Beneficial effects of pyruvate for a high-density perfusion process

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Running title: Pyruvate impact on perfusion

Abstract

High volumetric productivities can be achieved when perfusion processes are operated at high cell densities. Yet it is fairly challenging to keep high cell density cultures in a steady state over an extended period. Aiming for robust processes, in this study cultures were operated at a constant biomass specific perfusion rate (BSPR). The cell density was monitored with a capacitance probe and a continuous bleed maintained the cell density at the targeted viable cell volume (VCV). Despite our tightly controlled BSPR, a gradual accumulation of ammonium and changes in cell diameter were observed during the production phase for the three different monoclonal antibodies (mAbs). Although a lot of efforts in media optimization have been made to reduce ammonium in fed-batch process, less examples are known about how media components impact the cellular metabolism and thus the quality of monoclonal antibodies in continuous processes. In this work, we show that a continuous Na-pyruvate fed at 2 g/L/day strongly reduced ammonium production and stabilized fucosylation, sialylation and high mannose content for three different mAbs.

Keywords

Perfusion, CHO cell culture, pyruvate, glycosylation, mAb

1 Introduction

For decades the fed-batch process has been the dominant approach to produce recombinant proteins in mammalian cells. During this period the continuous improvement of cell lines, media formulation and feeding strategy could prolong the cell viability and push the maximal harvest titers beyond the 10 g/L limit (Huang et al., 2010; Takagi et al., 2017) – values that are clearly beyond anything that would have been seriously predicted when in 1986 the first commercial CHO cell-based fed-batch process achieved final titers of 0.05 g/L (Wurm, 2004). Today, the fed-batch process as a matured technique is widely used, but it offers limited prospects for further improvements while the production of recombinant proteins has become a strongly competitive field. The high pressure to reduce production costs drives innovation towards new approaches such as intensified fed-batches (Chen et al., 2018; Jordan et al., 2018; Xu et al., 2020; Yang et al., 2016), perfusion cultures and/or continuos production (J.-M. Bielser et al., 2018; Coolbaugh et al., 2021; Daniel Johannes Karst et al., 2018; Warikoo et al., 2012; Wolf et al., 2020). The highest volumetric productivity per reactor volume is promised by perfusion processes, while the low residence time of the protein in the reactor can be beneficial for some quality attributes (J. M. Bielser et al., 2019). Nevertheless, stabilization of the cellular metabolism is critical for the process to deliver a stable product output. (Bertrand et al., 2019; Hutter et al., 2017; Daniel J. Karst et al., 2017; Daniel J Karst et al., 2016). With the perspective of a manufacturer, it is not crucial to understand if all metabolic markers are stable, but the product quality attributes (QAs) must be under control with no major changes in time.

To control perfusion cell cultures, different online monitoring probes are available (Kiviharju et al., 2008). Capacitance probes have become a standard approach to monitor and control biomass. Any increase in biomass is detected instantly by the change in permittivity signal and can trigger or stop the cell bleed pump. In the system used in the present study, this pump continuously removes cells from the bioreactor, assuring a stable biomass providing the cell

66 growth rate can trigger a minimal bleed. The control of both, the biomass and the actual perfusion rate should allow a more precise regulation of the perfusion process over extended
68 durations.

Whereas the bleeding of the culture represents a straightforward method to achieve the steady
70 state, it still needs specific attention when optimizing the yield (Bielser et al., 2020). Since the protein is not usually recovered from the bleed, more bleed would lower product yields.
72 Accordingly, a large volume of bleed is not desired. Moreover, more bleed represents strong cell growth, whilst the highest productivity can be achieved in the stationary phase. A true
74 stationary phase for all the cells however is not desired in perfusion since a certain amount of cell growth is needed to replace dying cells and to maintain a constant biomass.

