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Development of an Integrated Bioprocess for production of potential Malaria vaccines with *Pichia pastoris*

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Abstract: *Pichia pastoris* was used for the expression of an artificial fusion protein D1M1 – a potential Malaria vaccine. A fully automated integrated bioprocess was set up which combined upstream and downstream operations and has been performed in alternating fed batch cycles with parallel protein capture by Expanded Bed Adsorption Chromatography. Design of Experiments was used to study the influence of procedural factors on protein recovery and purity. As a result it was achieved to purify the protein to a purity of up to 87 % and a recovery of 51 % was reached in a single downstream operation.

Keywords: Design of Experiments, Expanded Bed Adsorption Chromatography, In Situ Product Removal, Integrated Bioprocess, Malaria vaccine, *Pichia pastoris*, Sequential Processing.

1. INTRODUCTION

A fast removal of product from the fermentation broth and its producing cells can prevent inhibition provoked by product toxicity or degradation of the product itself (Freeman et al., 1993). Hence, In Situ Product Removal (ISPR) can help to intensify processes and is a step towards continuous biological production.

Expanded Bed Adsorption (EBA) chromatography integrates the conventional process steps of solid-liquid separation by centrifugation and/or micro filtration, protein adsorption and volume reduction of the protein solution in one unit operation. Consequently this technique saves considerably process time and investment costs without a loss in separation performance (Hubbuch et al., 2005). Thus, EBA offers the potential to be used as a tool for ISPR.

Immobilized Metal Affinity Chromatography (IMAC) is a separation technique for selective purification and isolation of proteins with natural or artificial surface-exposed histidine residues (Gaberc-Porekar et al., 2001). Accordingly the combination of EBA technology with an IMAC provides a powerful method for direct capture of proteins with affinity ligands from crude unclarified fermentation broth (Jahic et al., 2006).

Design of Experiments (DoE) was tested for the optimization of an Integrated Bioprocess which implements the specified methods.

2. MATERIALS AND METHODS

2.1 Integrated bioplant

The configuration of an integrated bioplant is shown in fig. 1. It consist of a 5 l BIOSTAT® ED bioreactor (Sartorius Stedim Systems, Germany) and an EBA chromatography system equipped with a STREAMLINE 50 column and 300 ml STREAMLINE Chelating media (GE Healthcare, Germany).

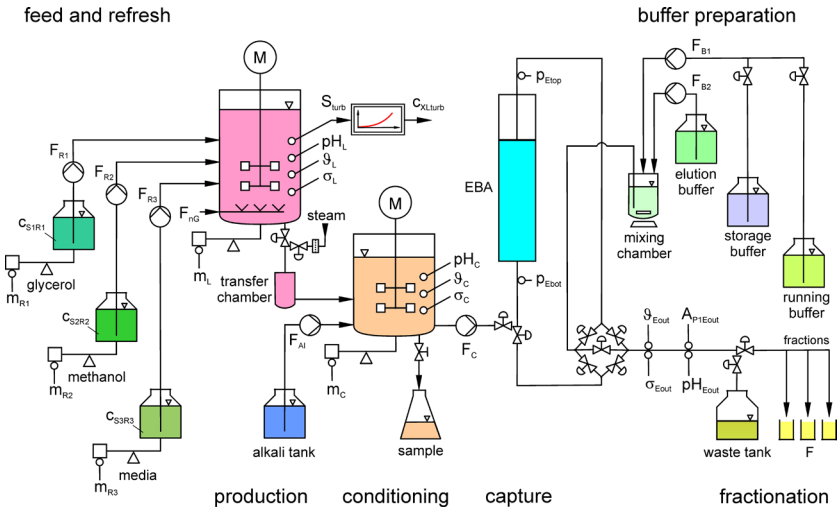

Fig. 1. Setup of an integrated bioplant with EBA for a fully automated process strategy with ISPR. Shown are production with feed and refresh, stirred conditioning vessel, capture sector with EBA column and buffer preparation as well as piping with valves and monitoring instrumentation. S_{turb}: turbidity of fermentation broth, c_{XLturb}: cell density estimated from turbidity signal, p_{Ebot}: pressure at EBA column bottom, p_{Etop}: pressure at EBA column top, A_{P1Eout}: UV absorption at 280nm in EBA outlet stream, F_K: flow rate, m_K: mass, pH_K: pH-value, ϑ_K: temperature, σ_K: conductivity, c_{JK}: concentration of compound J, all in subsystem K

