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Advanced automation strategies for reliable, reproducible cultivation runs in a sequential/parallel operated multi-bioreactor plant

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Abstract: A designed multi-bioreactor plant, comprising a 5 l cell breeding BIOSTAT® Bplus system and a sixfold 1 l BIOSTAT® Qplus system, was equipped with different, interlinked automation structures for systematic process controlling and reliable optimization procedures. Benefits obtained were time saving, assurance and improvement of quality and cost reduction in bioprocess production. In this report the concept for moving beyond the state-of-the-art by creating a fully automated multi-bioreactor plant is explained. The efficiency of the approach is demonstrated by investigation relating the influence of the dissolved oxygen tension pO_2 in combination with the methanol concentration c_{S2M} in Malaria vaccine production processes with *Pichia pastoris*.

Keywords: multi-bioreactor plant, fully automated cultivations, bioprocess control, Design of Experiments, Malaria vaccine production, *Pichia pastoris*

1. INTRODUCTION

Current pilot scale process development is often inefficient as companies use traditional methods to improve their operations. This leads to lengthy, expensive development procedures, as multiple runs must be carried out over many months before an acceptable end point is reached. Due to steadily increasing cost pressure for the production of recombinant proteins in the European pharmaceutical and biotechnology industry process development should be executed in a more systematic way for generating well defined product and process characteristic information.

A desired goal of the PAT framework of the FDA is to design and develop well understood processes that will consistently ensure a predefined quality at the end of the manufacturing process. In this regard the initiative encourages the use of process analysers, process control tools, and multivariate tools for design, data acquisition and analyses. To incorporate these ideas into a new biotechnological process a multi-bioreactor plant was designed to meet the outlined requirements (Fricke et. al, 2011; Fricke et. al, 2013). The plant enables fully automated Design of Experiments (DoE) optimization procedures via extended PAT solutions.

2. PROGRESS BEYOND STATE-OF-THE-ART

2.1 Expression system Pichia pastoris

A *Pichia pastoris* strain KM71H has been transformed with sequences of *Plasmodium falciparum* proteins Apical Membrane Antigen 1 (AMA1) and Merozoite Surface Protein 1 (MSP1) by the Biomedical Primate Research Centre (Faber et al., 2013), allowing secreted expression of artificial potential Malaria vaccines.

The scheme of the cloning process is shown in fig. 1.

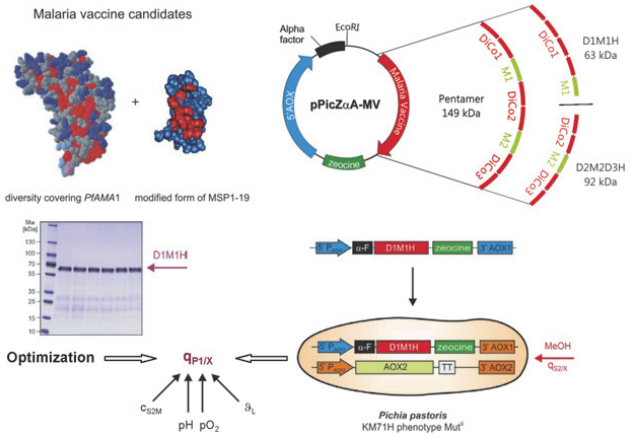

Fig. 1. Construction of a *Pichia pastoris* production strain.
Sources crystal structures: Remarque et al., 2008, Pizarro et al., 2002

The investigation described herein was conducted with the D1M1H protein with a theoretical size of 63 kDa.

Finally the goal is to manipulate the cell-specific target protein production rate $q_{P1/X}$ by varying process parameters, e.g. methanol concentration c_{S2M}, pH-value, dissolved oxygen tension pO_2 and liquid temperature ϑ_L for achieving an optimal volumetric productivity PRD.

2.2 Optimization effort

As shown in fig. 2 an optimization procedure follows a three step method. The experimental objective of the first step *screening* is to identify process parameters with a significant influence on the chosen quality criterion.