76 We applied our strategy to control the biomass at its corresponding perfusion rate to several clones and found that a bleed pump under the control of a capacitance probe worked reliably to
78 maintain the online permittivity signal. However, the offline cell count did not remain constant during the perfusion run. In some cases, it dropped significantly while the permittivity signal
80 remained stable. As it is known from fed-batch processes, the cell diameter can change and, especially in the stationary phase, there can be a substantial increase in the cellular volume (Pan
82 et al., 2017). The mechanisms that trigger these cell volume changes are still poorly understood. For fed-batch processes, these changes have no impact on the process and its control. For
84 perfusion however, the distinction between cell number and biomass becomes relevant when using a capacitance probe which cannot distinguish between an increase of biomass due to cell
86 division and the one due to an increase of cell diameter. In the latter case the bleed will maintain the biomass constant, resulting in a reduction of the absolute cell number. Perfusion cultures
88 for which a significant increase in cell volume was observed also showed a shift in the metabolism and more importantly in the protein quality.

This study aims to stabilize the cell diameter and the cellular metabolism as well as the protein quality. Our data demonstrate that pyruvate supplementation can improve the perfusion process in the desired direction.

2 Material and methods

2.1 Cell lines and culture media

The cell culture media used in this study is a proprietary chemically defined fed-batch platform medium derived from DMEM/F12 medium. Three different CHO-K1 cell lines expressing fusion (mAb 1 and mAb 3) or bispecific monoclonal IgG1 (mAb 2) antibody were used in this study. Their selection system is based on glutamine synthetase (Fan et al., 2013; Seabra et al., 2013). Therefore, the medium does not contain any glutamine.

2.2 Biomass specific perfusion rate

Cell specific perfusion rate (CSPR, pL/cell/day) is often used to described the amount of media flowrate perfused for a given cell concentration (Bausch et al., 2019). Equivalently here, the biomass specific perfusion rate (BSPR, L/VCV/day) defines the amount of medium flowrate used for a percentage of viable cell volume (as opposed to cell concentration). Viable cell volume (VCV, %) was calculated as presented in equation 1 (Metze et al., 2020) with the assumption that cells are spherical and with D the average cell diameter, V the cell culture volume and VCD the viable cell density.

$$VCV = \frac{\left(\frac{4}{3} \cdot \pi \cdot \left(\frac{D}{2}\right)^3 \cdot VCD\right)}{V} \cdot 100 \quad (1)$$

BSPR was then calculated by dividing the perfusion rate (P) by this viable cell volume:

$$BSPR = \frac{P}{VCV} \quad (2)$$

Volumetric productivity (VPR, g/L/day) was calculated using the mAb concentration (c_{mAb}) and the harvest rate (H).

$$VPR = c_{mAb} \cdot H \quad (3)$$

2.3 Perfusion bioreactors

Perfusion bioreactors (Sartorius, Goettingen, Germany) were operated according to Table 1. The DO was fixed at 50 % and the pH was maintained between 6.90 and 7.24 using CO_2 and a 1.1 M Na_2CO_3 solution. The working volume of the culture was set to 2 L.

The perfusion rate was started on day 0 and increased stepwise with the VCV increasing to keep the target BSPR constant. Once the VCV set-point was reached, the final perfusion set-point flowrate was fixed. Gravimetric control was used for the basal medium inlet flow. The harvest flow was controlled to keep the bioreactor weight constant. An on-line permittivity signal (BioPAT®Viamass, Sartorius, Goettingen, Germany) was used to control the bleed stream and, therefore, maintain the viable biomass at the target set-point (Davey et al., 1993; Downey et al., 2014; Metze et al., 2020). The permittivity set-point was defined and corrected during the run to maintain the target VCV and BSPR constant.

An alternating tangential flow filtration device (ATF2H, Repligen, Waltham, Massachusetts, USA) using polyethersulfone (PES) hollow fibers with a pore size of 0.2 µm (Repligen, Waltham, Massachusetts. USA) was used as the cell retention device.

