting along with a molecular weight marker was electrophoretically transferred onto poly vinyl difloride (PVDF) membrane (Millipore, USA) using semidry transfer unit (Biorad, USA) in duplicates. The blots were incubated either with anti HRP-II mouse MAb (1:2,000 dilution) or with human serum sample (1:500 dilution) for overnight at 4 °C in 3 % BSA solution prepared in PBS. For a negative control, a serum from an apparently healthy person was used at the same dilution. The membranes were washed with PBS-T [(PBS containing 0.05 % Tween-20(v/v)] thrice for 5 min each and then incubated either with 1:5,000 diluted goat anti-mouse Horse radish peroxidase conjugated secondary antibody (Sigma, USA) (ln case the blot reacted with MAb) or with 1:5,000 diluted anti-human IgM Horse radish peroxidase conjugate (Sigma, USA) in the case of the human clinical sample for 2 h at 37 °C dilution for 1 h at 37 °C. The membranes were thoroughly washed thrice with PBS-T for 5 min each and enzymatic colour development was done using DAB-H_2O_2 substrate-chromogen mixture.

Immuno chromatographic test

Furthermore, to confirm the functionality of rHRPII, the purified protein was tested with the commercially available BinaxNOW malaria ICT strip test (Binax, Inc, USA) that detects HRPII antigen as per manufacturer's instructions. The results were interpreted as described in the manual of the kit. Similarly a panel of thirty-six clinical human blood samples that were found positive for malaria parasite infection through microscopic examination (samples obtained from ISPAT General Hospital, Rourkela-769 005, India) and six healthy samples were tested with BinaxNOW malaria ICT strips and the obtained results were compared with that of the microscopic test and indirect ELISA.

Evaluation of diagnostic potential of recombinant HRPII protein using indirect ELISA

In order to assess the diagnostic potential of the recombinant HRP II protein produced in the present study, Indirect ELISA was performed on the sera obtained from forty-two previously tested blood samples (36 positive and 6

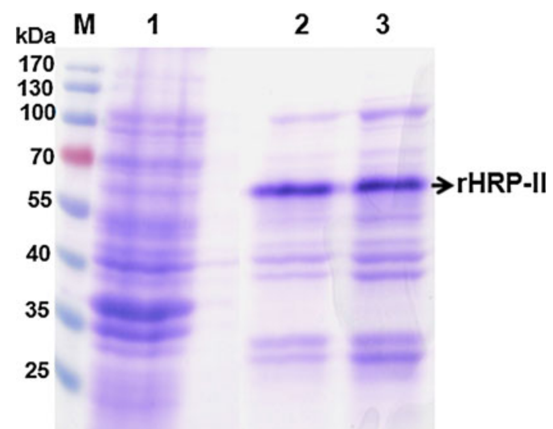

Fig. 1 SDS-PAGE analysis of *P. falciparum* expression at shake flask level *lane M* pre-stained protein marker, *lane 1* un-induced (negative control), *lane 2*, *3* lysates induced with 1 mM IPTG after 2 and 4 h, respectively

negative). The purified and desalted recombinant HRPII protein was coated onto MaxiSorp Immuno plates (Nunc, Denmark) at a concentration of 100 ng/well and blocked with 3 %BSA for overnight at 4 °C. The wells were reacted with the above sera samples in triplicates at 1:500 dilutions for 60 min at 37 °C. The wells were washed thrice for 3 min each with phosphate buffered saline containing 0.025 %Tween-20 (PBST) and incubated with anti-human IgM-HRP conjugate (1:5,000 dilution in 3 % BSA made in PBST) for 1 h at 37 °C. The plate was washed as above and enzymatic color development was done using 100 µl of liquid TMB/H_2O_2 substrate solution (Sigma,

