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Transfer of Fed-Batch to Semi-Perfusion Process Using Automated Small Scale Bioreactors

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Abstract

Continuous processes offer advantages over batch approaches, including improved product quality, increased yield, and cost savings. This application note describes the use of the Ambr® 15 automated, high throughput microscale bioreactor system with perfusion mimic capabilities for transfer of a fed-batch process to perfusion using a semi-perfusion protocol. Cell culture conditions, including medium and feed composition, glucose concentration, and the cell-specific perfusion rate, were explored and optimized. The protocol was then transferred to an Ambr® 15 system and process performances were compared.

Using the same platform medium and proprietary feeds, a powerful but low resource approach to transfer an existing CHO fed-batch process to a perfusion process using design of experiment (DoE) studies and perfusion-specific handling was established. The suitability of the process for an Ambr® 15 system with perfusion mimic as scale-down model has been demonstrated.

Application Note is excerpted from "A Protocol to Transfer a Fed-Batch Platform Process into Semi-Perfusion Mode: The Benefit of Automated Small-Scale Bioreactors Compared to Shake Flasks as Scale-Down Model," published in Biotechnol. Prog. DOI 10.1002/btpr.2757 in August 2018.

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Introduction

The use of perfusion to improve efficiency of monoclonal antibody (mAb) production is of great interest to the biopharmaceutical industry. Perfusion-based processes can offer advantages in terms of product yield, manufacturing costs, and flexibility compared to fed-batch processes. Intensified processes are based on the continuous addition of fresh medium and removal of spent medium, which results in high cell concentrations and productivity in relatively small bioreactors.

Clones selected under fed-batch conditions, however, may behave differently when transferred to a perfusion process. To maintain the culture in a steady state, the cell-specific perfusion rate (CSPR) is one of the key process parameters that must be monitored and controlled. The glucose concentration and the cell bleed rate are also essential. In addition to these considerations, the process must be successfully scaled-up, and critical process parameters, such as temperature, pH, or the oxygen transfer coefficient, must be determined for process transfers.

Conventional bench-scale methods for development of intensified processes are both time consuming and expensive to execute. In addition, the cell retention step in a perfusion process needs to be precisely and specifically adjusted during process scale-up and automation. While alternating tangential flow (ATF) or tangential flow filtration (TFF) are widely used in the industry for cell retention, these approaches are not available in the scale needed to be compatible with small volume (± 10 mL) high throughput microbioreactors used for clone screening and the initial development of perfusion processes. A more time- and cost-effective approach uses a microscale bioreactor solution capable of mimicking perfusion to enable fast track optimization of media and culture conditions.

This application note describes development and optimization of a semi-perfusion protocol and transfer of the process from classical shake flasks to an automated Ambr® 15 single-use, microscale stirred tank bioreactor system with perfusion mimic protocols.

As a first step, a standard medium formulation with proprietary feeds used in fed-batch processes was adapted to the nutritional requirements of perfusion culture. Critical process variables including CSPR, glucose concentration, and cell bleed were investigated for the semi-perfusion operation. The optimized semi-perfusion process in shake flasks was transferred to the Ambr® 15 system, which also offered advantages for future testing of critical process parameters in early development of continuous processes due to pH and dissolved oxygen (DO) control. The possibility of using the automated small-scale bioreactor system as a screening tool and scale-down model with increased process control compared to shake flasks was also evaluated. Ultimately, this work represents an easy perfusion-based protocol using the Ambr® 15 system to reduce costs and maximize the benefits of process intensification.

Materials

Cell Lines and Medium

A DG44 CHO cell line expressing an IgG1 mAb was used in this study. Stock medium for the seed culture (SM) and production medium as basal medium for production (PM) were used as well as Feed Medium A (FMA) for macronutrients (e.g., glucose) and Feed Medium B (FMB) for micronutrients (e.g., amino acids). All medium and feeds used are chemically defined.

Seed Culture

The shake flask was incubated in an incubation shaker at 36.8 °C and 7.5% pCO_2 with a shaking rate of 120 rpm and 85% humidity. Cells were passaged for five times every three to four days until inoculation of the production culture was done.

Methods

Semi-Perfusion Shake Flask Studies

Semi-perfusion 125 mL shake flask cultures with a working volume of 25 mL were inoculated from the seed train with a starting cell concentration of 2.5 million cells/mL. Each medium exchange took place in a 24-hour rhythm. For sample analysis, shake flasks were moved to the biological safety cabinet, and 1 mL was removed after sufficient shaking. The remaining cell suspension was transferred into a 50 mL Falcon tube by pipetting and centrifuged at 500 xg for five minutes at room temperature. Following centrifugation, 2 mL of the supernatant were taken for IgG quantification, and the remainder was discarded by decanting. The cell pellet was resuspended with 25 mL of pre-warmed, fresh medium by gentle pipetting, transferred into the shake flask, and placed back into the incubator. To use the least amount of time possible, the total duration of each centrifugation step did not exceed a total time of 15 minutes.

Design of Experiment Study

The experimental setup and the analysis of the results were performed using MODDE® 12 design of experiment (DoE) software. A full factorial design was used to evaluate the influence of different medium compositions on the viable cell concentration (VCC) as a single and quantitative response using a quadratic regression model, with FMA (three levels) and FMB (three levels) as the controllable and qualitative mixture factors, and PM (nine levels) as the fill factor (to 100%).

