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Model-based optimization of temperature and pH shift to increase volumetric productivity of a Chinese hamster ovary fed-batch process

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Industrial production processes, which utilize mammalian cells for the production of therapeutic proteins are routinely designed to include temperature and pH shifts. The process conditions are shifted away from growth promoting conditions towards a state of decreased metabolic activity. This results in the extension of the cultivation duration and therefore in a higher final product concentration. Although the correct timing of these shifts is essential for peak process performance, not many studies have been investigating this topic. In this work temperature and pH shift were optimized with a mechanistic model to increase the final product concentration in comparison to an established industrial fed-batch process. The major advantage of the mechanistic in comparison to a data-driven approach lies in the reduced number of experiments, which is needed. Therefore process development is faster, which decreases the time of the product to the market. Based on the optimization, an increased final product concentration of 14% was achieved in comparison to an already established industrial fed-batch process with the same cell line. Furthermore, the space-time-yield of the process did increase in comparison, resulting in a 20% increase of the final volumetric product concentration.

[**Key words:** Chinese hamster ovary; Model-based bioprocess optimization; Temperature shift; pH shift; Modeling; Biphasic cultivation]

With the expiration of patents on many top selling monoclonal antibodies, an increasing number of biosimilars will enter the biopharmaceutical market. An advantage of biosimilars over original biologics lies in their reduced developmental costs and therefore lower market price. There are however challenges associated with the development process of biosimilars. It includes cell line, upstream and downstream development, as well as Scale-up. The entire process can take up to eight years and is very complex, since a high quality duplicate of the original biologic is required (1). Process development does therefore not only face the challenge of ensuring a high product yield, but also a specific product quality. The most commonly applied tool for bioprocess improvement are Design of experiments (DoE). Since a statistical model is established based on the generated data, many experiments are required to establish a satisfactory model. This is particularly time consuming for cell culture processes, since the cells grow slowly and fed-batch processes usually last about two weeks. Another challenge lies in the extraction of process information from the generated data. Mechanistic models offer an alternative to this data driven approach. In contrast to data driven models, mechanistic models consist of physiologically meaningful terms. The plausibility of the determined model parameters can therefore be evaluated (2). Mechanistic models have been used to extract process information (3,4) and optimize process performance. Described strategies for process optimization include model based improvement of the feeding regime (5,6) and process design (7). One aspect of process design is the determination of ideal settings for process parameters such as temperature, pH and dissolved oxygen concentration (8). For mammalian cell culture processes temperature and pH are however routinely shifted from growth promoting conditions towards a state of decreased metabolic activity to extend the duration of the cultivation and therefore the final product yield. The beneficial effects of growing mammalian cells under hypothermic conditions have been reported for different products expressed in mammalian cells (9). The time point for the temperature shift is however not optimized (10–13) or optimized with an unstructured approach (14). There is only one systematic approach for this problem described. The timing of the temperature shift was optimized with a mechanistic model in order to increase the final product concentration in a batch process (7). The same concept is applicable for the decrease in pH, which extends the viability of the cells and has also been described for a batch process (8). The goal of this work is the optimization of pH and temperature shift to increase the final product concentration. This goal was achieved by setting up a model, which is able to describe fed-batch dynamics, since the majority of industrial Chinese hamster ovary (CHO) production processes are run as fed-batches. Based on the model simulations, the temperature and pH shift are improved in a similar fashion, as previously described (7). The main difference to this work is the optimization of the pH shift in addition to the temperature shift. Furthermore a fed-batch process is modelled rather

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than a batch process (7). Different parameter sets were established for each of the different cultivation conditions. These parameter sets reflect the influence of pH and temperature on the cells without describing the underlying principles. The temperature and pH shift is therefore modeled by a switch from one parameter set to the other. Different time points for the shift between parameter sets were then simulated for an industrial monoclonal antibody (mAb) production process to determine the ideal point for temperature and pH shift.