A concentrated glucose (400 g/L) feed was added continuously to maintain the respective concentration in the bioreactor at 4 g/L. The flow rate was calculated based on the glucose concentration measured during in process control (IPC) and was adjusted daily. A stock solution of 110 g/L Na-pyruvate (Sigma Aldrich, St. Louis, Missouri) was used to feed 2 g/L/day pyruvate starting from day 0.

2.4 Analytics and data treatment

Cell counts, viability, cell diameter, glucose, lactate, and ammonium were quantified by the BioProfile FLEX2 (Nova Biomedical, Zürich, Switzerland) for mAb 1 and mAb 2. For mAb 3 cell counts, viability and cell diameter measurements were done using a Vi-Cell analyzer

(Beckman Coulter, Brea, CA), while metabolites were quantified with the Vi-CELL MetaFLEX™ bioanalyte analyzer (Beckman Coulter, Brea, CA). Amino acids concentrations were measured by Ultra-Performance Liquid Chromatography (UPLC, Waters, Baden-Wättwil, Switzerland). Protein concentrations were measured using a protein affinity high performance liquid chromatography device (PA-HPLC, Waters, Baden-Wättwil, Switzerland) for the fusion monoclonal antibodies. For the bispecific IgG1 (mAb 2), titers were measured by surface plasmon resonance (Biacore C, GE Healthcare, Chicago, Illinois, USA). Protein quality of Phytips eluates (PhytipsVR, PhyNexus, San Jose, California, USA) were analyzed by reduced caliper (Caliper LabChip GXII, Perkin Elmer, Waltham, Massachusetts, USA) for the determination of clipping and by size exclusion high performance liquid chromatography (SE-HPLC, Waters, Baden-Wättwil, Switzerland) for aggregates. The glycosylation profiles were analyzed by separation of 2-aminobenzamide labeled glycans in Ultra-Performance Liquid Chromatography (2AB-UPLC, Waters, Baden-Wättwil, Switzerland) for mAb 1. For the mAb 2 and mAb 3, the glycan forms were detected through multi-attribute method (MAM) by high performance liquid chromatography-mass spectrometry (LC-MS, Vanquish™ Horizon UHPLC System and Q Exactive ™ Plus, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Data was analyzed using JMP statistical software (version 15).

3 Results

3.1 Biomass specific perfusion rate (BSPR) as a control parameter for perfusion

Three different monoclonal antibodies were produced in a comparable perfusion process. Once the target permittivity was reached (Figure 1C), bleed was activated and a strictly controlled perfusion rate was applied. For each cell line an operating biomass specific perfusion rate (BSPR) was selected (Table 1) based on preliminary experimental data. VCV was calculated

each day from the process data and the permittivity signal set-point adjusted to match the target VCV (Figure 1C and 1D). This control strategy successfully maintained a stable VCV within the reactor. However, the cell specific perfusion rates (CSPR) were not stable. At the beginning of the production phase mAb 1, mAb 2 and mAb 3 had a CSPR of 18.8, 35.6 and 36.7 pL/cell/day respectively (Figure 1B). The CSPR increased with time till a final value of 46.1 pL/cell/day for mAb 1, 43.1 pL/cell/day for mAb 2 and 94.4 pL/cell/day for mAb 3. This data demonstrated that controlling perfusion using a constant BSPR as defined above is necessary to maintain a VCV constant. This would not have been achieved if the CSPR was used instead. For all three cell lines the increase in CSPR can be explained by an increase in cell diameter observed during the production phase (Figure 2A). With the control strategy used here, a cell population with a growing diameter would increase the measured VCV. As this value is kept constant, the cell number will decrease (Figure 2B). Interestingly, a strong reduction in lactate concentration preceded the changes in cell diameter. The diameter change was concomitant to low lactate availability (below 0.15 g/L, Figure 2C) and to increasing ammonium concentrations (Figure 2D). These particular profiles of lactate and ammonium motivated us to explore the potential impact of a pyruvate supplementation on our process. It is known indeed that pyruvate reduces ammonium formation and cell growth arrest (Herzenberg et al., 1960).