Cell Bleed in Semi-Perfusion Experiments

The cell bleed in shake flasks was applied based on the defined conditions for each experiment and was calculated based on the daily VCC and the total volume of the culture (Vc). The calculated volume of cell suspension was removed by manual pipetting and replaced with fresh, pre-warmed medium.

Parallel, Automated Small-Scale Bioreactor

The automated small-scale bioreactor system Ambr® 15 with up to 48 disposable cell culture bioreactor vessels was used in the experiments. Working volume of each bioreactor is between 10 – 15 mL.

Each bioreactor was inoculated from the seed train with a seeding density of 2.5 million cells/mL. To achieve this higher seeding density, cells from the seed train were centrifuged at 500 xg at room temperature for five minutes and resuspended in pre-warmed medium for the semi-perfusion process. The total duration of each centrifugation step did not exceed a total of 15 minutes. Temperature set point was 36.8 °C and the pH was 7.1. Agitation of each bioreactor was set to 1,300 rpm and dissolved oxygen was controlled at 60%. A constant N_2 flow was set to 150 µL/min to allow gas transfer. The process lasted 12 days, with one vessel volume exchange of medium per day (VVD) performed by spinning down the cells in the vessel and resuspending the cell pellet in 10 mL fresh medium using the liquid handler of the Ambr® 15 system. Prior to each medium exchange, 2 mL of supernatant were stored at –20°C for IgG quantification. In addition, supernatant was taken after the daily media exchange; the volume varied to maintain the 10 mL working volume in each bioreactor.

Offline Analytics

Process specific values for pH, glucose, lactate, and osmolality were measured for every sample. With the VCC indicating the quantity of viable cells in the cultivation, the viability and the cell diameter were analyzed and the CSPR calculated.

The integral viable cell concentration (IVCC), also called cumulative cell hours, indicates the total amount of time the cells have produced mAb and is calculated by trapezoidal integration of the VCC over the time (t).

Analysis for IgG Quantification

The cell-free supernatants of all samples were stored in the freezer at –20 °C before IgG titer quantification with HPLC was performed. A mixture of 1 M Na_2SO_4, 0.5 M NaH_2PO_4 + 0.5 M Na_2HPO_4, and water (1:1:8) with a pH at 6.6 was used as the buffer. Prior to analysis, samples were thawed and diluted with water and filtered through a 0.2 µm syringe filters into autosampler cups using 1 mL syringes. The calibration curve was measured based on an IgG stock solution.

To calculate the indicated cumulative product, measured product concentrations were normalized to a volume of 1 L, resulting in the product amounts. The product amount of the supernatant after centrifugation, and the product amount of the resuspended cells after the exchange of medium, were factored into the calculation of the cumulative product, considering a 1 L working volume. By plotting the cumulative product against the integral viable cells (IVC), the productivity of each process is indicated.

Calculation of Cell-Specific Productivity and the Lactate Yield Coefficient From Glucose

The cell-specific productivity (Qp) was calculated to compare the key experiments from each bioreactor system, the shake flask and automated small-scale bioreactor, and the newly established semi-perfusion process. The lactate yield coefficient from glucose was calculated based on the specific glucose consumption and the specific lactate production, with the glucose concentration, the lactate concentration, and the geometric mean of the VCC.

Results and Discussion

DoE Studies for Medium Optimization

As a first step in this study, medium composition for the semi-perfusion process was optimized. Different blends from a CHO fed-batch platform medium and its proprietary feeds were investigated using DoE in shake flasks. FMA, FMB, and the PM were combined in different blends to develop a formulation that would meet the requirements of higher cell concentrations in the semi-perfusion process. The DoE was based on a full factorial central composite face-centered design (CCF) containing the three factors PM, FMA, and FMB; VCC was integrated as response factor. The experimental design was based on an optimization response surface methodology with a quadratic process model.

Criteria for optimization were VCC and viability of the cell population (Figure 1A). VCCs up to 45 million cells/mL were obtained, which is twice the number of cells compared to the previous fed-batch process. With the medium blends PF1_DoE4 and PF1_DoE8, viability and VCC were high over a period of 250 hours. In all other shake flasks, the viability decreased dramatically after 170 hours. These results indicate that addition of both feeds and 8% FMA is necessary for a stable process.

