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Seed Train Intensification Using an Ultra-High Cell Density Cell Banking Process

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Abstract: A current focus of biopharmaceutical research and production is seed train process intensification. This allows for intermediate cultivation steps to be avoided or even for the direct inoculation of a production bioreactor with cells from cryovials or cryobags. Based on preliminary investigations regarding the suitability of high cell densities for cryopreservation and the suitability of cells from perfusion cultivations as inoculum for further cultivations, an ultra-high cell density working cell bank (UHCD-WCB) was established for an immunoglobulin G (IgG)-producing Chinese hamster ovary (CHO) cell line. The cells were previously expanded in a wave-mixed bioreactor with internal filter-based perfusion and a 1 L working volume. This procedure allows for cryovial freezing at 260 $\times$ 10^6 cells mL^{-1} for the first time. The cryovials are suitable for the direct inoculation of N−1 bioreactors in the perfusion mode. These in turn can be used to inoculate subsequent IgG productions in the fed-batch mode (low-seed fed-batch or high-seed fed-batch) or the continuous mode. A comparison with the standard approach shows that cell growth and antibody production are comparable, but time savings of greater than 35% are possible for inoculum production.

Keywords: Chinese hamster ovary cells; cryopreservation; monoclonal antibodies; N−1 perfusion; process intensification; upstream processing

Citation: Müller, J.; Ott, V.; Eibl, D.; Eibl, R. Seed Train Intensification Using an Ultra-High Cell Density Cell Banking Process. *Processes* **2022**, *10*, 911. https://doi.org/10.3390/pr10050911

Academic Editor: Raja Ghosh

Received: 7 April 2022
Accepted: 1 May 2022
Published: 5 May 2022

1. Introduction

The prevalence of biopharmaceuticals in the pharmaceutical market has been steadily increasing in terms of approvals and sales, reaching USD 270 billion in global sales in 2020 [1]. Biopharmaceuticals include, among others, enzymes, hormones, blood-clotting factors, and vaccines. The largest and best-selling group, mAbs, is mainly produced in CHO cells [2]. As a result of continuous improvements to production cell lines and cell culture media in recent decades, product titers of up to 5 g L^{-1} in standard fed-batch processes have become state of the art in biopharmaceutical production, and maximum titers in the range of 10 g L^{-1} have already been achieved [3–5].

The focus of biopharmaceutical research and production has now shifted from improving cell lines and media to intensifying production processes to achieve time and cost savings. A frequently used approach is seed train intensification, especially through N−1 perfusion, in which perfusion cultivation is done as the final step of inoculum production to generate UHCDs exceeding 100 $\times$ 10^6 cells mL^{-1}. These cells can subsequently be used to inoculate a production bioreactor. On the one hand, the inoculum production steps can be reduced, and on the other hand a continuous process or a high-seed fed-batch process can be directly implemented with these cells instead of the otherwise usual low-seed fed-batch (standard fed-batch) process. High-seed fed-batch is defined as fed-batch processes, in which the inoculation cell density of the production process is increased to 4–10 $\times$ 10^6 cells mL^{-1}, compared to a maximum of 0.5 $\times$ 10^6 cells mL^{-1} in standard processes, thus avoiding unproductive phases [6]. Successful N−1 perfusions have already been achieved. Schulze et al. demonstrated that cell densities of up

to 100 × 10^6 cells mL^{-1} can be achieved in a wave-mixed perfusion bioreactor bag with an internal filter for an IgG-producing CHO DG44 cell line and that these cells are suitable as inoculum for batch experiments in stirred tank bioreactors (STRs) with a 15 mL working volume. Comparable maximum viable cell densities (VCDs) and slightly increased productivities were observed for cells from N−1 perfusion [7]. Stepper et al. used tangential flow filtration (TFF) in the cultivation of IgG-producing CHO DG44 cells in STRs with a cell density of up to 45 × 10^6 cells mL^{-1} (3.1 L and 16 L working volume), with the aim of subsequently starting a high-seed fed-batch experiment [8]. In addition, Xu et al. used STRs with alternating TFF (ATF) up to a volume of 200 L and reached about 10 × 10^6 cells mL^{-1} (mAb-producing CHO K1 cell lines) before inoculating the production bioreactor (STR, up to 1000 L) [9]. Wright et al. tested the growth performance of enzyme-producing CHO cells by inoculating their spin tube bioreactors from an N−1 perfusion STR (10 L working volume) with ATF at cell densities up to 100 × 10^6 cells mL^{-1} [10]. The fact that cell densities >200 × 10^6 cells mL^{-1} are also possible in the perfusion mode has already been shown by Clincke et al. (IgG-producing CHO cell line) in a wave-mixed bag with TFF [11] and by Müller et al. (IgG-producing CHO DP-12 cell line) in a wave-mixed perfusion bag with an internal filter [12]. In these two studies, however, no further bioreactors were inoculated with the cells from the perfusion process.

Besides N−1 perfusion, another starting point for the seed train intensification is the cell banking process. There are two approaches, both based on increasing the number of frozen cells compared to the standard cell bank, and both with a much longer tradition than N−1 perfusion: (1) the freezing of high volumes (large volume cell banks) and (2) the freezing of high cell densities (high cell density cell banks). More than thirty years ago, Ninomiya et al. first described an approach to freezing human–human and mouse–mouse hybridomas with cell densities of up to 150 × 10^6 cells mL^{-1} [13]. However, a serum-containing medium was used; now chemically defined media are state of the art, and dimethyl sulphoxide (DMSO) is almost exclusively used as the cryoprotective additive. In 2002, Heidemann et al. published an approach to freezing 50–100 mL cell suspension with 20–40 × 10^6 cells mL^{-1} in cryobags to directly inoculate 2 L stirred bioreactors [14]. Subsequently, further approaches were developed, in which bioreactors in the perfusion mode were used for cell bank production. Here, cryovials or cryobags with up to 110 × 10^6 cells mL^{-1} were frozen [10,15–17]. Similar approaches are also currently outlined by bioreactor and media manufacturers [18,19]. Both variants, high cell density as well as high volume, have the advantage of avoiding cell propagation in shake flasks and directly inoculating a larger bioreactor instead. Besides the resulting time and labor savings, another advantage is that fewer manual operations and fewer culture vessels are required, which reduces the risk of contamination. Furthermore, process steps in which pH and dissolved oxygen are not actively controlled are eliminated, and the cells are provided with more consistent conditions. When using cryobags, it must be noted that they have disadvantages compared to cryovials. The flexible material is less robust and special equipment is required for controlled freezing [20]. In fact, not every cryobag available on the market is suitable for freezing cells at −196 °C [21,22], and cryobags are much more expensive than cryovials.