MATERIALS AND METHODS

Cell line, preculture and cultivation conditions A CHO suspension cell line, producing mAbs of the IgG type was cultivated in chemically defined medium. Preculture of the cells was performed in shake flasks at 10% pCO_2, 36.5°C and 143 rpm for at least 2.5 weeks to simulate the seed train of an industrial production process. The 3 L bioreactors (Labfors, Infors, Bottmingen, Switzerland) were inoculated to achieve an initial cell density of $3 \cdot 10^5$ cells·mL^{-1}. Dissolved oxygen (dO_2) (VisiFerm, Hamilton, Franklin, MA, USA) and pH (EasyFerm, Hamilton) were monitored with inline probes. pCO_2 was monitored with an offgas sensor (BlueInOne, Bluesens, Herten, Germany). dO_2, pCO_2 and pH were independently controlled as described in a previous report (15). pH control was performed with 0.5 M NaOH (F_{base}) and 3% H_3PO_4 (F_{acid}). The dO_2 (40%) and pCO_2 (10%) were kept constant by the addition of O_2 and CO_2, respectively. Feed A (F_a) was continually fed from day 4 and feed B (F_b) started at day 6 of the cultivation. Both feeds contain mixtures of different amino acids. Glucose (F_{glc}) was fed continually after its concentration dropped below 2 g L^{-1} to reestablish glucose levels of 2 g L^{-1}. Four cultivations under static process conditions were conducted to generate different parameter sets for the model. EXP1 and EXP3 were conducted at 36.50°C, while EXP2 and EXP4 were conducted at 33°C. The pH setpoint was 7.0 for EXP1 and EXP3 and 6.90 for EXP2 and EXP4. No temperature or pH shift was performed in any of these cultivations. The different setpoints do however reflect the process at different phases throughout a fed-batch process with pH and temperature shift. In the original process, which was improved, the temperature shift is performed at 40 h, while the pH is kept at 6.90 after 50 h.

Analytical methods Viable cell counts (VCC) and dead cell counts (DCC) were measured with the Cedex HiRes automatic picture analyzer (Roche, Mannheim, Germany). The metabolites glucose and glutamine (Gln) were measured with the Cedex Bio HT Analyzer (Roche). Concentration of the mAbs was measured by high performance liquid chromatography (Ultimate 3000, Dionex, Sunnyvale, CA, USA) with a protein A sensor cartridge (Applied Biosystems, Bleiswijk, Netherlands).

Model equations Ordinary differential equations were used to describe the relevant states (VCC, mAb, glutamine, DCC) of the cultivation. The majority of equations are described with standard Monod terms, which are predominantly used in mechanistic models. The DCC, for example, is described according to another published model (16).

$$q_{Gln} = \frac{[Gln]}{[Gln] + K_{Gln}} \cdot \frac{VCC.i}{[VCC] + VCC.i} \tag{1}$$

$$\mu = Y_{VCC/Gln.max} \cdot \frac{DCC.i}{[DCC] + DCC.i} \cdot q_{Gln} \tag{2}$$

$$k_d = k_{d.max} \cdot \left(\frac{k_\mu}{k_\mu + \mu}\right) \cdot q_{Gln} \tag{3}$$

$$\frac{d[V]}{dt} = F_{base} + F_{acid} + F_{glc} + F_a + F_b \tag{4}$$

$$\frac{d[VCC]}{dt} = (\mu - k_d) \cdot [VCC] - \frac{d[V]}{dt} \cdot [VCC] \tag{5}$$

$$\frac{d[DCC]}{dt} = k_d \cdot [DCC] - \frac{d[V]}{dt} \cdot [DCC] \tag{6}$$

$$\frac{d[Gln]}{dt} = -q_{Gln} \cdot [VCC] + \frac{F_a}{V} \cdot (Gln_{F_a} - [Gln]) - \frac{d[V]}{dt} \cdot [Gln] - 0.0023 \cdot [Gln] \tag{7}$$

$$\frac{d[mAb]}{dt} = q_{mAb.max} \cdot [VCC] - \frac{d[V]}{dt} \cdot [mAb] \tag{8}$$