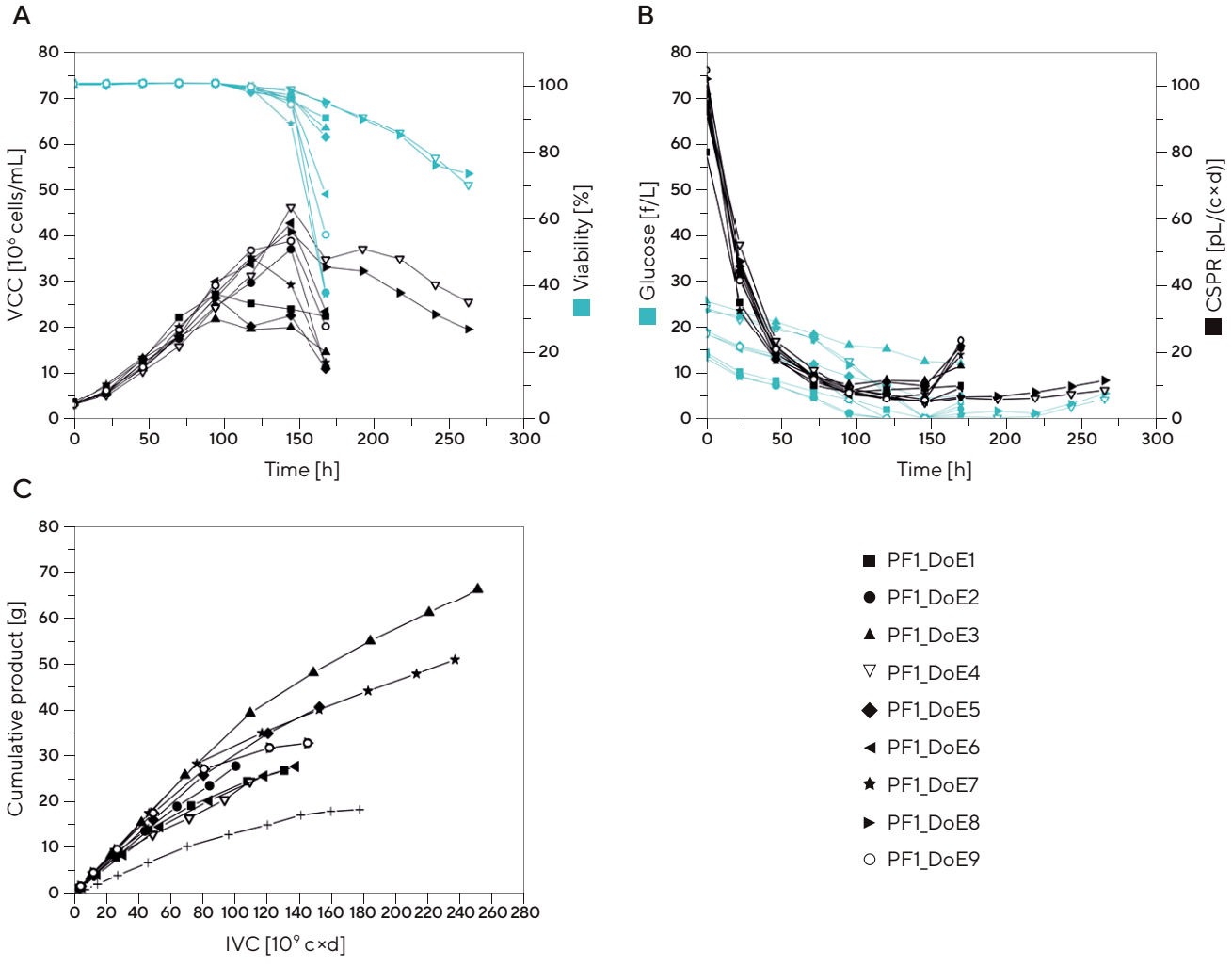

Figure 1: Results of DoE for semi-perfusion medium optimization with a mAb producing DG44 CHO cell line. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) of each shake flask run are shown. Center point PF1_DoE9 based on n = 3.

However, the increase of the FMA concentration was limited due to higher osmolality with increasing nutrient concentration. The glucose profile (Figure 1B) showed a lack of glucose for nearly every cultivation after 150 hours; lack of glucose corresponded with a strong decrease in viability and VCC. The correlation of the glucose concentration and the decreased viability and VCC indicated a possible glucose limitation during the cell cycle and other metabolic processes and requires further optimization.

The CSPR values (Figure 1B) indicated a lower limit at 50 pL/(c × day). The cumulative product represented the product concentrations before and immediately after centrifugation based on the integral of viable cells in the cell culture. Product yield of the antibody in relation to the IVC of each shake flask run is shown in Figure 1C. The cumulative product in the semi-perfusion processes showed a clear improvement up to 12 g compared to the normal fed-batch process with an average of 3 g in the corresponding working volume of 1 L.

The DoE response surface plot showed that with 8% FMA, a local optimum was reached (Figure 2). However, further increases of FMA and FMB to optimize the first screening DoE is likely to result in an osmolality exceeding 400 mOsmol/kg, which may reduce CHO production rates. The resulting local optimum was used as a satisfactory compromise between performance and osmolality.