A link between the upstream and the downstream sector was realized by implementing a stirred conditioning vessel with steam-sterilizable transfer line and has minimized a risk of contamination of the harvest flow.

An enhanced monitoring and control instrumentation enables adjustable process settings in upstream and downstream procedures. The whole process is operated with the SCADA-system MFCS/win® (Sartorius Stedim Systems, Germany).

2.2 Product, strain and media

Two surface proteins of the pathogen *Plasmodium falciparum*, the apical membrane antigen 1 (AMA1) and the merozoite surface protein 1 (MSP1) have been identified as potential Malaria vaccines. An artificial fusion protein D1M1 with a size of 73 kDa was designed from both to amplify the effect of vaccination (Faber et al., 2013).

The molecular information of this fusion protein has been cloned into *P. pastoris* strain KM71H by the Biomedical Primate Research Center (BPRC) of the Netherlands. A natural histidine-tag in the pro-domain of AMA1 facilitated immobilized metal affinity chromatography (IMAC) as a feasible method for protein recovery (Martens et al., 2011).

Modified FM22 minimal media complemented with 4 mll^{-1} PTM4 trace elements and 8 mgl^{-1} biotin was used for pre-culture and fermentation runs. Initial glycerol concentration was 20 gl^{-1} for each and 500 gl^{-1} in the reservoir for feeding during fed batch mode. A 790 gl^{-1} methanol reservoir was used for feeding during induction.

2.3 Sequential cultivation strategy

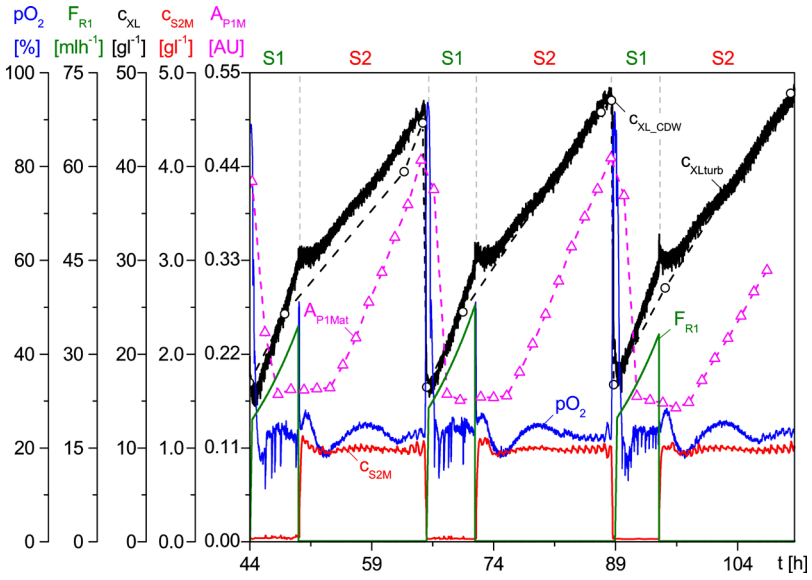

Fig. 2. Three sequences consisting of a fed batch on glycerol (S1) and production on methanol (S2). pO_2: dissolved oxygen tension, F_{R1}: glycerol feed rate, c_{XLturb}: cell density, estimated from turbidity measurement, c_{XL_CDW}: cell density, determined from cell dry weight (○), c_{S2M}: methanol concentration, A_{P1Mat}: UV absorbance of D1M1, measured at-line via HPLC (Δ)

The recommended cultivation strategy schedules a start up cycle composed of a batch phase and one initial sequence – consisting of a glycerol fed batch for cell breeding and a methanol fed batch for protein production – followed by an optional number of equivalent sequences to generate the raw material for the linked EBA chromatography.

