Title: Improvement of a fermentation process for the production of two *Pf*AMA1-DiCo-based malaria vaccine candidates in *Pichia pastoris*

Authors: Robin Kastilan[1], Alexander Boes[1], Holger Spiegel[1,*], Nadja Voepel[1], Ivana Chudobová[1], Stephan Hellwig[1], Johannes Felix Buyel[1,2], Andreas Reimann[1], Rainer Fischer[2,3]

Supplementary Text S1 - Mass spectrometry

Proteins were reduced, alkylated and digested in the polyacrylamide gel using trypsin (Promega, Mannheim, Germany) as previously described[1]. The resulting peptides were analyzed by nanoHPLC using an UltiMate 3000 HPLC system (LC PAcking, Dionex, Idstein, Germany) coupled to an amaZon ETD ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) with an ESI nanosprayer. The nanoHPLC system and the ion trap mass spectrometer were controlled using Bruker Compass HyStar v3.2 SR2 software. The liquid chromatography system comprised a reversed-phase pre-column (LC PAckings, Dionex) for sample desalting and a 15-cm PepMap 100 reversed-phase C18 column, 75 µm inner diameter (LC PAckings, Dionex), for peptide fractionation. The peptides were separated using a 45-min linear gradient from 96% (v/v) solution A (2% (v/v) acetonitrile, 0.1% (v/v) formic acid in high-purity water) and 4% (v/v) solution B (98% (v/v) acetonitrile, 0.1% (v/v) formic acid in high-purity water) to 50% (v/v) solution A and 50% (v/v) solution B at a flow rate of 300 nL min^{-1}. The electrospray was operated in positive ion mode with a spray voltage of –4000 V and 10 psi gas pressure. The end plate offset of the mass spectrometer was set to –500 V and Proteomics AutoMSMS Alternating Spectra CID-ETD Bruker trapControl v7.0 was used for data acquisition. Raw data files were evaluated using Compass DataAnalysis v4.0 SR5 software with embedded search engine Mascot Search v2.3.01 (Matrix Science Ltd, London, UK). The spectra were searched against the NCBInr fungi database and the cRAP database (including VAMAX1 and VAMAX2 sequences) using the following parameters: enzyme = trypsin, up to one missed cleavage permitted, no fixed modifications, variable modifications allowed = carbamidomethyl (C), oxidation (M) and propionamide (C), mass tolerance for precursor ion ±0.3 Da and fragment ion ±0.3 Da.

Supplementary Figure S1

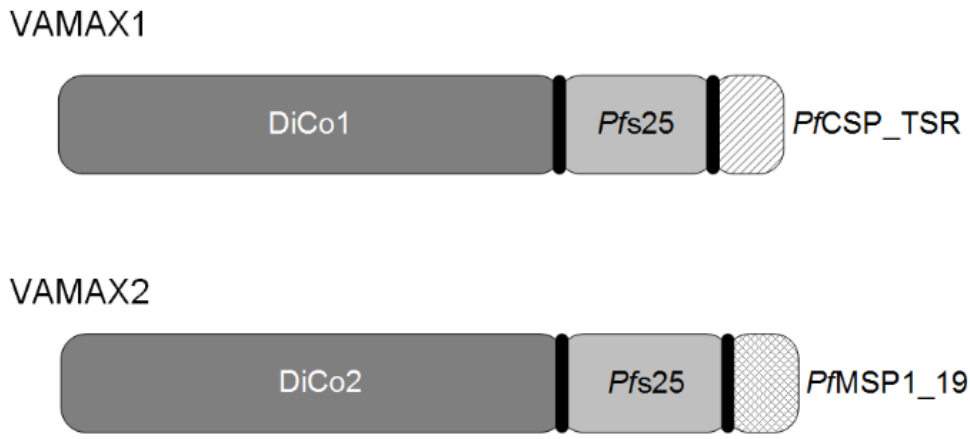


Figure S1 Schematic representation of the VAMAX1 and VAMAX2 fusion antigens. DiCo1 and DiCo2 = diversity-covering variants of *Pf*AMA1 (dark gray); *Pf*s25 = *Plasmodium falciparum* D7 sexual-stage surface antigen *Pf*s25 (light gray); *Pf*CSP_TSR = thrombospondin-related region from *P. falciparum* 3D7 circumsporozoite protein *Pf*CSP (hatched); *Pf*MSP1_19 = C-terminal 19 kDa fragment of the merozoite surface protein 1 from *P. falciparum* strain FUP (cross-hatched).

