

## Heterologous Expression and Characterization of *Plasmodium falciparum* Merozoite Surface Protein 1 (MSP1<sub>42</sub> kDa) in *Pichia pastoris* and its *in-vitro* Parasite Growth Inhibition Assay

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The C-terminal portion of *Plasmodium falciparum* Merozoite Surface Protein1<sub>42</sub>kDa known to have prophylactic potential was expressed using *Pichia pastoris*. Bioinformatics based B-cell and T-cell epitope predictions revealed uniform distribution of epitopes on MSP1<sub>42</sub> kDa protein. In order to have a broad spectrum protection with multiple epitopes, the complete MSP1<sub>42</sub> kDa was selected for expression using *P. pastoris*. The gene fragment encoding the MSP1<sub>42</sub> kDa was amplified from the *P. falciparum* genomic DNA and cloned into pPIC9K yeast transfer vector under the control of AOX1 promoter in fusion with the alpha secretory signal. The expression cassette was integrated into the *Pichia* genome *via* homologous recombination. Recombinant *Pichia* clones carrying multi-copy integrants of the transgene were selected based on geneticin tolerance. The positive *Pichia* clone was methanol induced to express the transgene and the expression of the recombinant MSP1<sub>42</sub> kDa was confirmed *via* immunoblotting and MALDI-TOF analysis. Purification strategy were developed and verified by SDS-PAGE electrophoresis. Immunization of rabbit with recombinant MSP1<sub>42</sub> kDa in Freund's adjuvant resulted in high antibody titers against the recombinant MSP-1<sub>42</sub> kDa. Rabbit produced anti-MSP1IgG were inhibited *in vitro* parasite growth, demonstrating that this inhibition anti-MSP1IgG mediated. The multi-copy recombinant *Pichia* transformant obtained in the present study has an immense industrial application for large scale production of MSP1<sub>42</sub> kDa recombinant protein for either prophylactic or diagnostic applications.

**Key words:** *Plasmodium falciparum*, MSP1<sub>42</sub> kDa, *Pichia pastoris*, Secreted expression, multi-copy integrants *In - vitro* parasite growth inhibition.

Malaria represents the world's greatest public health problem in terms of number of people

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affected, levels of morbidity and mortality. With as many as 300 to 500 million new cases each year, malaria accounts for the death of over 2 million people globally each year, and most are children<sup>1</sup>. Malaria continues to cause significant levels of morbidity and mortality in tropical regions. Current

control efforts are hindered by the emergence of parasite drug resistance and insecticide resistance of the mosquito host. The development of a malaria vaccine which protects against clinical disease caused by *Plasmodium falciparum* would significantly enhance existing control measure<sup>2</sup>. Among the four species of *Plasmodium* that infect human, the most threatening is *Plasmodium falciparum*. The extensive spread of drug-resistant *Plasmodium falciparum* strain as well as the insecticide-resistant mosquito necessitates the development of a malaria vaccine on an urgent basis<sup>3</sup>. Apicomplexa are a group of parasite protozoa, including the human malaria parasite *Plasmodium falciparum* (Pf) and *Toxoplasma gondii* (Tg), that use apical secretory organelles to invade host cells. Invasion of *Plasmodium* spp. merozoite into erythrocyte begins with an initial weak attachment of the merozoite to the red blood cell (RBC) surface through yet-unidentified parasite receptor-RBC interaction, followed by a reorientation that ultimately brings the apical end of the merozoite into close apposition with the RBC surface<sup>4,5</sup>. The pathogenic processes of malaria occur during blood stage infection when merozoite invade erythrocytes using multiple receptor-ligand interaction and replicate inside them. Hence an effective vaccine against the erythrocytic stage of *Plasmodium falciparum* would be expected to induce both high antibody titer and T-cell responses, which would limit parasite multiplication rates and thereby reduce morbidity and mortality<sup>6</sup>. Antigen on the merozoite surface such as merozoite surface protein 1 (MSP1) are thought to mediate initial attachment<sup>7</sup>. MSP1 is synthesized by intracellular *Plasmodium falciparum* schizonts as a ~200kDa glycosyl phosphatidyl inositol (GPI)-linked precursor, which is directed to the parasite's surface (a process requiring specific trafficking sequences<sup>8</sup>). Upon release of the free merozoite the precursor is cleaved to four fragments of 83, 30, 38 and 42kDa that remain associated and form a complex together with fragment of two other proteins, MSP6 and MSP7 on the merozoite surface. This multimeric complex is tethered to the merozoite surface by the C-terminal GPI-anchored 42kDa fragment (MSP1<sub>42</sub>kDa)<sup>9</sup>. Just before completion of erythrocyte invasion, the C-terminal 42kDa fragment, attached to the merozoite surface, is further processed into 33 and 19kDa fragments

with only the 19kDa fragment being carried into the new erythrocyte<sup>10</sup>. Indeed the antibodies against MSP1<sub>42</sub>kDa and MSP1<sub>19</sub>kDa are able to inhibit the invasion of erythrocytes *in-vitro* and also has shown protective efficacy *in-vivo*<sup>11-19</sup> hence both MSP1<sub>42</sub>kDa and MSP1<sub>19</sub>kDa are under consideration as erythrocyte stage vaccine. However, *P. falciparum* MSP1<sub>42</sub>kDa being a structurally complex protein requires a robust and reliable expression system for its economical bulk production for mass vaccination programmes. Till date, this protein has been successfully expressed using bacteria<sup>19,20</sup> mammalian cells<sup>21</sup>, baculovirus<sup>12</sup> and adenovirus<sup>22</sup>. However no studies are available on using *Pichia pastoris* that has gained lot of popularity as an alternate expression host for expressing MSP1<sub>19</sub>kDa protein.

Using *P. pastoris*, heterologous proteins can either be expressed intracellularly or secreted into the medium. Because *P. pastoris* secretes only low levels of endogenous proteins, and because its culture medium contains no added proteins, a secreted heterologous protein comprises the vast majority of the target protein in the medium. Thus, secretion serves as a major first step in purification, separating the foreign protein from the bulk of cellular proteins<sup>23</sup>. *P. pastoris* has the potential of performing many of the post translational modifications typically associated with higher eukaryotes. These include processing of signal sequences, folding, disulfide bridge formation, and both O- and N-linked glycosylation. The combination of strong regulated expression of target gene under control of the AOX1 promoter along with an appropriate media may result in strikingly high levels of foreign proteins in *P. pastoris*.

## MATERIALS AND METHODS

### Malaria parasite and yeast strain

*Plasmodium falciparum* culture was obtained from National Malaria Research Institute, Delhi-110 009 India. The parasite was grown at 37°C in RPMI medium (Sigma, USA) containing human RBCs; *Pichia pastoris* GS115 (Invitrogen, USA) was grown at 28°C in YPD medium (Yeast Extract Peptone Dextrose Medium). For growth on plates, 2 % agar was added to the media. Transformants were grown in media supplemented

with 250-1000 µg/ml Geneticin (Sigma, USA). For cloning procedures, *Escherichia coli* DH5α was used and grown at 37°C in LB medium supplemented with 100 µg/ml ampicillin.

#### Parasite culture and genomic DNA isolation

The *P. falciparum* culture was grown in RPMI complete medium consisting 5% AB<sup>+</sup>ve serum, 1% Gentamycin, a drop of freshly prepared human RBC in six well plate. The culture was incubated CO<sub>2</sub> incubator with 5% CO<sub>2</sub> at 37°C until the parasitemia reaches ring stage. The growth of the parasite was monitored by microscopic examination. The parasite culture was harvested and used for genomic DNA extraction. The DNA was isolated using QIA amp DNA mini kit (Qiagen, Germany) as per the instructions of the manufacturer. The DNA was finally eluted from the column in 100µl of elution buffer and stored at -80° C until used further.

#### Bio-Informatics study on *P.falciparum* MSP1<sub>42</sub> kDa protein for B-cell, T-cell epitope and glycosylation prediction

The prediction of linear B-cell epitopes based on seven different parameters *viz.*, hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic property was done using

B cepred tool with a threshold of 1.9, 2, 1.9, 2.4, 2.3, 1.8 and 1.9 respectively [24]. T-cell epitope prediction on MSP1<sub>42</sub> kDa protein was done using ProPred-I and ProPred-II online servers for MHC Class-I and MHC Class-II respectively based on quantitative matrices derived from published literature [25]. The N-linked glycosylation of MSP142kDa protein was studied using NetNGlyc1.0 server of Technical University of Denmark (<http://www.cbs.dtu.dk/services/NetNGlyc>).

#### DNA techniques

*E. coli* and *P. pastoris* cells were transformed by electroporation. Enzymes *Sna*B1 and *Not* I, *Sac* I and T4DNA ligase (Fermentas, USA), *Taq* DNA polymerase and reagents for PCR (Invitrogen, USA) were used as recommended by the supplier. Molecular biology protocols were carried out according to Sambrook and Russell [26]. DNA sequencing was performed by ABI DNA sequencer (Applied Biosystems, USA). DNA sequences were analyzed using DNA star software.

#### Cloning MSP1<sub>42</sub> kDa gene into pPICK9K yeast transfer vector

The 1125 bp DNA encoding the carboxyl terminal of MSP1<sub>42</sub> kDa gene from *P. falciparum* (Indian isolates) was amplified from the parasite genomic DNA using high fidelity *Pfu* polymerase (Fermentas, USA) employing the following primers having introduced *Sna*B I and *Not* I sites (bold and underlined sequences) in the forward and reverse primers respectively. MSP1<sub>42</sub> kDa forward: 5'-TATACG**TAGCAATATCTGT**CACAATGG-3' (Nucleotide position +1 to +19 of NCBI accession no FJ919374) and Reverse: 5'-TAGCGG**CCGCGTTAGAGGA**ACTGCAGAA-3' (Nucleotide position +1125 to +1108 of NCBI accession no FJ919374). The PCR conditions used were: 94°C for 50 sec, 53°C for 45 sec, 72°C for 45sec, for 40 cycles, and finally 72°C for 10 min. The amplified MSP1<sub>42</sub> kDa gene fragment was digested with *Sna*B I and *Not* I restriction enzymes and was cloned into pPIC9K yeast transfer vector (Invitrogen, USA) at the same restriction sites. The resulting vector pPICK9KMSP1<sub>42</sub> kDa had the MSP1<sub>42</sub> kDa gene in frame with the fused *Saccharomyces cerevisiae* alpha mating factor secretion signal under the control of the methanol-inducible *P. pastoris alcohol oxidase 1* (AOX1) promoter. The pPICK9KMSP1<sub>42</sub> kDa was initially transformed into *E. coli* DH5 α cells (Invitrogen, USA). For selection of the recombinant transformants, the bacterial cells were cultured in Luria-Bertani medium (Himedia, India) supplemented with 50µg/ml ampicillin and 50µg/ml of Kanamycin. The positive bacterial transformants were selected through restriction digestion of plasmid DNA using *Sna*B I and *Not* I enzymes and PCR analysis using above MSP1<sub>42</sub> kDa specific primers. The correct integration of the transgene within the vector was further confirmed through nucleotide sequencing using ABI sequencer.