Fig. 2 shows three reproducible sequences all consisting of a fed batch phase on glycerol for cell growth and metabolic change and a production phase where protein expression was induced by feeding methanol as sole carbon source (Cornelissen et al., 2003). Concentration of methanol c_{S2M} was measured and controlled to 1 gl^{-1}. Liquid temperature ϑ_L of 28°C and pH_L of 5.5 were also controlled, due to Fricke et al., 2013.

After 24 h from the start of the glycerol fed batch phase a partial harvest of the cultivation broth into the stirred conditioning vessel was executed. It was designed to retain a cell mass m_{XL} of 80 g in the reactor to reach a cell density c_{XL} of 20 gl^{-1} after refreshing with idle media to a liquid volume V_L of 4 l. This ensured equal initial conditions for every sequence to maintain reproducible production cycles as can be seen in fig. 2.

The developed cultivation strategy was fully integrated into S88 recipe structures which minimized the need for manual operations.

2.4 Integrated downstream procedure

STREAMLINE Chelating media was activated with 2 CV 0.1 M Cu^{2+}-solution followed by a washing step with 2 CV 50 mM NaAc-solution to rinse out unbound copper ions.

In advance of the actual chromatographic procedure the cell density of the harvested broth was automatically adjusted to 20 gl^{-1}, by diluting with running buffer B1 (0.5 M NaCl, 20 mM $NaH_2PO_4 \cdot 2H_2O$, 5 mM Imidazole, pH 7.4). Tween®20 was added to avoid a clocking of the flow distributor plate at the column inlet caused by accumulation of cells. In a second step of conditioning an automatic adjustment of broth pH-value to a desired setpoint was carried out by titrating with 1.5 M NaOH.

In parallel to the conditioning step of the cell broth, STREAMLINE media was equilibrated to reach a stable expanded state by pumping buffer B1 in an upward flow direction. Subsequently 5 l of the conditioned cultivation broth was applied to the column likewise. The remaining cells and particles were washed out with 10 CV (of the settled bed) buffer B1, in the upward direction and subsequently in the downward direction.

Elution was executed in the settled bed with buffer B2. The volume of eluate collected was further analyzed by SDS-PAGE.

The developed downstream strategy has been fully integrated into S88 recipe structures of the process control software MFCS/win. This process automation has minimized the need for error-prone manual operations during EBA chromatography runs. Additionally a BioPAT® MFCS/win module was successfully implemented into the recipe structure and assigned the respective set points of DoE experiments automatically to defined software controllers, e.g. pH controller. The batch structure of the process and the phase structure of the product capture are shown in fig. 3.