The MATLAB (MathWorks, Inc., Natick, MA, USA) ODE solver 15s was used to solve these equations. Parameters were optimized using the genetic algorithm function of MATLAB with a population size of 250 and 100 generations to minimize the squared error. The boundaries for the optimization were based on physiologically plausible values from which the algorithm could deviate by 100%. For each parameter set 50 optimization runs were conducted and the coefficient of variation between the different estimates was calculated. The number of estimations was chosen to replicate the variability as accurately as possible, while simultaneously only allowing a reasonable time interval for the estimations. A further increase in the number of simulations did also not increase variation. The final parameter set for each condition was the calculated mean of all estimations for EXP1 and EXP3. EXP4 showed higher deviations of the simulated data to the experimental data. Since EXP4 represents the final stage of the cultivation process with temperature and pH shift, a good fit towards the end of the cultivation was ensured by picking a parameter set manually. The sensitivity of the parameters was investigated by varying parameter values by 5% and determining the effect on the states, which was previously described (17).

The deviation between experimental data (y) and simulated data ($\hat{y}$) was calculated by the normalized root mean square error (NRMSE):

$$\text{NRMSE} = \frac{\sqrt{\sum \frac{(\hat{y}-y)^2}{n}}}{\max(y) - \min(y)} \tag{9}$$

Calculation of the specific product formation rate The specific product formation rate (q_{mAb}) and integral viable cell density (IVCD) were calculated as follows:

$$q_{mAb} = \frac{[mAb]_2 - [mAb]_1}{[IVCD]} \cdot [IVCD] \tag{10}$$

$$IVCD = \frac{([VCC]_2 \cdot V_2 + [VCC]_1 \cdot V_1)}{2} \cdot (t_2 - t_1) \tag{11}$$

RESULTS AND DISCUSSION

A model, which is able to describe fed-batch dynamics under varying process conditions was developed to rationally improve the time point of temperature and pH shift. Different model parameter sets were established for each of the different static cultivation conditions. Simulations of different time points for temperature and pH shift were then performed to evaluate the influence on VCC and mAb concentration.

Development of the model The main requirement for the model structure is its ability to describe cell physiology under the four different process conditions of the fed-batch process. Furthermore, the model is built only on offline data, which is standardly available for cell culture processes to allow general applicability. Therefore a generic structure, describing the trajectories of VCC, mAb concentration, glutamine concentration and DCC was setup. Since the goal of the model is the increase of the final mAb concentration, the focus was on describing VCC and mAb concentration well. One phenomenon, which is commonly not modeled is the decrease in uptake rates, like glutamine, with increasing cell density (18). The Monod term VCC inhibition constant (VCC.i) describes this decrease. All model parameters were determined through static experiments at constant process conditions. The results of these static cultivations are shown in Fig. 1. It is apparent, that the decrease in temperature decreases the maximal VCC, while the decrease in pH slightly increases it. Since a high maximal VCC has to be achieved in order to maximize the mAb concentration, the pH shift is performed before the temperature shift in the dynamic experiment with changing process conditions. The data from EXP2 was not further used, since it represents the temperature shift before the pH shift. Therefore parameter sets were only generated for EXP1, EXP3 and EXP4. All model parameters for the respective states and their variation coefficients between the different identification runs are shown in Table 1. All calculated coefficients of variation show similar values to a previous report (3). Furthermore the generated parameters for EXP1 and EXP3 are mostly in good agreement with values published in literature (5,19). This does not hold true for EXP4, which is however not surprising, since most published models describe cells at physiological conditions and EXP4 was