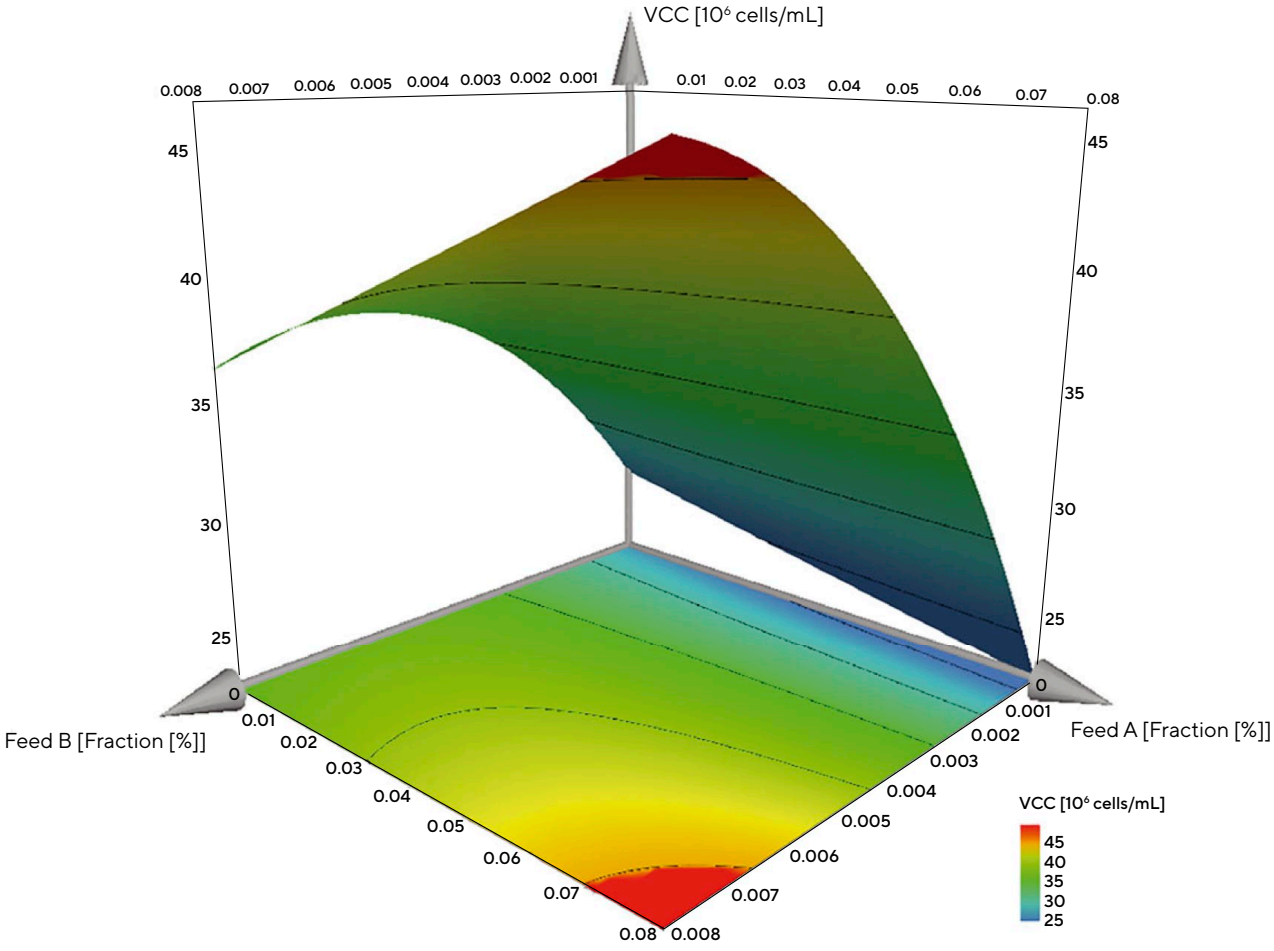

Figure 2: Response surface plot of DoE. The evaluation is based on VCC and different feed fractions (%) in the medium blends. The response of the target variable is color coded, ranging from blue (low) to red (high).

Optimization of Glucose Concentrations

One critical factor during process transfer from fed-batch mode to perfusion processes was the glucose concentration. As can be seen from the DoE study, it was necessary to optimize the amount of glucose in accordance with the higher cell concentrations obtained in the semi-perfusion process.

Different compositions of PM, FMA, and FMB together with glucose (added directly to the compositions) were analyzed. These compositions were tested to gain a better understanding of the correlation between each component. In a second step, glucose spikes were added directly to the culture after the daily medium exchange (Figure 3).

Both experiments showed similar culture behavior, achieving a longer stability and the favored 12 days of cultivation with high cumulative products. The control composition PF2_Glc1, without any additional glucose, showed the same glucose depletion after five cultivation days as in previous experiments. Best results in viability were achieved in PF2_Glc4, PF2_Glc5,