Robust production cell lines and progressive improvements in commercially available media have helped to simplify process development in recent years. However, further intensification of cell banking by freezing higher cell densities as well as inoculation at densities above 100 × 10^6 cells mL^{-1} as a result of perfusion have not yet been published. In fact, most processes continue to be based on standard cell banks and upstream processing through several passages in shake flasks. Therefore, this work aimed to determine the suitability of cells, which were produced through perfusion with a wave-mixed bioreactor, achieving cell densities of over 150 × 10^6 cells mL^{-1}, as inoculum for subsequent batch experiments. In addition, an approach in which CHO cells were frozen in the UHCD range over 200 × 10^6 cells mL^{-1} was investigated for the first time.

2. Materials and Methods

2.1. Cell Line and Medium

For all experiments, an IgG-producing ExpiCHO-S cell line (Gibco, Waltham, MA, USA) was used. As a basal medium, 0.66× concentrated High-Intensity Perfusion CHO medium (Gibco) was used for inoculum production, batch experiments in the shake flasks, and as a starting medium in the perfusion processes. With the switch to the perfusion mode, High-Intensity Perfusion CHO Medium 1× concentrated was used. The basal and perfusion medium were supplemented with 4 mmol L^{-1} L-glutamine and 0.1% Anti-Clumping Agent (Gibco). To maintain selection pressure, 400 nmol L^{-1} methotrexate (Sigma-Aldrich, St. Louis, MO, USA) was added to all passages of inoculum production except the first passage after thawing and the last passage before production trials.

2.2. Experimental Design

Figure 1 outlines the experimental design of the study. In the first stage of the study, a comparison between batch experiments with standard inoculum production and direct inoculation from cryovials was performed. In addition, cryovials with cell densities between 90 and 250 × 10^6 cells mL^{-1} were frozen, and the growth and production behavior of these frozen cells was compared to standard cryovials with 15–40 × 10^6 cells mL^{-1}. The second stage consisted of establishing perfusion experiments, checking cell growth and production performance by inoculating batch experiments over the course of a perfusion process, and finally freezing the UHCD-WCB. Lastly, the growth and production behavior of the UHCD-WCB was tested by directly inoculating batch experiments and a perfusion bioreactor.

2.3. Standard Inoculum Production in Shake Flasks

Standard inoculum production took place over a period of 7 d in disposable shake flasks (Corning, Corning, NY, USA). Cryovials (2 mL, Brand, Wertheim, Deutschland) with a VCD of 15 × 10^6 cells mL^{-1} were thawed and transferred to a 125 mL shake flask with a 40 mL working volume. The cells were passaged every second or third day with a VCD of 0.3–0.5 × 10^6 cells mL^{-1}, and 250 mL and 500 mL shake flasks were used with 80 mL and 160 mL working volumes, respectively. Shake flasks were incubated in a shaking incubator with a 120 rpm shaking speed at an amplitude of 25 mm, 37 °C, 8% CO_2, and 80% relative humidity.

2.4. Direct Inoculation with Cells from Cryovials

For direct inoculation of batch experiments (B-CV-15, B-90–B-250, B-UHCD) in shake flasks and the perfusion experiment P05 with cells from cryovials, either vials from WCBs with standard freezing cell densities of ≤40 × 10^6 cells mL^{-1} or cryovials from the freezing experiments and the UHCD-WCB with ≥90 × 10^6 cells mL^{-1} were used. For this purpose, the vials were removed from the cryotank and thawed for 1–2 min at 37 °C in a water bath, and then the cell suspension was transferred directly into the shake flask (2 mL cryovial) or the wave-mixed bioreactor (5 mL cryovial). B-CV-15 and B-UHCD were performed as triplicates, with batches B-90–B-250 as duplicates.