The *optimization* procedure provides a factor setting for an optimal Malaria vaccine productivity, in this case. The last step *robustness* testing investigates the sensitivity of the response to small changes in factor setting.

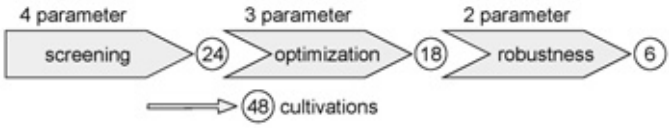

Fig. 2. Three stage DoE optimization strategy.

At all 48 cultivations have to be executed for an optimization study with an average length of 24 h each, exemplary shown in fig. 2. In this context a multi-bioreactor plant, consisting of two BIOSTAT® systems (Sartorius Stedim Biotech, Germany) for fast DoE-optimization was designed.

2.3 Experimental setup

The multi-bioreactor approach, as shown schematically in fig. 3, deals with the limitations of the status quo of lengthy, costly development procedures. In consideration of this bottle neck a two reactor system, where the cultivation of cells and the production process is decoupled, was designed.

The effectiveness is enabled by using a 5 l BIOSTAT® Bplus in combination with a sixfold 1 l BIOSTAT® Qplus reactor system. The 5 l cell breeding bioreactor provides consistent inoculum for the six 1 l screening bioreactors in a cyclic manner.

The seven bioreactors are highly instrumented with probes for measurements of cell density, MeOH concentration and O_2 and CO_2 offgas. At all 36 scales for accurate process balancing and automated processing are installed.

The plant has an industrial oriented sterile design with sterilizable transfer valves (GEMÜ GmbH & Co. KG, Germany) and quick connectors (Stäubli Tec-Systems GmbH, Germany).

By piloting the pressure valves and controlling pumps fully automated inoculation, cultivation, harvest and refresh operations as well as controlled media component additions were already realized.

2.4 Extended automation software solutions

The implementation of a fully automated two stage process strategy demands the organization of several tools. The overall installation, the digital pathways and the interaction between software and hardware devices are schematically illustrated in fig. 4.

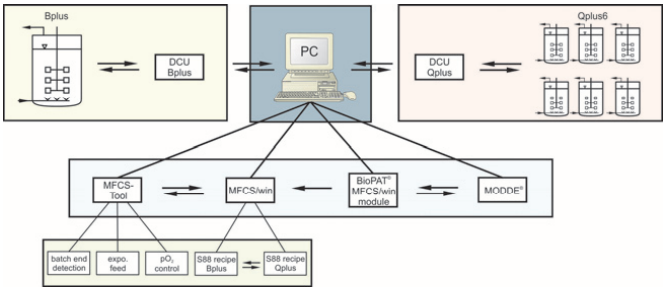

Fig. 4. Organization of extended software solutions in bioprocesses.

Main parts are the PC-system as well as the Digital Control Units (DCU) for the Bplus and the Qplus system.

The DCUs are operated remote-controlled with the SCADA-system MFCS/win (Sartorius Stedim Biotech, Germany), connected via an Ethernet switch to the PC-system.