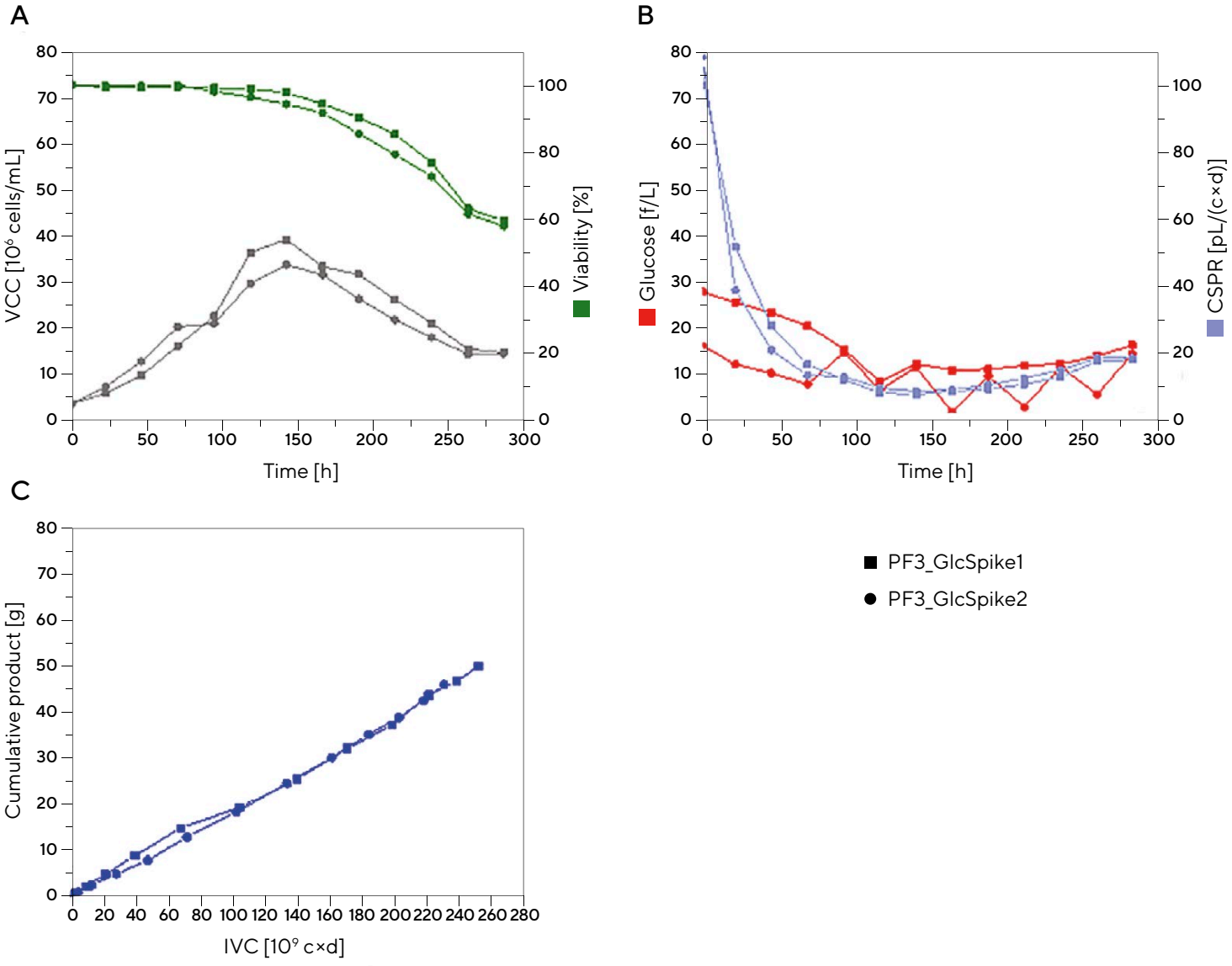

Figure 3: Performance of shake flasks with implementation of additional glucose spikes in the semi-perfusion processes of a mAb producing DG44 CHO cell line. For PF3_GlcSpike1 glucose was spiked with 4 g/L daily after Day 5 of cultivation. The medium composition for PF3_GlcSpike1 was 91.2% PM, 8% FMA, and 0.8% FMB. The glucose spike for PF3_GlcSpike2 was applied daily starting from Day 1 if the subtraction of the glucose consumption of the last 24 h from the actual glucose concentration was below 2 g/L. In that case, a spike of 4 g/L was carried out. The medium composition for PF3_GlcSpike2 was 100% PM in the first three days and 91.2% PM, 8% FMA, and 0.8% FMB in the following days. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the produced antibody in relation to the IVC (C) of each shake flask run are shown. Depicted are the mean values of duplicate measurements (n = 2).

and PF3_GlcSpike1, which contained 91.2% PM, 8% FMA, and 0.8% FMB. This medium composition was used in the following experiments and is referred to as the semi-perfusion medium.

Addition of glucose was highly beneficial for stability of the semi-perfusion process with VCCs up to 40 million cells/mL. At a CSPR of 50 pL/(c × day), a decrease in viability could be seen in the following days. Glucose additions starting from 2 g/L to 8 g/L were beneficial for higher CSPR values.

No difference was observed between the two approaches to adding glucose to the cell culture; as such, the glucose spike was further evaluated, as it offers advantages in easier handling and better adaption to batch-to-batch variations in cell growth. However, viability in both experimental setups decreased to 60% on the last two cultivation days. As viabilities below 70% are common harvest criteria, further improvement of the process stability and higher CSPR values are desirable.

Influence of the Cell Bleed on Cell Culture Stability

In order to move from a fed-batch culture to perfusion, a cell bleed was essential to maintain a stable VCC over prolonged process time, provided that the VVD of 1 is kept constant. In this study, different cell bleed strategies were tested instead of increasing the VVD. In addition, to further improve glucose concentrations and the correlation of cell bleed and glucose spikes, different combinations of bleed and glucose additions were evaluated. The cell bleed was combined with the daily media exchange.

In all approaches applying the cell bleed to the semi-perfusion shake flask cultures, peak VCCs of 25 million cells/mL or higher were achieved (Figure 4A). However, the first bleed condition (PF4_Bleed1 with a bleed VCC of 40 million cells/mL and a high glucose addition to 4 g/L from Day 5 on) showed a decrease in viability to 65% on the last cultivation day. This viability was lower than the desired viability of 70% at the end of the semi-perfusion culture. Therefore, the high bleed VCC is not beneficial for a stable semi-perfusion process with the given VVD of 1. Moreover, the VCC of PF4_Bleed1 was similar to the VCC achieved in PF4_Bleed4 with a bleed threshold of 25 million cells/mL. In both approaches, the CSPR fell below 50 pL/(c × day) after 110 hours of cultivation, resulting in a decrease of viability and inhibition of further cell growth to higher VCCs. These results indicate that the bleed should be applied at an earlier stage with lower target bleed VCCs.