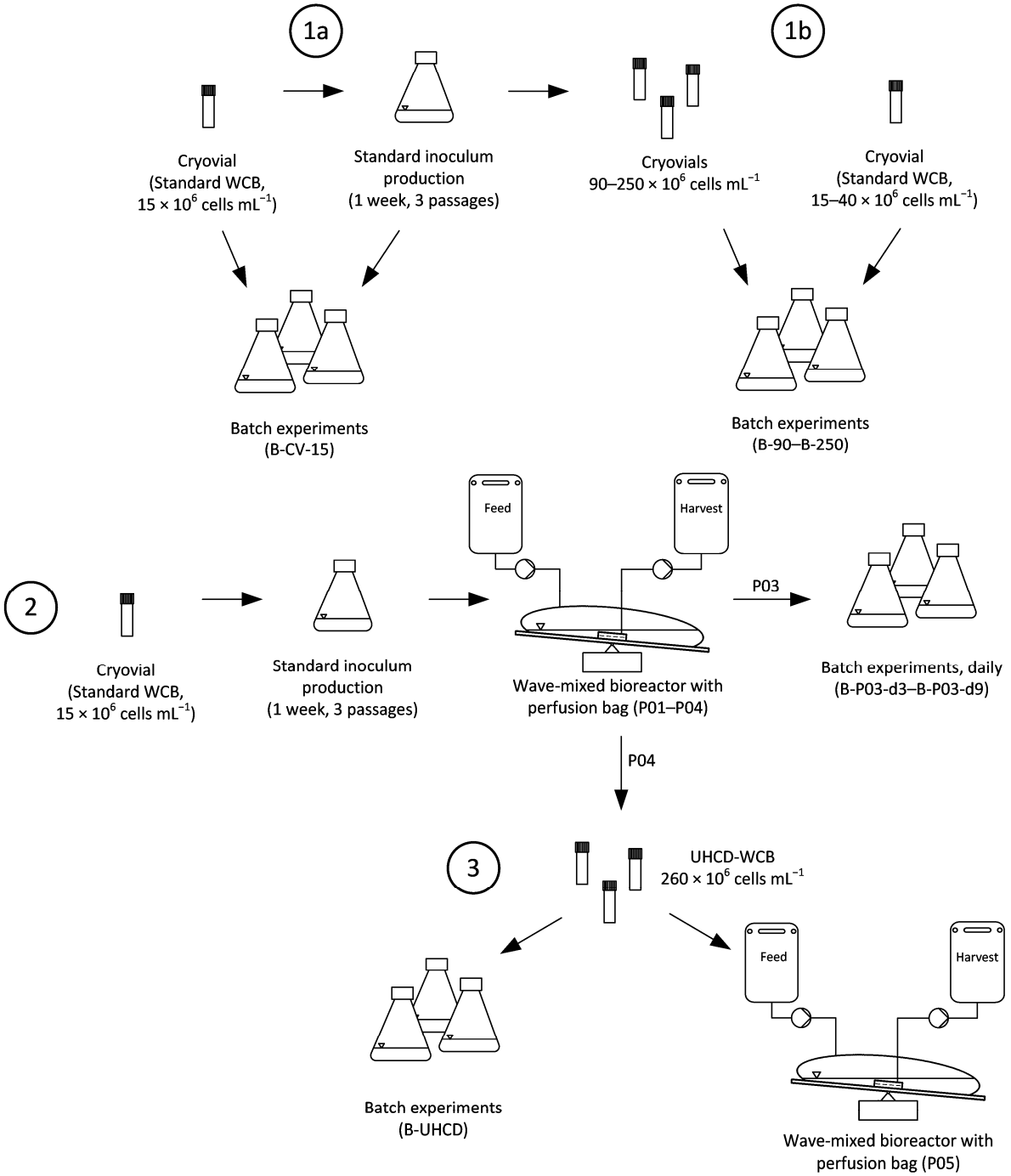


Figure 1. Schematic representation of the experiment design: (**1a**) Direct inoculation out of cryovials (B-CV-15) vs. standard inoculum production, freezing of different VCDs; (**1b**) Direct inoculation from standard cryovials vs. cryovials with higher cell densities (B-90–B250; 90–250 × 10^6 cells mL^{-1}); (**2**) Perfusion processes (P01–P04) with standard inoculum production, daily inoculation of batch experiments (B-P03-d3–B-P03-d9), and freezing of the UHCD-WCB; (**3**) Performance test of the UHCD-WCB in batch (B-UHCD) and perfusion (P05) experiments.

2.5. Batch Cultivation in Shake Flasks

All batch experiments were performed in 125 mL disposable shake flasks (Corning) with a 40 mL working volume. Shake flasks were incubated in a shaking incubator at a 120 rpm shaking speed, an amplitude of 25 mm, 37 °C, 8% CO_2, and 80% relative humidity.

A starting VCD of 1×10^6 cells mL^{-1} was targeted. Samples for atline analyses (Section 2.10) were taken daily. Batch experiments were terminated when viability fell below 40%.

2.6. Perfusion Cultivations in Wave-Mixed Bioreactors

All cultivations were carried out with the Ready to Process Wave 25 control unit from GE Healthcare (now Cytiva, Marlborough, MA, USA). 2 L wave-mixed bioreactors with integrated filter membrane (Flexsafe RM 2 L perfusion pro 1.2 µm; for P01–P04) from Sartorius (Göttingen, Germany) and Cytiva (Cellbag, 2 L, BC10, pHOPT, DOOPT II and Perfusion; for P05) were used. The inoculum was produced with the standard method (Section 2.3) for P01–P04; P05 was inoculated directly from a 5 mL UHCD cryovial. The processes were inoculated with a VCD of 0.6–1.2 $\times$ 10^6 cells mL^{-1}. Cultivations were performed at 1 L working volume, 37 °C, with overlay aeration of 0.2 vvm, pH $\leq$ 7.2 by addition of CO_2, and dissolved oxygen (DO) controlled to $\geq$40% by addition of O_2. The rocking rate (20–40 rpm) and rocking angle (6–12°) were manually adjusted with the growth of the cells and the accompanying oxygen demand. Samples were taken daily during the batch phase and twice daily during the perfusion phase. Perfusion was started on day two of the cultivation, between 3 and 6 $\times$ 10^6 cells mL^{-1}. Depending on the current VCD, the specific growth rate, and the time until the next sampling, the perfusion rate D was adjusted to ensure a minimum cell-specific perfusion rate (CSPR) of 55 pL $cell^{-1}$ d^{-1} (Equation (1)).

$$D = CSPR_{min} \times VCD_{next\ sample} \quad (1)$$

For P01 and P02, 10 L perfusion medium was prepared, and D was limited to maximal 3.1 vvd, for P05 to 7 vvd with 15 L perfusion medium. P03 (15 L perfusion medium) and P04 (10 L perfusion medium) had no perfusion rate limit since the cells were used for further experiments. In the bioreactors, a constant glucose concentration of 3 g L^{-1} was targeted by the continuous addition of a 200 g L^{-1} glucose solution. Perfusion experiments P01–P03 and P05 were terminated when the perfusion medium was depleted, and P04 ended with the freezing of the UHCD-WCB.

2.7. Cell Growth and Production Performance after N−1 Perfusion

In order to characterize the growth and production behavior of the cells grown in perfusion mode, a sterile cell suspension was taken daily from perfusion experiment P03 between day 3, at 7 $\times$ 10^6 cells mL^{-1}, and day 9, at 170 $\times$ 10^6 cells mL^{-1}. Batch experiments (B-P02-d3–B-P03-d9) in shake flasks were performed as duplicates according to Section 2.5.

2.8. Freezing Cells from Shake Flask Cultivations

To determine the maximum possible freezing cell density, the cells were expanded according to the standard inoculum production described in Section 2.3. Afterward, the cell suspension was concentrated by centrifugation at 500 g for 5 min and resuspended in ice-cold 0.66× concentrated supplemented High-Intensity Perfusion CHO medium with 10% DMSO. The following freezing cell densities were chosen: 90 $\times$ 10^6 cells mL^{-1}, 115 $\times$ 10^6 cells mL^{-1}, 150 $\times$ 10^6 cells mL^{-1}, 180 $\times$ 10^6 cells mL^{-1}, and 250 $\times$ 10^6 cells mL^{-1}. Immediately after resuspension and aliquotation in 2 mL cryovials (Brand), the cells were stored in freezing containers (Mr. Frosty, Nalgene, Waltham, MA, USA) for 24 h in a −80 °C freezer and subsequently transferred to the liquid nitrogen cryotank.

2.9. Freezing Cells from Perfusion Cultivation

The inoculum for the perfusion experiment (P04) was produced with the standard method (Section 2.3) with MTX addition in passages 2 and 3 as well as in the wave-mixed bag. The perfusion cultivation was performed as described in Section 2.6. The cells were grown for 6 d. At the start of the freezing process, the cell suspension in the wave-mixed bag was cooled to 10 °C on a water-cooled rocking platform (Sartorius), and the cell suspension was transferred to centrifuge tubes (175 mL, Falcon, Corning, NY, USA) afterward. From here on out, the cell suspension was kept on ice if possible. The entire freezing procedure is

shown in Figure 2. The suspension was centrifuged at 500 g for 5 min. The largest part of supernatant (75% of the centrifugation volume) was discarded; 25% of the centrifugation volume of ice-cold fresh medium containing 14% DMSO was added; and the cell pellet was resuspended. The cell suspension was centrifuged again; the complete supernatant was removed; and the cell pellet was resuspended with a volume of 1:1 of ice-cold medium containing 14% DMSO. The resulting DMSO concentration was 10.5% DMSO (v/v), and the VCD was 260 $\times$ 10^6 cells mL^{-1}.

