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RESEARCH ARTICLE

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Rapid intensification of an established CHO cell fed-batch process

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Abstract

Currently, the mammalian biomanufacturing industry explores process intensification (PI) to meet upcoming demands of biotherapeutics while keeping production flexible but, more importantly, as economic as possible. However, intensified processes often require more development time compared with conventional fed-batches (FBs) preventing their implementation. Hence, rapid and efficient, yet straightforward strategies for PI are needed. In this study we demonstrate such a strategy for the intensification of an N-stage FB by implementing N-1 perfusion cell culture and high inoculum cell densities resulting in a robust intensified FB (iFB). Furthermore, we show successful combination of such an iFB with the addition of productivity enhancers, which has not been reported so far. The conventional CHO cell FB process was step-wise improved and intensified rapidly in multi-parallel small-scale bioreactors using N-1 perfusion. The iFBs were performed in 15 and 250 ml bioreactors and allowed to evaluate the impact on key process indicators (KPI): the space–time yield (STY) was successfully doubled from 0.28 to 0.55 g/L d, while product quality was maintained. This gain was generated by initially increasing the inoculation density, thus shrinking process time, and second supplementation with butyric acid (BA), which reduced cell growth and enhanced cell-specific productivity from ~25 to 37 pg/(cell d). Potential impacts of PI on cell metabolism were evaluated using flux balance analysis. Initial metabolic differences between the standard and intensified process were observed but disappeared quickly. This shows that PI can be achieved rapidly for new as well as existing processes without introducing sustained changes in cellular and metabolic behavior.

KEYWORDS

butyric acid, CHO cell culture, intensified fed-batch, N-1 perfusion, process intensification

1 | INTRODUCTION

The demand and market size of biopharmaceuticals is steadily increasing. Monoclonal antibodies (mAbs) in particular experienced an excessive growth during the last decade.[1] With an estimated market value of over US$ 300 billion by 2025, they are nowadays by far the best selling drugs worldwide.[2] To further meet this demand, the industry's mindset is shifting from batch to continuous production

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concepts[3] and relevant industry leaders foster the transition towards process intensification.[4] The common goal is to increase productivities and flexibility while ideally keeping operational and capital expenditures (OPEX, CAPEX) considerably low.

During the last decade many different upstream process strategies have emerged in the field of process intensification. Many of these novel strategies are utilizing perfusion cultivation to generate high cell densities (HCD, >100 × 10^6 c/ml) during the N-1 stage for the inoculation of subsequent iFB.[5–9] This implies two alterations compared with conventional biomanufacturing: the inoculum preparation and condition of the cells are different (batch vs. perfusion) and the iFB starts at higher inoculation cell concentrations than a standard FB (sFB). This results in a shortened time frame where production is low due to low cell densities and, thus, avoiding to have an idle-state in the production bioreactor.[10] iFBs can improve the STY in two ways: (a) By running shorter processes reaching similar titer,[9] thus, increasing yearly net operation time of the production facility, that is, facilitating to run more batches per year in the large scale production bioreactors. Option (b) is to keep the process duration equal by modifying the process, for example, the feed regimen.[6] In this way the final titer can be increased if the cell viability and productivity remains above critical levels, which allows to run less bioreactors per year to meet production goals. However, this is not really a choice and current iFB strategies often result in option (a), since cell growth and viability decrease earlier, yet productivities are increased. In order to better understand critical growth limiting factors in iFBs and therefore to find possible starting points for further STY increase, detailed analysis would be needed. Currently, detailed knowledge on these differences is still missing, like changes in the gene expression profile or metabolic flux distribution.

Next to these designs on the process strategy, KPIs such as growth, longevity or productivity might benefit from additional changes in process parameters. This can be temperature[11] or pH shifts[12] or the supplementation of production enhancers, for example, short chain fatty acids (SCFA),[13] that are known to be potentially advantageous. The potential of SCFAs, for example, valeric,[14] valproic[15] or butyric acid,[16] to stimulate protein production is well known.[17] The accepted mode of action is, that SCFAs inhibit the deacetylation and dephosphorylation of histones, which makes the DNA more accessible ultimately improving transcription rates and cell-specific productivities. These effects are linked to a reduction in cell growth due to an arrest in the G0/G1 phase of the cell cycle. During the last 20 years, several studies evaluated these impacts in small lab-scale experiments[18–20] and conventional processes, that is, batch[14] and fed-batches.[15] Recently, with respect to CHO cell process intensification, SCFAs revived greater application to further optimize these processes: Valeric acid was exploited to improve the production of factor VIII[21] and also IgG[22] in a perfusion process. However, to the best of our knowledge, the application of SCFAs in iFBs has not been studied yet. The effect of SCFAs may be different in iFBs, since these processes reach the stationary phase (high G0/G1 ratio) earlier on and the described decrease in cell viability upon SCFA addition might outweigh productivity enhancing effects.

The application of such process strategic changes, and even more likely the combination, thereof, can affect the physiological state of the cell and product quality. This might lead to unexpected hurdles or bottlenecks in the intensification strategy and longer development times. The problem of implementing such an intensified process or transforming it from the established sFB is to avoid changes in the physiological state of the cells and, ultimately, the final product quality. The goal of this study is obtain more insight into differences in STY, product quality and CHO cell physiology between the sFB and iFB process as well as for an iFB process with addition of butyric acid.

This was approached in a step-wise manner. Initially, a screening study in 15 ml bioreactors was conducted to evaluate the effect of changing the N-1 inoculum source from a batch to a HCD perfusion culture with previously automized perfusion rate control based on online capacitance measurements (of up to 100 × 10^6 c/ml).[23] At the same time, two different inoculation concentrations for the iFB (2.5 and 5 × 10^6 c/ml) were investigated with respect to cell growth, viability and cell diameter as well as product titer and STY. Subsequently, the most promising iFB (5 × 10^6 c/ml) was transferred into 250 ml bioreactors, which were used for the final comparison. At the same time, further intensification potential was evaluated by including an approach with BA supplementation. Preliminary studies revealed BA to have a favorable effect on the cell line of this study.

By following this step-by-step intensification strategy, the volumetric productivity was almost doubled in this study to 0.55 g/L d, starting from an established sFB at 0.28 g/L d. For this purpose, the inoculation concentration was increased from 0.3 to 5 × 10^6 c/ml using N-1 perfusion, and an increase in the average cell-specific productivity from about 24 to almost 37 pg/c d was achieved through BA supplementation. Simultaneously, the presented data emphasize that process intensification at the N-stage itself does not cause changes in final product titer and that comparable product quality can be expected. Initial differences in cellular physiology and metabolism between sFB and iFB quickly disappear during the process and only minor differences could be observed at time of harvest.

2 | MATERIALS AND METHODS

2.1 | Cell lines and medium

For all cultivations a CHO DG44 cell line (Sartorius) expressing a monoclonal antibody (mAb, IgG1) was used. All experiments were carried out using the same chemically defined media (Sartorius) being seed medium (SMD) for the seed train, production medium (PM) with feed medium A and B (FMA, FMB) for fed-batch runs and an optimized mixture, thereof, as perfusion medium (PF-M).[24]

2.2 | Seed train using N-1 perfusion

Cells from a cryovial were passaged every 3–4 days and kept in an incubator at 36.8°C, 7.5% pCO_2, 85% humidity and 120 rpm

(CERTOMAT® CT plus, Sartorius) until the inoculation of the N-1 perfusion processes. They were operated in 2 L single-use bag bioreactors based on rocking motion (RM; BIOSTAT® RM 20|50, Sartorius) with a working volume of 1 L. Process parameters were used according to Schulze et al.[23] with automized cell-specific perfusion rate (CSPR) control of 50 pl/c d. The processes were operated for up to 7 days before cells were used for the inoculation of subsequent N-stage cultivations in Ambr®15 and Ambr®250 bioreactors. They were sampled daily (5 ml) for offline analysis.

2.3 | N-stage process intensification

All FB cultivations used the same volumetric feeding scheme starting with 4% (vol/vol) FMA and 4% (vol/vol) FMB. Daily feeding started with the depletion of L-Glutamine, which occurred after 3, 2, and 0.5 days for the sFB, iFB_2.5 and iFB_5, respectively. Glucose was added (400 g/L stock solution) if required towards 5 g/L. Regardless of the process and bioreactor system used, identical process conditions were applied ($T = 36.8°C$, pH 7.1 via CO_2 gassing, DO 60%). Processes lasted maximally for 12 days in total or nine consecutive feeding days unless the viability fell below 70%, after which the STY was evaluated.