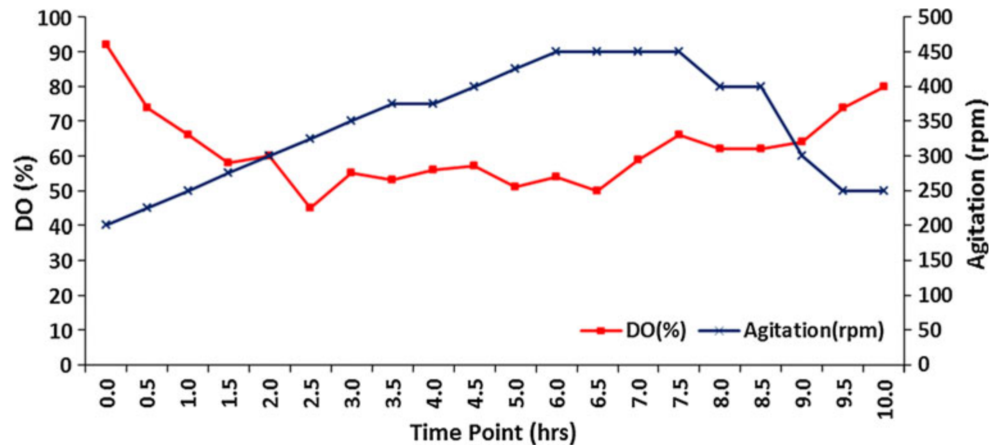

Fig. 2 Agitation (rpm), dissolved oxygen (DO) profiles relative to fermentation time (h) dissolved oxygen (DO %; 30–75 %), agitation speed (rpm) are plotted with respect to time. Different events of batch fermentation are indicated

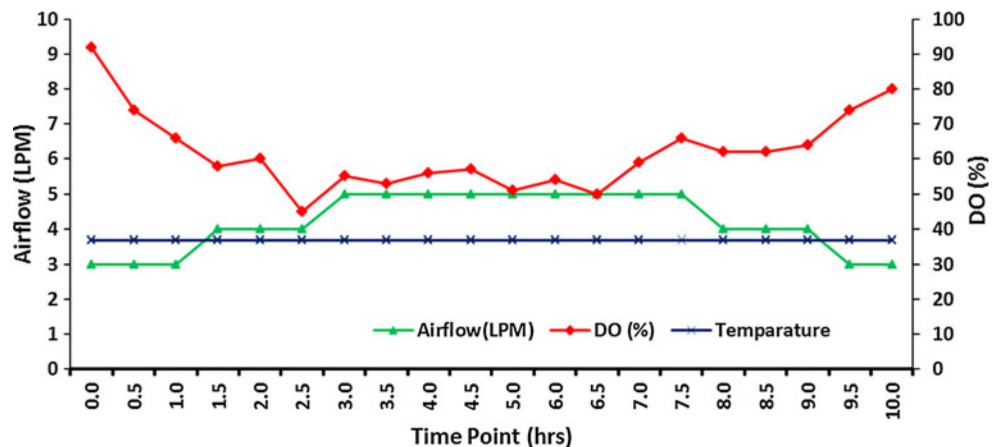

Fig. 3 Airflow, dissolved oxygen (DO), temperature profiles relative to fermentation time (h) airflow (LPM), dissolved oxygen (DO %; 30–70 %) and temperature are plotted with respect to time

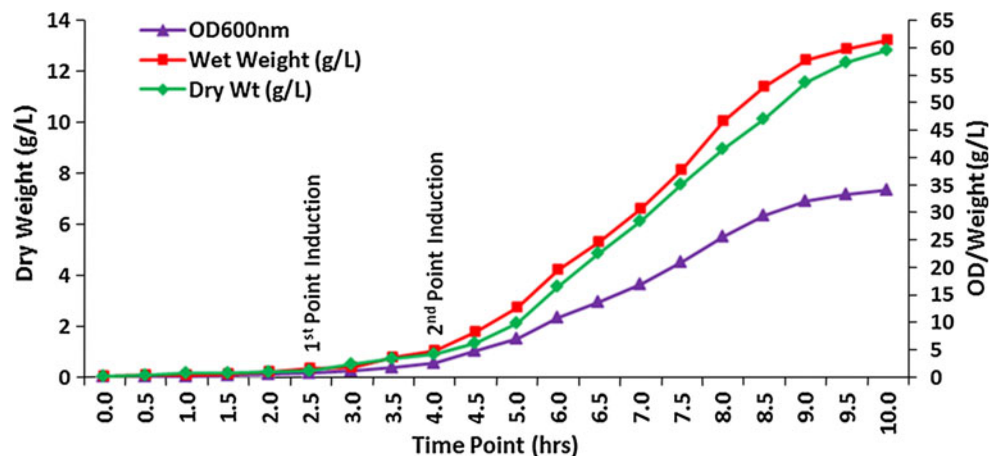

Fig. 4 Biomass analysis during rec. HRPII batch fermentation. Optical density (OD) in combination with wet weight (g/L) and Dry weight (g/L) are plotted against time