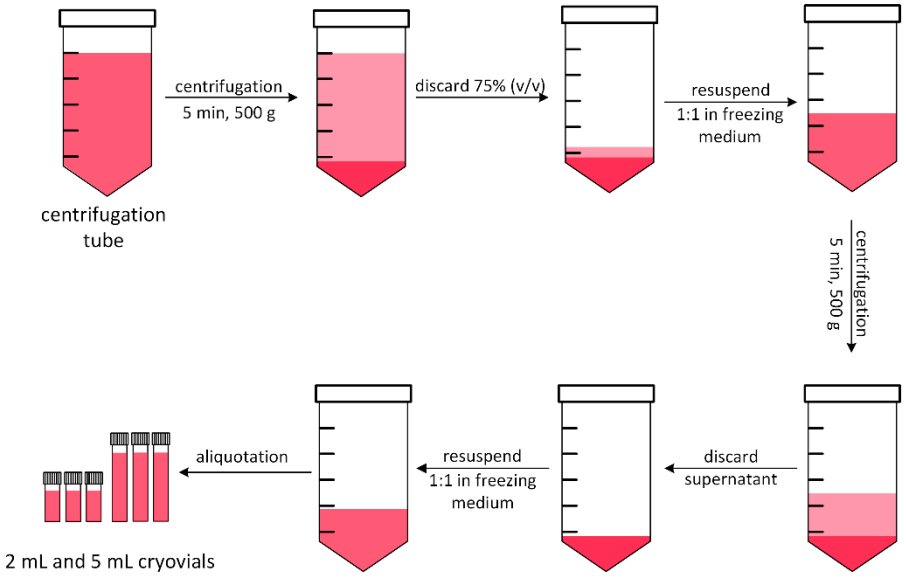


Figure 2. Scheme of the preparation of the cell suspension for freezing of the UHCD-WCB.

The cell suspension was transferred to 2 mL and 5 mL cryovials (Brand) containing 1.5 mL and 4.5 mL cell suspensions and stored in the freezing container (Mr. Frosty, Nalgene) for 24 h at −80 °C. The UHCD-WCB was transferred to the cryotank at −196 °C for long-term storage.

2.10. Analytical Methods

During cultivation, VCD, total cell density, viability, cell diameter, compactness, and the aggregation rate were determined using a Cedex HiRes analyzer (Roche Diagnostics, Basel, Switzerland). For the determination of the concentration of glucose, glutamine, ammonium, lactate, and IgG, the Cedex Bio analyzer (Roche Diagnostics) was used.

2.11. Statistical Evaluation of Experiments

In diagrams showing a positive control in multiple determination, the arithmetic mean value is shown with a tolerance interval with a coverage p of 90% and a confidence α of 90%, calculated according to Howe [23] and Guenther [24].

3. Results

3.1. Direct Inoculation of Batch Experiments with Cells from Cryovials

A preliminary study was performed to compare the growth and production performance of ExpiCHO-S cells in shake flasks inoculated from both a standard inoculum production and directly from cryovials with 15 $\times$ 10^6 cells mL^{-1}. The courses of VCD, viability, and glucose and IgG concentration are shown in Figure 3. As expected, there was a lag phase after thawing, so the growth rate of 0.0344 $\pm$ 0.0028 h^{-1} in the experiments inoculated from cryovials (B-CV-15_1, B-CV-15_2, B-CV-15_3) was 16.0% lower on the first day than that of experiments inoculated from shake flasks using standard inoculum production, therefore being inoculated in the exponential growth phase (0.0409 $\pm$ 0.0013 h^{-1}, control). On day 2, the growth rate was still 8.4% lower. Due to the higher growth rates on days 3 and

4, an almost identical peak VCD was achieved after 4 d with 14.2 ± 0.2 × 10^6 cells mL^{-1} compared to 14.6 ± 0.4 × 10^6 cells mL^{-1} for the control group. Due to the initial lag phase, however, the growth curve is slightly offset in time, so the death phase also occurred later. The viability was still 35–88% on day 5, whereas it was already ≤12% for classical inoculum production. Along with the later death phase, glucose as the main carbon source was depleted later.