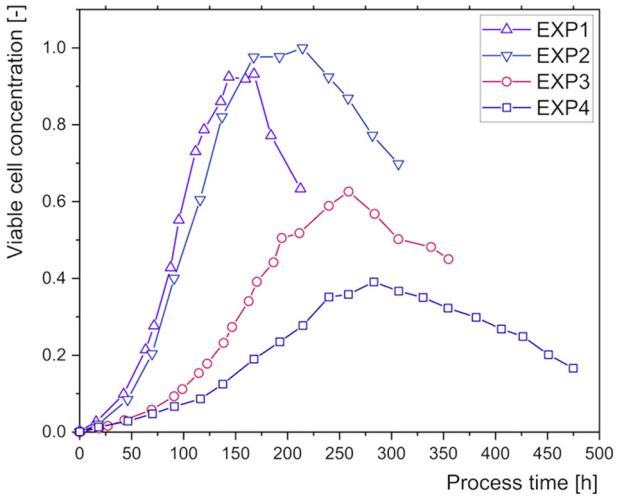

FIG. 1. VCC of the conducted static cultivations. Parameter sets were generated for all of the cultivations based on these runs.

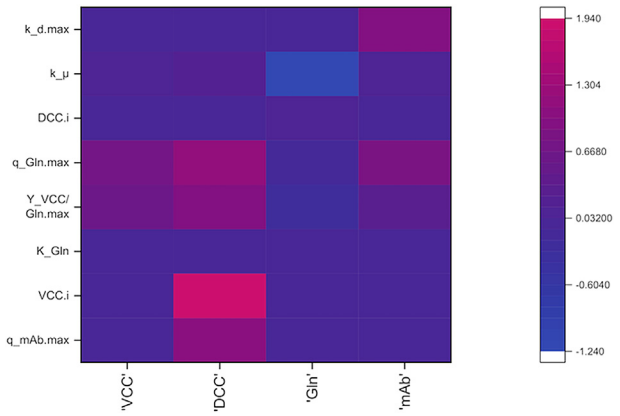

FIG. 2. Sensitivity analysis for the static run at pH 7.0 and 36.50°C (EXP1). Low values indicate a negative correlation between the parameter and the respective state, while positive values indicate a positive correlation. High values show a strong influence of the parameter on the respective state, while values close to zero show no influence of the parameter on the respective state.

run at non optimal conditions for cell growth. The highest coefficients of variation are those for the parameters glutamine saturation constant (K_{Gln}), maximum death rate ($k_{d.max}$), intrinsic death rate (k_{μ}) and DCC inhibition constant (DCC.i). To assess the impact of these parameters on the states, a sensitivity analysis was conducted (Fig. 2). K_{Gln} only influences the state glutamine to a limited extent. This is due to the fact, that this parameter only influences the simulated glutamine concentration at very low levels, which are usually not occurring in a fed-batch process. The parameters $k_{d.max}$ and k_{μ} are related to cell death and therefore influence the DCC most. Throughout the majority of the process, the DCC is however at very low levels and influences VCC levels only towards the end of the process. DCC.i is also related to cell death, but it shows very low sensitivity in regards to all simulated states. Overall it can therefore be said, that the parameters showing the highest variation coefficients do either not influence the VCC and mAb concentration or only influence it throughout a very limited time frame of the process. Therefore all parameters were estimated and none set to fixed values.

The NRMSE between the model simulations and experimental data for EXP1 and EXP3 is below 10% for all states. VCC as well as mAb concentration show even lesser errors of around 5%. EXP4 shows generally higher NRMSEs. VCC and glutamine concentration are still satisfactorily described with an error of approximately 8%, while the mAb concentration and DCC are described with errors of 17% and 17.72%, respectively. Although the overall error for the mAb concentration is high, simulated and experimental data fit well throughout the end (350–500 h) of the cultivation. This discrepancy can be explained by an adaptation phase from preculture to bioreactor conditions. Throughout the preculture process of approximately three weeks, the cells were kept at a pH of around 7.10 and at 36.50°C. In the bioreactor they were then introduced to a pH of 6.90 and 33°C. This represents an abrupt environmental change, which the cells respond to. Since an adaptation phase is not included in the model, the initially lower productivity results in the high overall NRMSE. Since EXP4 represents the final part of a cultivation with pH and temperature shift, the initial product concentration is not relevant for the improvement of the shifts. The offset throughout the early cultivation does therefore not interfere with the improvement of temperature and pH shift.