Supplementary Figure S2

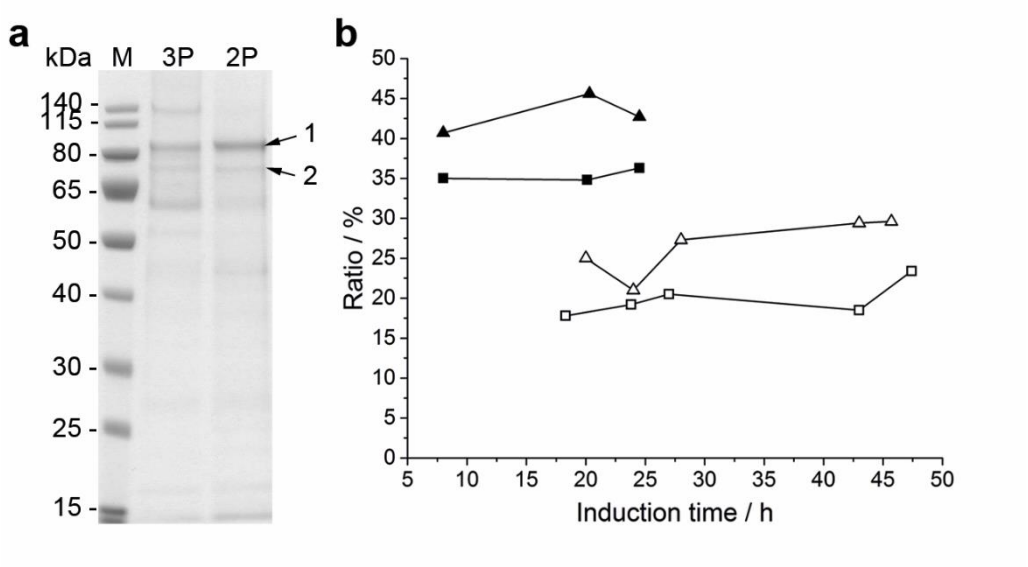


Figure S2 Investigation of potential proteolytic activity in the fermentation broth during the course of induction. **(a)** Samples of the supernatant during the induction phase of both strategies (three-phase = 3P, two-phase = 2P) were analyzed by LDS-PAGE and Coomassie-staining followed by the assessment of the background-corrected intensity of protein band 1 (VAMAX1) and protein band 2 (degradation product of VAMAX1) using AIDA Image Analyzer software (Raytest). Representative samples shown here from the end of the fermentation runs. M: marker. **(b)** To detect potential proteolytic activity in the fermentation broth the intensity ratio of band 1 to band 2 was plotted during the induction phase. Filled symbols: three-phase strategy. Open symbols: two-phase strategy. Squares: VAMAX1 fermentations. Triangles: VAMAX2 fermentations.