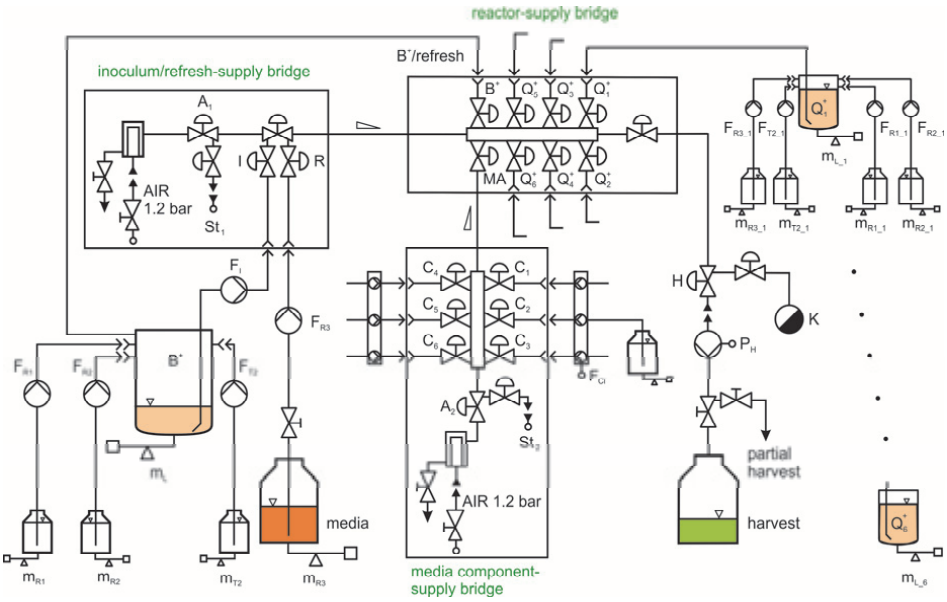

Fig. 3. Concept of a two-stage multi-bioreactor plant.
control valves: A_j: air supply j; B^+: cell breeding; C_i: media component i; H: harvest; I: inoculation; MA: media addition; Q_k: screening reactor k; R: refresh *sterilization devices*: K: condenser; St_j: steam supply j

For the realization of complex automation tasks the in-house designed visual basic MFCS-Tool for on-line batch end detection, exponential feeding processes and pO_2-control, MFCS/win with the ANSI/ISA-88.01 recipe structures for the Bplus and the Qplus as well as a newly developed BIOPAT® MFCS/win DoE module (Sartorius Stedim Biotech, Germany) is installed.

The data exchange between the MFCS-tool and MFCS/win guarantees fully automated cultivation processes. The DoE procedure is planned and conducted with the software tool MODDE® (MKS Umetrics AB, Sweden) and the BIOPAT® MFCS/win DoE module. A linking of these enables an automated setup of designed experiments in MFCS/win

3. APPLICATION FIELD IN BIOPROCESSING

3.1 Experimental design

In fig. 5 the experimental design of the conducted sequential/parallel cultivation strategy is shown. The cell breeding procedure follows a typical three step *Pichia pastoris* cultivation strategy with a batch and a fed batch procedure on glycerol c_{S1M}, and an induction phase on methanol c_{S2M}.

The repeated process starts with a cell density c_{XL} of 5 gl^{-1} in the batch phase, where the cells grow with the maximum specific growth rate μ_{1max}.

During the whole process, the dissolved oxygen tension pO_2 is controlled on a given set point of 25 % by cascading the stirrer speed N_{St}.

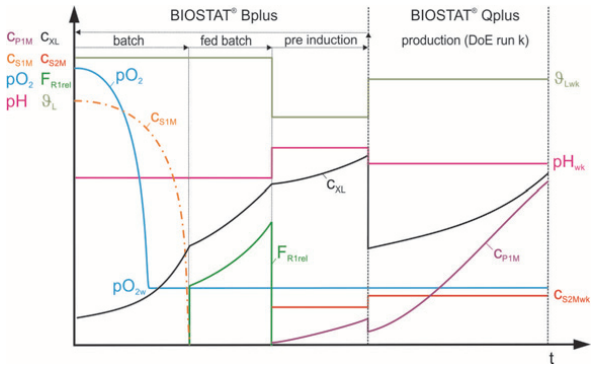

Fig. 5. Experimental Design for the two-stage sequential/ parallel cultivation runs.

After an on-line batch end detection, a substrate limited fed batch phase on glycerol starts with an exponential increase of glycerol feed F_{R1} to control the cell specific growth rate μ_{1w}. The glycerol feed ends automatically at a previous defined cell density, estimated online from an in-line turbidity measurement.