2.4 | Ambr®15

Initially, the automated small-scale bioreactor system Ambr®15 with sparged cell culture vessels (Sartorius) was used to compare the impact of the N-1 mode for a conventional FB process inoculated from a N-1 batch (sFB Std) and N-1 perfusion (sFB Perf). Besides, the latter one was used to inoculated two iFBs at 2.5 and 5×10^6 c/ml, respectively. Agitation was set to 1050 rpm (down) and air was used as ballast gas (150 μl/min). The initial starting volume of all approaches was 14 ml and was reduced towards 12.5 ml by sampling before the first feed to ensure similar volume profiles for all approaches, thus, identical transfer and mixing rates. A volume of 20 μl antifoam (2% Antifoam C, Sigma) was added every second day. Daily samples of 475 μl were used for offline analysis.

2.5 | Ambr250® high throughput

Based on Ambr®15 experiments, iFBs were transferred into the high throughput, automated bioreactor system Ambr®250 (Sartorius). Three approaches were investigated. Using N-1 perfusion, an iFB was inoculated at 5×10^6 c/ml as control (iFB_5) and a modified iFB was started at 5×10^6 c/ml (iFB_5 + BA) which was supplemented with 2.5 mM BA (Sigma-Aldrich) after 2.5 days, that is, the end of the exponential phase. This point in time was identified in preliminary studies to be the optimal one with respect to the STY (see Supplementary file S1). Also, a sFB was inoculated at 0.3×10^6 c/ml from a conventional batch seed. Agitation was set to 855 rpm and DO was controlled using a gassing cascade using enriched oxygen if required. The initial starting volume of all approaches was 200 ml and was reduced towards 194 ml by sampling before the first feed to ensure similar volume profiles for all approaches, thus, identical transfer and mixing rates. 50 μl antifoam (2% Antifoam C, Sigma-Aldrich) were added every day. Samples for offline analysis were taken daily before feeding. Moreover, the processes were also sampled after feeding finished for respectively 2 days during the exponential and stationary growth phase to allow for precise calculations of cell-specific exchange rates.

2.6 | Offline analytics

Cell growth (VCC, viability and average cell diameter) were measured using a Cedex HiRes Cell Counter (Roche). The pH, pO_2, pCO_2, as well as metabolite levels (glucose, lactate, ammonia, and L-glutamine) and osmolality, were measured using a BioProfile® FLEX2 (Nova a). The remaining sample volume was centrifuged (15,000× g, RT, 5 min) and the supernatant was stored at −20°C for subsequent analytics.

At definite time points, cells from the Ambr®250 experiment were also prepared for cell cycle analysis. For this purpose, 5×10^6 cells were centrifuged (300× g, RT, 5 min), the cells were fixed by resuspending the pellet carefully with ice cold 70% ethanol and were stored at 4°C for subsequent flow cytometric analysis. The fixed cells were stained with propidium iodide (Invitrogen) and analyzed with the Intellicyt iQue® Screener (Sartorius).

The product titer was determined via HPLC using a Dionex UltiMate 3000 HPLC System (Thermo Scientific) and a Yarra 3 μm SEC 3000 (Phenomenex). As described previously,[25] the space–time yield (STY) was calculated as follows:

$$\mathrm{STY} = \frac{c_{\mathrm{IgG,final}}}{t_{\mathrm{final}}} \tag{1}$$

where t_{final} (d) and $c_{\mathrm{IgG,final}}$ (g/L) are the time of harvest and the product concentration at harvest, respectively, where the harvest time is the latest point where the viability is still above 70%. Final product quality profiles at the end of Ambr®250 FB cultivations in terms of N-Glycan profiles were analyzed using capillary gel electrophoresis on a microchip (LabChipGXII Touch 24, PerkinElmer). Samples were denatured, digested via PNGase F and stained according to the manufacturer's protocol (Glycan Profiling Assay Release and Labeling Kit, PerkinElmer).

For the Ambr®250 experiments, supernatant was also used for spent media analysis of extracellular sugars, amino and organic acids and ammonia using NMR (Spinnovation Biologics BV) during the exponential growth and stationary phase of the respective approaches.

Final samples of the raw supernatant of the Ambr®250 experiments were also subjected for analysis of host cell impurities with respect to host cell proteins (HCP) using an HCP-ELISA kit (CYG-F550,

Cygnus Technologies)[26] and extracellular DNA assay using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, Waltham).[27]

2.7 | Cell-specific metabolic rates

Based on spent media analysis, daily cell-specific metabolic rates $q_{S,i}$ were calculated for individual intervals between two consecutive feedings (24 h interval):

$$q_{S,i} = \frac{\Delta c_{S,i}}{\Delta IVCC_i} \quad (2)$$

where $\Delta c_{S,i}$ (mol/L) is the difference of the concentration of a certain compound, for example, glucose, and ΔIVCCi the corresponding integral viable cell concentration (IVCC).

Principal component analysis (PCA; SIMCA®, Sartorius Stedim Data Analytics) was used to identify major variations among each observation of each approach, which comprised all metabolic rates as well as growth and IgG production rates at each specific day.

2.8 | Flux balance and variability analysis

FBA was applied to examine potential differences in the flux distribution between the conventional and intensified fed-batch process. In order to characterize and compare similar time points in both processes, the process time after the feed start was considered: for the exponential and stationary phase, the iFB was investigated after 1.5 and 4.5 days, while the sFB was investigated at day 4 and 7, respectively.

A basic CHO metabolic model was used in this study.[28] Few reactions were modified based on cross-comparison with the latest GSM for CHO cells[29] and examination of the included lumped reactions with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Moreover, the reaction of mAb formation was updated based on the relative amino acid composition of the protein produced in this study. The relative biomass composition was adopted from Pan et al. (NI phase[28]), but absolute values were tailored considering the cellular dry weight (DW) of the cell line used in this study with respect to the respective cell size in the exponential and stationary phase. Since the available sample volumes from the 250 ml bioreactor were not sufficient to generate an appropriate amount of biomass, the required dry weights were interpolated based on the linear regression between cell size and DW of two representative 5 L STR fed-batch cultivations (procedure described in the Supplementary file S1). The cellular metabolic rates for glucose, amino and organic acids and ammonia, as well as IgG and biomass production rate measured in this study, were used as constraints for the exchange rates of the model. Geometric FBA[30] was conducted using the COBRA Toolbox v3.0 with Matlab 2020a (The MathWorks, Inc.) with the growth rate as objective function to find an unique solution. Flux ranges of the solution space were investigated using FVA.[31]

3 | RESULTS AND DISCUSSION

3.1 | Seed intensification

For inoculation of the sFB, cells from a conventional N-1 seed were used, that is, batch-wise passaging in shake flasks. For the iFBs, cells from N-1 perfusion cultivation were used. Cell concentrations in perfusion cultivation reached 100×10^6 c/ml within less than 7 days. The respective conventional seeds were split several times not exceeding VCCs of 7×10^6 c/ml (Figure 1). Throughout both approaches, the cells remained viable and in an appropriate state for the inoculation of subsequent N-stages. During the N-1 perfusion processes, the viability decreased slightly towards 95% when used for inoculation. In a previous study we have shown that an apoptotic population was absent and that the inoculum of this culture had no consequences for the N-stage cultivation.[23] The cell diameter grew over time in the N-1 perfusion up to 16 μm while it ranged between 14 and 15 μm for the batch seed.