Improvement of pH and temperature shift After the establishment of the three parameter sets it was necessary to determine, whether or not an adaptation phase after the shift needs to be incorporated into the model. For this reason, a dynamic experiment was designed, which includes a pH and a temperature shift. The design was based on the available data, with the goal to increase the final mAb concentration in comparison to the original process (Fig. 5). Since the cultivation of the cells at pH 7.0 only results in a brief phase of faster initial growth (Fig. 1), the pH shift should be performed rather early. Day 4 (93 h) was chosen for the pH shift to allow a smooth and stable transition from preculture to bioreactor cultivation. The temperature shift was

TABLE 1. Determined parameter values and coefficients of variation.

Parameter	Parameter value			Coefficients of variation (%)			Literature value[a]
	EXP1	EXP3	EXP4	EXP1	EXP3	EXP4	
$k_{d.max}$ (h^{-1})	0.044	0.003	0.016	25.55	4.42	13.68	0–0.063
k_{μ} (h^{-1})	0.001	0.002	0.001	19.72	23.83	16.41	$6.9 \cdot 10^{-4}$–$1 \cdot 10^{-4}$
DCC.i (10^9 cells · L^{-1})	0.750	0.297	0.560	17.98	32.73	23.63	N/A
$q_{Gln.max}$ (mmol · 10^{-9} cells · h^{-1})	0.034	0.057	0.022	5.94	4.75	6.55	0.029–0.103
$Y_{VCC/Gln.max}$ (10^9 cells · $mmol^{-1}$)	1.755	1.173	1.344	1.70	4.24	0.91	13.70–70.21
K_{Gln} (mmol · L^{-1})	0.061	0.432	0.169	24.03	25.17	10.82	0.08–0.3
VCC.i (10^9 cells · L^{-1})	3.632	2.870	1.621	10.22	2.67	11.51	N/A
$q_{mAb.max}$ (g · 10^{-9} cells · h^{-1})	0.001	0.001	0.001	1.93	2.42	5.74	$1.25 \cdot 10^{-3}$

$k_{d.max}$, maximum death rate; k_{μ}, intrinsic death rate; DCC.i, DCC inhibition constant; $q_{Gln.max}$, specific maximum glutamine uptake rate; $Y_{VCC/Gln.max}$, yield of VCC from glutamine; K_{Gln}, glutamine saturation constant; VCC.i, VCC inhibition constant; $q_{mAb.max}$, specific maximum mAb production rate.

[a] Adapted from Frahm et al. (5) and Ulonska et al. (19).