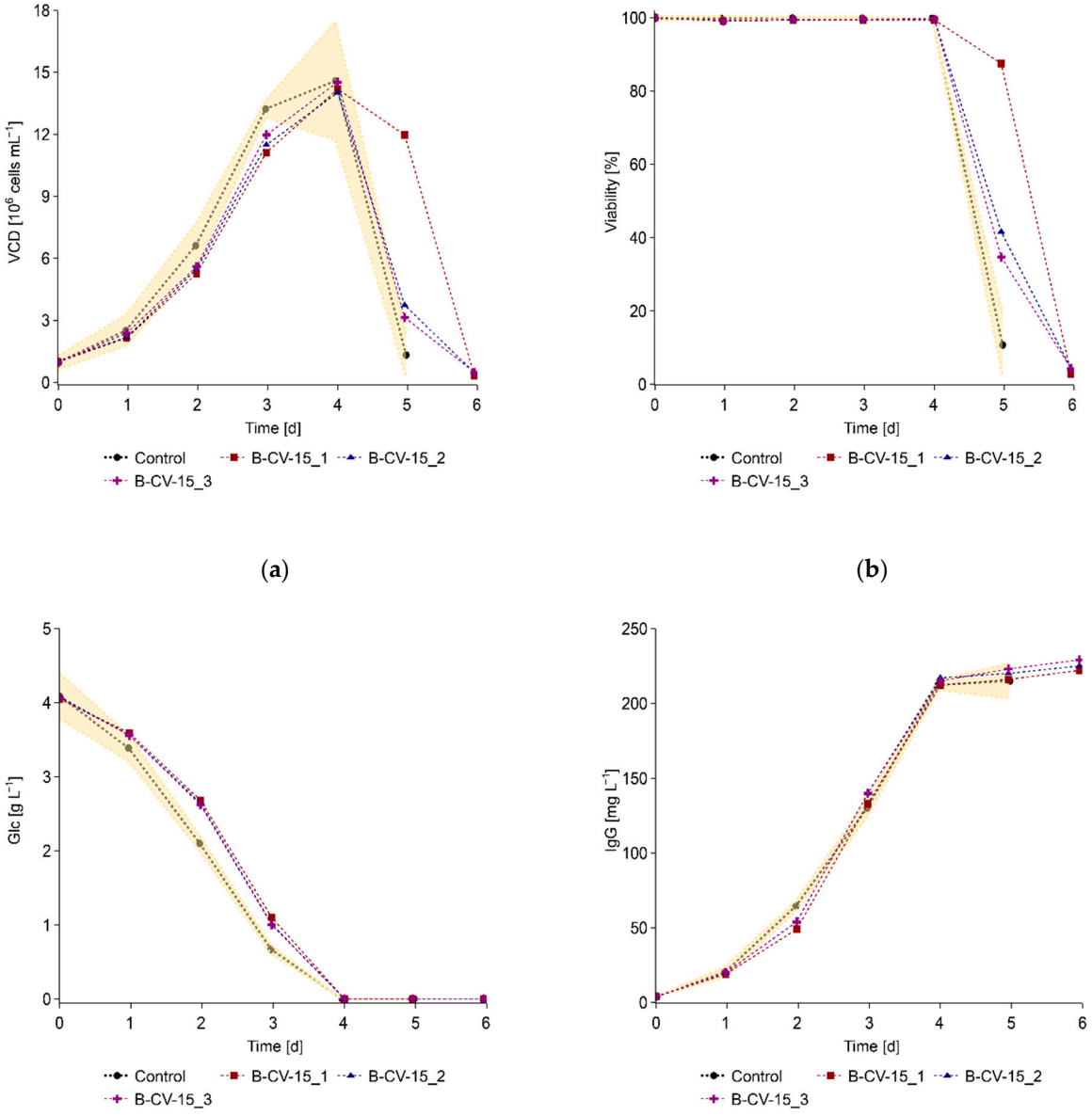


Figure 3. Course of (**a**) VCD, (**b**) viability, (**c**) glucose (Glc), and (**d**) IgG concentration during the batch experiments inoculated directly from cryovials (nomenclature: B = Batch, CV = inoculated out of cryovial, 15 = VCD in cryovial (×10^6 cells mL^{-1})). Control: batch experiments with standard inoculum production (n = 3).

IgG production was similar. The titers on day 5 were comparable for both approaches, with 220 ± 3 mg L^{-1} for direct inoculation from cryovials and 215 ± 2 mg L^{-1} for standard inoculum production. The courses of the glucose consumption rate and the IgG production rate are shown in the Appendix A (Figure A1).

Since comparable VCDs and IgG titers were achieved for batch experiments with direct inoculation from cryovials compared to standard inoculum production after five days of batch cultivation, cryovials with different VCDs were frozen (Section 2.8) and used again for directly inoculating shake flasks. The growth curves and viability courses as well as the concentration courses of glucose and IgG are shown in Figure 4.

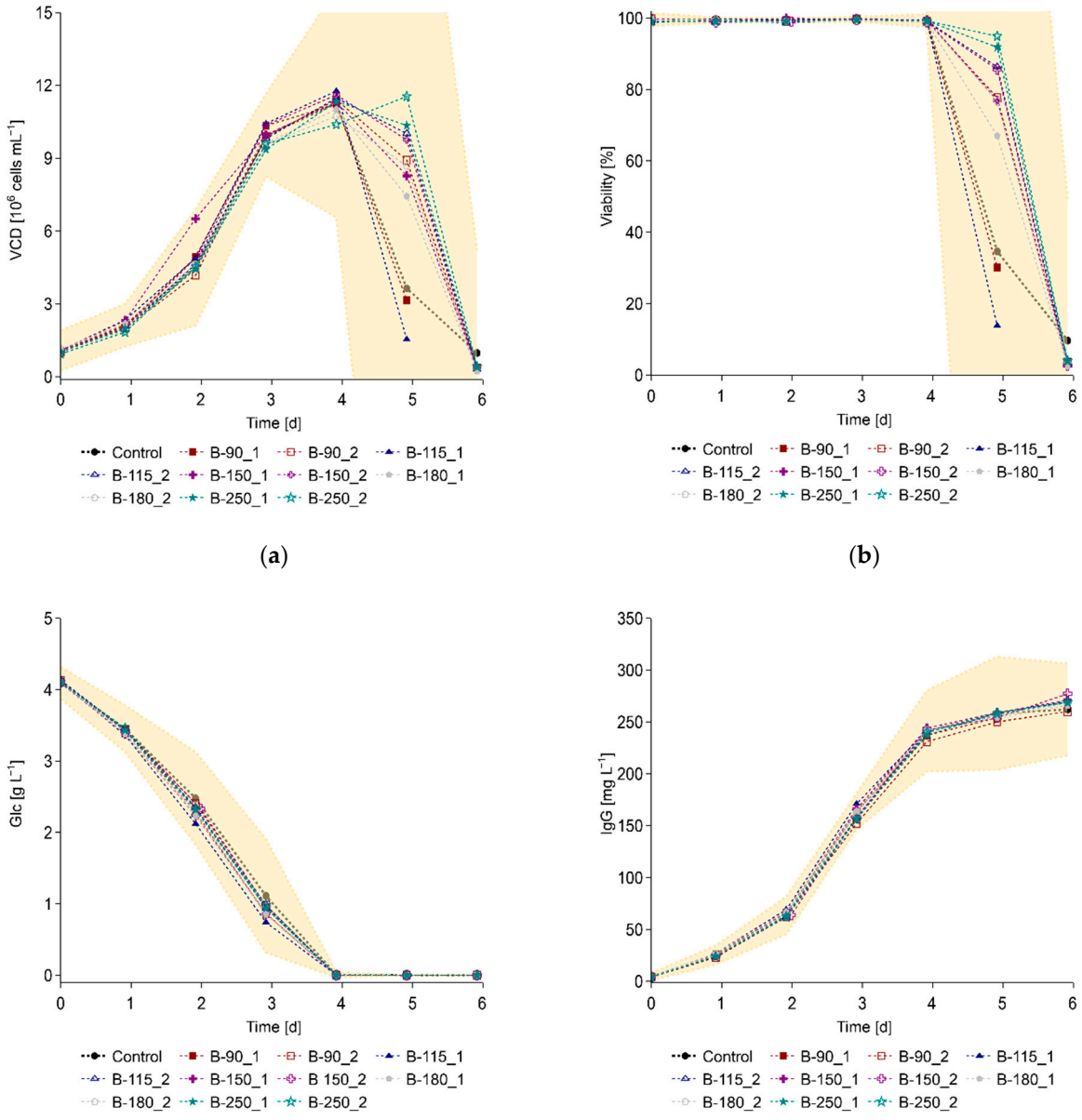


Figure 4. Course of (**a**) VCD, (**b**) viability, (**c**) glucose (Glc), and (**d**) IgG concentration during the batch experiments inoculated directly from cryovials containing high VCDs from 90 to 250 $\times$ 10^6 cells mL^{-1} (nomenclature: B = Batch, 90/115/150/180/250 = VCD in cryovial ($\times 10^6$ cells mL^{-1})). Control: batch experiments inoculated directly from cryovials containing lower VCDs (15–40 $\times$ 10^6 cells mL^{-1}, n = 3).