In the pre induction phase the methanol concentration c_{S2M} is controlled to a given set point of 0.5 gl^{-1} for about 12 h. The resulting metabolic change of the cells avoids expression delays in subsequent production phases in the Qplus system.

A defined volume of cell suspension is then transferred consecutively into the emptied six screening reactors, where the process parameters are varied for DoE investigations. After harvest and transfer procedures the rest of cultivation cell broth in the cell breeding reactor is refreshed for a new cycle.

The at-line measurement of the target product expression is realized by linking monitoring systems to the existing process. The used HPLC- and SIA-methods are explained previously (Pohlmann et al., 2011).

The verification of the functionality could be already proved and is described elsewhere (Fricke et al., 2013).

3.3 Sequential cell breeding cultivations

Fig. 6 shows four nearly identical cell breeding cycles in the BIOSTAT® Bplus system. As explained before, each cycle starts with a glycerol batch (S1, unlimited), followed by a fed batch (S1, limited) and a pre induction on methanol (S2, unlimited).

The four cycles show comparable courses for the cell density. In addition the cell breeding cultivation process can be claimed as reproducible by obtaining the plotted process factors.

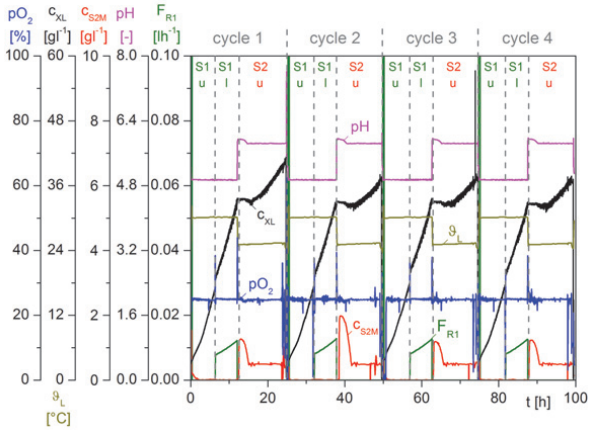

Fig. 6. Cell breeding cycles in the BIOSTAT® Bplus reactor. c_{XL}: cell density, calculated from turbidity measurement, ϑ_L: cultivation temperature, pO_2: dissolved oxygen tension, c_{S2M}: methanol concentration, F_{R1}: glycerol feed rate

3.4 Reproducible production cultivations

Fig. 7 shows four Malaria vaccine production runs with equal process parameter settings. The runs were conducted in different screening reactors and two cycles at all.

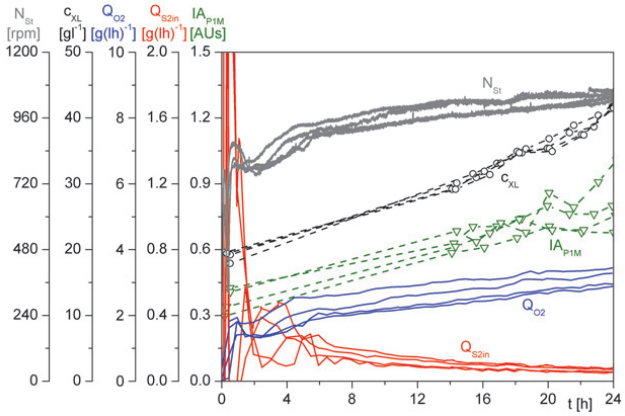

Fig. 7. Parallel production runs with identical factor settings. c_{XL}: cell density, reconstructed from cell dry weight (○), IA_{P1M}: integral of target protein UV absorption (▽), N_{St}: stirrer speed, Q_{O2}: oxygen supply rate, Q_{S2in}: methanol supply rate

Small variations in the product level and in the oxygen and methanol supply rate courses could be obtained. However the time courses show nearly same slopes, which indicate a similar behaviour of *P. pastoris*, related to PRD, oxygen and methanol uptake respectively. The time courses of the cell densities fit very well for all cultivations.