3.2 | N stage intensification: 15 ml bioreactors

The impact of replacing the conventional seed by perfusion was investigated. Both approaches revealed very similar growth profiles with peak VCCs of 20×10^6 c/ml and maintained high final viabilities

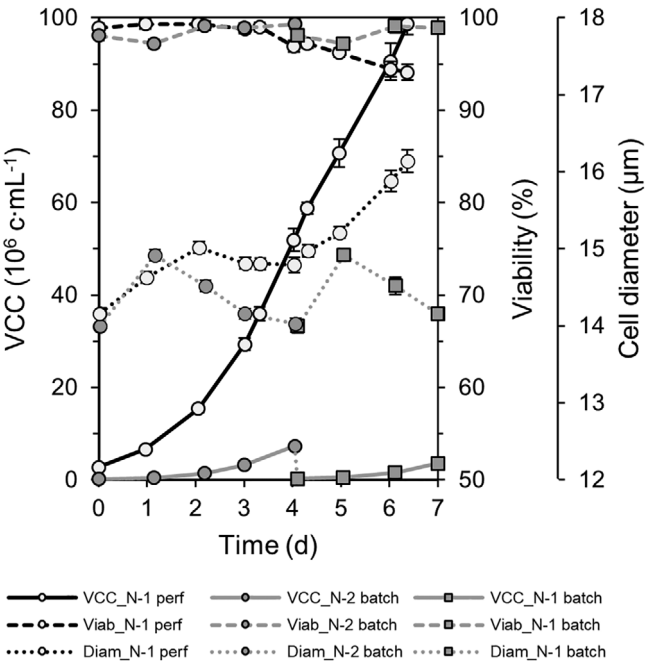

FIGURE 1 Comparison of the precultures for the two seed strategies used for inoculation of the Ambr®15 and Ambr®250. Conventional batch (N-2: 2× shake flask; N-1: 1× shake flask and 1× RM bioreactor) and intensified seed using N-1 perfusion (2× RM bioreactor). The parameters VCC, viability and cell diameter of N-1 perf are shown as mean ($n = 2$), such that error bars represent min/max values

(Figure 2(a,b)). Still, the seed train mode introduced differentially conditioned cells which is shown by the increased initial cell diameter (Figure 2(c)) of the approach inoculated from N-1 perfusion that decreased quickly within the first 3 days, such that afterwards cells of same size were cultivated. Final IgG titers of about 3.4 g/L and a STY of 0.29 g/L d were obtained (Figure 2(d,e)). Thus, it was concluded that the N-1 mode can be intensified using perfusion, without significantly influencing N-stage performance. Following, the sFBs can be compared against the two iFBs inoculated at 2.5 and 5.0 × 10^6 c/ml from N-1 perfusion, either. As expected, increasing inoculation cell concentration led to an earlier peak in VCC. As a result, cell viabilities of the intensified processes started to decline earlier, thus, causing faster process terminations after 11 and 8.5 days for the 2.5 and 5 × 10^6 c/ml approach due to the applied harvest criteria of 70% viability. The observed earlier peak in VCC followed by a decrease in cell viability in iFB set-ups is consistent with results observed by Padawer et al.[32] In contrast to the presented data, Brunner et al. also described a general increase in peak VCC compared to sFBs, but this might be related to an increased feed rate for their iFB, the value of which was not indicated.[33] In our study, the feed regimen was not changed as it proofed to suffice and no nutrients were limited. The cell diameter of the iFBs started to increase again after reaching a minimum of 15 µm. As similar titers were reached in shorter process times (Figure 2(d)), the STY was significantly improved by 16% and 36% for the 2.5 and 5 × 10^6 c/ml approach, respectively (Figure 2(e)). This first screening experiment showed that the different type of preculture used (batch vs. perfusion) resulted in an initial small difference in cell diameter, which quickly disappeared with N-stage culture time. The STY was increased with process intensification at comparable final titers, peak VCCs and final viabilities which is relevant for process related impurities. Thus, replacement of the N-1 batch by N-1 perfusion in combination with the use of intensified fed-batches is advantageous as it permits the use of less and smaller preculture steps. It was pursued in the subsequent experiment for a more detailed characterization of underlying mechanisms.

3.3 | Process transfer into 250 ml bioreactor with further improvement

The most promising approach, that is, inoculation at 5 × 10^6 c/ml (iFB_5), was transferred to 250 ml bioreactors in order to allow for more frequent and larger sampling to better characterize similarities and differences in metabolism between the intensified process and the sFB using FBA. Additionally, to further improve the intensified process supplementation with the SCFA, butyric acid (BA) was explored. This compound is known to be able to improve the cell-specific productivity[16] and was, therefore supplemented (iFB_5 + BA)

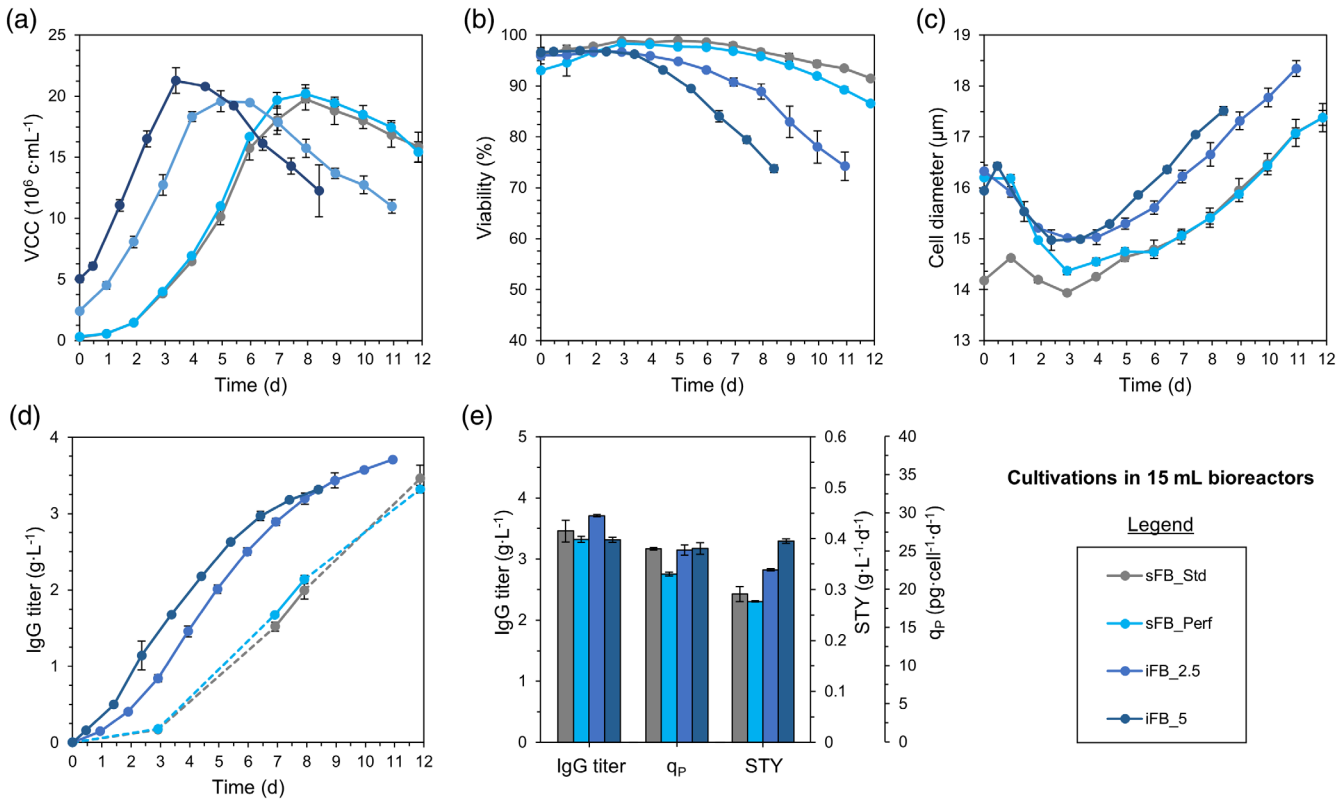

FIGURE 2 Comparison of fed-batch cultures in the Ambr®15 inoculated at different cell concentrations and from different precultures. N^{-1} perfusion was used to start two iFBs at 2.5 and 5 × 10^6 c/ml and one sFB at 0.3 × 10^6 c/ml. Another sFB was inoculated at 0.3 × 10^6 c/ml from a conventional seed. (a) VCC, (b) viability, (c) cell diameter, (d) IgG titer with increasing process time and (e) final IgG titer, average cellspecific productivity and space–time yield. All cultures were done in triplicate

at 2.5 mM at day 2.5 (see Figures S1 and S2 for preliminary BA experiments), that is, at the transition from exponential to stationary phase (Figure 3(a)). Both approaches iFB_5 and iFB_5 + BA were compared to the sFB for several KPIs with respect to growth and productivity characteristics.

The sFB, as well as the iFB displayed a similar growth pattern (Figure 3(a,b)), as was also observed in the 15 ml bioreactor, indicating successful process scale-up and good comparability between the two bioreactor systems. Supplementation of 2.5 mM BA at day 2.5 affected the VCC and viability, resulting in early process termination and harvest after 6.5 days.