Higher VVDs might support stable cell populations with higher VCCs without the need to bleed; however, this significantly increases medium consumption and costs.

Every bleed condition with a target bleed concentration of 20 million cells/mL (PF4_Bleed2 and PF4_Bleed3) or 25 million cells/mL (PF4_Bleed4) showed viabilities above 70% on the last day of cultivation with similar peak VCCs of approximately 30 million cells/mL. In addition, the glucose concentration was not limited in the semi-perfusion medium over the complete process time (Figure 4B). Interestingly, PF4_Bleed2, with no additional glucose spiking, showed no glucose limitation and was characterized by the highest viability (> 80%). This indicates that the bleed in a semi-perfusion process enables constant CSPR values above the critical CSPR and is fundamental for culture stability.

Furthermore, the highlighted condition was constantly bled to the smallest investigated bleed level of 20 million cells/mL. According to previous observations, the decrease of CSPR below 50 pL/(c × day) in each shake flask run was aligned with a lower viability in the following culture days. Cumulative product was increased significantly to 10 g in comparison to the fed-batch process (Figure 4C).

Medium optimization and corresponding cell bleed adaptation led to successful semi-perfusion cultures with final viabilities of more than 70% after 12 days of cultivation. Based on these findings, the medium formulation was defined as 91.2% PM, 8% FMA, and 0.8% FMB with a target bleed concentration of 20 million cells/mL.

The following experiments demonstrate how this semi-perfusion process can be transferred into an automated small-scale bioreactor system. This enables more process control and facilitates future investigations of the new process. Due to the direct transfer of the optimized process into the small-scale bioreactor, the advantage of the automated bioreactor as scale-down model for process optimization over the classical shake flask approach was investigated.

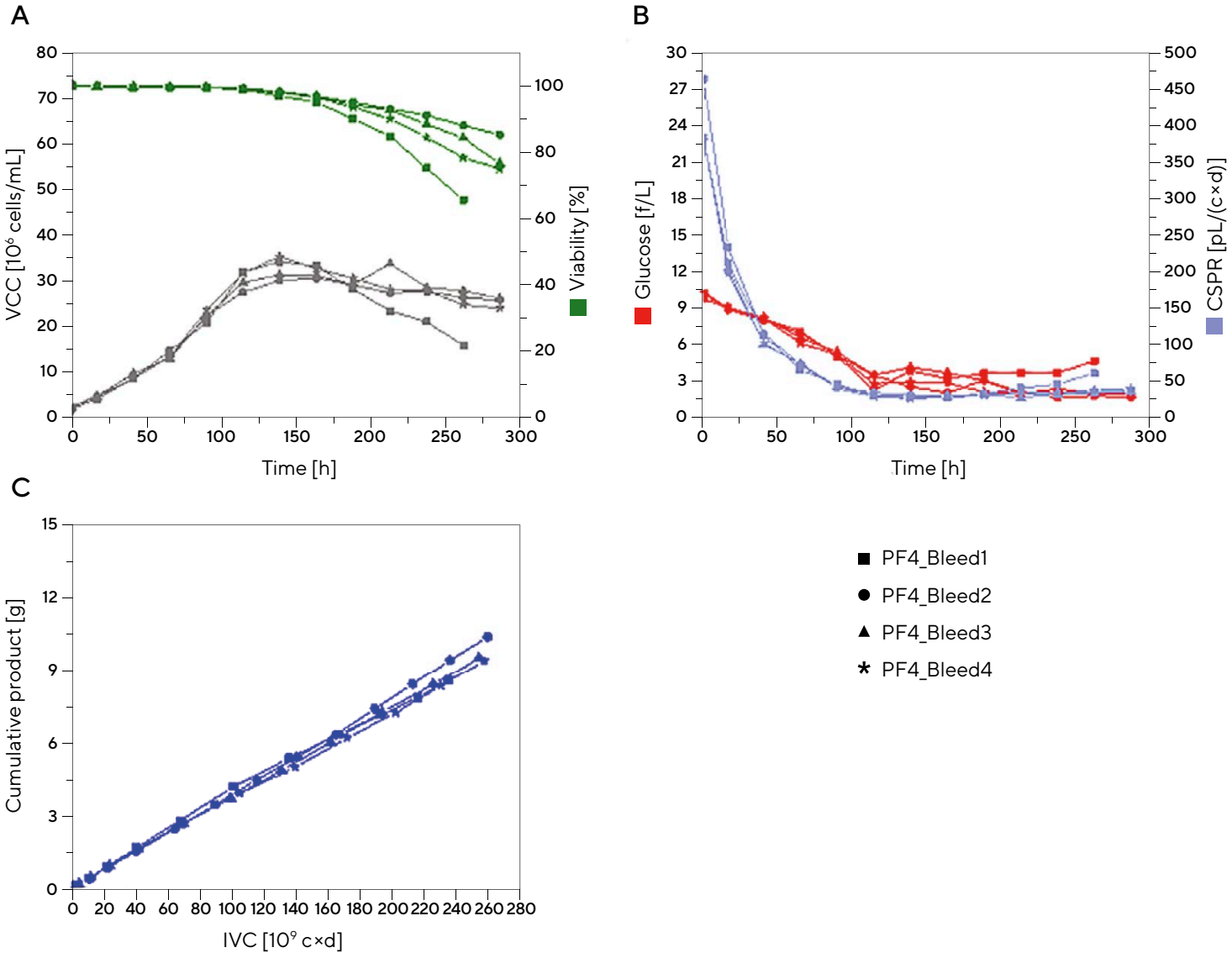

Figure 4: Implementation of cell bleed in the semi-perfusion processes of a mAb producing DG44 CHO cell line. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) of each shake flask run are shown. Depicted are the mean values of duplicate measurements (n = 2).