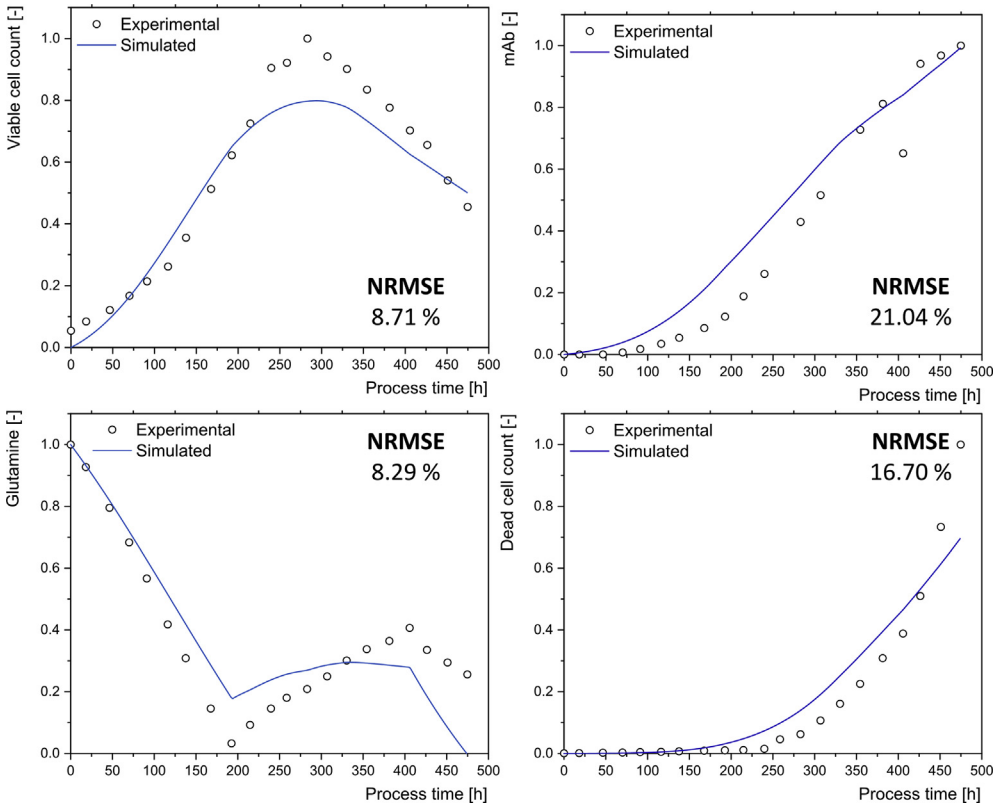

FIG. 3. Dynamic experiment. The pH Shift was performed after 12 h and the temperature shift after 105 h. Parameter sets were changed at 93 h for the pH and after 198 h for the temperature. Lines represent the simulated data and the symbols the experimentally obtained data.