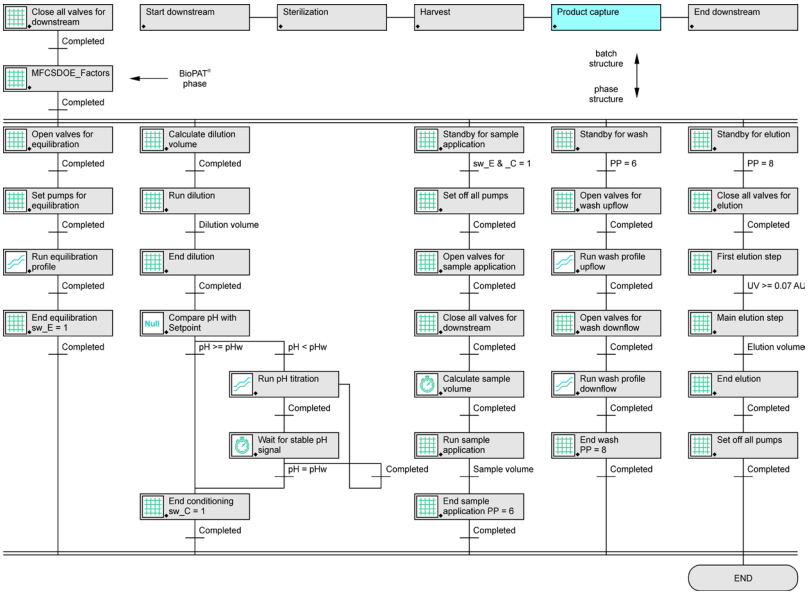

Fig. 3. S88 recipe structure with an integrated BioPAT® phase

2.5 Analytical procedures for product quantification

The total protein concentration in all contemplated samples has been determined by the Bradford method using Bradford Kit (BioRad Laboratories, Germany).

For the estimation of target protein purity P_{P1F},

$$P_{P1F} = \frac{A_1}{\sum_{j=1}^{n} A_j}, \quad (1)$$

each sample has been prepared by SDS-PAGE with Coomassie staining. Pictures of the gel were taken with Quantity One® software (BioRad Laboratories, Germany) and were further analyzed with TotalLab Quant® image analysis software. The software detected the bands of the generated protein pattern and calculated their individual area A_j by means of intensity. The area of the band belonging to the product was named A_1 and all following A_j, $j \in [2, n]$, ending with the maximum number of bands n.

The amount of product m_{P1F},

$$m_{P1F} = P_{P1F} \cdot V_F \cdot c_{PtotF}, \quad (2)$$

was calculated from total protein concentration c_{PtotF}, the eluate volume V_F and the previously estimated product purity P_{P1F}.

The mass recovery of the target protein R_{P1E},

$$R_{P1E} = \frac{m_{P1F}}{m_{P1C}} = \frac{P_{P1F} \cdot V_F \cdot c_{PtotF}}{P_{P1C} \cdot V_C \cdot c_{PtotC}}, \quad (3)$$

was estimated from the protein mass m_{P1F} in the eluate fraction divided by the protein mass m_{P1C} applied from conditioning tank C.

2.6 Process optimization

Design of Experiments

DoE is a method for planning and evaluation of experimental series. The impact of multiple interrelated input variables (factors) x_i on one or more output variables (responses) y_j can be investigated simultaneously. The utilization of DoE in biotechnology expands rapidly and many successful applications have been performed, e.g. Mandenius et al., 2008, Loegering et al., 2011, Fricke et al., 2013. MODDE® 9.0 software (MKS Umetrics AB, Sweden) was used as a state of the art toolbox for data processing and visualization.

As shown in fig. 4, five procedural factors, x_1, x_2, …, x_5, have been investigated in the downstream process.

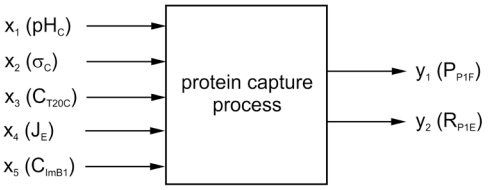

Fig. 4. Protein capture factors and responses. x_1: pH-value pH_C, x_2: salt concentration σ_C, x_3: Tween®20 concentration C_{T20C}, all adjusted in conditioning vessel and running buffer, x_4: EBA flux J_E through the column and x_5: Imidazole concentration C_{ImB1} of running buffer

The effects of the studied factors on protein purity P_{P1F} (y_1) of the eluted fraction and mass recovery R_{P1E} (y_2) of the chromatographic step were regarded as the most evident quality responses for process evaluation.

Split optimization strategy

To save time and chemicals during the required screening experiments an optimization strategy for an analytical scale was conducted with an AEKTA™purifier 100 (GE Healthcare, Germany). It reduced the experimental effort of 20 screening experiments with the original process from 24 days to 4 days with the FPLC-setup.

3. RESULTS AND DISCUSSION

3.1 AEKTA™ screening experiments

2^{5-1} factorial screening

A 2^{5-1} fractional factorial screening design schedules 16 experiments with different factor settings augmented with 4 center point experiments at intermediate factor levels.