#### Integration of pPICK9KMSP1<sub>42</sub> kDa DNA into *P. pastoris* genome and screening of multi-copy integrants

The recombinant plasmid DNA pPICK9KMSP1<sub>42</sub> kDa was linearized by digesting with *Sac* I enzyme to integrate the transgene at His4 locus on the *Pichia* genome and also to generate HIS<sup>+</sup>, Mut<sup>+</sup> transformants in *P. pastoris* GS115 cells. Ten microgram of the linear DNA was

used to transform fresh electro competent *P. pastoris* cells via electroporation using Bio-Rad Gene Pulsar Xcell™ electroporation system (Bio-Rad laboratories, Inc USA.) at 1492V, 25µF capacitance and 200°C resistance. After transformation, cells were plated on SD-His plate (1.34% yeast nitrogen base, 2% dextrose, 0.01% complete amino acid supplement minus Histidine, 1M sorbitol supplement, and 2% agar), and incubated at 30°C for 2 days. The parent plasmid DNA pPIC9K without insert, linearized with *Sac* I was also transformed similarly for negative control. The colonies obtained were streaked on fresh SD-His plates.

In order to select for transformants containing multiple integrants 100 transformed colonies bearing the chromosomally integrated copies of the pPICK9KMSP1<sub>42</sub>kDa were screened for multiple copy integrants through replica plating on YPD plates containing different concentrations of Geneticin (250 µg/ml, 500 µg/ml, 750 µg/ml and 1000 µg/ml). Plates were incubated at 30°C for three days and the growth obtained was scored with plus (+) and minus (-) for the presence or absence of growth respectively on the selection plate as previously described<sup>23</sup>. To further confirm the transformants having multiple copy integrants, a total of three *Pichia* clones were randomly selected from each group of geneticin tolerance. PCR was performed on the genomic DNA isolated from the above clones by employing MSP1<sub>42</sub>kDa gene specific primers as described earlier<sup>27</sup>. Three clones that were found positive through genomic DNA PCR were further sequence confirmed using alpha factor forward and MSP1<sub>42</sub>kDa reverse primers. Single sequence confirmed *Pichia* clone that also showed high level resistance to Geneticin (1000 µg/ml) was selected further for subsequent expression study.

#### **Optimization of MSP1<sub>42</sub>kDa protein expression in *Pichia pastoris***

In order to have an optimum expression of the recombinant MSP1<sub>42</sub>kDa protein, different parameters viz, medium, temperature, pH, methanol, induction and harvest period were tested as described earlier<sup>23</sup>. The optimized medium and culture conditions were used for subsequent expression of the target recombinant protein in shake flask culture. Briefly, the glycerol stocks of the above *Pichia* clone was inoculated into 250 ml

of YPD (1% Yeast extract, 2% bacto peptone and 2% Dextrose) taken in 2000 ml conical flask along with negative control (*Pichia* transformed with pPICK9K without insert) and were incubated at 28°C in a shaker incubator at 250 rpm until the culture reached an A<sub>600</sub> of 2-3. The cells were harvested by centrifugation at 3,000 × g for 10 min at room temperature and the cell pellets were resuspended in required volume of fresh YPM induction medium (1% Yeast extract, 2% bacto peptone and 2% methanol) so that the final OD at A<sub>600</sub> was 3. Incubation was continued at 28°C as above for four days. To sustain induction, required volume of methanol was added to the culture once in every 24 hour. Culture supernatants were collected at different time points ranging from 24-96 h (24 h, 48 h, 72 h and 96 h) and the protein was precipitated using equal volume of chilled acetone by centrifuging at 10000 X g for one hour at 4°C. The protein pellets were resuspended in PBS and further analyzed by running them on 10% polyacrylamide gel electrophoresis under denaturing conditions<sup>26</sup>. The gels were subsequently stained with Coomassie Brilliant Blue R-250 (Sigma, USA). The protein concentration was determined through Bradford assay against BSA standards<sup>28</sup>.

#### **Characterization of recombinant MSP1<sub>42</sub>kDa protein through Immunoblotting**

The expressed MSP1<sub>42</sub>kDa protein was reacted with four different human acute phase sera collected from culture confirmed *P. falciparum* infected patients via immunoblotting. The recombinant protein after separation on SDS-PAGE gel was incubated with 1:500 diluted sera samples individually for one hour at 37°C. The blot was washed six times in washing buffer (PBS containing 0.025% Tween-20) and further reacted with anti-human IgM horse reddish peroxidase conjugate (Sigma, USA) at 1:5000 dilution for one hour at 37°C. After excessive washing of the blot, the enzymatic color development was done using H<sub>2</sub>O<sub>2</sub>/DAB substrate-chromogen.

#### **MALDI-TOF/MS analysis of yeast-derived recombinant MSP1<sub>42</sub>kDa protein**

The MSP1<sub>42</sub>kDa protein expressed from *P. pastoris* was further confirmed using MALDI-TOF/MS study. The Coomassie stained recombinant MSP1<sub>42</sub>kDa recombinant protein was excised from the gel and the gel slices were washed

thrice with 200 µl destaining solvent (50% v/v ACN in 25mM NH<sub>4</sub> HCO<sub>3</sub>) with constant vortexing of 10 min. The washed gel pieces were dehydrated with 200µl of 100% ACN and dried in speed-vac centrifuge (Thermo, USA). The protein in the gel slices were subjected to trypsin digestion for overnight. The peptides were extracted and lyophilized for complete removal of the solvent. The pellets obtained were reconstituted sterile triple distilled water and was subsequently purified using Mini Tip C18 micro tips (Sigma, USA). The purified tryptic digest (1 µl) along with equal volume of HCCA matrix was spotted on the MALDI plate. Mass spectrometric analysis was performed using MALDI-TOF instrument (Bruker Microflex LRF-20, Flex Control workstation, Bremen, Germany) equipped with delayed extraction (150 ns) and a UV ionisation laser (N2, 337 nm) with a 3-ns pulse width. The accelerating voltage was 20 kV and the grid voltage was set to 19 kV and laser repetition rate was 20 Hz. For the peptide mass fingerprinting, the instrument was operated in the reflector mode with an average of hundred shots per spectra. Seven hundred laser shots were accumulated and the spectra were evaluated using the Flex Analysis Software (Bruker Daltonics). The MS spectra obtained was submitted to MASCOT search *via* Bio tools version 3.1. The search parameters used were partial methionine oxidation, one missed cleavage and the database selected was NCBI.

#### **Development of purification method for yeast derived rec.MSP1 protein**

After 72.0 h, methanol induced culture was harvested. The culture supernatant was separated from the cells by centrifugation at 5,000g for 30 min at 4°C in a swinging bucket rotor using a Sorvall evolution RC centrifuge (Thermo Scientific, USA). Following centrifugation, supernatant was filtered through a 0.45 µm filter. Upon filtration the filtrate was further concentrated approximately ten times using a 10 kDa Molecular weight cut off (MWCO) membrane (Millipore USA). Sodium azide and PMSF were added at the concentrations of 0.05 % and 10 mM, respectively, to the final concentrate and stored at -80°C until used further for purification. Development of purification method on the of anion exchange chromatography was carried out using a 5 mL DEAE Sepharose anion exchange column (Toyo, Japan) in an AKTA

explorer system (GE Healthcare, USA) as per the instructions given by the manufacturer. A five ml volume of protein sample was loaded onto a DEAE Sepharose column pre-equilibrated with sterile PBS. For efficient binding of the protein the flow through was reloaded tries on to the column at same flow rate as above. The unwanted proteins were extensively washed using sterile PBS. Following the washing, target proteins were eluted with 0–1M NaCl linear gradient (in sterile PBS) at a flow rate of 1 ml/min. The concerned target protein peaks were collected, concentrate and analyzed by SDS-PAGE for their purity. The concentration of rec. MSP1 protein was determined using the Bradford assay<sup>28</sup>.

#### **Immunization study of yeast derived MSP1 recombinant protein**

Healthy adult male New Zealand White rabbits tested sero negative for *Plasmodium falciparum* was immunized intramuscularly with 100 µg purified rec. MSP1 protein in combination with Freund's complete adjuvant (FCA) (Sigma, USA). Subsequent immunizations were carried out with 100 µg purified rec. MSP1 protein combination with Freund's incomplete adjuvant (FIA) (Sigma, USA) at 21 and 42 days of post immunization. Preimmune sera were collected preceding to immunization, 21, 42 and 60 days and stored at -80°C until further utilization.

#### ***In-vitro* growth inhibition assay**

In vitro parasite growth inhibition assay using pre-immune and immune (tertiary and quaternary bleed) rabbit sera were evaluated for their ability to inhibit parasitic growth in vitro as described previously<sup>34</sup>. Briefly, rabbit sera were heat inactivated at 58°C for 40 min and absorbed with fresh normal human erythrocytes with a equal amount of complete media (90% Incomplete media + 10 inactivated human serum). Human erythrocytes were washed in incomplete media (95.7 % RPMI 1640 + 40 µg/l Gentamycin sulfate + 4.3% Sodium bicarbonate) before use. For in vitro assays purified and dialyzed with PBS rabbit derived anti-MSP1IgG was again dialyzed overnight against RPMI 1640 culture medium. Parasite cultures were synchronized by sorbitol lysis. Infected human erythrocytes were adjusted to an initial parasitemia of 0.5% and a hematocrit of 1.5% in culture medium containing 0.5% complete media. Rabbit preimmune or rabbit derived anti-MSP1IgG were added to

infected erythrocyte cultures in a 6-well plate. Cultures were incubated for 72 h, and the parasitemia of Giemsa-stained thin smears of cultured erythrocytes was determined by microscopy.