The fact of comparable start values for c_{XL} and IA_{P1M} verifies the sequential/parallel cultivation approach.

4. INVESTIGATION IN PROCESS OPTIMISATION

4.1 Scientific background

A common feature of the induction phase is that the methanol concentration in the bioreactor must be carefully monitored. Stratton et al. stated that high levels of methanol in cell broth at high oxygen supply can result in an abundance of formaldehyde in *Pichia pastoris* culture. High formaldehyde concentrations result in high levels of hydrogen peroxide (Jahic et al., 2002), which can cause cell damages.

In this regard the experiments, described in the following, should investigate the influence of the dissolved oxygen tension pO_2 in combination with the inductor methanol c_{S2M} on Malaria vaccine production.

4.2 Performance index "Productivity"

The conducted experiments were evaluated by calculating the target secretion productivity PRD_k,

$$\begin{aligned} PRD_k = & [V_{Lkn} \cdot c_{P1Mkn} \cdot (\rho_Z - \alpha_{Z/X} \cdot c_{XLkn}) \\ & - V_{Lk0} \cdot c_{P1Mk0} \cdot (\rho_Z - \alpha_{Z/X} \cdot c_{XLk0}) \\ & + \sum_{j=1}^{n} \Delta V_{Skj} \cdot c_{P1Mkj} \cdot (\rho_Z - \alpha_{Z/X} \cdot c_{XLknj})] \\ & \cdot [V_{Lkn} \cdot \rho_Z \cdot (t_{kn} - t_{k0})]^{-1}, \end{aligned} \quad (1)$$

of each cultivation run k. Data, taken at the end t_{kn} and at the beginning t_{k0} of each screening run k, and the amount of produced protein taken out by each sample time t_{kj} in between, was considered.

The target protein concentration c_{P1Mk},

$$c_{P1Mk} = P_{P1Mk} \cdot c_{PtotMk}, \quad (2)$$

was calculated off-line from target protein purity P_{P1Mk} determined via SDS-PAGE analysis, evaluated with Quantity One software (BioRad Laboratories, Germany), and total protein concentration c_{PtotM} of the culture supernatant, determined using Bradford Kit (BioRad Laboratories, Germany).

4.3 Preliminary investigation

Pre-tests were conducted cyclically in two blocks in six parallel screening runs, where the pO_2 was varied in between 1 % (O_2 transfer limited) to 45 %. For tracing pO_2 courses at low setpoints Oxygold G probes (HAMILTON Bonaduz AG, Suisse) were used.

Time courses for the at-line measured UV absorption A_{P1M}, cell density c_{XLturb}, pH-value, dissolved oxygen tension pO_2, liquid volume V_L, cultivation temperature ϑ_L and the determined productivity PRD (dashed boxes) of the 12 production runs are shown in fig. 8.