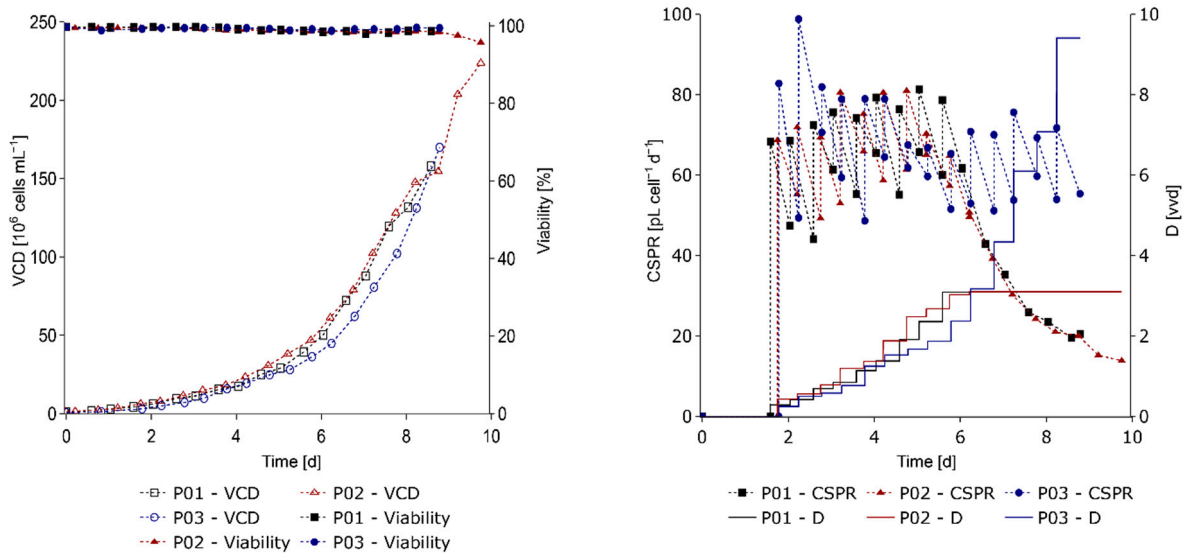
Growth was independent of the freezing VCD until day 4. Growth rates from day 0 to day 3 ranged from 0.0310 $\pm$ 0.0027 h^{-1} for the batch experiments inoculated from cryovials containing 180 $\times$ 10^6 cells mL^{-1} to 0.0325 $\pm$ 0.0023 h^{-1} for the batch experiments inoculated from cryovials containing 90 $\times$ 10^6 cells mL^{-1}. Viability remained >98% in all shake flasks

until day 4. Maximum VCDs were comparable and ranged from 10.7 × 10^6 cells mL^{-1} (B-180_2) to 11.8 × 10^6 cells mL^{-1} (B-115_1). Differences could be seen based on the differently progressed death phases on day 5. The shake flasks with the highest freezing VCD were the most viable (92% and 95% for the duplicate), and the control group had the lowest viability (35 ± 31%). No change was detected in glucose consumption and IgG production. On day 4, glucose was depleted in all experiments. On day 5, when the first batch experiments were stopped due to low viability, IgG concentrations in all shake flasks were between 250 mg L^{-1} and 269 mg L^{-1}. The courses of the glucose consumption rate and the IgG production rate can be found in the Appendix A (Figure A2).

Although these results show that a higher freezing cell density minimally delays growth and the death phase occurs a few hours later, high viability is obtained after thawing regardless of the freezing cell density. The peak VCDs are comparable, and IgG production also shows no differences. Since it could be shown that the establishment of a UHCD-WCB with >200 × 10^6 cells mL^{-1} is possible with regard to cell growth and IgG production, the expansion of a sufficiently large number of cells was considered in the next step. Therefore, perfusion experiments were performed.

3.2. UHCD Perfusion Cultivations and Inoculation of Batch Experiments out of a Perfusion Process

The second part of the study aimed to check whether the growth and production behavior of the cell suspension changes with increasing VCD during a perfusion process with complete cell retention. For this purpose, a perfusion process was established, whereby the first two cultivations P01 and P02 were not used for further experiments, whereas daily batch experiments were inoculated from P03. The growth and viability curve as well as the course of the perfusion rate and the CSPR of the perfusion experiment are shown in Figure 5.



(**a**) (**b**)

Figure 5. (**a**) Course of the VCD and viability of P01, P02, and P03, and (**b**) course of perfusion rate D and the CSPR of P01, P02, and P03.

The wave-mixed bioreactors were inoculated with 1.0 (P01), 1.2 (P02), and 0.6 × 10^6 cells mL^{-1} (P03). Perfusion was started after 1.6–1.8 d, and the perfusion rate was gradually increased. For the first two perfusion processes P01 and P02, the maximum perfusion rate was limited to 3.1 vvd, causing the CSPR and thus the growth rate μ to decrease after about 6 d. For P03, D was increased to a maximum of 9.4 vvd between 8.2 d

and 8.8 d to provide the cells with the best possible conditions, as P03 was used for daily inoculation of batch experiments. The maximum cell density of P01 was 158 × 10^6 cells mL^{-1} with 98.6% viability after 8.6 d, of P02 224 × 10^6 cells mL^{-1} with 95.7% viability after 9.8 d, and of P08 170 × 10^6 cells mL^{-1} with 99.4% viability after 8.8 d. The growth rate from the start of cultivation to the limit of the perfusion rate was comparable for all three cultivations: 0.0267 ± 0.0100 h^{-1} for P01 (until 6.0 d), 0.0295 ± 0.0089 h^{-1} for P02 (until 5.8 d), and 0.0259 ± 0.0096 h^{-1} for P03 (until 8.8 d).

The cell growth and product formation of batch experiments inoculated from the perfusion cultivation P03 were characterized. The growth and viability curves as well as the courses of the glucose and IgG concentration of the experiments are shown in Figure 6. The experiments that were started on days 3 and 4 were considered as the positive control. It was found that the growth rate on the first day of the batch experiments increased during the perfusion cultivation until day 7, at 62 × 10^6 cells mL^{-1}. The growth rate on the first day was 0.0301 h^{-1} and 0.0289 h^{-1} for the shake flask duplicate inoculated on day 7. The control group, on the other hand, had a growth rate of 0.0254 ± 0.0034 h^{-1}, which was 14% lower.

However, viability remained >99% for all experiments until day 4. Glucose consumption and product formation also showed a slight increase for the above-mentioned experiments. For example, glucose concentration in B-P03_d7 on day 2 was already 17% lower than the control, and the IgG titer was 17% higher. The specific consumption and production rates can be seen in Appendix A (Figure A3). Nevertheless, comparable peak VCDs were achieved, from 11.5 × 10^6 cells mL^{-1} (B-P03-d6_2) to 12.4 × 10^6 cells mL^{-1} (B-P03-d7_2). The final IgG titers also showed only minor differences. The lowest value was measured for B-P03-d3_2 (included in the control group) with 254 mg L^{-1}, and the highest value for B-P03-d6_1 with 284 mg L^{-1}. Although high viability can be assumed even with higher VCDs, since higher VCDs are not necessary for freezing a sufficiently large WCB, a cell density of about 100 × 10^6 cells mL^{-1} at the time of freezing was aimed for to establish the UHCD-WCB. Perfusion cultivation P04 was run for 6 d and afterward used to freeze the UHCD-WCB at 260 × 10^6 cells mL^{-1} (Section 2.9).