## RESULTS AND DISCUSSION

### Malaria parasite culture

Ring-forms of *P. falciparum* were noticed in the culture initially (fig 1A) followed by which the rings became small trophozoites (fig 1B) and finally schizonts (fig 1C) were observed. Approximately 80-90% of the parasites were in the ring stage of development at 48 h and 72 h during which the culture was harvested for genomic DNA extraction.

### Bio-informatics study on *P. falciparum* MSP1<sub>42</sub> kDa protein B-cell, T-cell epitope and glycosylation prediction

The linear B-cell epitope prediction on MSP1<sub>42</sub> kDa protein based on different parameters viz, hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic property revealed more than ten potential B-cell specific epitopes that were uniformly distributed all throughout the protein (fig 2). The protein regions

having values greater than threshold value and also qualifying at least more than four parameters were considered to be potential epitopes. For T-cell epitope predication, human MHC-I and MHC-II binding regions were identified based on several MHC-Class I alleles (A, B and C) and MHC-Class II alleles (DR, DQ and DP). Numerous peptides within MSP1<sub>42</sub> kDa protein that crossed threshold peptide score (3%) were found distributed all throughout the protein as seen in figure 3A and 3B. The N-linked glycosylation study of MSP1<sub>42</sub> kDa protein sequence revealed the presence of two N-linked glycosylation points at amino acid position 281(NISQ) and 326(NPTC) as seen in figure 4. In order to have more number of B-cell and T-cell epitopes for a balanced humoral and cell mediated immune response, whole sequence of MSP1<sub>42</sub> kDa protein was selected for recombinant expression rather than partial sequence.

### Cloning, integration of MSP1<sub>42</sub> kDa gene into *P. pastoris* genome and screening of multi-copy integrants

The PCR product of DNA encoding the carboxyl terminal of MSP1<sub>42</sub> kDa gene (1125bp) of *P. falciparum* was inserted at *Sna*B I - *Not* I sites into pPICK9K yeast transfer vector under AOX1

**Table 1.** List of tryptic peptides that showed homology to that of *P. falciparum* MSP1<sub>42</sub> kDa protein in the Peptide mass fingerprint obtained from MALDI-TOF study

Sl. No	Position (start-end)	Measured mass (m+H) <sup>+</sup>	Theoretical mass (m <sup>+</sup> )	Mass error (ppm)	Miss cleavage	Peptide sequence
1	57-63	831.4869	830.4796	35.9	0	K.LLNSEQK.N
2	75-82	933.54	932.5327	94.0	0	K.ESVENDIK.F
3	167-174	934.4567	933.4495	28.8	0	K.INDCNVEK.D
4	209-216	946.5316	945.5243	11.8	1	K.LINDDTKK.D
5	64-71	966.5943	965.587	33.6	1	K.NTLKSYK.Y
6	175-183	1040.545	1039.538	-27.5	1	K.DEAHVKITK.L
7	156-164	1106.751	1105.744	128	0	K.IDDYLINK.A
8	184-193	1117.757	1116.75	132	1	K.LSDLKAIDDK.I
9	165-174	1133.705	1132.698	126	1	K.AKINDCNVEK.D
10	189-198	1177.686	1176.679	34.1	1	K.AIDDKIDLFK.N
11	278-288	1310.7802	1309.7729	106	1	K.CLLNYKQEGDK.C
12	72-82	1337.8788	1336.8715	138	1	K.YIKESVENDIK.F
13	57-68	1400.7978	1399.7905	-9.26	1	K.LLNSEQKNTLLK.S
14	29-42	1706.0537	1705.0465	141	0	K.YFLDGLSDLMQFK.H
15	28-42	1834.1509	1833.1436	132	1	R.KYFLDGLSDLMQFK.H
16	222-238	1845.0938	1844.0866	24.9	0	K.LLSTGLVQNFNPTIISK.L
17	138-155	2153.3422	2152.3349	82.7	0	K.FLPFLTNIETLYNNLVNK.I
18	239-257	2231.4258	2230.4185	132	1	K.LIEGKFQDMLNISQHQCVK.K
19	75-93	2249.3054	2248.2981	109	1	K.ESVENDIKFAQEGISYYEK.V
20	284-309	2722.4742	2721.4670	146	1	K.ESVENDIKFAQEGISYYEK.V.C

promoter in fusion with *S. cerevecea* alpha secretory signal at N-terminus. The resultant pPICK9KMSP1<sub>42</sub>kDa (fig 5), linearised with *Sac* I restriction enzyme after transformation in *P. pastoris* via electroporation yielded 238 His<sup>+</sup> transformants per 10µg of DNA used. Upon antibiotic screening of randomly selected 140 transformants at different concentrations of Geneticin, 93 colonies grew at lowest concentration of Geneticin (250 µg/ml). Whereas the selection plates with 500 µg/ml, 750 µg/ml and 1000 µg/ml Geneticin had 86, 85 and 75 colonies respectively (fig 6). As both *Kan<sup>R</sup>* and MSP1<sub>42</sub>kDa gene fragment are integrated together, resistance to Geneticin would give a rough idea on the copy number of the integrated MSP1<sub>42</sub>kDa gene. Resistance to higher concentrations of geneticin is directly proportional to the copy numbers of kanamycin resistance gene (*Kan<sup>R</sup>*) integrated which confers resistance to Geneticin in yeast cells. As reported earlier by us [27], high copy integrants are due to the multiple gene insertion events at a single locus in *Pichia* cells that happens spontaneously with a low frequency of all selected His<sup>+</sup> transformants. Hence the observed less number of high copy integrants is justified.

Genomic DNA PCR from selected yeast transformants (three from each category) using MSP1<sub>42</sub>kDa gene specific primers resulted in amplification of approximately 1125 bp product as expected. This confirmed the presence of MSP1<sub>42</sub>kDa gene fragment within the *Pichia* genome. As seen in figure 7 only five out of twelve DNA samples amplified the expected 1125 bp DNA. The failure of amplification in the remaining samples may be attributed to the poor quality of the DNA wherein the impurities would have inhibited the PCR amplification. Multiple copy integrant growing of MSP1<sub>42</sub> kDa gene was selected based on antibiotic sensitivity assay and genomic DNA PCR.

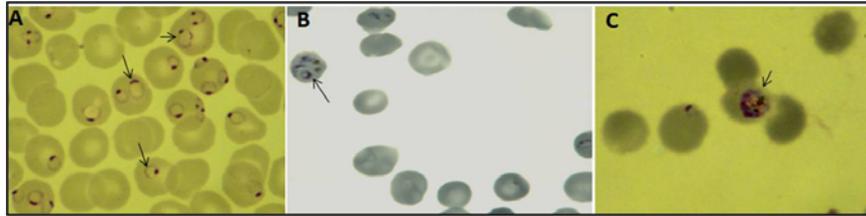
#### **Optimization of MSP1<sub>42</sub>kDa protein expression in *Pichia pastoris***

We have employed *S. cerevisiae* alpha secretory signal (SS) upstream to the target gene in the pPICK9KMSP1<sub>42</sub> kDa construct. The kex2 protease of *Pichia* cleaves the fusion tag of the expressed protein resulting in the release of matured fully processed MSP1<sub>42</sub> kDa protein. The His<sup>+</sup> Mut<sup>+</sup> positive *Pichia* transformant was selected for

inducing the expression of the target gene. An optimum expression of the MSP1<sub>42</sub>kDa recombinant protein of size ~ 42 kDa was noticed after 72 h of post methanol induction (Fig 8). Whereas no specific protein bands were detected in pPICK9K vector transformed yeast and non-induced positive transformant in this region. Studies conducted to improve the expression level with different induction period and methanol concentrations revealed that 72 h of post-induction with 2% methanol concentration is optimum for better expression of MSP1<sub>42</sub> kDa protein at shake flask level (Data not shown). Upon quantification, the concentration of the MSP1<sub>42</sub>kDa protein expressed was found to be 45 mg/L of the culture supernatant. However, the level of expression could not be further elevated above this scale with either increased methanol concentration or increased duration of incubation. With the above optimized expression condition, MSP1<sub>42</sub>kDa was secreted into the medium as a soluble protein. In the present study, the purification tag was avoided in the construct as the previous study by Khan *et al* [29] have shown that inclusion of metal ion affinity-tag (Histidine) onto MSP1<sub>42</sub>kDa affects the specificity of the humoral and cellular immune responses due to differences in the protein fold and epitope accessibility. In light of these findings the soluble secretory MSP1<sub>42</sub>kDa expressed in the present study is highly advantageous.

#### **Characterization of recombinant MSP1<sub>42</sub>kDa protein through Immuno-blotting**

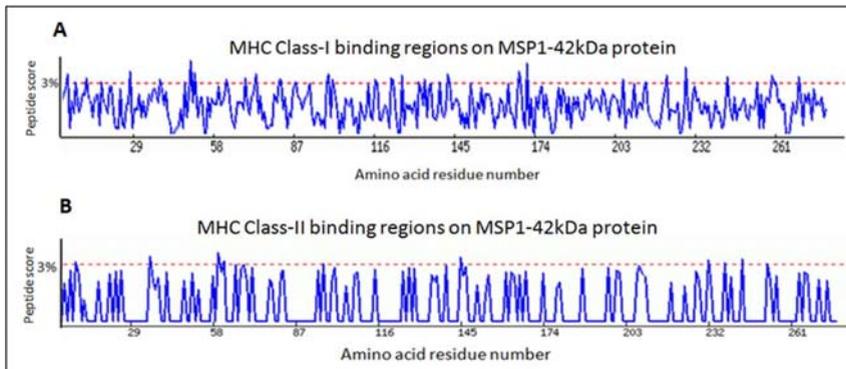
The recombinant protein obtained from positive *Pichia* transformants after methanol induction was separated on PAGE gel under denaturing conditions and subsequently transferred onto PVDF membranes. Immuno-blotting using four different human patient sera reacted with the recombinant MSP1<sub>42</sub>kDa protein resulting positive signals (Fig 9). Earlier report [30] has reported that the native conformation of the C-terminal region of MSP-1 of *Plasmodium* is crucial for the protective immune response. Hence the conformational integrities of the yeast derived MSP1<sub>42</sub>kDa is established on the basis of its reactivity with *P. falciparum* infected patient sera. It is important to note that the bacterial expressed MSP1<sub>42</sub>kDa resulted in considerable loss of reactivity with the patient sera due to the reduction of the disulde bonds in the recombinant protein<sup>31</sup>.