The moment each of the three processes reached peak VCC, that is, cell growth rate became smaller than the death rate, the cell diameter started to increase. For the iFBs this moment is reached earlier, while BA supplementation additionally amplified this effect (Figure 3(c)). To further study the phenomena, cell cycle analysis was performed (Figure 4). During the exponential phase, the ratios between the different cell cycle phases were comparable between sFB and iFB, except for a slight increase in the G2/M phase in the iFB. This is in accordance with the initially increased average cell diameter of the iFB as cells reach their largest size at the end of G2 phase before dividing into two new daughter cells (M phase). During the stationary phase for all cultures the number of cells in the G1/G0 phase increased similarly as expected. BA supplementation resulted in a slight increase in the G0/G1 population (85%), compared to sFB (82%) and iFB (80%) processes and a decrease in the G2/M population to 3%, in contrast to 7% in the sFB and 5% in the iFB. Although this change is not significant it does agree with literature, where the time an individual cell remains in the G1 phase was reported to be increased by up to 25%, while simultaneously accelerating the apoptosis rate. The fact that the change is small may be related to the fact that the percentage of cells in G1 is already high. This underlines that the time of administration has to be determined critically[14,20,34] as done in this study at the end of the exponential phase. The fact that the IgG production increased (Figure 3(d,e)) without a significant increase in G1 implies that BA can increase productivity without affecting the cell cycle. So far, these two observations were reported to occur concurrently and thus this strategy was not explicitly considered to be useful also in the beginning of the stationary phase. However, with respect to STY our data suggest that BA addition can be advantageous even in iFBs. When on average all approaches resulted in similar final product titers of about 3.5 g/L, the STY was improved by 50% with the iFB_5 and even

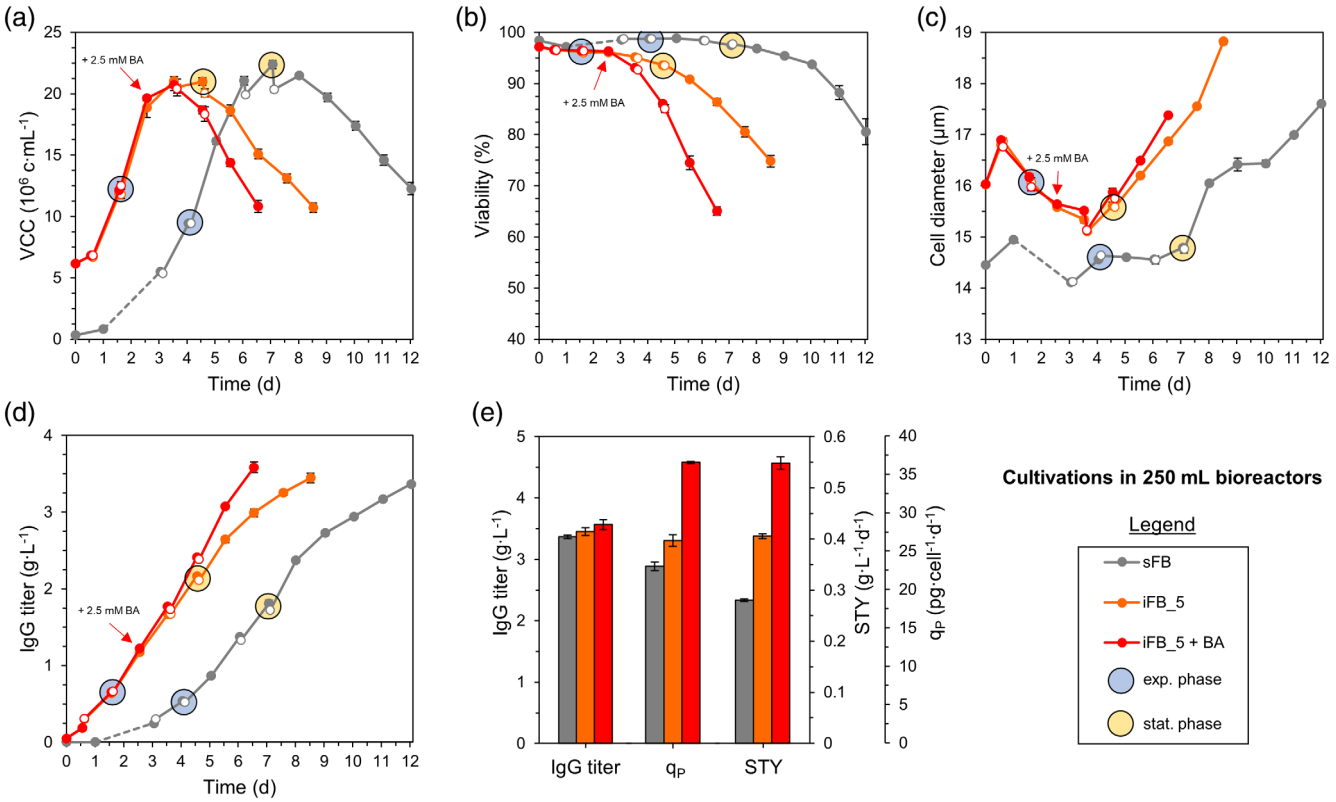

FIGURE 3 Comparison of a standard fed batch, intensified fed batch started at 5×10^6 c/ml and an intensified fed batch started at 5×10^6 c/ml with addition of 2.5 mM butyric acid after 2.5 days in the Ambr®250. Results ($n = 3$) are displayed for (a) VCC, (b) viability, (c) cell diameter, (d) IgG titer with increasing process time and (e) final IgG titer, average cell-specific productivity and space–time yield. White symbols indicate samplings directly after the feed used for accurate calculation of metabolic rates. These were required for the metabolic characterization of the iFB versus sFB in the exponential (blue circle) and stationary phase (yellow circle)

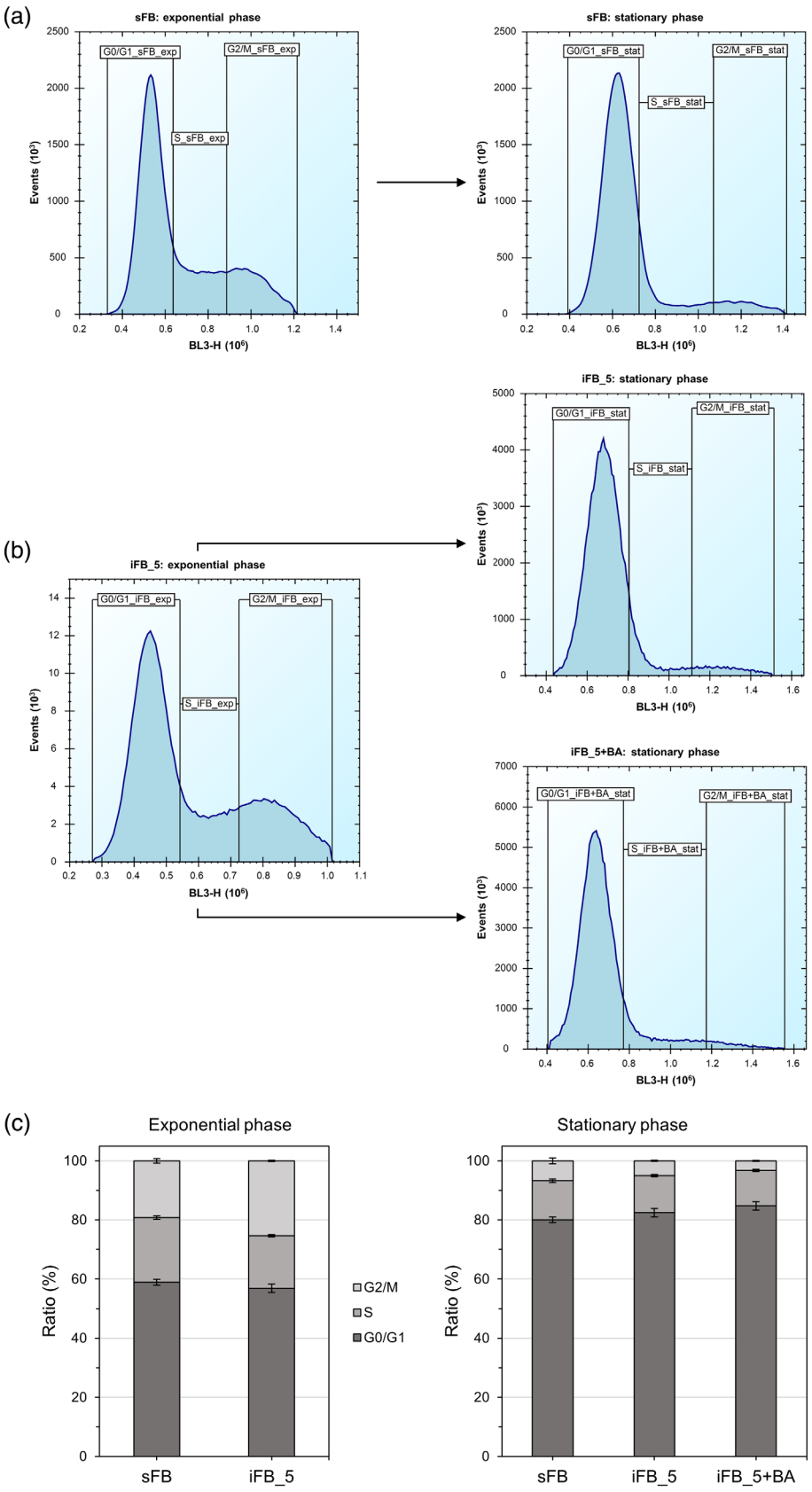

FIGURE 4 Cell cycle analysis in the Ambr®250. Histograms for the (a) sFB, (b) iFB_5 and iFB_5 + BA in the (c) exponential and stationary phase. Events of the individual replicates of the approaches were accumulated during the gating procedure. BL3-H represents the fluorescent intensity of detected PI used to stain cellular DNA