Use of Automated Small-Scale Bioreactor System to Control pH and DO

Following conversion of the fed-batch process to a semi-perfusion process in shake flasks, the process was transferred to an automated small-scale bioreactor system. This transfer enabled greater process control of relevant process parameters (e.g., DO, pH), enabled better process understanding through monitoring of key performance indicators, and offered the possibility to establish automated medium exchanges.

Due to improved process control of the parameter set points, and especially the pH control at 7.1 in the automated bioreactor, higher cell growth rates were expected. Therefore, additional glucose was spiked (2 g/L to each bioreactor) whenever the resulting glucose concentration for the following day was calculated to be below 2 g/L based on the specific glucose consumption per cell. The glucose spike was implemented to maintain a stable culture over the total cultivation time.

Figure 5 shows the results of the process transfer to the automated system. For all critical parameters, the favored criteria were achieved. The culture was stable over the entire culture time of 12 days. A concentration of 20 – 30 million cells/mL was achieved and kept as steady-state concentration with the help of precise cell bleeds (Figure 5A).
Viability exceeded 98% for half of the cultivation time and decreased to 95% once the previous detected critical CSPR of 50 pL/(c × day) (Figure 5B) was reached. Therefore, it was shown that maintaining CSPR above 50 pL/(c × day) resulted in a stable cell culture with high viabilities.

In the bioreactor system, cell viability was significantly higher than in any other shake flask experiment; additionally, the process was more stable than in uncontrolled shake flasks. The process was controlled to the pH set point of 7.1 and the DO set point of 60% over the complete culture time (Figure 5D). Comparing the pH values to the shake flask experiments showed a marked improvement of control.

In addition, an oscillation of DO could be detected in the automated bioreactors over the cultivation period; this was a result of high cell densities creating an increased oxygen demand that triggered a more active control loop and subsequent oscillations in the DO signal.