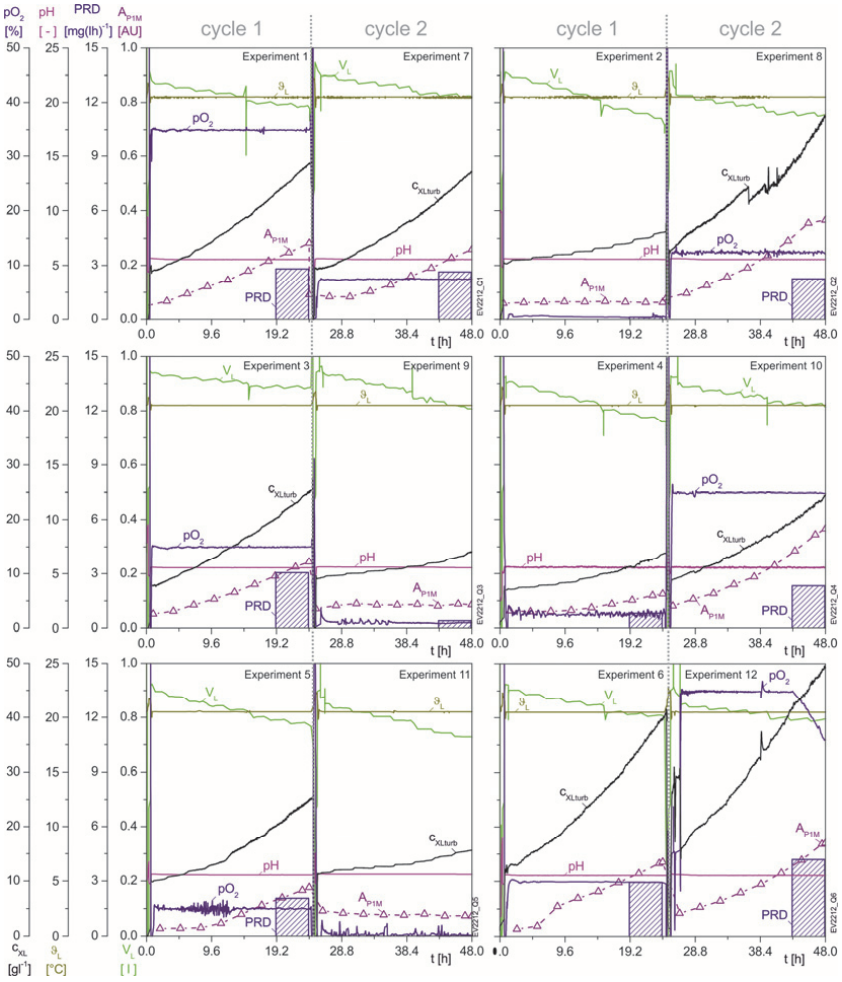

Fig. 8. Time courses of 12 production runs in the Qplus with different pO_2. c_{XLturb}: cell density, calculated from turbidity measurement, A_{P1M}: target protein UV absorption (Δ), measured at-line with HPLC, ϑ_L: cultivation temperature, pO_2: dissolved oxygen tension, V_L: liquid volume

In these experiments only the pO_2-setpoint was changed. The pH-value, the temperature ϑ_L, and the methanol concentration c_{S2M} were kept constant. The measured target protein concentration c_{P1M} was used to calculate the related performance index in (1). With a first view to the PRD significant differences are obtained.

Fig. 9 depicts the results in a state diagram. The PRD_k of cultivation k, target protein concentration c_{P1Mkn}, total protein concentration $c_{PtotMkn}$ and purity of the product P_{P1Mkn}, all measured in the final samples at t_{kn} in the media phase, show nearly same courses by varying the pO_2.

Above a dissolved oxygen tension of about 7 % the values reach already their maximum. This experiment is not a definitive statement about optimal expression conditions, since all operating parameters were kept constant, except for pO_2.

In the next step a two-parameter optimization procedure with pO_2 and c_{S2M} is required for having a deeper insight into target protein expression.

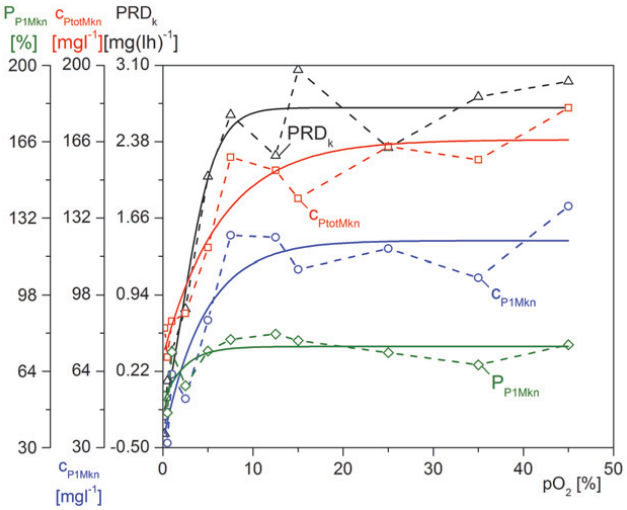

Fig. 9. State diagram of the preliminary tests.
P_{P1Mkn}: final product purity (◊), c_{P1Mkn}: final target protein concentration (○), c_{PtotMk}: final total protein concentration (□) all of cultivation run k