**Fig. 1.** Microscopic examination of *P. falciparum* parasite at different stages of growth: (A) Ring-forms of stage (B) trophozoite stage (C) Schizonts stage

<b>Hydrophilicity</b>	<sup>1</sup> AI <sup>1</sup> SV <sup>1</sup> TM <sup>1</sup> DN <sup>1</sup> LS <sup>1</sup> GF <sup>1</sup> EN <sup>1</sup> YD <sup>1</sup> VI <sup>1</sup> LK <sup>1</sup> PL <sup>1</sup> AG <sup>1</sup> VY <sup>1</sup> SL <sup>1</sup> KK <sup>1</sup> QI <sup>1</sup> EN <sup>1</sup> IF <sup>1</sup> FN <sup>1</sup> LN <sup>1</sup> DL <sup>1</sup> NS <sup>1</sup> RL <sup>1</sup> KK <sup>1</sup> RY <sup>1</sup> FL <sup>1</sup> DV <sup>1</sup> ES <sup>1</sup> DL <sup>1</sup> MQ <sup>1</sup> FK <sup>1</sup> HIS <sup>1</sup> NEY <sup>1</sup> I <sup>1</sup> ED <sup>1</sup> SK <sup>1</sup> LI <sup>1</sup> <u>NSE<sup>1</sup>Q<sup>1</sup>NT<sup>1</sup>LL<sup>1</sup>KS<sup>1</sup></u> YK <sup>1</sup> YI <sup>1</sup> KE <sup>1</sup> SV <sup>1</sup> EN <sup>1</sup> DI <sup>1</sup> KFA <sup>1</sup> QEG <sup>1</sup> IS <sup>1</sup> YK <sup>1</sup> VL <sup>1</sup> AK <sup>1</sup> YK <sup>1</sup> DD <sup>1</sup> LE <sup>1</sup> SI <sup>1</sup> KK <sup>1</sup> VI <sup>1</sup> EE <sup>1</sup> KE <sup>1</sup> FP <sup>1</sup> SS <sup>1</sup> PT <sup>1</sup> PP <sup>1</sup> SP <sup>1</sup> AK <sup>1</sup> TD <sup>1</sup> E <sup>1</sup> Q <sup>1</sup> K <sup>1</sup> ES <sup>1</sup> K <sup>1</sup> FL <sup>1</sup> PL <sup>1</sup> TN <sup>1</sup> IE <sup>1</sup> TL <sup>1</sup> VN <sup>1</sup> LN <sup>1</sup> VN <sup>1</sup> KID <sup>1</sup> DY <sup>1</sup> LN <sup>1</sup> LK <sup>1</sup> AK <sup>1</sup> IN <sup>1</sup> D <sup>1</sup> <u>CNV<sup>1</sup>E<sup>1</sup>K<sup>1</sup>DE<sup>1</sup>AH<sup>1</sup>V<sup>1</sup>K<sup>1</sup>IT<sup>1</sup>KL<sup>1</sup>SDL<sup>1</sup>KA<sup>1</sup>IDD<sup>1</sup>K<sup>1</sup>IDL<sup>1</sup>FK<sup>1</sup>NP<sup>1</sup>YD<sup>1</sup>FE<sup>1</sup>AI<sup>1</sup>KK<sup>1</sup>L<sup>1</sup>ND<sup>1</sup>TK<sup>1</sup>K<sup>1</sup>DM<sup>1</sup>L<sup>1</sup>G<sup>1</sup>KL<sup>1</sup>ST<sup>1</sup>GL<sup>1</sup>VQ<sup>1</sup>N<sup>1</sup>FN<sup>1</sup>TI<sup>1</sup>SK<sup>1</sup>LIE<sup>1</sup>G<sup>1</sup>KF<sup>1</sup>Q<sup>1</sup>DM<sup>1</sup>LN<sup>1</sup>IS<sup>1</sup>QH<sup>1</sup>CV<sup>1</sup>KK<sup>1</sup>Q<sup>1</sup>C<sup>1</sup>PEN<sup>1</sup>SG<sup>1</sup>CF<sup>1</sup></u> HL <sup>1</sup> DE <sup>1</sup> EE <sup>1</sup> CK <sup>1</sup> CL <sup>1</sup> NY <sup>1</sup> KQ <sup>1</sup> EG <sup>1</sup> DK <sup>1</sup> CV <sup>1</sup> EN <sup>1</sup> PT <sup>1</sup> CN <sup>1</sup> EN <sup>1</sup> GG <sup>1</sup> CD <sup>1</sup> AD <sup>1</sup> AT <sup>1</sup> CE <sup>1</sup> ED <sup>1</sup> SG <sup>1</sup> SS <sup>1</sup> R <sup>1</sup> KK <sup>1</sup> IT <sup>1</sup> CE <sup>1</sup> CT <sup>1</sup> K <sup>1</sup> PD <sup>1</sup> SV <sup>1</sup> PL <sup>1</sup> FD <sup>1</sup> G <sup>1</sup> IF <sup>1</sup> CS <sup>1</sup> SS <sup>1</sup> <sup>274</sup>
<b>Flexibility</b>	<sup>1</sup> AI <sup>1</sup> SV <sup>1</sup> TM <sup>1</sup> DN <sup>1</sup> LS <sup>1</sup> GF <sup>1</sup> EN <sup>1</sup> YD <sup>1</sup> VI <sup>1</sup> LK <sup>1</sup> PL <sup>1</sup> AG <sup>1</sup> VY <sup>1</sup> SL <sup>1</sup> KK <sup>1</sup> QI <sup>1</sup> EN <sup>1</sup> IF <sup>1</sup> FN <sup>1</sup> LN <sup>1</sup> DL <sup>1</sup> NS <sup>1</sup> RL <sup>1</sup> KK <sup>1</sup> RY <sup>1</sup> FL <sup>1</sup> DV <sup>1</sup> ES <sup>1</sup> DL <sup>1</sup> MQ <sup>1</sup> FK <sup>1</sup> HIS <sup>1</sup> NEY <sup>1</sup> I <sup>1</sup> ED <sup>1</sup> SK <sup>1</sup> LI <sup>1</sup> <u>NSE<sup>1</sup>Q<sup>1</sup>NT<sup>1</sup>LL<sup>1</sup>KS<sup>1</sup></u> YK <sup>1</sup> YI <sup>1</sup> KE <sup>1</sup> SV <sup>1</sup> EN <sup>1</sup> DI <sup>1</sup> KFA <sup>1</sup> QEG <sup>1</sup> IS <sup>1</sup> YK <sup>1</sup> VL <sup>1</sup> AK <sup>1</sup> YK <sup>1</sup> DD <sup>1</sup> LE <sup>1</sup> SI <sup>1</sup> KK <sup>1</sup> VI <sup>1</sup> EE <sup>1</sup> KE <sup>1</sup> FP <sup>1</sup> SS <sup>1</sup> PT <sup>1</sup> PP <sup>1</sup> SP <sup>1</sup> AK <sup>1</sup> TD <sup>1</sup> E <sup>1</sup> Q <sup>1</sup> K <sup>1</sup> ES <sup>1</sup> K <sup>1</sup> FL <sup>1</sup> PL <sup>1</sup> TN <sup>1</sup> IE <sup>1</sup> TL <sup>1</sup> VN <sup>1</sup> LN <sup>1</sup> VN <sup>1</sup> KID <sup>1</sup> DY <sup>1</sup> LN <sup>1</sup> LK <sup>1</sup> AK <sup>1</sup> IN <sup>1</sup> D <sup>1</sup> <u>CNV<sup>1</sup>E<sup>1</sup>K<sup>1</sup>DE<sup>1</sup>AH<sup>1</sup>V<sup>1</sup>K<sup>1</sup>IT<sup>1</sup>KL<sup>1</sup>SDL<sup>1</sup>KA<sup>1</sup>IDD<sup>1</sup>K<sup>1</sup>IDL<sup>1</sup>FK<sup>1</sup>NP<sup>1</sup>YD<sup>1</sup>FE<sup>1</sup>AI<sup>1</sup>KK<sup>1</sup>L<sup>1</sup>ND<sup>1</sup>TK<sup>1</sup>K<sup>1</sup>DM<sup>1</sup>L<sup>1</sup>G<sup>1</sup>KL<sup>1</sup>ST<sup>1</sup>GL<sup>1</sup>VQ<sup>1</sup>N<sup>1</sup>FN<sup>1</sup>TI<sup>1</sup>SK<sup>1</sup>LIE<sup>1</sup>G<sup>1</sup>KF<sup>1</sup>Q<sup>1</sup>DM<sup>1</sup>LN<sup>1</sup>IS<sup>1</sup>QH<sup>1</sup>CV<sup>1</sup>KK<sup>1</sup>Q<sup>1</sup>C<sup>1</sup>PEN<sup>1</sup>SG<sup>1</sup>CF<sup>1</sup></u> HL <sup>1</sup> DE <sup>1</sup> EE <sup>1</sup> CK <sup>1</sup> CL <sup>1</sup> NY <sup>1</sup> KQ <sup>1</sup> EG <sup>1</sup> DK <sup>1</sup> CV <sup>1</sup> EN <sup>1</sup> PT <sup>1</sup> CN <sup>1</sup> EN <sup>1</sup> GG <sup>1</sup> CD <sup>1</sup> AD <sup>1</sup> AT <sup>1</sup> CE <sup>1</sup> ED <sup>1</sup> SG <sup>1</sup> SS <sup>1</sup> R <sup>1</sup> KK <sup>1</sup> IT <sup>1</sup> CE <sup>1</sup> CT <sup>1</sup> K <sup>1</sup> PD <sup>1</sup> SV <sup>1</sup> PL <sup>1</sup> FD <sup>1</sup> G <sup>1</sup> IF <sup>1</sup> CS <sup>1</sup> SS <sup>1</sup> <sup>274</sup>