3.2 Impact of pyruvate supplementation on cell culture and metabolite profiles

Two different conditions were studied for each molecule. The first condition can be considered as the control and was described in section 3.1. The second condition was supplemented with a continuous Na-pyruvate feed at a constant addition rate of 2 g/L/day throughout the run. The same VCV was targeted in the supplemented runs as in the control runs. Figure 3A illustrates for mAb 1 how the VCV and perfusion rate were aligned in both conditions. On the other hand, in this example, pyruvate supplementation diminished the cell diameter drift. While for the control condition the cell diameter continually increased and achieved a final value of 23 µm,

the pyruvate feed could stabilize the cell diameter after day 13 (Figure 3C). Consequently, the viable cell density was also stabilized at 60 x 10^6 VCs/mL (Figure 3B). Moreover, the cell growth rate was stable instead of decreasing as indicated by the bleed flowrate that is representative of the excessive biomass produced (Figure 3E).

Other impacts of pyruvate feeding were seen on different metabolite concentrations. Pyruvate prevented lactate reduction and decreased alanine catabolism (Figure 4). In the control conditions, lactate accumulated rapidly on day 1. It was then gradually reduced to almost undetectable concentrations (about 0.10 g/L) from day 10 onwards (Figure 4B). When lactate was absent from the process, ammonium started accumulating to up to 5 mM (Figure 4C).

Na-pyruvate fed at 2 g/L/day strongly affected both metabolites. As shown in Figure 4B, the lactate profile stayed above 0.35 g/L, while the ammonium reached a steady state at 1.5 mM (Figure 4D). The pyruvate also affected alanine and glycine concentrations in the supernatant, with a strong increase of the first and a reduction of the latter (Figure 4D-E). Other amino acids were not impacted.

3.3 Impact of pyruvate supplementation glycosylation profile

In this section the impact of the tested pyruvate supplementation strategy on some quality attributes is presented. While no difference was observed for aggregates (Figure 5D) and clipped forms (Figure 5E) concentrations, glycosylation profiles were significantly impacted. In the control conditions, glycoprofiles were changing over time during the production phase. For example when producing mAb 1, fucosylation (Figure 5A) and sialylation (Figure 5B) levels decreased slowly during the run, while high mannose forms increased from 1% to 4% (Figure 5C). In contrast, with the addition of the pyruvate feed, fucosylation (Figure 5A) and sialylation were stable at 97% and 48% (Figure 5B), respectively and the high mannose level was reduced and maintained around 1.5% (Figure 5C).

Similar effects of pyruvate were also found for mAb 2 and mAb 3 (Table 2). For the mAb 2, an increase of fucosylation and a reduction of high mannose were observed in the presence of the pyruvate feed. This molecule is not sialylated. The same trends were found for the different glycosylation sites (A, B and C) on mAb 3. An increase in sialylation and a reduction of high mannose were detected when pyruvate was supplemented. No fucosylation was observed for A and B sites, while an increase at C site was obtained when pyruvate was added to culture. Only one of the sites (site C) contained fucosylated glycoforms which were slightly higher but much more stable under pyruvate supplemented conditions.

To compare the variation of glycoforms during the production phases, the coefficient of variation (CV) of the given glycoforms was compared between the control and the pyruvate supplemented condition. Considering the CV for each condition, the ratio between the pyruvate feed culture and control was always below 1 for mAb 1, mAb 2 and mAb 3 (Table 2). This indicates that less variability during the steady state was observed for fucosylation, sialylation and high mannose in the presence of pyruvate feed.