USA). The reaction was stopped by adding 50 µl of 1 M sulfuric acid and the absorbance were recorded at A_{490nm} using a microplate reader (Biotek, USA). The samples showing at least OD values twice that of the negative control plus 2 standard deviation (SD) value were considered as positive.

Results and discussion

Expression of recombinant HRP-II protein at shake flask level

Three different media, viz, Lauria Bertani broth, Terrific broth and modified Terrific broth were tested for expressing rec. HRPII protein at shake flask level as described previously. Amongst three media tested, modified Terrific broth was found to give better expression of recombinant HRP II (data not shown) with a total protein yield of 60.2 mg/L and; hence, the same medium was used for the subsequent fermentation process. As also witnessed with many other proteins [25, 26], modified Terrific broth was found to be optimum for producing recombinant HRPII. As seen in Fig. 1, a distinct band of 62 kDa protein corresponding to the expected size of HRP II was noticed upon induction. As composition of media plays an important role for the optimal cell growth, solubility and stability of the expressed product, selection of an appropriate medium is important in Bio-process scale up studies. 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 modified Terrific broth that are often critical for protein folding, solubility, stability of HRP-II protein. However, attempts were not made to evaluate more media formulations in the present study for better expression of rec.HRP-II protein.

Batch fermentation

Batch fermentation was employed in the present study using modified Terrific broth medium for large scale expression of *P. falciparum* recombinant HRP II protein. The real time profiles of DO, airflow, temperature and agitation with respect to time are depicted in Figs. 2 and 3. As evident in Fig. 1, during the fermentation process, with increase in the fermentation time, the oxygen demand also increased, thus resulting in decreased DO levels. To meet the oxygen requirement or demand, the DO % was maintained between 30 and 75 %, either by increasing agitation (200–450 rpm), or by air (3–5 LPM). After reaching high cell densities, the DO levels were maintained by supplementing pure oxygen (5–10 %) in the air. A constant pH of 7.0 was maintained throughout the fermentation process by injecting either

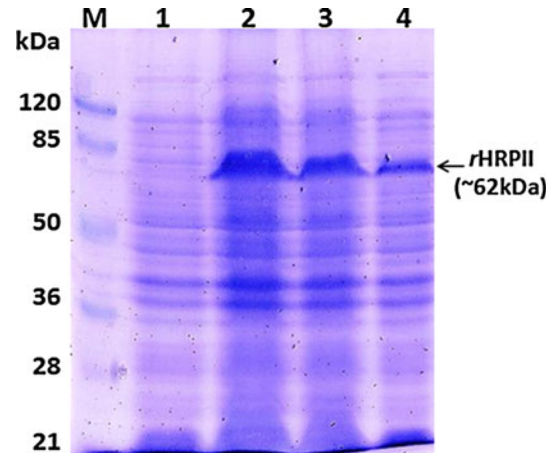

Fig. 5 SDS-PAGE analysis of culture lyate at different time points of fermentation *lane M* protein marker (Fermentas catalogue no SM0441), *lane 1* un-induced culture lysate, *lane 2* culture lysate after 7.5 h of post induction (final harvest), *lane 3* culture lysate after 5 h of post induction, *lane 4* culture lysate after 2.5 h of post induction