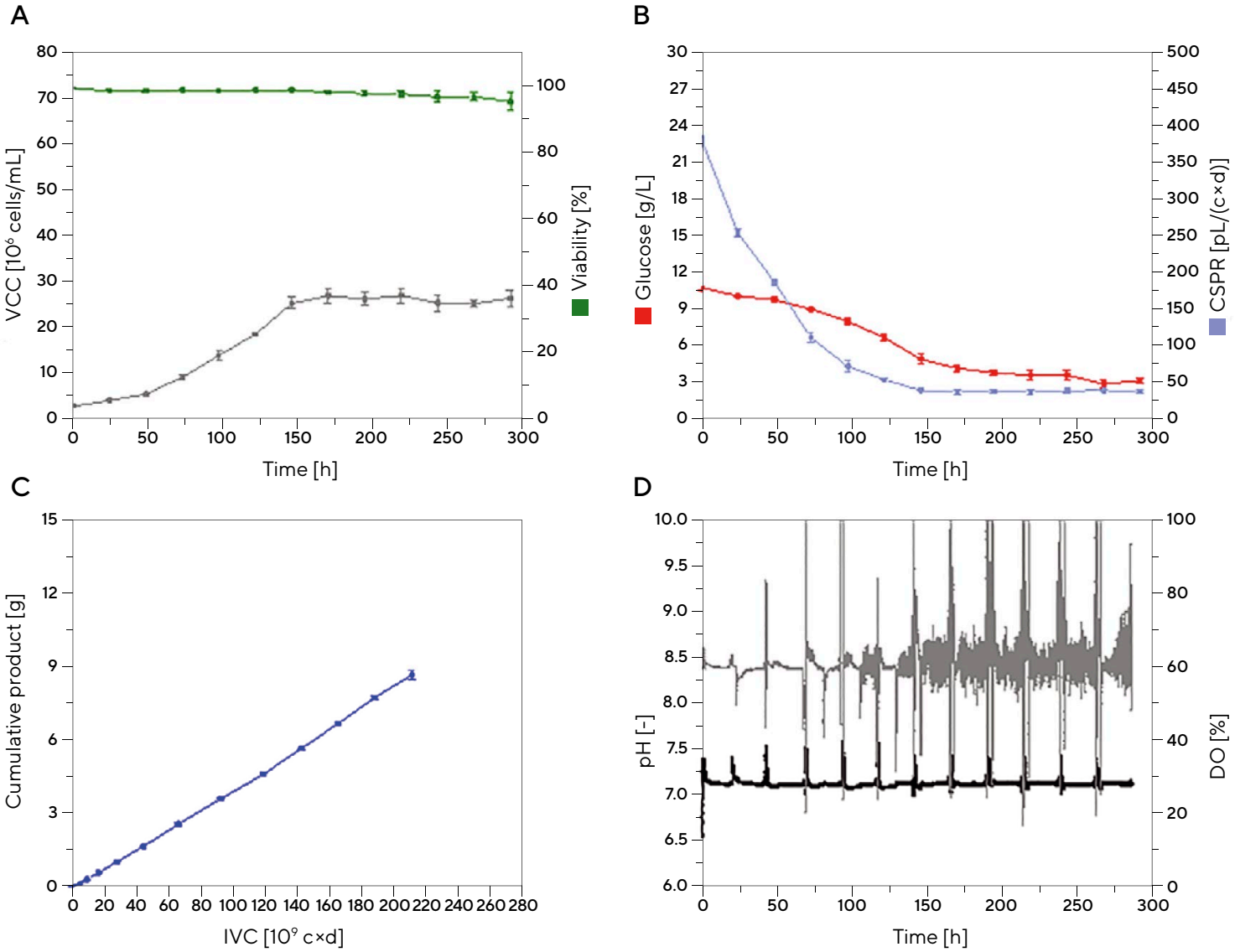

Figure 5: Transfer of the semi-perfusion processes of a mAb producing DG44 CHO cell line to an automated small-scale bioreactor system. The VCC and viability (A), the glucose concentration and CSPR (B), and the product yield of the antibody in relation to the IVC (C) are shown. Error bars are reported for each value. The controlled parameters, the pH value and the DO of the process, are shown here (D). Mean values of triplicate measurements (n = 3) are depicted.

Based on the generated data for DO and pH control, experienced users will develop a sound understanding of process behavior at larger scales. Therefore, the use of small-scale bioreactors is highly favored to gain deep knowledge for intensified bioprocesses. However, in a large-scale bioreactor, the process will be implemented as a fully continuous process with a suitable cell retention device. The change from centrifugation in small-scale bioreactors to cell retention in large-scale bioreactors will enable culture control and process monitoring over the complete process time.

Application of glucose spikes whenever the glucose concentration was below the threshold of 2 g/L led to a sufficient nutrient supply for the semi-perfusion cell culture. The cumulative product of 8.7 g in the semi-perfusion process was three-fold higher than in the corresponding fed-batch process. The resulting standard deviations showed the three bioreactors performed consistently throughout the process. The product amount over the total culture time can be further improved by cultivating the semi-perfusion culture in the controlled system for longer periods in the future.

The possibility of longer cultivations was enabled by the higher stability and high viability in the controlled bioreactors. Due to the consistent and controlled semi-perfusion process in the small-scale bioreactors, conclusions can be compared and used to improve process understanding for continuous processes and implementation of perfusion in suitable bioreactors with cell retention devices. Furthermore, the results can be used to predict and optimize the scale-up to production scales.

Finally, the fed-batch process, the successful implementation of semi-perfusion processes in shake flasks, and the process transfer in the automated small-scale bioreactor can be compared. This comparison can be based on the lactate production compared to glucose consumption (YLac/Glc), the specific productivity, the increase in cell diameter, the cumulative product as well as the IVC, and the end viability of each process.

YLac/Glc allows deeper information about the cell state and the metabolism in the cells. Glucose is used in different pathways based on the metabolic state and the nutrients in the environment, while lactate is a waste product in unfavorable pathways that can inhibit the cell growth. Therefore, a low YLac/Glc indicates efficient cell metabolism with low lactate production or lactate consumption by the cells. In the beginning of each process, YLac/Glc is significantly lower in the semi-perfusion process, indicating a more efficient metabolism than in the corresponding fed-batch cultivation. The biggest positive effect was achieved in the automated small-scale bioreactor with values around 0; the ratio of glucose consumption and lactate production indicated a highly productive and healthy cell culture. These findings were supported by the lactate production over time in each of the three cultures. At the start of the original historic fed-batch, a significantly higher lactate production was detected before the cells switched into lactate consumption. Furthermore, the increase in cell diameter (indicating apoptotic cells) was much less for the established semi-perfusion process compared to the historic fed-batch, indicating a more viable cell culture. Another benefit of the semi-perfusion process is the low osmolality (~310 mOsmol/kg at the end of the process in comparison to ~360 mOsmol/kg in the fed-batch). The higher osmolality can support the increase in cell diameter in the fed-batch.

Finally, the average specific productivity was increased to a final productivity of 38.88 pg/(c × day) in the automated small-scale bioreactor. The cumulative product and the IVC increased significantly in the semi-perfusion processes during the same culture period compared to the fed-batch. The automated small-scale bioreactor performed better regarding the end viability of 95.23% compared to the semi-perfusion in shake flask or the fed-batch process. These results indicated that the Ambr® 15 platform performed best in the studies in terms of product yield and viability of the cell culture. The benefit of the automated small-scale bioreactor as scale-down model was clearly shown compared to the shake flasks.

Conclusion

The ability to mimic perfusion during cell line screening and initial process development is essential to identify suitable clones, as well as key parameters, and to optimize culture conditions for process intensification. The strategy described in this application note utilized the Ambr® 15 automated, high throughput microscale bioreactor system to transfer an existing CHO fed-batch process into a semi-perfusion process leveraging DoE studies. In addition, suitability of the process for a multi-parallel controlled microbioreactor system as scale-down model has been demonstrated, indicating that early stage development of a semi-perfusion process can be done in a controlled system.

In this study, conversion of a fed-batch process to semi-perfusion was successfully demonstrated by using the same platform medium and its proprietary feeds. The product yield, normalized to 1 L of the corresponding fed-batch process, increased from 3 g to a cumulated product amount of 10 g in the semi-perfusion process for the same cultivation duration.

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