Supplementary Figure S3

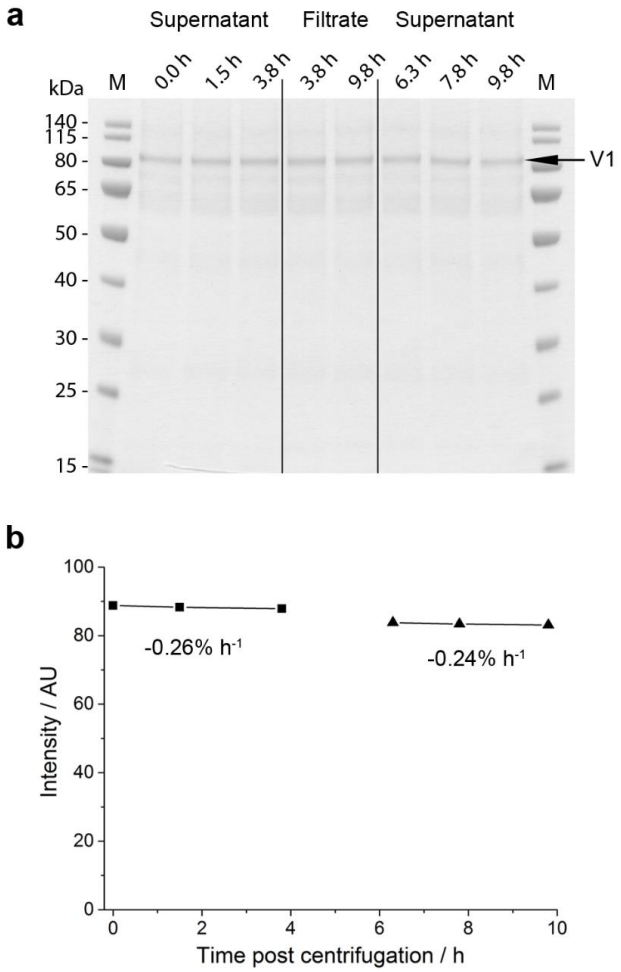


Figure S3 Stability of VAMAX1 (V1) in the supernatant after solid–liquid separation by centrifugation. At the end of the two-phase process, the cells were semi-continuously separated from the fermentation broth by centrifugation with a CARR Powerfuge P6 at 5,500 × *g* and a feed rate of 0.75 kg min^{-1}. The cell density was considerably reduced to an OD_{600} of 2.48 ± 0.01 (n = 3) in the supernatant. **(a)** The stability testing of VAMAX1 was performed during two consecutive filtration experiments starting directly (0.0 h) and 6.3 h after centrifugation, respectively, at room temperature. Samples of the supernatant were taken at the indicated times post-centrifugation and were analyzed by LDS-PAGE and Coomassie-staining. **(b)** The Coomassie-stained bands of VAMAX1 were evaluated by densitometric analysis using AIDA Image Analyzer software. The background-corrected integral of each band was plotted over time post-centrifugation. During the first filtration experiment, the apparent decrease in band intensity (representing product loss) was –0.26% per hour, calculated using a linear regression function (R^2 = 0.97). For the second filtration experiment, the decrease in band intensity was –0.24% per hour (R^2 = 0.97).

Supplementary Table S1 – Mass spectrometry data

Candidate / Protein band	Accession	Protein	Score	Peptides	Sequence coverage
VAMAX1					
Band 1	VAMAX1_Dico1_ pfs25FKO_CSP_TSR	VAMAX1	103.1	4	5.9%
	gi|328352741	Endochitinase [*Komagataella phaffii* CBS 7435]	137.0	2	8.0%
Band 2	VAMAX1_Dico1_ pfs25FKO_CSP_TSR	VAMAX1	396.1	8	12.9%
VAMAX2					
Band 1	VAMAX2_Dico2_ pfs25FKO_MSP1_19	VAMAX2	313.2	8	12.4%
	gi|328352741	Endochitinase [*K. phaffii* CBS 7435]	273.0	3	10.0%
	gi|254564921	Cell wall protein with similarity to glucanases [*K. phaffii* GS115]	237.0	4	15.0%
	gi|254570078	Protein of the SUN family (Sim1p, Uth1p, Nca3p, Sun4p) that may participate in DNA replication [*K. phaffii* GS115]	193.0	2	9.0%
Band 2	VAMAX2_Dico2_ pfs25FKO_MSP1_19	VAMAX2	539.1	12	21.5%
	gi|254564921	Cell wall protein with similarity to glucanases [*K. phaffii* GS115]	196.0	3	11.0%
	gi|254570078	Protein of the SUN family (Sim1p, Uth1p, Nca3p, Sun4p) that may participate in DNA replication [*K. phaffii* GS115]	171.0	2	9.0%

Supplementary Data S1

Sequences of the putative host cell proteins identified by mass spectrometry (Supplementary Table S1) were used as BLAST queries against the UniProtKB database with the following parameters:

- Threshold e-value: 0.001
- Matrix: auto
- Filtering: none
- Gapped: yes
- Hits: 100

Alignment with an endochitinase from *Saccharomyces cerevisiae* (UniProtKB entry P29029) found by using gi|328352741 (endochitinase [*Komagataella phaffii* CBS 7435]) as a BLAST query:

P29029 CHIT_YEAST - **Endochitinase** Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)

E-value: 6.8e-133
Score: 1067
Ident.: 48.0%
Positives : 66.1%
Query Length: 686
Match Length: 562

```
F2QUR1 F2QUR1_KOMPC      18      AFDNSAKNNVALYWGQNSAGSQERLSYYCQSDSVDIVLLSFLYIFPANPLGLDFSNACGD      77
                                 AFD SA  N+A+YWGQNSAG+QE L+ YC+S   DI LLSFL  FP   LGL+F+NAC D
P29029 CHIT_YEAST        20      AFDRSANTNIAVYWGQNSAGTQESLATYCESSDADIFLLSFLNQFPT--LGLNFANACSD      77

F2QUR1 F2QUR1_KOMPC      78      QFPSGLLKCDTIAEDIQTCQSLGKKVLLSLGGATGTYGFSSDSEAEDFAEVLWDTF-LGG     136
                                  F  GLL C  IAEDI+TCQSLGKKVLLSLGGA+G+Y FS DS+AE FA+ LWDTF  G
P29029 CHIT_YEAST        78      TFSDGLLHCTQIAEDIETCQSLGKKVLLSLGGASGSYLFSDDSQAETFAQTLWDTFGEGT     137

F2QUR1 F2QUR1_KOMPC      137     STDERPFGDSILDGIDYDAENNNPTGYTALSAKLREFYASDPSRTYYIAAAPQCPYPDAS     196
                                    ERPF  +++DG D+D ENNN  GY+AL+ KLR  +A + ++ YY++AAPQCPYPDAS
P29029 CHIT_YEAST        138     GASERPFDSAVVDGFDFDIENNNEVGYSALATKLRTLFA-EGTKQYYLSAAPQCPYPDAS     196

F2QUR1 F2QUR1_KOMPC      197     VGDVLANADVDFVFIQFYNNYCALASTSFNWATWLDYAQNTSPNPNVKLYVGLPGGPTGA     256
                                 VGD+L NAD+DF FIQFYNNYC++ S  FNW TWL YAQ  SPN N+KL++GLPG  + A
P29029 CHIT_YEAST        197     VGDLLENADIDFAFIQFYNNYCSV-SGQFNWDTWLTYAQTVSPNKNIKLFLGLPGSASAA     255

F2QUR1 F2QUR1_KOMPC      257     SSGYVG-TDVVKQRIDEIGASSSLGGIMLWDASQGFSNQVDGGNYVDAMKSILNGLGSVD     315
                                  SGY+  T +++  I +I +SSS GGI LWDASQ FSN+++G  YV+ +K++L
P29029 CHIT_YEAST        256     GSGYISDTSLLESTIADIASSSSFGGIALWDASQAFSNELNGEPYVEILKNLL-------     308

F2QUR1 F2QUR1_KOMPC      316     ASTTSSSQAAATSQTTSTLATSISSTPGSSSTVSSSSSLSSSSSPSSSSSSTTIWWTPDT     375
                                    TS+SQ A T  T +T  TS +ST  +S++ +S+S   ++ S +S+ S + +  +P
P29029 CHIT_YEAST        309     ---TSASQTATT--TVATSKTSAASTSSASTSSASTSQKKTTQSTTSTQSKSKVTLSP--     361

F2QUR1 F2QUR1_KOMPC      376     PQSSSSSVAAVSSETSSVLTSSVSTTQNSHGEVDSEGTTLTGTSTIWWTPSEAQSYETSS     435
                                   ++SS++    ++T+  LTSS + T++S G   +E T  +   T   T   +Q    +S
P29029 CHIT_YEAST        362     --TASSAIKTSITQTTKTLTSS-TKTKSSLGTTTTESTLNSVAITSMKTTLSSQ---ITS     415

F2QUR1 F2QUR1_KOMPC      436     LSSVSSIPTGNKDVSSILVITDVTDSLT-STKESSDSALTISTSLSSSPSLADSSRDGET     494
                                  + V+   T    VSS  + T +T +L+ +TK SS  +L  +T+ + SP+   +S  G T
P29029 CHIT_YEAST        416     AALVTPQTTTTSIVSSAPIQTAITSTLSPATKSSSVVSLQTATTSTLSPTTTSTS-SGST     474

F2QUR1 F2QUR1_KOMPC      495     STVVQVTSST                                                      504
                                 S+    + ST
P29029 CHIT_YEAST        475     SSGSTSSDST                                                      484
```