4.4 Optimization with Design of Experiments

After consideration of the pre-tests pO_2 was varied between 7 to 25 %. The centre-point experiments were executed at a pO_2 of 16 %. In addition the methanol concentration was varied between 1 to 10 gl^{-1}.

Table 1. DoE search domain and results

Exp. No.	pO_{2w} [%]	c_{S2Mw} [gl^{-1}]	PRD [$mg(lh)^{-1}$]
1	7	1	2.50
2	7	10	1.67
3	16	5.5	3.87
4	16	5.5	3.78
5	25	1	4.13
6	25	10	3.56

Tab. 1 shows the search domain and the results of the conducted DoE procedure. This experimental setup represents a screening procedure, described already in chapter 2.2.

The values of the centre-point experiments 3 and 4 show a high conformity.

Analysis and evaluation of data was done with the software program MODDE® [Eriksson et al., 2008].

Thereby, the quadratic polynomial model for PRD,

$$PRD = a_0 + a_1 \cdot c_{S2M} + a_2 \cdot pO_2 + a_{22} \cdot pO_2^2, \quad (3)$$

was fitted to the experimental data via Multiple Linear Regression (MLR) by generating the model coefficients,

$$a_0 = -0.030\ mg(lh)^{-1}, \quad a_1 = -0.078\ mg(lh)^{-1} \cdot lg^{-1},$$
$$a_2 = 0.43\ mg(lh)^{-1} \cdot \%^{-1}, \quad a_{22} = -0.011\ mg(lh)^{-1} \cdot \%^{-2},$$

where a_0 is the constant term, a_1 and a_2 describe the linear and a_{22} the quadratic dependencies.

The relation of observed vs. predicted PRD of the conducted investigations is plotted in fig. 10.

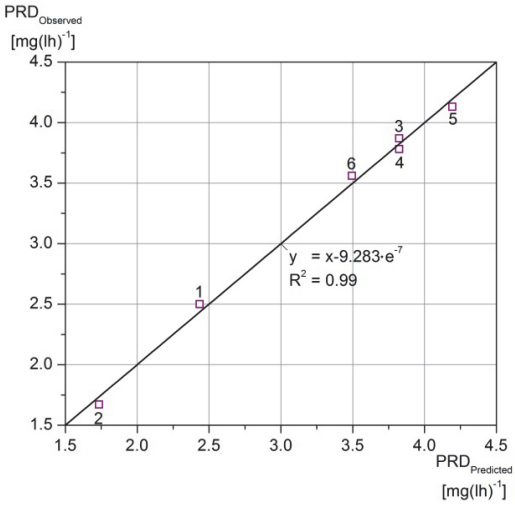

Fig. 10. Observed vs. predicted productivity.

A high value for the goodness of fit R^2 of 0.99 indicates a very well fitting of the model to the data observed. The goodness of prediction Q^2 of 0.94 is close to the determination coefficient R^2, indicating a low predicted variation.

High significance of the model can be obtained after consideration of ANalysis Of VAriance (ANOVA) (tab. 2) for a significance level of 95 %. The low *p*-value for regression (0.007) confirms the analysis. The model shows no lack of fit with a p_{lof}-value of 0.29.