doubled by addition of BA due to a reduction in process duration and, therefore, operation time from 12 (sFB) to 6.5 days (iFB_5 + BA). This is related to the fact, that the cell-specific productivity was significantly increased after BA supplementation (Figure 3(d)) resulting in an average value of 37 pg/c d compared to 23 and 26 pg/c d for the sFB and iFB (Figure 3(e)), respectively. A comparable increase in

the cell-specific productivity, accompanied by reduced cell growth and viability upon butyrate treatment, has been reported by others.[13]

These values can be used to estimate the economic benefit of the described process changes for the annual capacity utilization of a 2000 L production bioreactor (Table 1). Next to the aforementioned improvement in STY, shrinking the process duration while keeping the feed regimen equal implicates a reduction in feed media utilization by up to 140 L per run. This is advantageous considering the respectively required production and storage capacities. Thus, in order to harvest similar final volumes in each process, the starting volume can be increased. Ultimately, the doubled STY of iFB_5 + BA results in a potential 70% annual increase of product generated with a single production bioreactor. Of course, such improvement has to be backed up by downstream capabilities. Alternatively, the number of required bioreactor runs to produce a targeted product amount per year can be reduced as described by Stepper et al.[6] allowing to use the 2000 L bioreactor to produce other products, thus making a facility more flexible.

TABLE 1 Comparison of the annual capacity utilization in a 2000 L single-use production bioreactor for the three processes

	sFB	iFB_5	iFB_5 + BA
Process duration (days)	12	8.5	6.5
Applied feeds (−)	9	8	6
Start volume (L)	1333	1400	1500
Additives (L)	540	500	400
Harvest volume (L)	1873	1900	1900
Final IgG titer (g/L)	3.37	3.45	3.57
STY (g/L/d)	0.28	0.41	0.55
STY (%)	–	+45	+96
Mass per batch (kg)	6.31	6.56	6.78
Batch productivity (kg/d)	0.42	0.57	0.71
Feasible batches per year (−)	24	31	38
Mass per year (kg)	152	203	258
Increase of mass per year (%)	–	+34	+70

Note: Maximum operation time of 350 days per year and a downtime of 3 days between runs were assumed to calculate the number of feasible batches per year. Additives include feed medium A and B, as well small proportions of glucose stock solution and antifoam.

To assess possible differences in product quality, the produced IgG was subjected to N-glycan analysis (Figure 5(a)) and compared with the glycan profile of the sFB. The iFB revealed neglectable small alterations, whereas for the approach treated with BA small, but systematic changes could be detected. While galactosylation (higher G0 and G0F) and fucosylation (lowered G1F', G1F, and G2F) decreased, the mannosylated isoforms increased. These changes might translate into alterations in antibody activity and mode of action. A study investigating the impact of in vitro trimmed mannosylated mAb1 isoforms on its effector functions, showed that while antibody-dependent cellular cytotoxicity (ADCC) was increased by about 6-fold, complement-dependent cytotoxicity (CDC) was decreased by 50%.[35] Still, the respectively investigated mannosylation ratios were 62%–94%,[35] which is not comparable to the observed significant, but small increase from 1.5% to 4.5% in this study. Yet, even with such low ratios faster clearance of the proportion of a mannosylated mAb compared with its complex and hybrid isoforms were observed within 30 days post injection in humans.[36] Afucosylated isoforms can also improve ADCC as it increases the affinity between IgG1 Fc and FcγRIII of immune effector cells.[37] Hong et al. also reported similar results as observed in this study, that is, decreased galactosylation and fucosylation, after BA treatment of mAb producing CHO cells.[16] Still, a mechanistical explanation for the observed impact of carboxylic acid on glycosylation patterns remains elusive. Ultimately, it needs to be considered for every individual biopharmaceutical product if and to which extent changes in the glycosylation profile are acceptable. If process changes are implemented in an existing process it then needs to be decided whether new studies on pharmacokinetic and -dynamics are required.

In contrast to the processes presented here, many similar studies show iFBs that last as long as the respective sFB causing distinct differences in the final viability, that is, below 50%.[6,32,33] On the one hand, the last days of such cultivations are not as productive anymore, such that the final STY is impaired. On the other hand, it is very likely that host cell impurities accumulate in the cell culture fluid during the last days of such processes. Nevertheless, even with respect to the respectively shortened process in this study final viabilities of the two iFBs were considerably lower than for the sFB (Figure 3(b)). To

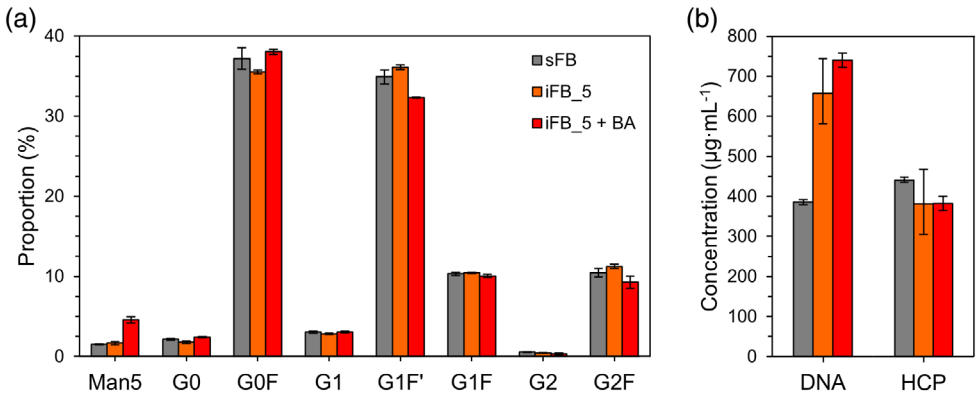

FIGURE 5 Final (a) IgG glycosylation profile and (b) potential process impurities in the cell culture fluid, that is, DNA and HCP, for the Ambr®250 cultivations. Results are displayed as means of the respective triplicates, error bars represent one SD

determine if these reduced viabilities consequently lead to higher process impurities, HCP and DNA levels were quantified (Figure 5(b)). HCP levels were very comparable between the approaches (410 ± 30 μg/ml) and within the range reported for other FB processes.[38] This is important as long exposure of the product to proteases bears the risk of uncontrollable degradation and product quality loss. In this study, a difference in the final viability of up to 25% was observed between sFB and iFB + BA, but the loss in viability occurred rather fast after BA supplementation, that is, within 4 days, resulting in a reasonably small time period for the accumulation of such proteases. Still, the amount of extracellular DNA almost doubled, but this, however, should be within feasible boundaries considering DNA to be removed by multiple log-stages within the first downstream unit operations, especially protein A capture and anion exchange chromatography.[39,40]

In conclusion, the most favorable iFB identified in the Ambr®15 to be inoculated at 5×10^6 c/ml was successfully transferred into the Ambr®250 with very similar process performance. Additionally, supplementation of BA in this iFB proofed to be an efficient modification for process intensification by reducing the process duration while maintaining product quantity and quality, thus, significantly increasing the STY. If these processes, for example, through the observed small changes in the glycan profile, are disadvantageous in terms of product quality still remains to be further examined and finally accessed via functional assays (e.g., binding affinities, target specificity, mode of action, or stability/half-life in vivo). With respect to the presented data, impact on downstream product purification should be considerably low, but this needs to be confirmed in further studies as well.