3.3. Evaluation of a Perfusion-Based UHCD-WCB

3.3.1. Batch Experiments in Shake Flasks

As described in Section 2.4, cryovials from the UHCD-WCB were also thawed and used to directly inoculate shake flasks. Here, a triplicate with three cryovials was performed. Batch experiments inoculated directly from cryovials frozen with low VCD (15 × 10^6 cells mL^{-1}) served as the positive control, which were also performed in triplicate. The growth and viability curves as well as the courses of glucose and IgG concentration of the experiments are shown in Figure 7. The shake flasks inoculated directly from the UHCD-WCB had a slightly prolonged lag phase. On day 1, the growth rate was 0.0266 ± 0.0022 h^{-1} and therefore 23% lower compared to 0.0344 ± 0.0028 h^{-1} in the positive control. A viability drop on day 1 was also observed, but the viability remained high at 97.8 ± 1.0% and increased again to >99% in all experiments during the cultivation. The maximum VCD achieved in the batch experiments inoculated with the UHCD-WCB was 13.0 ± 0.3 × 10^6 cells mL^{-1}, which was slightly lower (−9%) than in the control group (14.2 ± 0.2 × 10^6 cells mL^{-1}). According to the shorter lag phase in the positive control, the death phase had already begun on day 5 (55 ± 23% viability), while at this time the viability of the batches from the UHCD-WCB was still 96 ± 1%. Although product formation was also delayed in accordance with the delayed growth, the final product titers achieved were comparable: 225 ± 3 mg L^{-1} in the positive control and 222 ± 0 mg L^{-1} for the cells from the UHCD-WCB. On days 1 and 2, in contrast to the experiments from Parts 1 and 2 of the study, the glucose concentration was lower than in the control in all three shake flasks, although the VCDs were lower. The specific glucose consumption rates were 53% higher than for the control group on day 1 and 34% higher on day 2. The course of the glucose consumption rate can be seen in Appendix A (Figure A4). To check the morphol-

ogy of the cells, microscopic images of the cells were taken after thawing a cryovial with 15×10^6 cells mL^{-1}, a cryovial with 260×10^6 cells mL^{-1}, and after one week of standard inoculum production. No abnormalities in cell morphology were observed (Figure A5).

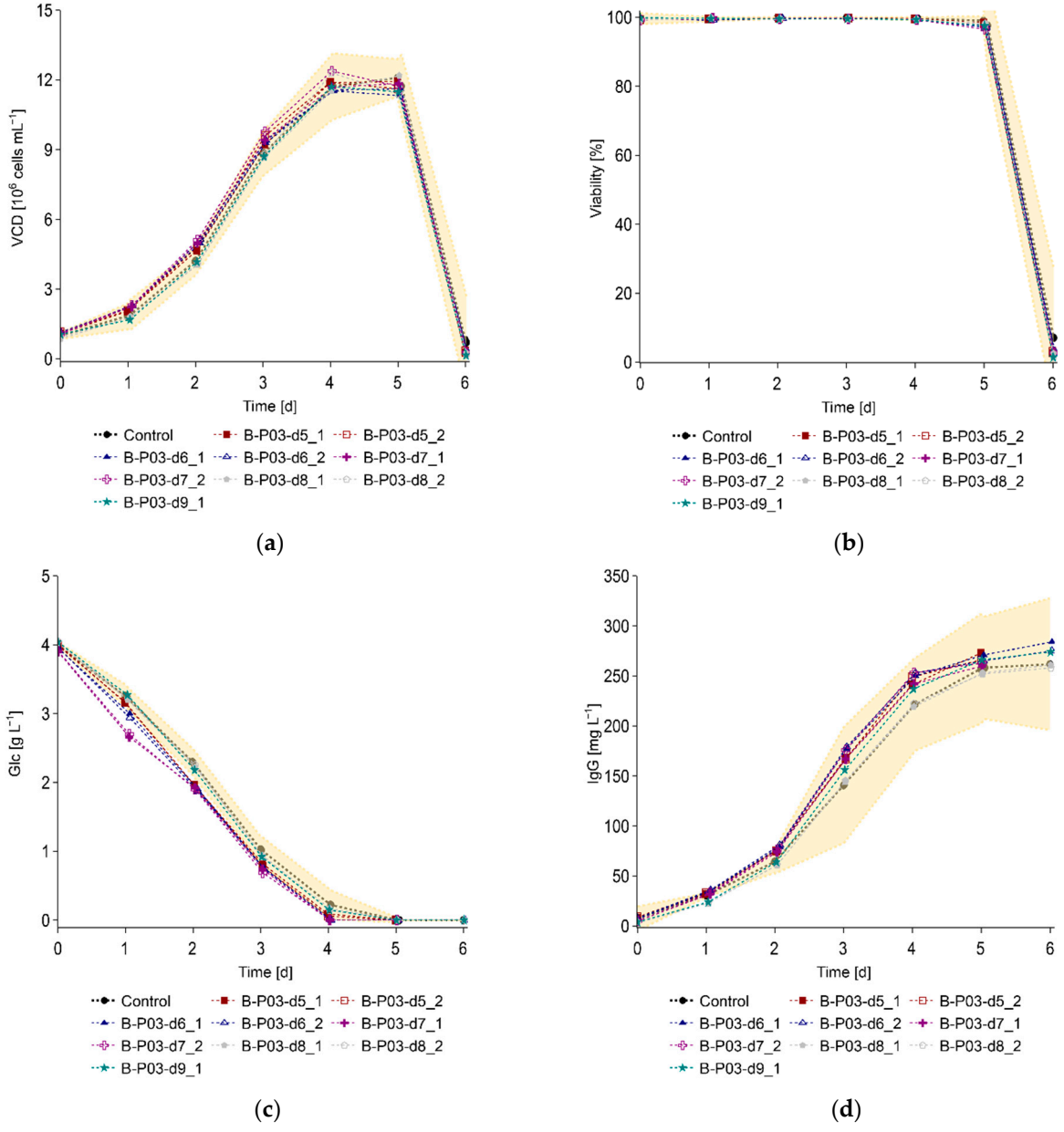


Figure 6. Course of (**a**) VCD, (**b**) viability, (**c**) glucose (Glc), and (**d**) IgG concentration during the batch experiments inoculated from perfusion cultivation (nomenclature: B = Batch, P03 = Source of inoculum, d5–d9 = day of cell harvest from P03). Control: batch experiments inoculated from P03 on days 3 and 4 ($n = 4$).