10 % ammonia or 1 M HCl as required. A constant temperature (37 °C) was observed throughout the fermentation process (Fig. 3). The culture was induced with IPTG after 2.5 and 4 h of growth when the corresponding OD was 0.793, and 2.652, respectively, as seen in Fig. 4. As reported earlier by Srivastava et al. [23], double induction helps in sustaining the induction for a long time with increased biomass obtained as a result of fermentation. As seen in Fig. 4, there is a clear relationship between OD, wet weight and dry weight with respect to incubation period. A clear increase in the bio mass (wet weight and dry weight) and OD were noticed as the fermentation progressed. At end of the batch, a final wet cell biomass of 61.34 g/L and dry cell biomass of 12.81 g/L was attained (Fig. 4). The OD reached up to 34 before harvesting the induced culture as seen in Fig. 4. After batch fermentation, a final dry mass of 17.81 g/L was attained after 7.5 h of post induction. The correlation between OD_{600} and Wet cell weights was 1 OD_{600} equal to 1.8 g/L and a dry cell weight of 0.52 g/L. On SDS-PAGE analysis of the biomass, obtained after fermentation, a good expression of rHRP-II was observed at 62 kDa region as seen in Fig. 5. The band intensity increased with respect to fermentation time and was proportional to the obtained biomass. Recombinant protein production in heterologous hosts like bacteria, mammalian cells and yeast can be increased to many folds by using various fermenter culture systems, that can be classified into three groups: batch, fed-batch and continuous fermentation. Among all the three, batch fermentation is the most widely accepted process due to time saving, cost effective, and low labor involved [25]. In batch fermentation, monitoring of pH and DO parameters simultaneously gives better control over the growth condition of the culture. Glucose is the

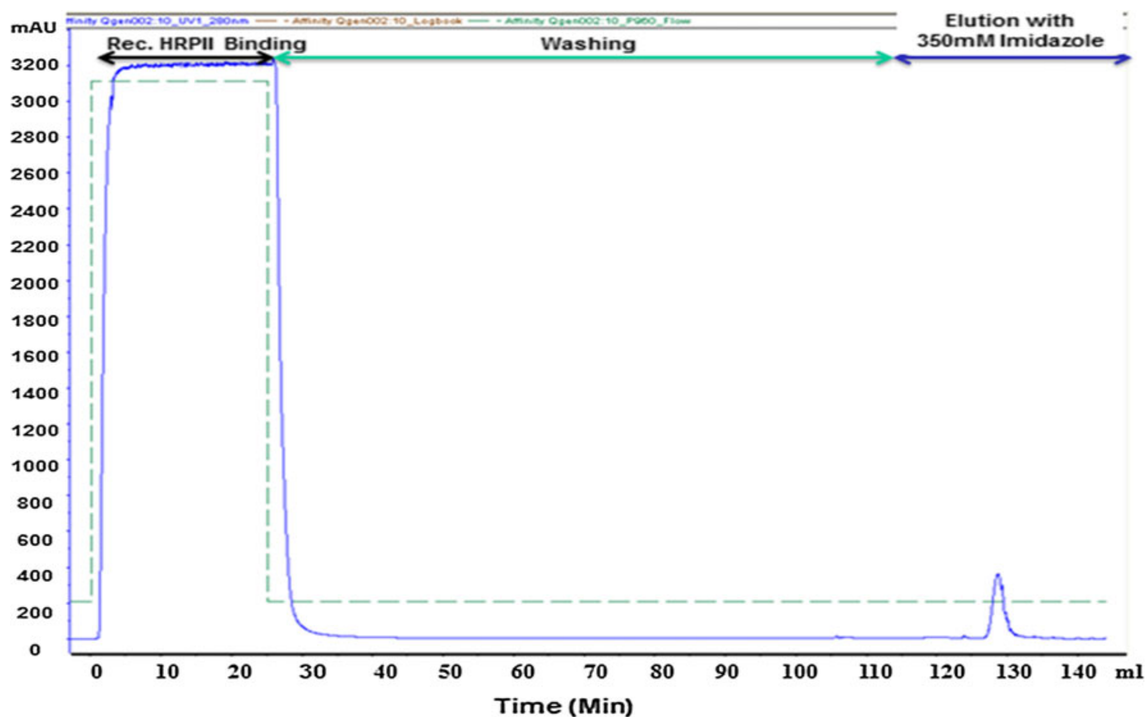

Fig. 6 Chromatogram of affinity purification of recombinant HRP-II protein using the AKTA explorer FPLC system