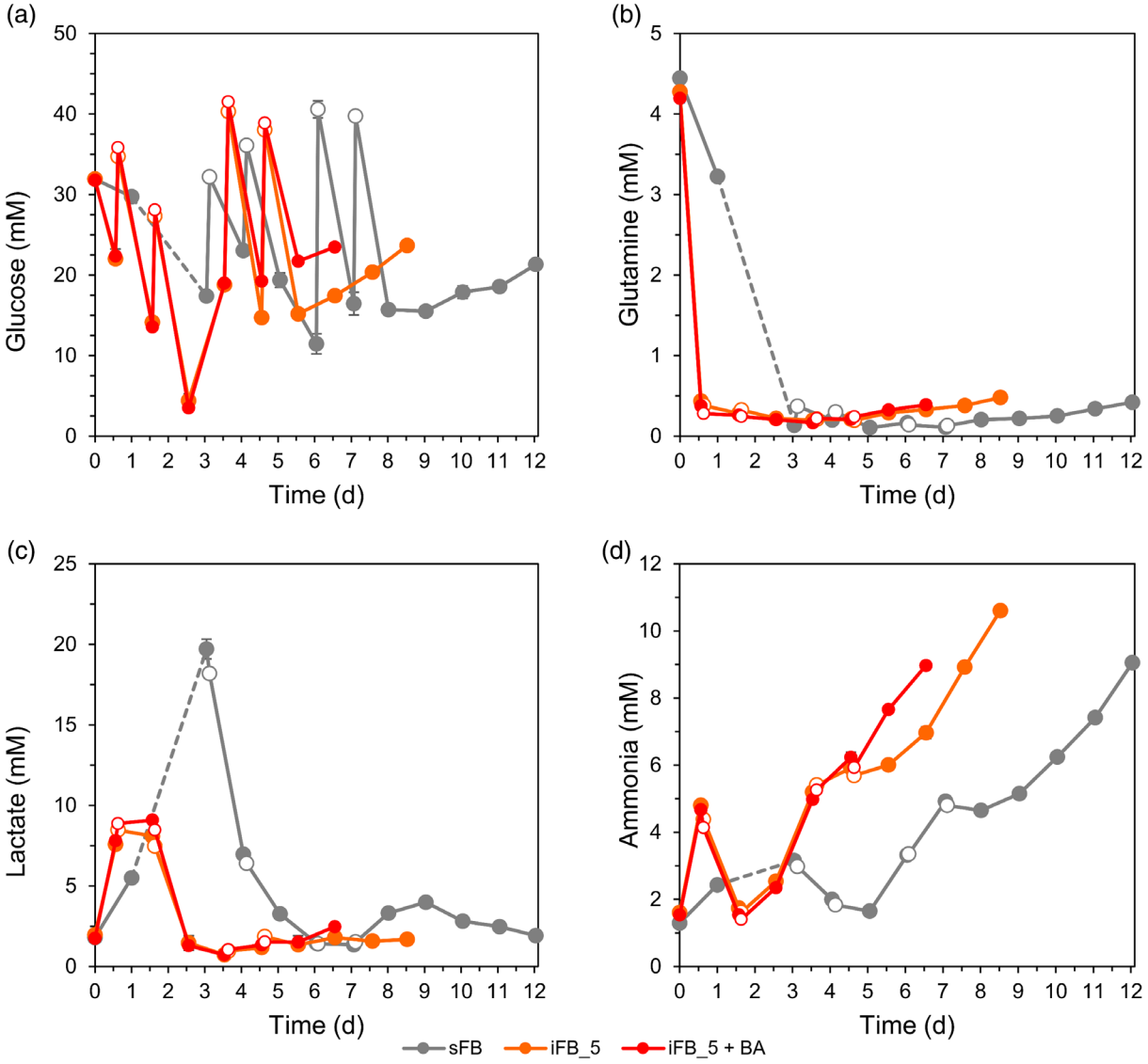

FIGURE 6 Concentration profiles of (a) glucose, (b) glutamine, (c) lactate, and (d) ammonia of the experiments conducted in the Ambr®250. Data are represented as mean of triplicates with respective SD as error bars. White symbols indicate samplings directly after the feed used for accurate calculation of metabolic rates

3.4 | Nutrient profiles of intensified fed-batches

In order to characterize the impact of process intensification on a metabolic level, nutrients and metabolites were measured extracellularly during the process. An overview of the concentrations of all measured components can be found in the Table S5. The concentrations of key nutrients glucose and glutamine, as well as byproducts lactate and ammonia, are depicted in Figure 6. Applying the standard feeding scheme with FMA and FMB for the iFBs, but earlier in time (feed start after 12 h compared to day 3 for the sFB), efficiently maintained glucose levels within the boundaries of the sFB except for day 2.5, after which the first glucose feed was applied. Feeding was started as soon as the glutamine concentration fell below 0.5 mM, that is, after 0.5 days for the iFBs and day 3 for the sFB. Subsequently, glutamine concentrations remained low as it was not contained in the feed. Lactate peak concentration was found to be doubled (20 mM) for the sFB compared with iFB (<10 mM) before feed onset. Afterwards, it was consumed and stayed below 5 mM for all processes. After a local maximum after feed start, ammonia was consumed temporarily before it accumulated to about 10 mM after the stationary phase towards the end of each process. Earlier accumulation of such inhibiting substances, like lactate and ammonia, in iFBs compared to conventional processes was reported before, for example, by Padawer et al.[32] The osmolality (data not shown) started for all approaches at 315 ± 5 mOsmol/kg and increased until the process end towards about 360 mOsmol/kg, such that no differentiating impact of these values is expected.

3.5 | Metabolic rates

In order to characterize metabolic differences, thus, derive potential individual cellular requirements, between the approaches, metabolic exchange rates were calculated for the exponential and stationary phase (see blue and yellow circled points in Figure 3), respectively. In the exponential phase (Figure 7(a)), the cells of the iFB were initially consuming twice as much glucose as the cells of the sFB which distinctly indicates an elevated energy demand and points towards a substantially enhanced glycolytic flux. At the same time, there was hardly any lactate uptake, but ammonium consumption was increased for the iFB. In parallel, IgG productivity was increased for the iFB_5 (0.35 pmol/c d) compared with the sFB (0.29 pmol/c d). In contrast, during the stationary phase (Figure 7(b)), all metabolic rates converged. Both lactate and ammonia were neither produced nor consumed in this phase for both approaches. IgG productivity was found to be identical at 0.29 pmol/c d for the sFB and iFB_5, but BA supplementation caused an increase in cell specific productivity to 0.35 pmol/c d. Still, the growth rate of the sFB was slightly higher at this point in the stationary phase which is in accordance with other studies, also reporting sustained differences in growth characteristic during the stationary phase (P4 in Brunner et al.[33]).

In order to compile all rates of the approaches, oversee their incorporated variations and compare them between each other, PCA

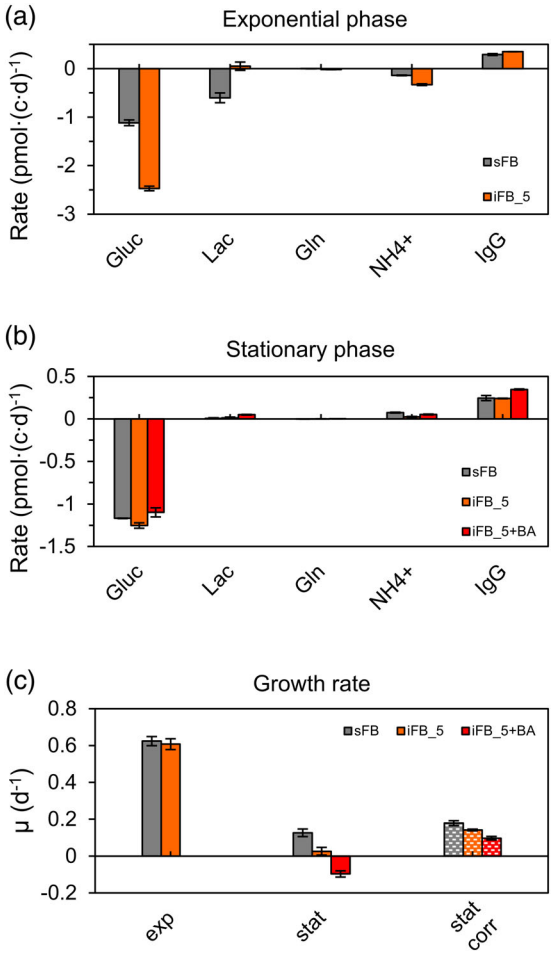

FIGURE 7 Cell-specific rates in the Ambr®250 for the metabolization of glucose, lactate, glutamine and ammonia and production of IgG in the (a) exponential and (b) stationary phase as well as (c) phase-specific growth rates, which also contain corrected values generated from closing the nitrogen balance for the metabolic model used for FBA. The exponential phase (d1.5) does not contain rates for iFB_5 + BA as BA was administered after 2.5 days