Fig. 5 shows the design region for a 2^{5-1} fractional factorial screening. All factors were investigated at a low and at a high level. This design scheduled 16 experiments with different factor settings augmented with 4 center point experiments at intermediate factor levels.

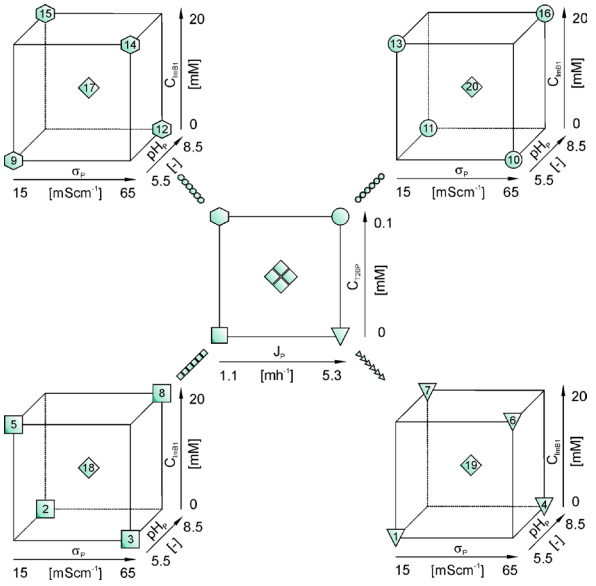

Fig. 5. Design region of a 2^{5-1} fractional factorial screening. The type of symbol on the square denotes the level of factor J_P and C_{T20P}. The position of the symbols on the cubes indicates the level of factors ϑ_P, pH_P and C_{ImB1}.

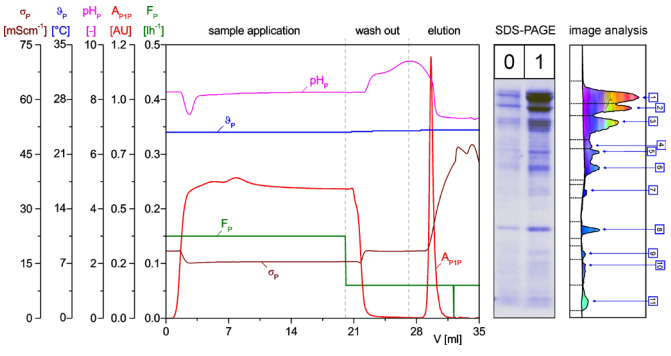

Fig. 6. Time courses of AEKTA™ process variables and associated SDS-PAGE analysis of selected screening runs. σ_P: conductivity, ϑ_P: temperature, pH_P: pH-value, A_{P1P}: UV absorption at column outlet, F_P: chromatography flow rate

As an example fig. 6 shows the result of one screening run. A Chromatogram of selected process variables is displayed. An SDS-PAGE of the applied sample (0) as well as the eluted fraction (1) and the resulting chromatogram rendered with TotalLab Quant® software including the indication of the detected bands is pictured right hand. It features a high and defined peak in the adsorption signal during elution which hypothesizes a good chromatographic result. But SDS-PAGE revealed a bad quality of the eluate with some content of product but many impurities, as confirmed by the image.

An application of MODDE® 9.0 software (MKS Umetrics AB, Sweden) using a multiple linear regression (MLR) with a confidence interval of 90 % for an investigation of model coefficients identified the variables J_P and C_{T20P} as non significant. It is supposed that a flux influences the porosity of an expanded bed but as obviously not that of a packed bed. The detergent Tween®20 assumedly influences cell-cell interactions which were not present when using fermentation supernatant. In this particular case DoE was able to approve the hypothesis by eliminating these parameters from the model.

Result of this screening was a narrower search space for a further and more detailed investigation of three significant factors for pH-value pH_P, Imidazole concentration C_{ImB1} and salt concentration σ_P, in a full factorial pre-optimization with the same FPLC-setup.