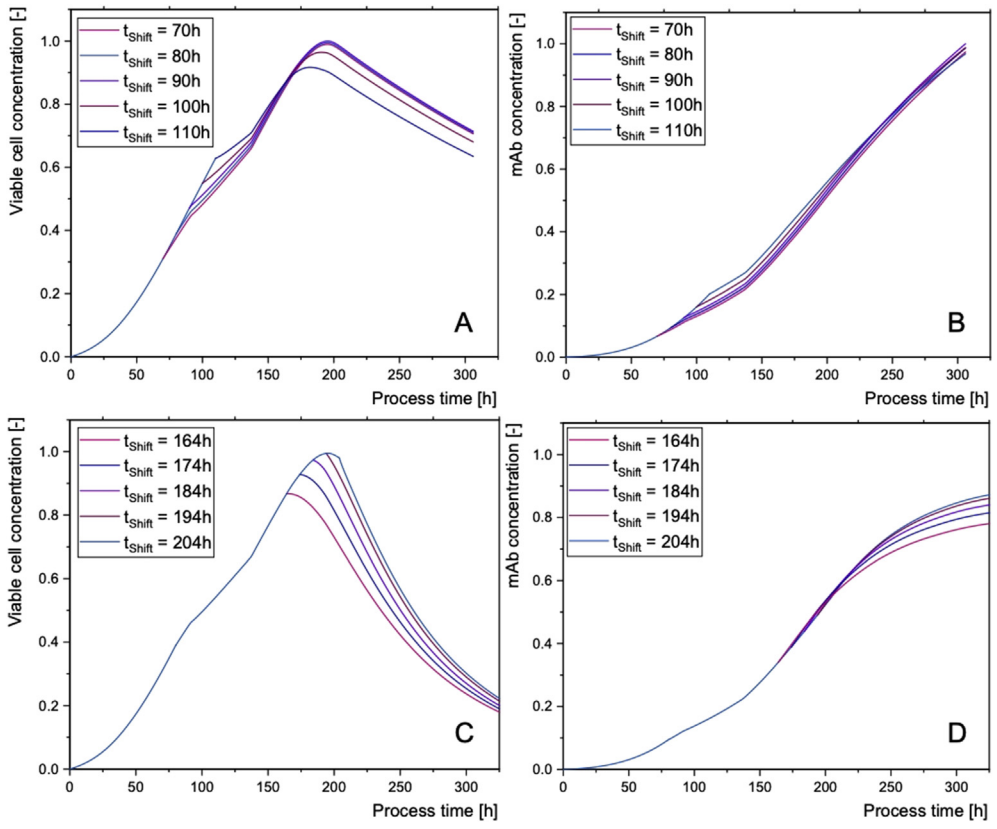

FIG. 4. Simulations to determine improved time points for pH and temperature shift. VCC trajectories (A, C) and mAb concentration (B, D) based on different time points for the pH (A, B) and temperature shift (C, D).

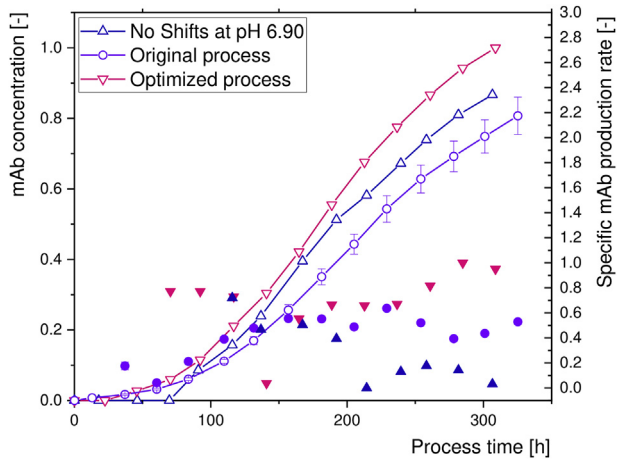

FIG. 5. Volumetric and specific productivity of the different process designs. Open symbols correspond to the volumetric productivity and closed symbols to the specific productivity. Comparison of mAb concentrations and specific mAb production rates from the optimized and original process and the static run at pH 6.90. Error bars for the original process are calculated from duplicates.

performed on day 8 (198 h), since the data from the static cultivation at pH 6.90 showed the highest VCC at that point. Based on the results from this dynamic experiment the adaptation time was determined by creating the best fit to the experimental data by minimizing the NRMSE between experimental and simulated data (Fig. 3). This resulted in an adaptation time for the pH shift of 72 h and for the temperature shift of 84 h. A transition phase between two parameter sets is more realistic physiologically, but since the model is designed as an easy to use tool for process optimization, this simplification was made. As shown in Fig. 3, the NRMSE of VCC, as well as mAb concentration is below 10%, which shows that the simplification is a good approximation.

An error in the feeding regime for glutamine in EXP3 resulted in the glutamine spike, which is visible in Fig. 3. For this reason, the NRMSE is at 25.27%. The VCC is however fitting very well, showing the robustness of the model to minor errors in the feeding regime. The DCC is also showing a high discrepancy between simulated and experimental data. Until 200 h, when the DCC is negligible, simulated and experimental data agree well. After this point and the shift to parameter set 3, the simulated DCC is higher than the experimental data. The reason for this is likely, that throughout EXP4 the cells are not at physiologically favorable conditions. It has been previously reported (20) that the induction of apoptosis or necrosis is dependent on the severity of the stress, which cells are exposed to, as well as the duration of the stress. In the case of EXP4 the process conditions can already represent mild stressful conditions and any further added stress throughout the cultivation could more easily lead to necrosis, rather than apoptosis. Since the dynamic cultivation was run at more physiological conditions, these cells could go more towards apoptosis than necrosis. This is in alignment with previous reports (21), who showed that different process strategies influence the amount of fragmented DNA, which is released from apoptotic cells. They showed that the same viability of the different processes did therefore not reflect the amount of apoptotic cells. The trypan blue exclusion assay for distinguishing between live and dead cells does however only classify necrotic cells as dead. Early apoptotic cells do not get stained, because the trypan blue cannot enter the intact cell membrane. Only throughout the final stages of apoptosis, secondary necrosis sets in and the apoptotic cells loose membrane integrity (22). Furthermore, necrotic cells, as well as late stage apoptotic cells eventually disintegrate and are not detected as dead cells anymore (20).