was conducted (Figure 8). It indicates clusters of the respective triplicates at different points during the processes, where the first two principal components cover already 76% of the total variation. While clear differences can be observed at the beginning of the exponential phase (iFB_5: d1.5; sFB: d4), the observations converged subsequently, resulting in two joint clusters S1 and S2 for the stationary phase, irrespectively, of the seed source or inoculation concentration. Moreover, BA supplementation caused the respective cultures to be separated from S2 early on. Stepper et al.[6] recently reported similar results within PCA of gene expression data for a standard (inoculated at 0.7×10^6 c/ml) and two intensified FBs (inoculated at 10×10^6 c/ml) where phase-specific clusters formed but progression throughout them was accelerated for the intensified processes. Yet, they only used N-1 perfusion to inoculate the processes, such that the influence of this stage on the transcriptome profile and its progression in the N-stage cannot be evaluated as done in this study.

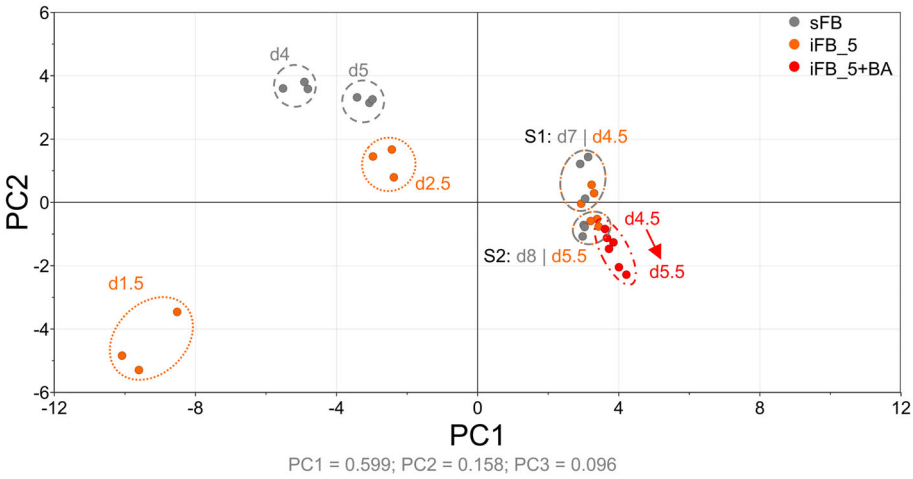

FIGURE 8 Principal component analysis (PCA) for the exchange rates used for the metabolic charactericzation of sFB and iFB in the exponential (sFB: d4, d5; iFB: d1.5, d2.5) and stationary phase (sFB: d7, d8; iFB: d4.5, d5.5). Additionally, metabolic rates of the iFB supplemented with BA were considered in the stationary phase

3.6 | Metabolic characterization using FBA and FVA

Based on the metabolic rates, geometric FBA was used to further explore metabolic differences and similarities between the sFB and iFB in their exponential and stationary phase. FVA identifies the minimum and maximum range of each reaction flux that still satisfies the constraints. It was used to identify overlapping fluxes between the approaches which means these fluxes cannot be identified as being different (Figure 9, Tables S3 and S4). Applying a χ^2-test, it was verified that the nitrogen balances closed in the exponential phase.[41] For the stationary phase, the nitrogen balance did not close for all three approaches. This was attributed to a systematic error in determining the specific growth rate, which in turn is due to difficulties in calculating the specific death rate due to more pronounced cell lysis in the stationary phase. For the sFB and iFB_5, only a small correction of the specific growth rate was required to close the nitrogen balance (Figure 7(c)), while for the iFB_5 + BA a large correction would have been needed. Thus, we decided to use the nitrogen balance to calculate the specific growth rate for the sFB and iFB_5 and to not include the iFB_5 + BA in the comparison using FBA. In general, the fluxes of glycolysis, TCA and PPP were identified to overlap in both phases between standard and intensified FB, therefore, they cannot be differentiated based on FVA.

In the exponential phase (Figure 9(a)), 88 out of 196 fluxes were found to be different between the iFB_5 and sFB. The calculated fluxes showed that twice as much ATP was produced in the iFB_5 and, related to this, that the oxidative phosphorylation reaction fluxes of NADH and $FADH_2$ and the calculated oxygen consumption were doubled. This finding can be of relevance for subsequent scale-up to production scale with respect to bioreactor design, k_La and DO control. In this case, oxygen in the gas outlet was not measured, such that the calculated specific oxygen uptake rates of the FBA model cannot be verified with experimental data. Still, with the given bioreactor design and DO control strategy, cells were sparged with a constant air flow and varying proportions of oxygen added flow as needed. However, the k_La is not exactly known which is why the oxygen transfer and, related with that, the oxygen uptake rate cannot be precisely calculated. Since identical bioreactors were used and operated at the same stirring speed and working volume also the k_La will be comparable and the oxygen flow is representative for the specific oxygen uptake rate at comparable cell densities. This oxygen flow was at about 0.02 vvm for the sFB and at 0.036 vvm for the iFB, confirming the increased oxygen uptake rate of the cells in the iFB. The main sources to cover the increased demand of required reduction equivalents NADH are either glycolysis and TCA or the PPP with subsequent transhydrogenation of NADPH. Based on FVA, it cannot be distinctly assigned which of these pathways is responsible for the increased NADH generation in the iFB_5. However, most likely the NADH production is covered by glycolysis and the citric acid cycle and these pathways are also responsible for the increase in NADH production in the iFB_5. This in accordance with the observation, that the energy demand of highly proliferating CHO cells in the exponential phase is mainly covered by the TCA cycle indicated by an increased activity of pyruvate dehydrogenase (PDH).[42] Further studies[43,44] exemplified that glucose carbon is predominantly shuttled via glycolysis rather than PPP during the exponential phase, for example, shown by Carinhas et al. with less than 10% being diverted into latter one.[45] Also, the higher specific production rate of the byproducts lactate and alanine in the iFB_5 indicates a higher glycolytic flux at this point as already observed with the calculated lactate rate in Figure 7. In the sFB, however, the metabolic switch from lactate production to consumption[46] already occurred and byproduct formation had stopped at this point. To a certain extent, the different ratios in glucose uptake will simply rely on the difference in cell size (Figure 3(c)). Considering this, it is obvious that cell duplication of larger cells then requires more biomass precursors, for example, lipids that are composed of fatty acids. Their synthesis requires NADPH, that is generated via anaplerotic catalyzation of malate to pyruvate (iFB_5: 2.11 pmol/c d vs. sFB: 1.17 pmol/c d). Pyruvate is then finally converted into lactate in the cytosol and this might explain why at a comparable process phase, that is, in the exponential phase, still lactate production is found for the iFB_5. Similarly, a fraction of the increased amount of ATP is used in the enhanced production of biomass precursors,