<b>Accessibility</b>	<sup>1</sup> AI <sup>1</sup> SV <sup>1</sup> TM <sup>1</sup> DN <sup>1</sup> LS <sup>1</sup> GF <sup>1</sup> EN <sup>1</sup> YD <sup>1</sup> VI <sup>1</sup> LK <sup>1</sup> PL <sup>1</sup> AG <sup>1</sup> VY <sup>1</sup> SL <sup>1</sup> KK <sup>1</sup> QI <sup>1</sup> EN <sup>1</sup> IF <sup>1</sup> FN <sup>1</sup> LN <sup>1</sup> DL <sup>1</sup> NS <sup>1</sup> RL <sup>1</sup> KK <sup>1</sup> RY <sup>1</sup> FL <sup>1</sup> DV <sup>1</sup> ES <sup>1</sup> DL <sup>1</sup> MQ <sup>1</sup> FK <sup>1</sup> HIS <sup>1</sup> NEY <sup>1</sup> I <sup>1</sup> ED <sup>1</sup> SK <sup>1</sup> LI <sup>1</sup> <u>NSE<sup>1</sup>Q<sup>1</sup>NT<sup>1</sup>LL<sup>1</sup>KS<sup>1</sup></u> YK <sup>1</sup> YI <sup>1</sup> KE <sup>1</sup> SV <sup>1</sup> EN <sup>1</sup> DI <sup>1</sup> KFA <sup>1</sup> QEG <sup>1</sup> IS <sup>1</sup> YK <sup>1</sup> VL <sup>1</sup> AK <sup>1</sup> YK <sup>1</sup> DD <sup>1</sup> LE <sup>1</sup> SI <sup>1</sup> KK <sup>1</sup> VI <sup>1</sup> EE <sup>1</sup> KE <sup>1</sup> FP <sup>1</sup> SS <sup>1</sup> PT <sup>1</sup> PP <sup>1</sup> SP <sup>1</sup> AK <sup>1</sup> TD <sup>1</sup> E <sup>1</sup> Q <sup>1</sup> K <sup>1</sup> ES <sup>1</sup> K <sup>1</sup> FL <sup>1</sup> PL <sup>1</sup> TN <sup>1</sup> IE <sup>1</sup> TL <sup>1</sup> VN <sup>1</sup> LN <sup>1</sup> VN <sup>1</sup> KID <sup>1</sup> DY <sup>1</sup> LN <sup>1</sup> LK <sup>1</sup> AK <sup>1</sup> IN <sup>1</sup> D <sup>1</sup> <u>CNV<sup>1</sup>E<sup>1</sup>K<sup>1</sup>DE<sup>1</sup>AH<sup>1</sup>V<sup>1</sup>K<sup>1</sup>IT<sup>1</sup>KL<sup>1</sup>SDL<sup>1</sup>KA<sup>1</sup>IDD<sup>1</sup>K<sup>1</sup>IDL<sup>1</sup>FK<sup>1</sup>NP<sup>1</sup>YD<sup>1</sup>FE<sup>1</sup>AI<sup>1</sup>KK<sup>1</sup>L<sup>1</sup>ND<sup>1</sup>TK<sup>1</sup>K<sup>1</sup>DM<sup>1</sup>L<sup>1</sup>G<sup>1</sup>KL<sup>1</sup>ST<sup>1</sup>GL<sup>1</sup>VQ<sup>1</sup>N<sup>1</sup>FN<sup>1</sup>TI<sup>1</sup>SK<sup>1</sup>LIE<sup>1</sup>G<sup>1</sup>KF<sup>1</sup>Q<sup>1</sup>DM<sup>1</sup>LN<sup>1</sup>IS<sup>1</sup>QH<sup>1</sup>CV<sup>1</sup>KK<sup>1</sup>Q<sup>1</sup>C<sup>1</sup>PEN<sup>1</sup>SG<sup>1</sup>CF<sup>1</sup></u> HL <sup>1</sup> DE <sup>1</sup> EE <sup>1</sup> CK <sup>1</sup> CL <sup>1</sup> NY <sup>1</sup> KQ <sup>1</sup> EG <sup>1</sup> DK <sup>1</sup> CV <sup>1</sup> EN <sup>1</sup> PT <sup>1</sup> CN <sup>1</sup> EN <sup>1</sup> GG <sup>1</sup> CD <sup>1</sup> AD <sup>1</sup> AT <sup>1</sup> CE <sup>1</sup> ED <sup>1</sup> SG <sup>1</sup> SS <sup>1</sup> R <sup>1</sup> KK <sup>1</sup> IT <sup>1</sup> CE <sup>1</sup> CT <sup>1</sup> K <sup>1</sup> PD <sup>1</sup> SV <sup>1</sup> PL <sup>1</sup> FD <sup>1</sup> G <sup>1</sup> IF <sup>1</sup> CS <sup>1</sup> SS <sup>1</sup> <sup>274</sup>
<b>Turns</b>	<sup>1</sup> AI <sup>1</sup> SV <sup>1</sup> TM <sup>1</sup> DN <sup>1</sup> LS <sup>1</sup> GF <sup>1</sup> EN <sup>1</sup> YD <sup>1</sup> VI <sup>1</sup> LK <sup>1</sup> PL <sup>1</sup> AG <sup>1</sup> VY <sup>1</sup> SL <sup>1</sup> KK <sup>1</sup> QI <sup>1</sup> EN <sup>1</sup> IF <sup>1</sup> FN <sup>1</sup> LN <sup>1</sup> DL <sup>1</sup> NS <sup>1</sup> RL <sup>1</sup> KK <sup>1</sup> RY <sup>1</sup> FL <sup>1</sup> DV <sup>1</sup> ES <sup>1</sup> DL <sup>1</sup> MQ <sup>1</sup> FK <sup>1</sup> HIS <sup>1</sup> NEY <sup>1</sup> I <sup>1</sup> ED <sup>1</sup> SK <sup>1</sup> LI <sup>1</sup> <u>NSE<sup>1</sup>Q<sup>1</sup>NT<sup>1</sup>LL<sup>1</sup>KS<sup>1</sup></u> YK <sup>1</sup> YI <sup>1</sup> KE <sup>1</sup> SV <sup>1</sup> EN <sup>1</sup> DI <sup>1</sup> KFA <sup>1</sup> QEG <sup>1</sup> IS <sup>1</sup> YK <sup>1</sup> VL <sup>1</sup> AK <sup>1</sup> YK <sup>1</sup> DD <sup>1</sup> LE <sup>1</sup> SI <sup>1</sup> KK <sup>1</sup> VI <sup>1</sup> EE <sup>1</sup> KE <sup>1</sup> FP <sup>1</sup> SS <sup>1</sup> PT <sup>1</sup> PP <sup>1</sup> SP <sup>1</sup> AK <sup>1</sup> TD <sup>1</sup> E <sup>1</sup> Q <sup>1</sup> K <sup>1</sup> ES <sup>1</sup> K <sup>1</sup> FL <sup>1</sup> PL <sup>1</sup> TN <sup>1</sup> IE <sup>1</sup> TL <sup>1</sup> VN <sup>1</sup> LN <sup>1</sup> VN <sup>1</sup> KID <sup>1</sup> DY <sup>1</sup> LN <sup>1</sup> LK <sup>1</sup> AK <sup>1</sup> IN <sup>1</sup> D <sup>1</sup> <u>CNV<sup>1</sup>E<sup>1</sup>K<sup>1</sup>DE<sup>1</sup>AH<sup>1</sup>V<sup>1</sup>K<sup>1</sup>IT<sup>1</sup>KL<sup>1</sup>SDL<sup>1</sup>KA<sup>1</sup>IDD<sup>1</sup>K<sup>1</sup>IDL<sup>1</sup>FK<sup>1</sup>NP<sup>1</sup>YD<sup>1</sup>FE<sup>1</sup>AI<sup>1</sup>KK<sup>1</sup>L<sup>1</sup>ND<sup>1</sup>TK<sup>1</sup>K<sup>1</sup>DM<sup>1</sup>L<sup>1</sup>G<sup>1</sup>KL<sup>1</sup>ST<sup>1</sup>GL<sup>1</sup>VQ<sup>1</sup>N<sup>1</sup>FN<sup>1</sup>TI<sup>1</sup>SK<sup>1</sup>LIE<sup>1</sup>G<sup>1</sup>KF<sup>1</sup>Q<sup>1</sup>DM<sup>1</sup>LN<sup>1</sup>IS<sup>1</sup>QH<sup>1</sup>CV<sup>1</sup>KK<sup>1</sup>Q<sup>1</sup>C<sup>1</sup>PEN<sup>1</sup>SG<sup>1</sup>CF<sup>1</sup></u> HL <sup>1</sup> DE <sup>1</sup> EE <sup>1</sup> CK <sup>1</sup> CL <sup>1</sup> NY <sup>1</sup> KQ <sup>1</sup> EG <sup>1</sup> DK <sup>1</sup> CV <sup>1</sup> EN <sup>1</sup> PT <sup>1</sup> CN <sup>1</sup> EN <sup>1</sup> GG <sup>1</sup> CD <sup>1</sup> AD <sup>1</sup> AT <sup>1</sup> CE <sup>1</sup> ED <sup>1</sup> SG <sup>1</sup> SS <sup>1</sup> R <sup>1</sup> KK <sup>1</sup> IT <sup>1</sup> CE <sup>1</sup> CT <sup>1</sup> K <sup>1</sup> PD <sup>1</sup> SV <sup>1</sup> PL <sup>1</sup> FD <sup>1</sup> G <sup>1</sup> IF <sup>1</sup> CS <sup>1</sup> SS <sup>1</sup> <sup>274</sup>
<b>Exposed Surface</b>	<sup>1</sup> AI <sup>1</sup> SV <sup>1</sup> TM <sup>1</sup> DN <sup>1</sup> LS <sup>1</sup> GF <sup>1</sup> EN <sup>1</sup> YD <sup>1</sup> VI <sup>1</sup> LK <sup>1</sup> PL <sup>1</sup> AG <sup>1</sup> VY <sup>1</sup> SL <sup>1</sup> KK <sup>1</sup> QI <sup>1</sup> EN <sup>1</sup> IF <sup>1</sup> FN <sup>1</sup> LN <sup>1</sup> DL <sup>1</sup> NS <sup>1</sup> RL <sup>1</sup> KK <sup>1</sup> RY <sup>1</sup> FL <sup>1</sup> DV <sup>1</sup> ES <sup>1</sup> DL <sup>1</sup> MQ <sup>1</sup> FK <sup>1</sup> HIS <sup>1</sup> NEY <sup>1</sup> I <sup>1</sup> ED <sup>1</sup> SK <sup>1</sup> LI <sup>1</sup> <u>NSE<sup>1</sup>Q<sup>1</sup>NT<sup>1</sup>LL<sup>1</sup>KS<sup>1</sup></u> YK <sup>1</sup> YI <sup>1</sup> KE <sup>1</sup> SV <sup>1</sup> EN <sup>1</sup> DI <sup>1</sup> KFA <sup>1</sup> QEG <sup>1</sup> IS <sup>1</sup> YK <sup>1</sup> VL <sup>1</sup> AK <sup>1</sup> YK <sup>1</sup> DD <sup>1</sup> LE <sup>1</sup> SI <sup>1</sup> KK <sup>1</sup> VI <sup>1</sup> EE <sup>1</sup> KE <sup>1</sup> FP <sup>1</sup> SS <sup>1</sup> PT <sup>1</sup> PP <sup>1</sup> SP <sup>1</sup> AK <sup>1</sup> TD <sup>1</sup> E <sup>1</sup> Q <sup>1</sup> K <sup>1</sup> ES <sup>1</sup> K <sup>1</sup> FL <sup>1</sup> PL <sup>1</sup> TN <sup>1</sup> IE <sup>1</sup> TL <sup>1</sup> VN <sup>1</sup> LN <sup>1</sup> VN <sup>1</sup> KID <sup>1</sup> DY <sup>1</sup> LN <sup>1</sup> LK <sup>1</sup> AK <sup>1</sup> IN <sup>1</sup> D <sup>1</sup> <u>CNV<sup>1</sup>E<sup>1</sup>K<sup>1</sup>DE<sup>1</sup>AH<sup>1</sup>V<sup>1</sup>K<sup>1</sup>IT<sup>1</sup>KL<sup>1</sup>SDL<sup>1</sup>KA<sup>1</sup>IDD<sup>1</sup>K<sup>1</sup>IDL<sup>1</sup>FK<sup>1</sup>NP<sup>1</sup>YD<sup>1</sup>FE<sup>1</sup>AI<sup>1</sup>KK<sup>1</sup>L<sup>1</sup>ND<sup>1</sup>TK<sup>1</sup>K<sup>1</sup>DM<sup>1</sup>L<sup>1</sup>G<sup>1</sup>KL<sup>1</sup>ST<sup>1</sup>GL<sup>1</sup>VQ<sup>1</sup>N<sup>1</sup>FN<sup>1</sup>TI<sup>1</sup>SK<sup>1</sup>LIE<sup>1</sup>G<sup>1</sup>KF<sup>1</sup>Q<sup>1</sup>DM<sup>1</sup>LN<sup>1</sup>IS<sup>1</sup>QH<sup>1</sup>CV<sup>1</sup>KK<sup>1</sup>Q<sup>1</sup>C<sup>1</sup>PEN<sup>1</sup>SG<sup>1</sup>CF<sup>1</sup></u> HL <sup>1</sup> DE <sup>1</sup> EE <sup>1</sup> CK <sup>1</sup> CL <sup>1</sup> NY <sup>1</sup> KQ <sup>1</sup> EG <sup>1</sup> DK <sup>1</sup> CV <sup>1</sup> EN <sup>1</sup> PT <sup>1</sup> CN <sup>1</sup> EN <sup>1</sup> GG <sup>1</sup> CD <sup>1</sup> AD <sup>1</sup> AT <sup>1</sup> CE <sup>1</sup> ED <sup>1</sup> SG <sup>1</sup> SS <sup>1</sup> R <sup>1</sup> KK <sup>1</sup> IT <sup>1</sup> CE <sup>1</sup> CT <sup>1</sup> K <sup>1</sup> PD <sup>1</sup> SV <sup>1</sup> PL <sup>1</sup> FD <sup>1</sup> G <sup>1</sup> IF <sup>1</sup> CS <sup>1</sup> SS <sup>1</sup> <sup>274</sup>