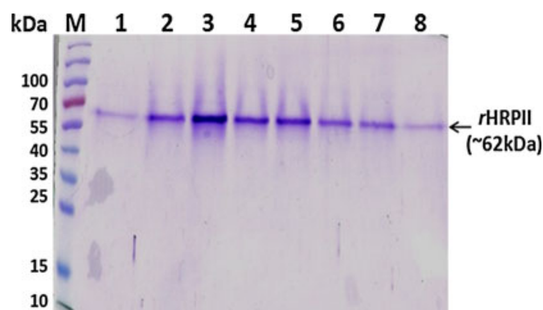

Fig. 7 SDS-PAGE analysis of affinity purified rHRP-II protein *lane M* protein marker (Fermentas catalogue no SM0441), *lanes 1–8*: different fractions collected from the elution peak

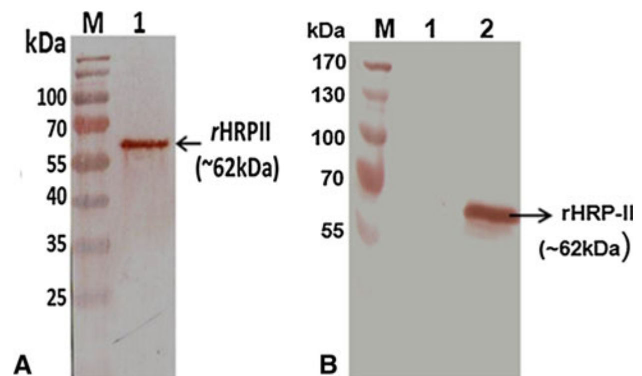

Fig. 8 Immunoblot analysis of rHRP-II protein **a** using anti-HRP-II monoclonal antibodies *lane M* protein marker (Fermentas catalogue no SM0441), *lane 1* purified rHRP-II protein **b** using *P. falciparum* infected clinical positive human serum *lane M* protein marker (Fermentas catalogue no SM0441), *lane 1* cell lysate of un-induced culture (negative control), *lane 2* purified rHRP-II protein

chief carbon source in several media, and, when excessively used, leads to the formation of acetate that reduces the pH of the fermentation broth. If glucose is replaced with glycerol, then acetate is not produced and high cell density can be achieved without decrease in pH. In addition, glycerol also has antifoaming properties. Hence, we presume that the odified Terrific broth supplemented with glycerol in the present study has helped in achieving high biomass and also rec.HRP-II protein yield.

Purification of *P. falciparum* recombinant HRP-II protein

The rHRP-II protein expressed as a fusion protein with His-tag at its amino acid terminal was successfully affinity-purified using a Ni–NTA column. In the present study, the protein was purified from the soluble fraction. As seen in Fig. 6, the bound rHRP-II protein gave a broad peak between 126 and 133 mL elution volumes at a flow rate of 1 mL/min. Elutes were collected from 125 mL of elution volume to 140 mL of elution volume. The different fractions of wash and elutes were analyzed by SDS-PAGE. The purified rHRP-II protein fractions was separated on denaturing SDS-PAGE gel and found to migrate with a molecular mass of ~62 kDa as seen in Fig. 7. Upon

downstream processing, the final concentration of purified desalted recombinant protein was 304.60 mg/L in the batch fermentation process which was approximately five fold more than shake flask culture.

Western blot analysis

Western blotting using anti-HRP-II monoclonal antibodies confirmed the authenticity of the purified protein. The purified recombinant HRPII gave a positive signal with HRP-II specific monoclonal antibodies as seen in Fig. 8a. Further the reactivity of the HRP-II protein with the human clinical sample was established using a known positive human sample for its ability to recognize HRP-II specific IgM as seen in Fig. 8b (Lane 2). Protein fractions from the un-induced cell lysate did not show any signal (Fig. 8, Lane 1). Similarly the healthy sample did not show any signal with the purified rHRP-II protein (data not shown). The immunoblot analysis has proved the reactivity of the purified protein with human acute phase sera collected

Table 1 Comparative evaluation of rHRP-II based IgM ELISA with other standard tests