4 Discussion

Control of perfusion bioreactors at high cell density can be challenging due to fluctuating cell concentrations (J. Bielser et al., 2020; Vits & Hu, 1992). A small cell diameter increase can significantly impact the VCV calculation (equation 1). Therefore an operating CSPR defined at smaller cell diameter, will be not able to sustain the nutrient requirements for the same amount of bigger cells (Gibco, 2020), representing a risk of process instability. For this reason, a new control strategy was introduced in Figure 1. The definition of a biomass specific perfusion rate (BSPR) allows a fine control of the viable biomass, independently of cell status.

Cell diameter changes is a phenomenon well known in fed-batch (Ansorge et al., 2010; Pan et al., 2019, 2017), where it is associated with cell cycle arrest and increase in productivity. At

BSPR near the nutrient limitation, we can see the same event occurring in perfusion cell culture. Interestingly, the increase in cell diameter for three different clones expressing three different mAbs (Figure 2A) occurs together with reduction in lactate concentration (Figure 2C) and strong increase in ammonium production (Figure 2D). It has been reported that during the cell size increase in fed-batch process there is a higher tricarboxylic acid (TCA) flux (Pan et al., 2017), whose regulation is important to keep cell in a stable process (Martínez-Reyes & Chandel, 2020). An optimized extracellular level of TCA components can avoid potential bottlenecks in the Krebs cycle (Zhang et al., 2020). Here we show that an increase of pyruvate concentration (the entry point of citric acid cycle) with an external feed leads to stabilization of perfusion cell culture as shown by the cell diameter for three different cell lines (Figure 3C and Supplementary Figure 1C). In all three cases the CV (%) ratio between Na-pyruvate and control condition is always under 1 (data not shown).

Interestingly, the stabilization of cell diameter occurred together with the increase of lactate concentration and reduction in NH_4^+ production. It has been already shown in fed-batch that pyruvate supplementation can modulate the formations of these by-products (Li et al., 2012). Our study confirms that, also in perfusion, pyruvate supplementation can reduce the catabolism of alanine into pyruvate (Figure 4D and Supplementary Figure 1F), which is responsible of NH_4^+ production. Pyruvate supplementation had a direct impact on the lactate concentration (Figure 4B). Both pyruvate and lactate are known for their role as a redox buffer that equilibrates the NADH/NAD^+ ratio of the cells (Rabinowitz & Enerbäck, 2020), with a direct link to the mitochondrial energy metabolism.

Here we showed a stabilization of fucosylation and sialylation profile together with strong reduction in high mannose level for all three mAbs (Figure 5, Table 2). Increase in high mannose level can negatively impact the efficacity, pharmacokinetics, and stability of monoclonal antibodies (Mastrangeli et al., 2020). It is well-known that high ammonium level

can inhibit the sialyltransferase (Blondeel & Aucoin, 2018) and N-acetylglucosaminyltransferase I (Shi & Goudar, 2014), leading to reduction in sialylation and increase in high mannose species. Here we showed that pyruvate can reduced NH_4^+ level, leading to higher percentage of fucosylation and sialylation and lower percentage of high mannose.

5 Conclusions

Stability of perfusion cell cultures can be described at different levels: process parameters (pH, DO, temperature etc.), flowrates (media addition, harvest, bleed, other feeds), impurity concentrations (host cell proteins, DNA etc.), product specific attributes (glycosylation, aggregates etc.), extra and intra cellular metabolites concentration are some of them. Product concentration, impurities and quality attributes are critical for a manufacturing process to be considered under control. Achieving such a stable state with higher cell densities and optimized media consumption have proven to be sometimes challenging. Limitation of one key nutrient can be sufficient to impact cell metabolism. In this work, a cell diameter drift was described and identified as being a visible phenotypic change for three clones all expressing different monoclonal antibodies. This phenomenon seemed to be linked to a strong lactate reduction. This work then showed the pyruvate as key media component to prevent the cell culture drift as the carbon source via lactate was probably compensated. The most visible effects were demonstrated with the clone producing mAb 1, but all three benefited from this supplementation strategy as glycosylation variations were minimized. This work demonstrates the beneficial effect of pyruvate addition on perfusion cell cultures that were close to limiting conditions.