<b>Polarity</b>	<sup>1</sup> AI <sup>1</sup> SV <sup>1</sup> TM <sup>1</sup> DN <sup>1</sup> LS <sup>1</sup> GF <sup>1</sup> EN <sup>1</sup> YD <sup>1</sup> VI <sup>1</sup> LK <sup>1</sup> PL <sup>1</sup> AG <sup>1</sup> VY <sup>1</sup> SL <sup>1</sup> KK <sup>1</sup> QI <sup>1</sup> EN <sup>1</sup> IF <sup>1</sup> FN <sup>1</sup> LN <sup>1</sup> DL <sup>1</sup> NS <sup>1</sup> RL <sup>1</sup> KK <sup>1</sup> RY <sup>1</sup> FL <sup>1</sup> DV <sup>1</sup> ES <sup>1</sup> DL <sup>1</sup> MQ <sup>1</sup> FK <sup>1</sup> HIS <sup>1</sup> NEY <sup>1</sup> I <sup>1</sup> ED <sup>1</sup> SK <sup>1</sup> LI <sup>1</sup> <u>NSE<sup>1</sup>Q<sup>1</sup>NT<sup>1</sup>LL<sup>1</sup>KS<sup>1</sup></u> YK <sup>1</sup> YI <sup>1</sup> KE <sup>1</sup> SV <sup>1</sup> EN <sup>1</sup> DI <sup>1</sup> KFA <sup>1</sup> QEG <sup>1</sup> IS <sup>1</sup> YK <sup>1</sup> VL <sup>1</sup> AK <sup>1</sup> YK <sup>1</sup> DD <sup>1</sup> LE <sup>1</sup> SI <sup>1</sup> KK <sup>1</sup> VI <sup>1</sup> EE <sup>1</sup> KE <sup>1</sup> FP <sup>1</sup> SS <sup>1</sup> PT <sup>1</sup> PP <sup>1</sup> SP <sup>1</sup> AK <sup>1</sup> TD <sup>1</sup> E <sup>1</sup> Q <sup>1</sup> K <sup>1</sup> ES <sup>1</sup> K <sup>1</sup> FL <sup>1</sup> PL <sup>1</sup> TN <sup>1</sup> IE <sup>1</sup> TL <sup>1</sup> VN <sup>1</sup> LN <sup>1</sup> VN <sup>1</sup> KID <sup>1</sup> DY <sup>1</sup> LN <sup>1</sup> LK <sup>1</sup> AK <sup>1</sup> IN <sup>1</sup> D <sup>1</sup> <u>CNV<sup>1</sup>E<sup>1</sup>K<sup>1</sup>DE<sup>1</sup>AH<sup>1</sup>V<sup>1</sup>K<sup>1</sup>IT<sup>1</sup>KL<sup>1</sup>SDL<sup>1</sup>KA<sup>1</sup>IDD<sup>1</sup>K<sup>1</sup>IDL<sup>1</sup>FK<sup>1</sup>NP<sup>1</sup>YD<sup>1</sup>FE<sup>1</sup>AI<sup>1</sup>KK<sup>1</sup>L<sup>1</sup>ND<sup>1</sup>TK<sup>1</sup>K<sup>1</sup>DM<sup>1</sup>L<sup>1</sup>G<sup>1</sup>KL<sup>1</sup>ST<sup>1</sup>GL<sup>1</sup>VQ<sup>1</sup>N<sup>1</sup>FN<sup>1</sup>TI<sup>1</sup>SK<sup>1</sup>LIE<sup>1</sup>G<sup>1</sup>KF<sup>1</sup>Q<sup>1</sup>DM<sup>1</sup>LN<sup>1</sup>IS<sup>1</sup>QH<sup>1</sup>CV<sup>1</sup>KK<sup>1</sup>Q<sup>1</sup>C<sup>1</sup>PEN<sup>1</sup>SG<sup>1</sup>CF<sup>1</sup></u> HL <sup>1</sup> DE <sup>1</sup> EE <sup>1</sup> CK <sup>1</sup> CL <sup>1</sup> NY <sup>1</sup> KQ <sup>1</sup> EG <sup>1</sup> DK <sup>1</sup> CV <sup>1</sup> EN <sup>1</sup> PT <sup>1</sup> CN <sup>1</sup> EN <sup>1</sup> GG <sup>1</sup> CD <sup>1</sup> AD <sup>1</sup> AT <sup>1</sup> CE <sup>1</sup> ED <sup>1</sup> SG <sup>1</sup> SS <sup>1</sup> R <sup>1</sup> KK <sup>1</sup> IT <sup>1</sup> CE <sup>1</sup> CT <sup>1</sup> K <sup>1</sup> PD <sup>1</sup> SV <sup>1</sup> PL <sup>1</sup> FD <sup>1</sup> G <sup>1</sup> IF <sup>1</sup> CS <sup>1</sup> SS <sup>1</sup> <sup>274</sup>
<b>Antigenic Propensity</b>	<sup>1</sup> AI <sup>1</sup> SV <sup>1</sup> TM <sup>1</sup> DN <sup>1</sup> LS <sup>1</sup> GF <sup>1</sup> EN <sup>1</sup> YD <sup>1</sup> VI <sup>1</sup> LK <sup>1</sup> PL <sup>1</sup> AG <sup>1</sup> VY <sup>1</sup> SL <sup>1</sup> KK <sup>1</sup> QI <sup>1</sup> EN <sup>1</sup> IF <sup>1</sup> FN <sup>1</sup> LN <sup>1</sup> DL <sup>1</sup> NS <sup>1</sup> RL <sup>1</sup> KK <sup>1</sup> RY <sup>1</sup> FL <sup>1</sup> DV <sup>1</sup> ES <sup>1</sup> DL <sup>1</sup> MQ <sup>1</sup> FK <sup>1</sup> HIS <sup>1</sup> NEY <sup>1</sup> I <sup>1</sup> ED <sup>1</sup> SK <sup>1</sup> LI <sup>1</sup> <u>NSE<sup>1</sup>Q<sup>1</sup>NT<sup>1</sup>LL<sup>1</sup>KS<sup>1</sup></u> YK <sup>1</sup> YI <sup>1</sup> KE <sup>1</sup> SV <sup>1</sup> EN <sup>1</sup> DI <sup>1</sup> KFA <sup>1</sup> QEG <sup>1</sup> IS <sup>1</sup> YK <sup>1</sup> VL <sup>1</sup> AK <sup>1</sup> YK <sup>1</sup> DD <sup>1</sup> LE <sup>1</sup> SI <sup>1</sup> KK <sup>1</sup> VI <sup>1</sup> EE <sup>1</sup> KE <sup>1</sup> FP <sup>1</sup> SS <sup>1</sup> PT <sup>1</sup> PP <sup>1</sup> SP <sup>1</sup> AK <sup>1</sup> TD <sup>1</sup> E <sup>1</sup> Q <sup>1</sup> K <sup>1</sup> ES <sup>1</sup> K <sup>1</sup> FL <sup>1</sup> PL <sup>1</sup> TN <sup>1</sup> IE <sup>1</sup> TL <sup>1</sup> VN <sup>1</sup> LN <sup>1</sup> VN <sup>1</sup> KID <sup>1</sup> DY <sup>1</sup> LN <sup>1</sup> LK <sup>1</sup> AK <sup>1</sup> IN <sup>1</sup> D <sup>1</sup> <u>CNV<sup>1</sup>E<sup>1</sup>K<sup>1</sup>DE<sup>1</sup>AH<sup>1</sup>V<sup>1</sup>K<sup>1</sup>IT<sup>1</sup>KL<sup>1</sup>SDL<sup>1</sup>KA<sup>1</sup>IDD<sup>1</sup>K<sup>1</sup>IDL<sup>1</sup>FK<sup>1</sup>NP<sup>1</sup>YD<sup>1</sup>FE<sup>1</sup>AI<sup>1</sup>KK<sup>1</sup>L<sup>1</sup>ND<sup>1</sup>TK<sup>1</sup>K<sup>1</sup>DM<sup>1</sup>L<sup>1</sup>G<sup>1</sup>KL<sup>1</sup>ST<sup>1</sup>GL<sup>1</sup>VQ<sup>1</sup>N<sup>1</sup>FN<sup>1</sup>TI<sup>1</sup>SK<sup>1</sup>LIE<sup>1</sup>G<sup>1</sup>KF<sup>1</sup>Q<sup>1</sup>DM<sup>1</sup>LN<sup>1</sup>IS<sup>1</sup>QH<sup>1</sup>CV<sup>1</sup>KK<sup>1</sup>Q<sup>1</sup>C<sup>1</sup>PEN<sup>1</sup>SG<sup>1</sup>CF<sup>1</sup></u> HL <sup>1</sup> DE <sup>1</sup> EE <sup>1</sup> CK <sup>1</sup> CL <sup>1</sup> NY <sup>1</sup> KQ <sup>1</sup> EG <sup>1</sup> DK <sup>1</sup> CV <sup>1</sup> EN <sup>1</sup> PT <sup>1</sup> CN <sup>1</sup> EN <sup>1</sup> GG <sup>1</sup> CD <sup>1</sup> AD <sup>1</sup> AT <sup>1</sup> CE <sup>1</sup> ED <sup>1</sup> SG <sup>1</sup> SS <sup>1</sup> R <sup>1</sup> KK <sup>1</sup> IT <sup>1</sup> CE <sup>1</sup> CT <sup>1</sup> K <sup>1</sup> PD <sup>1</sup> SV <sup>1</sup> PL <sup>1</sup> FD <sup>1</sup> G <sup>1</sup> IF <sup>1</sup> CS <sup>1</sup> SS <sup>1</sup> <sup>274</sup>