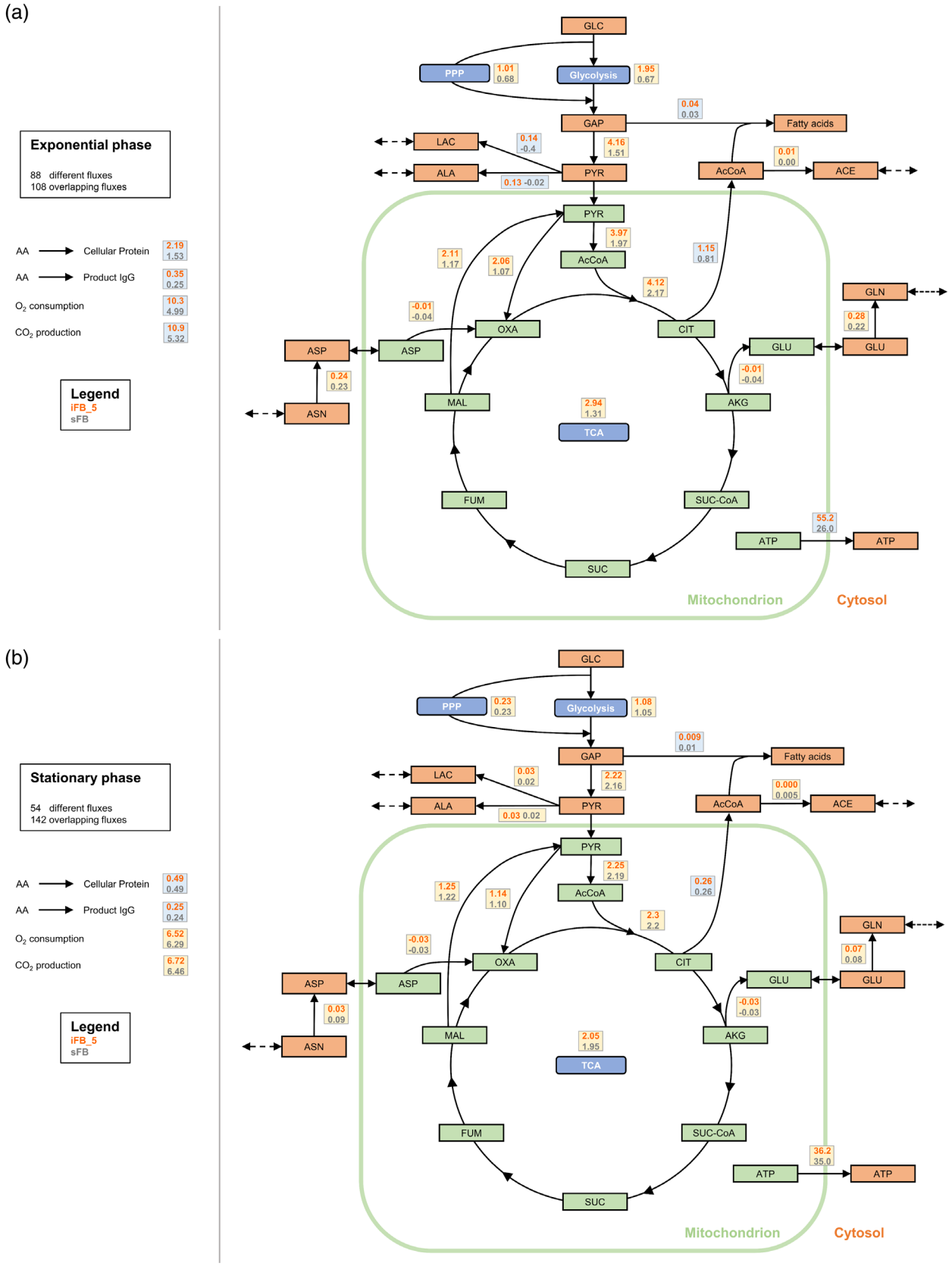

FIGURE 9 Metabolic characterization of the sFB and iFB_5 in the Ambr®250 using FBA. Metabolic fluxes of the two processes were compared in the (a) exponential and (b) stationary phase. According to the FVA, overlapping fluxes are displayed by yellow boxes, while distinctly different ones are encoded by blue boxes. The unit of all fluxes is pmol/(c d). The average flux of the TCA cycle is represented by the average fluxes between the flux from citrate (CIT) to α-ketoglutarate (AKG) and the flux from succinate (SUC) to fumarate (FUM). Figure adapted from Pan et al. (2017)[28]

especially cellular protein, required to meet demands for the slightly higher biomass formation rates. Although the specific growth rates were similar between processes the cells in the iFB were a bit larger resulting in slightly higher biomass formation rates. Accordingly, the fluxes related to the formation of protein, fatty acids, carbohydrates, and nucleotides were different between iFB_5 and sFB. Furthermore, the specific productivity was slightly higher in the iFB also requiring a bit of extra ATP. However, both the increase in biomass formation and in productivity seem not high enough to explain the increase in ATP formation. In contrast to this study's observations, Brunner et al.[33] reported an increased metabolic activity in the exponential phase (P1) for the sFB characterized by an elevated ATP production and glucose demand compared with their respective iFB inoculated at 5×10^6 c/ml. The difference to the aforementioned study is, that N-1 perfusion was used to inoculate both sFB and iFB_5, while in this study conventional N-1 and N-1 perfusion was used for the sFB and iFB_5, respectively.

In the stationary phase (Figure 9(b)), only 54 fluxes remained different based on FVA indicating that the metabolic states of the cells in both sFB and iFB_5 converge with progressing cultivation time. Also, the remaining different fluxes only had very small differences and were almost overlapping. The byproduct formation almost stopped and the fluxes of the main pathways glycolysis, TCA and PPP were very similar between sFB and iFB_5.

Remarkably, the flux changes from exponential to stationary phase were larger for the iFB_5, where the total metabolic turnover was decreased. In contrast, fluxes for the sFB increased generally slightly from the exponential to stationary phase. Here, the ATP generation was increased by 35%. While during the stationary phase about 16% of the total produced protein was IgG for both sFB and iFB_5, this ratio increased to about 50% in the exponential phase demonstrating efficient and similar product synthesis in both approaches. Concluding, the identified and characterized metabolic differences mainly rely on the initial difference in cell size, that fades out with time, but the flux ratios between the sFB and iFB_5 correlate. Recognizing this strengthens the implementation of an iFB with the proposed strategy to be straightforward and robust.

4 | CONCLUSION

Intensified processes allow for increased productivities, thus they are legitimately predestined to meet upcoming demands of biopharmaceutical manufacturing. The full potential of process intensification has not been explored yet and emerging processes of the future will be designed to exceed current biological and technical boundaries. At the same time, it is important to gain a better understanding of the impact of intensification on a cellular, that is, metabolic, level as well as on product quality, while keeping process intensification as simple and straightforward as possible.

This study demonstrates rapid and successful implementation of an iFB based on an existing production process (sFB) in multi-parallel small-scale bioreactors. Different inoculation concentrations were evaluated and the best performing iFB was further characterized and compared to the sFB on a metabolic level using FBA. This allowed to identify and assess differences between the processes. Our data show, that metabolic differences appear initially in the exponential phase but are within manageable borders and also disappear quickly.

Consistent with previous reports, the volumetric productivity was efficiently improved with increased inoculation density (+50%). From a metabolic perspective, the processes differed in the exponential, but converged quickly in the stationary phase, thus, supporting the hypothesis that the IgG is processed in a similar manner. Further improvement was then achieved by supplementing the iFB with a productivity enhancer (BA). For the first time, we show that this leads to an increase in the cell-specific productivity but without being correlated to an increased arrest of the cell cycle in the G0/G1 phase that is usually observed when cells are exposed to BA concentrations. The N-glycosylation profile and extracellular DNA content revealed only very minor differences that are likely to be acceptable. Ultimately, this strategy can improve the annual capacity utilization of large-scale production bioreactors by up to 70%. This data highlights how beneficial additional process modifications, beyond technical and strategic process intensification itself, can be. In the future, it would be particularly interesting to gain a better understanding of the impact of BA on cellular mechanisms, for example, through transcriptomic analyses. Such analyses could help to identify certain genes involved in the observed distinct increase in productivity shortly after BA supplementation and provide further insight on underlying cellular mechanisms. In addition, screening and explicit evaluation of other substances and molecules could be considered,[47] but also physical parameters, such as temperature or pH shifts should be subject of further investigations.

The changes in process strategy described in this study can also be of particular interest for upcoming processes to produce biosimilars, as it will increase margins and put even more pressure on competitors, for example, before biosimilars for blockbuster drugs such as Humira® will enter the (United States) market.[48] It is to be emphasized, that all experiments described can be conducted rapidly, with the result that the intensification of an existing process can be evaluated within only a few weeks using multi-parallel small-scale bioreactors. This allows for a significant and rapid increase in production capacity. Further improvements are within reach using systems biology tools, for example, FBA and transcriptomics, as a guiding tool in further screenings.

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AUTHOR CONTRIBUTIONS

Markus Schulze: Conceptualization (lead); investigation (lead); methodology (lead); software (lead); supervision (lead); validation (lead); visualization (lead). **Julia Niemann:** Supervision (supporting). **Rene Wijffels:** Supervision (equal). **Jens-Christoph Matuszczyk:**

Conceptualization (equal); methodology (equal); resources (lead); supervision (equal). **Dirk E. Martens:** Conceptualization (equal); investigation (supporting); methodology (equal); supervision (lead); validation (equal).

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

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SUPPORTING INFORMATION

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