Central Composite Face Centered (CCF) pre-optimization

A CCF design scheduled 14 runs with different factor settings complemented with 3 center point experiments at an intermediate factor level.

A calculated optimal factor setting was located at pH_P of 7, and C_{ImB1} of 8 mM. This setting would result in a predicted purity P_{P1P} of 82 % and recovery R_{P1P} of 85 % per run.

3.2 Final in situ EBA optimization experiments

During the pre-optimization pH_C and C_{ImB1} were determined as the most valuable factors for a further investigation in a preparative EBA scale. In addition a promising EBA optimization design for these factors could be defined as follows.

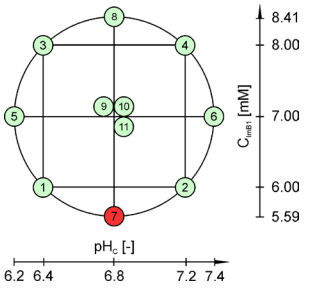

Fig. 7. CCC design for EBA optimization

The final optimization of the product purity P_{P1F} and the product recovery R_{P1E} was performed while operating the original integrated bioprocess with ISPR by the EBA. The variation of the significant factors pH_C and C_{ImB1} was proposed by a Central Composite Circumscribed (CCC) design with three additional center points as illustrated in fig. 7.

The screening range for pH_C was chosen from 6.2 to 7.4 and for C_{ImB1} from 5.59 mM to 8.41 mM. 11 experiments were necessary to achieve this design. A sequential cultivation, shown in fig. 8, supplied the EBA plant with raw material for each optimization run. EBA optimization conditions with factor values which have actually been set and purification results are listed in tab. 1.

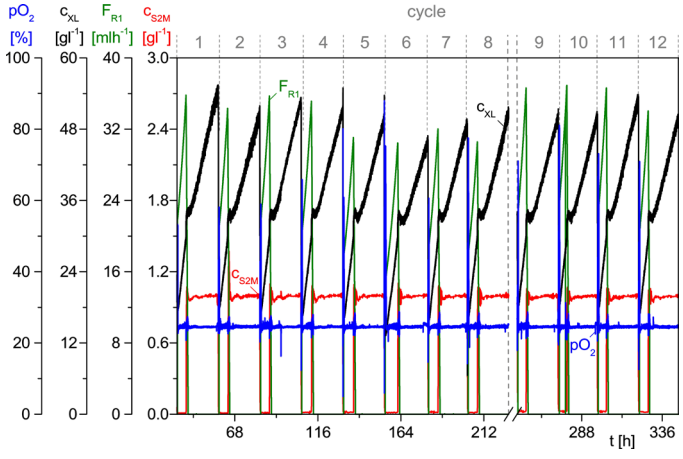

Fig. 8. Time course of a sequential production process of D1M1 in 12 reproducible cycles. pO_2: dissolved oxygen tension, F_{R1}: glycerol feed rate, c_{S2M}: methanol concentration, c_{XL}: cell density.

Tab. 1: EBA factor settings, coded values (in parentheses), and screening results of the CCC design for each run k

Run k	pH_{Ck} [-]		C_{ImB1k} [mM]		P_{P1Fk} [%]	R_{P1Ek} [%]
1	6.40	(-1)	6.00	(-1)	72.5	31.8
2	7.01	(+1)	6.00	(-1)	92.0	20.4
3	6.45	(-1)	8.00	(+1)	79.0	14.4
4	7.17	(+1)	8.00	(+1)	85.5	22.6
5	6.23	(-1.41)	7.00	(0)	74.1	57.5
6	7.21	(+1.41)	7.00	(0)	84.8	32.9
7	6.81	(0)	5.59	(-1.41)	87.3	50.6
8	6.87	(0)	8.41	(+1.41)	95.7	19.6
9	6.84	(0)	7.00	(0)	86.0	52.5
10	6.82	(0)	7.00	(0)	80.4	40.2
11	6.87	(0)	7.00	(0)	82.2	41.5