SI. No	Sample ID	Sex/age	Microscopy	HRP II Ag detection BinaxNow malaria ICT	Anti-HRP II IgM detection by ELISA using rHRP-II
1	60	M/35	P. falciparum (+)	P. falciparum (+)	+
2	29	F/40	P. falciparum & Vivax (+)	P. falciparum & Vivax (+)	−
3	24	F/28	P. falciparum (+)	P. falciparum (+)	+
4	77	M/45	P. falciparum (+)	P. falciparum (+)	+
5	51	M/47	P. falciparum (+)	P. falciparum (+)	+
6	58	M/47	P. falciparum (+)	P. falciparum (+)	+
7	25	M/40	P. falciparum (+)	P. falciparum (+)	+
8	64	F/53	P. falciparum (+)	P. falciparum (+)	+
9	05	M	P. falciparum (+)	P. falciparum (+)	+
10	06	M	P. falciparum (+)	P. falciparum (+)	+
11	07	M/11	P. falciparum (+)	P. falciparum (+)	+
12	12	M	P. falciparum & Vivax (+)	P. falciparum & Vivax (+)	−
13	19	M/17	P. falciparum & Vivax (+)	P. falciparum & Vivax (+)	+
14	20	F/51	P. falciparum & Vivax (+)	P. falciparum & Vivax (+)	+
15	23	F/6	P. falciparum (+)	P. falciparum (+)	+
16	27	M	P. falciparum (+)	P. falciparum (+)	+
17	28	F/12	P. falciparum (+)	P. falciparum (+)	+
18	29	M	P. falciparum & Vivax (+)	P. falciparum & Vivax (+)	+
19	31	M	P. falciparum (+)	P. falciparum (+)	+
20	32	M	P. falciparum (+)	P. falciparum (+)	+
21	34	M/18	P. falciparum (+)	P. falciparum (+)	+
22	36	M	P. falciparum (+)	P. falciparum (+)	+
23	37	M/3	P. falciparum (+)	P. falciparum (+)	+
24	40	M	P. falciparum & Vivax (+)	P. falciparum & Vivax (+)	+
25	41	M	P. falciparum (+)	P. falciparum (+)	+
26	41	M	P. falciparum (+)	P. falciparum (+)	+
27	43	M	P. falciparum (+)	P. falciparum (+)	+
28	44	M	P. falciparum (+)	P. falciparum (+)	+
29	29	F/23	P. falciparum (+)	P. falciparum (+)	+
30	46	M/62	P. falciparum (+)	P. falciparum (+)	+
31	47	M	P. falciparum (+)	P. falciparum (+)	+
32	48	M/12	P. falciparum (+)	P. falciparum (+)	+
33	49	F/66	P. falciparum (+)	P. falciparum (+)	+
34	50	M	P. falciparum (+)	P. falciparum (+)	+
35	51	F	P. falciparum (+)	P. falciparum (+)	+
36	52	M	P. falciparum (+)	P. falciparum (+)	+

M Male, *F* female

from a culture *P. falciparum* infected patient that further strengthens the authenticity of the expressed protein.

Immuno chromatographic test

The functionality of the purified desalted recombinant HRP-II, was verified with thirty-six selected positive clinical samples and six healthy samples using commercially available BinaxNow ICT strips. All the samples were found positive with the Immunochromatographic strip test as seen in Table 1. The purified recombinant HRP-II protein was also recognized by the above ICT strip Fig. 9b, whereas the protein derived from un-induced negative control did not show any signal as seen in Fig. 9a.