**Fig. 2.** B-cell epitope prediction: B-cell epitope prediction on MSP1<sub>42</sub>kDa protein based on different parameters viz, hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic property. The predicted B-cell linier epitopes are in blue underlined



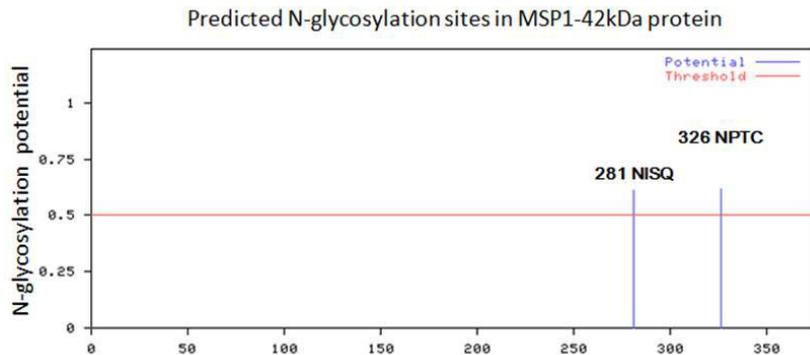
**Fig. 3.** T-cell epitope prediction on MSP1<sub>42</sub>kDa protein : (A) Human MHC-Class I binding regions (B) MHC-Class II binding regions. Amino acid regions crossing the peptide score threshold of 3% are considered to be potential T-cell epitopes.

<sup>32</sup>. Hence our derived MSP1<sub>42</sub> kDa is more beneficial over bacterially expressed proteins that have been reported earlier.

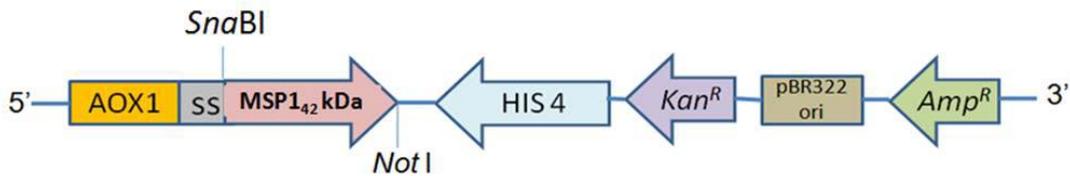
**MALDI-TOF/MS analysis of yeast-derived recombinant MSP1<sub>42</sub> kDa protein**

As no MSP-1 specific monoclonal antibodies were available in the present study to

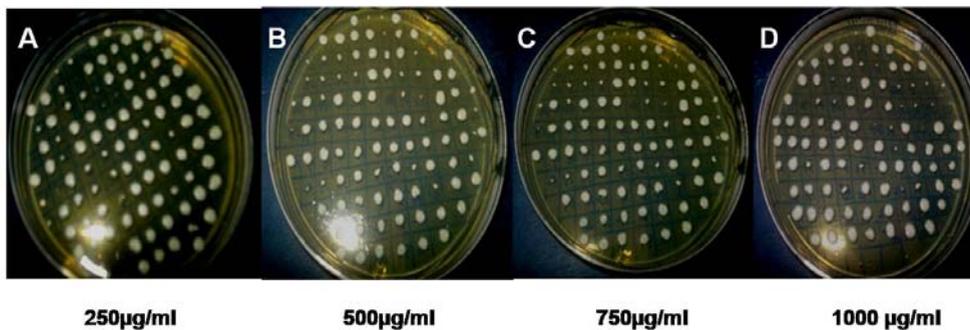
characterize the yeast expressed recombinant protein, we have employed an advanced MALDI-TOF/MS technique to further confirm its authenticity as described earlier<sup>33</sup>. The 42kDa protein band corresponding to recombinant MSP1<sub>42</sub> kDa protein was enzymatically digested as described under methodology section. As shown



**Fig 4.** Prediction of N-linked glycosylation on MSP1<sub>42</sub> kDa protein: Blue lines indicate the potential N-linked glycosylation points at amino acid position 281(NISQ) and 326(NPTC)



**Fig. 5.** Schematic representation of pPIC9KMSP1<sub>42</sub> kDa plasmid DNA: The expression cassette consists of *P.falciparum* MSP1<sub>42</sub> kDa gene cloned under AOX1 promoter and has Alpha secretory signal (SS) at amino terminal end. The plasmid has ampicillin resistance gene (Amp<sup>R</sup>) for selection in bacteria and kanamycin resistance gene (Kan<sup>R</sup>) that confers resistance to Geneticin for selection in yeast

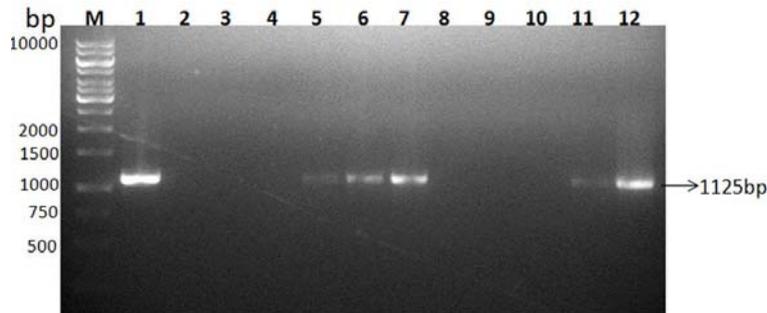


**Fig. 6.** Geneticin sensitivity assay: *Pichia* transformants were selected on different concentrations of geneticin (250µg/ml, 500µg/ml, 750µg/ml and 1000µg/ml) via replica plate method. Growth obtained were scored as ‘+’ or ‘-’ after three days.

in figure 10, peptide mass profiles were obtained from the destained band. Twenty peptides (range 800-2750 Da) were used for peptide mass database search which identified MSP1<sub>42</sub>kDa protein with 55% sequence coverage of the published *P. falciparum* MSP1<sub>42</sub>kDa protein sequence (Table 1) upon mascot search thus establishing the identity of the yeast expressed MSP1<sub>42</sub>kDa.