The resulting models for the product purity P_{P1F} [%],

$$P_{P1F} = a_0 + a_1 \cdot pH_C + a_2 \cdot C_{ImB1} + a_{12} \cdot pH_C \cdot C_{ImB1} + a_{22} \cdot C_{ImB1}^2 \quad (4)$$

with the coefficients

$a_0 = -736.32$ %, $a_1 = 155.47$ %, $a_2 = 69.41$ % mM^{-1},
$a_{12} = -20.67$ % mM^{-1}, $a_{22} = 5.29$ % mM^{-2}

and the EBA-recovery of product R_{P1E} [%],

$$R_{P1E} = b_0 + b_1 \cdot pH_C + b_2 \cdot C_{ImB1} + b_{11} \cdot pH_C^2 + b_{22} \cdot C_{ImB1}^2 \quad (5)$$

with the coefficients

$b_0 = -4210.61$ %, $b_1 = 1197.12$ %, $b_2 = 52.97$ % mM^{-1},
$b_{11} = -87.23$ %, $b_{22} = -4.55$ % mM^{-2},

are valid in the investigated optimization space.

Fig. 9 displays the coefficient plots (with scaled and centered coefficients for the models), the corresponding summaries of fit and the contour plots for both responses. The p-values for regression 0.003 (P_{P1F}) and 0.012 (R_{P1E}) support the analysis. The models show no lack of fit with p_{lof} -values of 0.74 and 0.90 respectively.

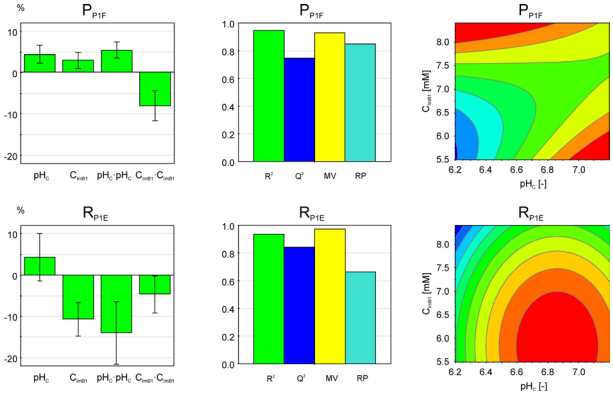

Fig. 9. Final EBA single optimization results

With little likelihood different response functions with non-identical model structures deliver the same optimal settings. Accordingly, two unequal optimal parameter sets are available.

At the end only one factor setpoint for each parameter can be transferred to the process. A factor setting which leads to a good purity may result in a bad recovery and vice versa. That is, why a compromise needs to be found were both responses scale as good as possible.

To find this combined optimum a total response y_{nP1E},

$$y_{nP1E} = \frac{P_{P1F} - P_{P1F\,min}}{P_{P1F\,max} - P_{P1F\,min}} + \frac{R_{P1E} - R_{P1E\,min}}{R_{P1E\,max} - R_{P1E\,min}}, \quad (6)$$

was introduced which is normalized between 0 and 2. A MATLAB® script has been develop to confirm the idea of the combined response y_{nP1E}. The results obtained matched with the results obtained from the MODDE® software. Fig. 10 shows the surface plot of y_{nP1E} as it appears in the investigated search space. The approach proposed an optimal pH value of 6.88 and an optimal Imidazole concentration of 5.59 mM.

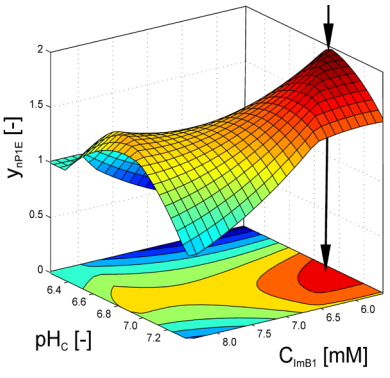

Fig. 10. Combined response y_{nP1E} of EBA optimization

The factor adjustment of run 7, a star point of the CCC optimization design in fig. 2, lies close to the derived optimum. The process settings, the result of the cultivation cycle, and corresponding EBA capture are shown in fig. 11 and tab. 2.