Fig. 3 shows that the VCC declines after approximately 200 h of the cultivation. Assuming that a viable cell stays either viable or dies, this decline should correspond to the measured DCC. To quantify the decline in viable cells, each VCC data point after 200 h was subtracted from the maximal VCC. A comparison of the DCC with those values shows that the amount of measured dead cells is 3.5 times lower than the calculated decreasing VCC values. The simulated DCC therefore matches the declining VCC better than the measured DCC values. A calculation of the NRMSE between the declining VCC and the simulated DCC results in an error of only 5.60%. The model could therefore be augmented to include disintegrated cells to more accurately describe cell death. Since the dead cells only influence the VCC at the very end of the process, it did not interfere with the goal to model VCC and mAb concentration well. Furthermore, analytics to confirm the disintegrated cells, like DNA measurements, would need to be available. Since the aim was to establish the model solely on standardized analytics in cell culture, no further modification of the model was performed.

To predict the time point for the shift in pH, simulations were performed at varying time points to assess the influence of different shifting times on VCC and mAb concentration (Fig. 4). Fig. 4A shows a decrease in maximal VCC, if the pH shift is performed after 90 h. There is only minor variation in the maximal VCC, if the pH is shifted between 70 and 90 h. It is therefore critical to not shift the pH too late, since an earlier shift only slightly influence the maximal VCC. Based on this result, the pH shift was set to 80 h, before different time points for the temperature shift were evaluated. Fig. 4C and D shows the outcomes for different time points, at which the temperature was shifted. Contrary to the shifting of the pH, an early shift to the lower temperature will result in decreased maximal VCC and therefore final mAb concentration. The temperature should ideally be lowered between 164 and 174 h.

Both model predicted time points match the manually determined shifting points from the dynamic run, showing that processes can be improved solely using experimental offline data. The benefit of the modeling approach lies however in the prediction of time windows, which will lead to similar process performance. This is in accordance with the quality by design principle (QbD), which requires extended process understanding for bioprocess development.

A comparison between an established industrial fed-batch process (original process), where the temperature is shifted earlier in the process, shows that the delayed temperature shift for the dynamic run increased the final product yield by 14% (Fig. 5). The increased mAb concentration of the dynamic run is in good agreement with the model predictions shown in Fig. 4C and D, which show that a later shift in temperature results in a higher final VCC and mAb concentration. By delaying the temperature shift, the highest mAb concentration is furthermore achieved 24 h prior to the original process, increasing the space-time-yield. Considering the mAb concentration of the original process at the time point the optimized process ended, an increase of 20% can be achieved. Fig. 5 also shows that the static experiment at pH 6.90 results in a similar final mAb concentration as the original process, further highlighting the necessity to time especially the temperature shift well. The specific mAb production rates (Fig. 5) underline this, since the early temperature shift decreases the specific mAb production rate initially. It further highlights the importance of the temperature shift, since the process, where the temperature is not lowered shows the lowest specific production rates throughout the final 100 h of the process.

In this work temperature and pH shift were improved to increase the final mAb concentration in comparison to an industrial fed-batch process. The improvement was based on predictions from a simple mechanistic model, where all parameters can be estimated from standardized cell culture analytics. The major advantage of the mechanistic approach is the reduced number of

experiments, which is needed. This speeds up process development and therefore decreases the time of the product to the market. A comparison between the improved and the original fed-batch process, where the temperature shift was performed early, shows that by delaying the decrease in temperature, an increased final product concentration of 14% can be achieved. The difference is calculated between the upper end of the error bar of the original process and the optimized process. A further benefit of the improved process is the increased space-time-yield, since the highest mAb concentration is achieved 24 h earlier in the improved process. When the mAb concentration of the original and optimized process are compared at the end point of the optimized process (309 h), an increase of 20% can be achieved.

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