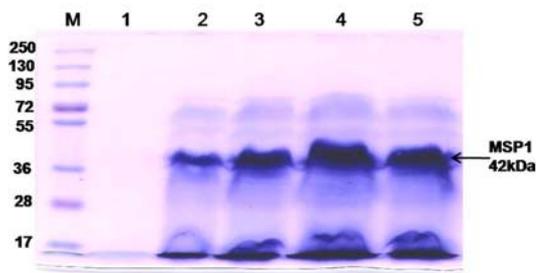
**Development of purification method of recombinant MSP1<sub>42</sub>kDa protein**

Anion exchange chromatography  
 The anion-exchange chromatography was selected seeing that rec.MSP1 is recognized to bind to an anion exchanger. The sterile PBS buffer was used in the present study offers a high binding competence of rec. MSP1 protein and also good



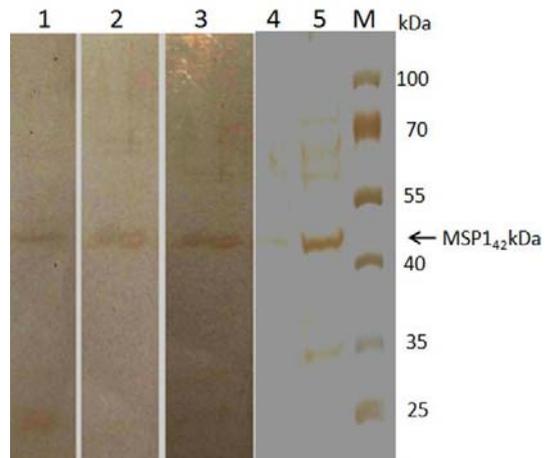
Lane M: 1Kb DNA ladder (Fermentas)  
 Lane 1-3: transformants from 250µg/ml, lane 4-6: 500µg/ml, lane 7-9: 750µg/ml and lane 10-12: 1000µg/ml

**Fig. 7.** Genomic DNA PCR: Pichia transformants were subjected to genomic DNA PCR using MSP1<sub>42</sub>kDa specific primers



Lane M: Pre-stained protein marker (Cat no: SM1811, Fermentas)  
 Lane 1: Uninduced Pichia transformant (Negative control)  
 Lane 2, 3, 4 and 5: Methanol induced transformant after 24h, 48h, 72h and 96h of post induction respectively

**Fig. 8.** SDS-PAGE profile of MSP1<sub>42</sub>kDa protein from culture supernatant of positive Pichia transformant after methanol induction



Lane M: Pre-stained protein marker (Cat no: SM 0671, Fermentas)  
 Lane 1, 2, 3, 4: reacted with *P.falciparum* infected known positive sera samples

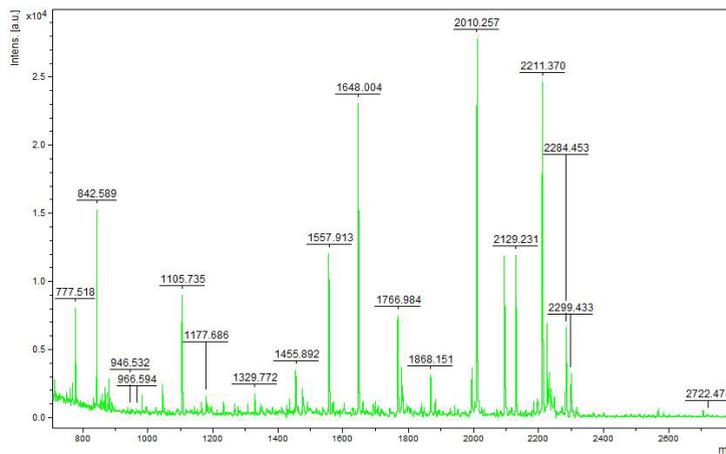
**Fig. 9.** Immunoblots of yeast derived MSP1<sub>42</sub>kDa protein using human sera samples

resolution. Following the binding four column volume gentle wash was applied with sterile PBS. Consequently elution of protein on the anion-exchange column was succeeded in the optimized range of 200-225 mM NaCl as shown in (Fig. 11). Fractions corresponding to the protein peak eluted using gradient NaCl showed a single protein band on SDS-PAGE as seen in (Fig. 12). The concentration of the purified protein (50.0 mg / L) was estimated by the Bradford assay.

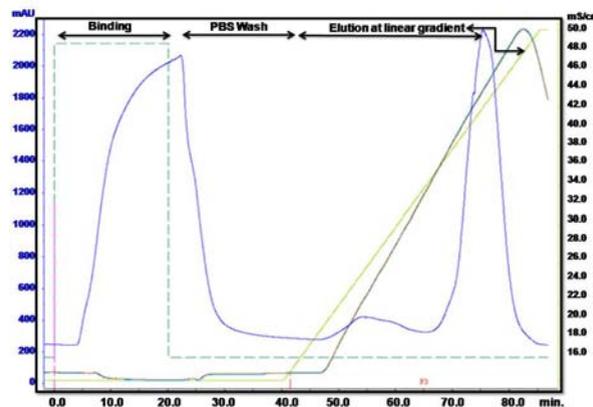
#### Immunization of rabbits with yeast derived MSP1 recombinant protein

To evaluate the elicitation of *P. falciparum* parasite inhibiting antibodies against the yeast expressed rec. MSP1 protein, rabbits were

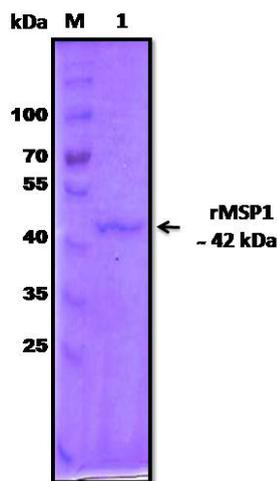
immunized intramuscularly. Collected Pre immune and post immunized, sera (42 and 60 days) were assayed for anti-MSP1 antibody titers. An antibody titer were interestingly increased in the both immunized rabbits with recombinant MSP1 while not in immunized with Freund's adjuvant plus sterile PBS ( control rabbit). High antibody titer of 1:150000 after six weeks of booster injection and 1:160000 after nine weeks of booster injection were observed (data not shown). The end point titer was set at 0.21, which were more than four standard deviations. These results clearly show that the recombinant MSP1 protein expressed in *Pichia pectoris* was highly immunogenic and inducing a specific antibody response.



**Fig. 10.** MALDI-TOF/MS spectrum of yeast derived MSP1<sub>42</sub> kDa protein tryptic digests in reflectron mode using HCCA as matrix



**Fig. 11.** Chromatogram of an anion exchange base purification of recombinant MSP1<sub>42</sub> kDa protein using the AKTA explorer FPLC system



Lane M: Pre-stained protein marker (Cat no: SM1811, Fermentas) Lane 1: Eluted peak fraction of purified recombinant MSP1<sub>42</sub>kDa protein

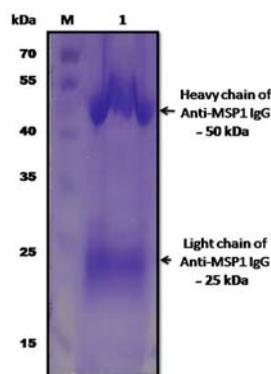
**Fig. 12.** SDS-PAGE analysis of purified recombinant MSP1<sub>42</sub>kDa protein by an anion exchange chromatography

#### ***In-vitro* parasite growth inhibition**

Immune sera of immunized rabbit was obtained after the third and fourth of immunization for distinguish promising effects on in vitro parasite growth. In this present in vitro growth inhibition assay, parasite growth inhibition was obtained for purified anti-MSP1IgG (Fig.13) of both rabbit vaccinated sera at a final concentration of 300 µg/ml [data not shown]. A maximum parasitemia was noted for the preimmune rabbit sera and control immunized sera because control rabbit was immunized with adjuvant. To binary confirmation with the intention of the observed parasite growth inhibition was rabbit derived anti-MSP1 IgG mediated, inhibition experiments for the two clinically known positive samples were also performed [data not shown]. High levels of growth inhibition (˜ 95%) were obtained. The experiments were executed using purified rabbit derived anti-MSP1 IgG three times with identical results (data not shown).

#### **CONCLUSIONS**

The MSP-1 protein expressed on the surface of invasive merozoites of Plasmodium is an important target for the development of an effective malaria vaccine. The bulk economical



Lane M: Pre-stained protein marker (Cat no: SM1811, Fermentas)

Lane 1: Eluted peak fraction of affinity purified anti-MSP1IgG

**Fig. 13.** SDS-PAGE analysis of purified Anti-MSP1IgG protein by affinity chromatography

production of this glycosylated MSP1<sub>42</sub>kDa protein requires a simple and reliable eukaryotic system. In the present study, *Pichia pastoris*, a most popular eukaryotic host has been successfully employed for expressing *P. falciparum* MSP1<sub>42</sub>kDa protein. Purification strategy was developed and purity of protein verified by SDS-PAGE electrophoresis. Immunization of rabbit with recombinant MSP1<sub>42</sub>kDa in complete and incomplete Freund's adjuvant were revealed high antibody titers against the recombinant MSP-1<sub>42</sub>kDa. Rabbit derived anti-MSP1IgG inhibited in vitro parasite growth, indicating that this inhibition was anti-MSP1IgG mediated. The multi-copy recombinant *Pichia* transformant obtained in the present study has an immense industrial application for large scale production of MSP1<sub>42</sub>kDa recombinant protein for either prophylactic or diagnostic applications.

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