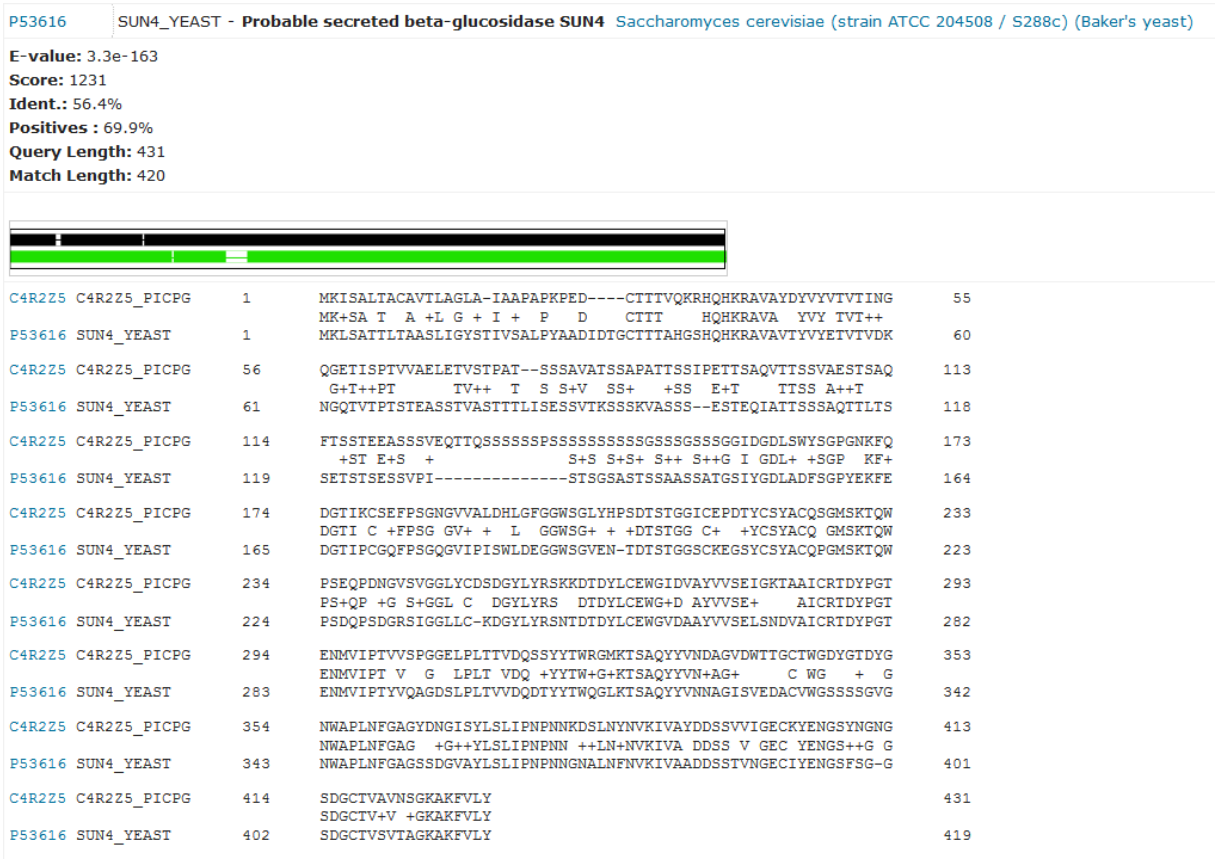
Alignment with a putative family 17 glucosidase SCW10 from *S. cerevisiae* (UniProtKB entry Q04951) found by using gi|254564921 (cell wall protein with similarity to glucanases [*K. phaffii* GS115]) as a BLAST query:

Q04951 SCW10_YEAST - **Probable family 17 glucosidase SCW10** Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)

E-value: 3.5e-124
Score: 961
Ident.: 55.5%
Positives : 67.8%
Query Length: 348
Match Length: 389

```
C4QVL7 C4QVL7_PICPG      26      HQHDKRGVVVVTK----TIVVDG----------------------------STVEATAA      52
                                 H+H+KR VV  T     T+VV G                              ST+E T +
Q04951 SCW10_YEAST       24      HKHEKRDVVTATVHAQVTVVVSGNSGETIVPVNENAVVATTSSTAVASQATTSTLEPTTS      83

C4QVL7 C4QVL7_PICPG      53      AQV---QEHAETFAESTPSAVV--SSSSAPSSASSASAPASSGSFSA----GTKGVTYSP     103
                                 A V   Q+   T   S  ++ V  S+SS+PSS+SS S+ ASS + S+    G KG+TYSP
Q04951 SCW10_YEAST       84      ANVVTSQQQTSTLQSSEAASTVGSSTSSSPSSSSSTSSSASSSASSSISASGAKGITYSP     143

C4QVL7 C4QVL7_PICPG      104     YQAGGGCKTAEEVASDLSQLTGYEIIRLYGVDCNQVENVFKAKAPGQKLFLGIFFVDAIE     163
                                 Y   G CK+  +VASDL QLTG++ IRLYGVDC+QVENV +AK   QKLFLGI++VD I+
Q04951 SCW10_YEAST       144     YNDDGSCKSTAQVASDLEQLTGFDNIRLYGVDCSQVENVLQAKTSSQKLFLGIYYVDKIQ     203

C4QVL7 C4QVL7_PICPG      164     SGVSAIASAVKSYGSWDDVHTVSVGNELVNNGEATVSQIGQYVSTAKSALRSAGFTGPVL     223
                                   V  I SAV+SYGSWDD+ TVSVGNELVN G AT +Q+G+YVSTAKSAL SAG+TG V+
Q04951 SCW10_YEAST       204     DAVDTIKSAVESYGSWDDITTVSVGNELVNGGSATTTQVGEYVSTAKSALTSAGYTGSVV     263

C4QVL7 C4QVL7_PICPG      224     SVDTFIAVINNPGLCDFADEYVAVNAHAFFDGGIAASGAGDWAAEQIQRVSSACGG-KDV     282
                                 SVDTFIAVINNP LC+++D Y+AVNAHA+FD   AA  AG W  EQI+RV +ACGG KDV
Q04951 SCW10_YEAST       264     SVDTFIAVINNPDLCNYSD-YMAVNAHAYFDENTAAQDAGPWVLEQIERVYTACGGKKDV     322

C4QVL7 C4QVL7_PICPG      283     LIVESGWPSKGDTNGAAVPSKSNQQAAVQSLGQKIGSSCIAFNAFNDYWKADGPFNAEKY     342
                                 +I E+GWPSKGDT G AVPSK+NQ+AA+ S+    GSS   F AFND WK DG +  EKY
Q04951 SCW10_YEAST       323     VITETGWPSKGDTYGEAVPSKANQEAAISSIKSSCGSSAYLFTAFNDLWKDDGQYGVEKY     382

C4QVL7 C4QVL7_PICPG      343     WGILDS                                                          348
                                 WGIL S
Q04951 SCW10_YEAST       383     WGILSS                                                          388
```

Alignment with a probable secreted β-glucosidase SUN4 from *S. cerevisiae* (UniProtKB entry P53616) found by using gi|254570078 (Protein of the SUN family [*K. phaffii* GS115]) as a BLAST query:



Supplementary Reference:

1. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J.V. & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1**, 2856-60, doi:10.1038/nprot.2006.468 (2006).