Table 2. Analysis of Variance for Malaria vaccine PRD

	SS	DF	MS	*p*-value
Model	457.4	3	152.5	0.007
Residual	0.021	2	0.010	
Lack of fit	0.017	1	0.017	0.290
Pure error	0.004	1	0.004	
Total	459.5	5	0.919	

SS: sum of squares, DF: degrees of freedom, MS: mean square

Fig. 11 shows response surface plots of the DoE results. Thereby the circles represent the DoE and the squares the pretest results. The model is extrapolated up to 40 % pO_2 for visualizing the values measured in the pretest. For the factor methanol c_{S2M} only a small influence on the response in the defined search domain can be obtained.

However, especially in the pO_2 range of 1.0 to 7.5 % the designed model fits very well the experimental data, both the pretest as well as the DoE results for PRD. In the range of 10 to 25 % significant differences of the experimental values are obtainable. The quadratic model equation does not describe the expression behavior above a dissolved oxygen tension of 25 % so far.

To keep in mind, the screening DoE procedure should only expose significant influences of factors and factor settings to the response PRD.

So, in summary can be claimed that for optimal production of the potential Malaria vaccine D1M1H a pO_2 value above 16 % should be used in cultivations. Further investigations will be required to gain a deeper insight in optimal vaccine production related to optimal pO_2 and c_{S2M} factor settings.

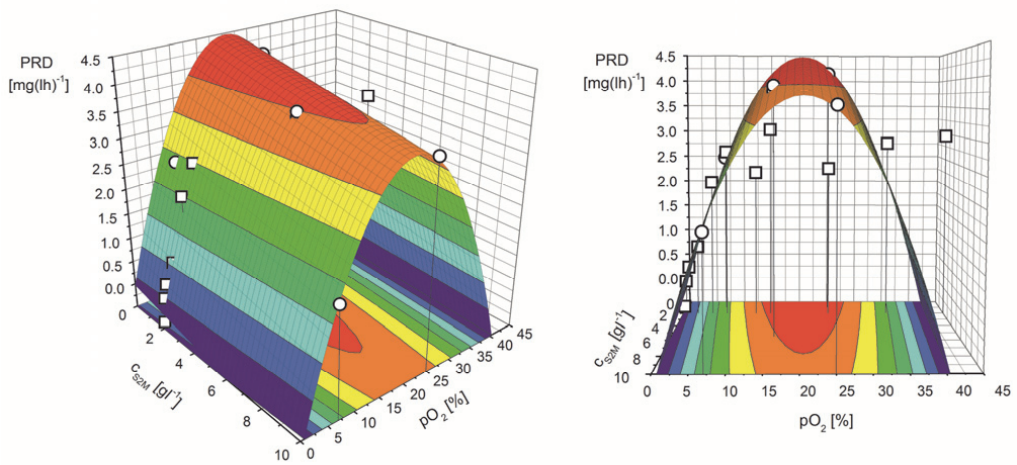

Fig. 11: DoE results for the expression of a potential Malaria vaccine. The circles represent the results from the DoE procedure, in addition the PRD values of the pretest are plotted as squares.

5. CONCLUSIONS

This article reports on a fully automated multi-bioreactor plant equipped with extended PAT for process observation and proper automation tools for effective determination of optimal expression conditions.

Investigations regarding the influences of dissolved oxygen tension pO_2 and methanol concentration c_{S2M} on the production of a potential Malaria vaccine D1M1H with *Pichia pastoris* were used as process examples. In a first screening application a promising research area for an optimal factor setting could be determined in only two cultivation approaches. At all 18 *Pichia pastoris* cultivations with different factor settings were conducted in 7 days.

The coupling of two bioreactor systems and the use of the sequential/parallel operation mode lead to significant time saving. Due to a high automation level, minimal manual process interventions are required. This supports reliability and reproducibility of conducted experiments.

In conclusion, the designed fully automated multi-bioreactor plant benefits a high cultivation run throughput. This leads to up scalable, cost-efficient approaches for kinetic as well as optimization studies in biopharmaceutical production processes (Fricke et al., 2011, Fricke et al., 2013).

6. ACKNOWLEDGEMENTS

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