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7 Reference

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8 Tables

Table 1. Perfusion bioreactor settings

Cell line	Inoculation density (10^6 cells/mL)	BSPR (L/VCV/day)	VCV target (%)	Temperature (°C)	Perfusion rate (VVD)
mAb 1	10	0.08	31	36.5 †	2
mAb 2	10	0.06	24	36.5 †	2
mAb 3	2	0.14	8.8	36.5 ‡ 33 §	1.2

† all run, ‡ day 0 to day 4, § day 5 to end run

Table 2. Glycosylation profile. The average and the coefficient of variation (CV, %) for total fucosylation (A), sialylation (B) and high mannose (C) are shown for 3 cell lines producing each a different mAb. A CV ratio was calculated between the supplemented condition and the control (ctrl).

A.

		Fucosylation (%)				
		Mean		CV (%)		
		ctrl	Na-pyruvate 2 g/L/day	ctrl	Na-pyruvate 2 g/L/day	Ratio
mAb 1		95.50	97.62	1.62	0.74	0.46
mAb 2		92.50	96.44	3.49	1.16	0.33
	Site A	-	-	-	-	-
mAb 3	Site B	-	-	-	-	-
	Site C	98.03	99.30	34.92	10.39	0.30

B.

		Sialylation (%)				
		Mean		CV (%)		
		ctrl	Na-pyruvate 2 g/L/day	ctrl	Na-pyruvate 2 g/L/day	Ratio
mAb 1		45.38	48.23	4.52	2.58	0.57
mAb 2		-	-	-	-	-
	Site A	84.84	88.75	1.66	0.76	0.46
mAb 3	Site B	91.53	94.76	0.91	0.39	0.43
	Site C	3.73	5.48	9.70	7.99	0.82

C.

		High mannose (%)				
		Mean		CV (%)		
		ctrl	Na-pyruvate 2 g/L/day	ctrl	Na-pyruvate 2 g/L/day	Ratio
mAb 1		2.90	1.38	40.68	34.14	0.84
mAb 2		5.35	2.02	54.72	44.74	0.82
	Site A	0.29	0.18	22.43	19.81	0.88
mAb 3	Site B	0.53	0.32	30.42	11.31	0.37
	Site C	0.68	0.39	36.44	25.76	0.71

9 Figure Legends

Figure 1. Cell culture profiles for three different mAbs in 3.5 L perfusion bioreactors with the biomass specific perfusion rate (A), the cell specific perfusion rate (B), the permittivity (C) and the viable cell volume (D). Dotted lines represent the BSPR and VCV set-points. The initial cell growth phase is highlighted in grey (C and D).

Figure 2. Cell diameter and metabolic profiles for the three different mAbs tested in the perfusion process: cell diameter (A), viable cell density (B), lactate concentration (C) and ammonium concentration (D.). The cell growth phase is represented in grey.

Figure 3. Impact of pyruvate feeding on cell culture profile for mAb 1. Cell culture data for the control and supplemented run with viable cell volume (A), viable cell density (B), cell diameter (C), perfusion rate (D), bleed (E) and the volumetric productivity (F).

Figure 4. Effect of pyruvate feeding on metabolite profiles obtained for mAb 1. glucose concentration (A), lactate (B), ammonium (C), alanine (D) and glycine (E).

Figure 5. Effect of pyruvate feeding on quality attributes for mAb 1: fucosylation (A), sialylation (B., high mannose (C), aggregates (D) and clipping (E)

Supplementary Figure 1. Impact of pyruvate feed on mAb 2 and mAb 3. A different VCV control was applied for mAb 2 and mAb 3 (A) No big difference was observed for VCD (B), cell diameter (C) and lactate concentration (D). Pyruvate supplementation influences the ammonium (E), alanine (F) and glycine (G) concentration