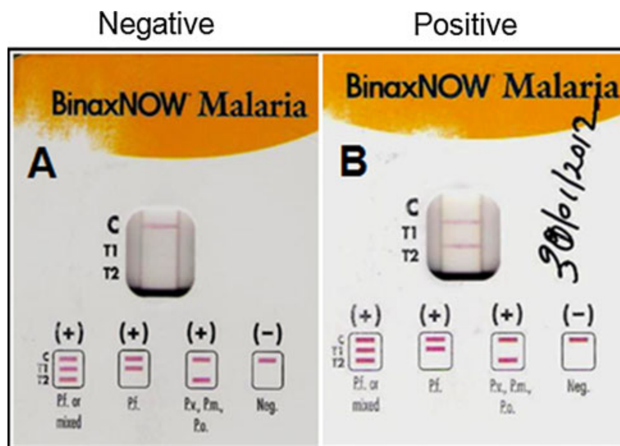

Fig. 9 Reactivity of purified rHRPII with BinaxNow ICT strip test system. This system detects HRP-II of *P. falciparum* and lactate dehydrogenase (LDH) of other Plasmodium sp. Development of signal at '*C*' and '*T1*' line indicate a positive reaction for *P. falciparum* infection. Appearance of signal only at '*C*' line indicates a negative reaction

Evaluation of diagnostic potential of recombinant HRP-II protein using indirect ELISA

Evaluation of the diagnostic potential of recombinant HRPII protein to detect *P. falciparum* specific IgM antibodies was carried out through Indirect ELISA. We have tested 36 clinically investigated human positive sera samples that were known positive for *P. falciparum* infection. Out of 36 sera samples tested, thirty-four samples were found to be positive for IgM antibodies by indirect ELISA as seen in Fig. 10. The healthy samples had no significant OD. The ELISA results have confirmed the immunoreactivity of this recombinant protein as it selectively reacts only with positive sera samples and not with negative ones. The obtained ELISA results are in concordance with that of the other two tests like microscopic examination and the BinaxNow malaria ICT strip test as depicted in Table 1. Our data clearly suggests that the recombinant HRPII protein obtained from the fermentation and downstream process has diagnostic capability and finds immense application for Invitro diagnostics. However, the present study on diagnosis is preliminary. This is because our main objective was focused on high yield production and purification of recombinant HRP-II protein. Further evaluation of the diagnostic potential of recombinant HRPII protein was beyond the scope of this study.

Conclusion

A simple batch fermentation method has been suggested to produce recombinant *P. falciparum* HRPII protein in *E.coli.* With the use of optimized media in fed batch fermentation there was an increase in the biomass and protein yield in comparison to the shake flask expression. The batch fermentation process developed in the present study

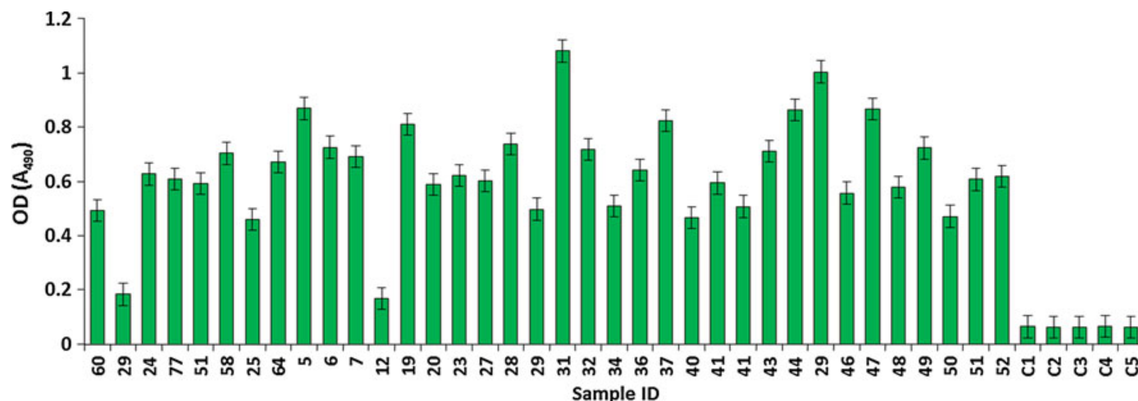

Fig. 10 rHRP-II protein based IgM antibody capture ELISA using known positive *P. falciparum* infected clinical sera samples

yielded 304.60 mg/L *P. falciparum* HRP-II protein that accounts for a nearly five fold increase compared with that of conventional shake flask cultivation. The final purified recombinant HRP-II protein obtained in this study recognized *P. falciparum* specific antibodies from previously tested clinical sera samples. Our data clearly suggests that the fermentation and downstream process developed in the present study is useful in bulk production of recombinant HRP-II protein that has immense diagnostic applications.

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