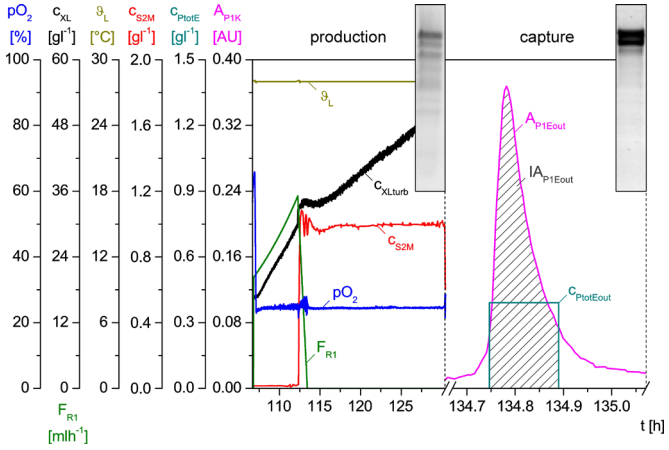

Fig. 11. Optimized two stage integrated bioprocess belonging to run 7 of the CCC design. pO_2: dissolved oxygen tension, F_{R1}: flow rate of glycerol stock, c_{XLturb}: cell density, estimated from turbidity measurement, ϑ_L: cultivation temperature, c_{S2M}: methanol concentration, $c_{PtotEout}$: total protein concentration in elution collector, A_{P1Eout}: UV absorbance of D1M1, measured at column outlet, IA_{P1Eout}: integral of A_{P1Eout}

Tab. 2: Process settings and results of optimization of an integrated bioprocess

ϑ_L	28.0	[°C]	J_E	200	[cmh^{-1}]
pH_L	5.5	[-]	C_{ImB1}	5.6	[mM]
c_{S2M}	1.0	[gl^{-1}]	c_{P1F}	0.3	[gl^{-1}]
pH_C	6.8	[-]	m_{P1F}	0.2	[g]
C_{T20C}	0.1	[mM]	P_{P1F}	87.3	[%]
σ_C	35.0	[$mScm^{-1}$]	R_{P1E}	50.6	[%]

To confirm this single optimization result a quasi robustness test was performed in six cycles of an integrated bioprocess without variation in the factor setpoints compared to the process shown in fig. 11.

Fig. 12 shows a SDS-PAGE of each of the six cycles, where average purities and recoveries comply well with the optimization results.

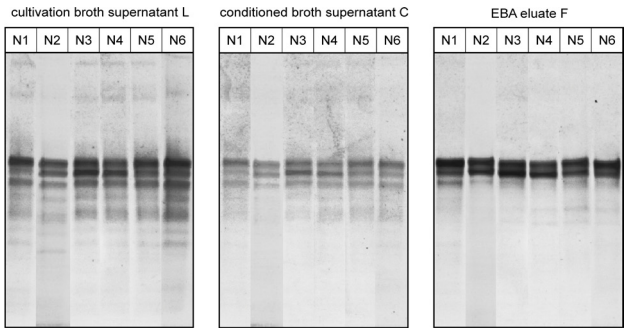

Fig. 12. SDS-PAGE of the repeated optimized integrated bioprocess

4. CONCLUSION

The integration of an EBA chromatography as primary downstream operation into a sequential production process of the vaccine candidate D1M1 enables a fast and reproducible single-step in situ separation of the recombinant protein from cells, host proteins and media components. Results obtained from scale-down screening experiments show that a pre-determination of significant factors and a restricted screening range simplifies and accelerates an optimization procedure in an integrated preparative scale.

5. ACKNOWLEDGEMENT

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