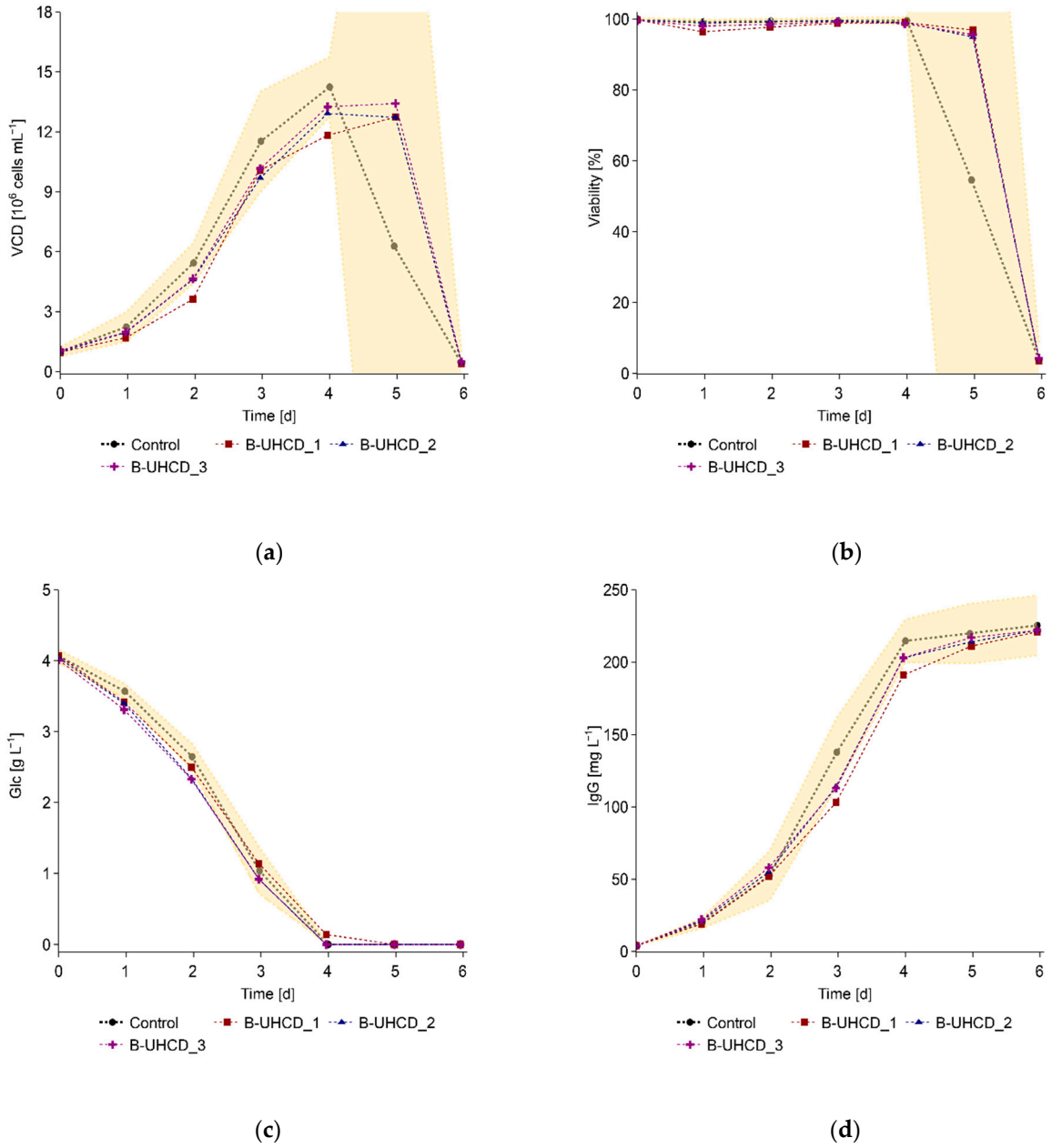
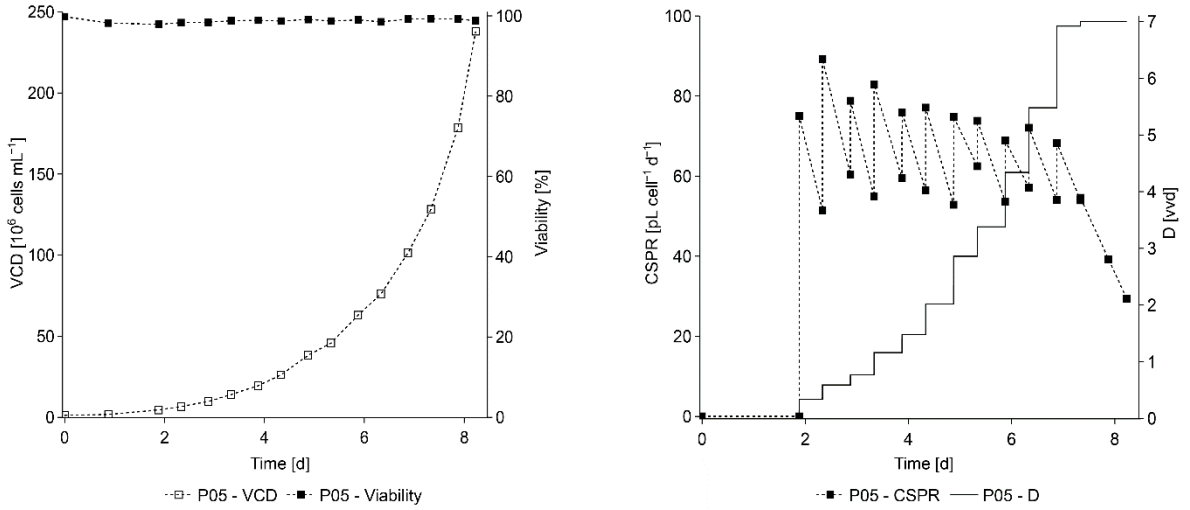


Figure 7. Course of (**a**) VCD, (**b**) viability (**c**) glucose (Glc), and (**d**) IgG concentration during the batch experiments inoculated directly from the UHCD-WCB (nomenclature: B = Batch, UHCD = inoculated from UHCD-WCB). Control: batch experiments inoculated directly from cryovials containing lower VCDs (15×10^6 cells mL^{-1}, $n = 3$).

3.3.2. Perfusion Experiment

After successfully confirming the suitability of the UHCD-WCB as the inoculum for batch experiments, a wave-mixed perfusion bioreactor with a 1 L working volume was inoculated with a starting VCD of 1.2×10^6 cells mL^{-1} from a 5 mL cryovial. The growth and viability curve as well as the course of the perfusion rate and CSPR are shown in Figure 8. As expected, the growth rate was only 0.0185 h^{-1} on the first day of cultivation due to the lag phase but subsequently increased to values >0.0300 h^{-1}. The viability drop, which was determined in the previous shake flask experiments, was only slight at 97.9% viability on day 2, and subsequently, the viability was >98% until the end of cultivation.



(**a**) (**b**)

Figure 8. (**a**) Course of the VCD and viability of P05, and (**b**) course of D and the CSPR of P05, inoculated directly from the UHCD-WCB.

In contrast to P03, in which the perfusion rate was increased until the end of cultivation, a maximum perfusion rate of 7 vvd was set for this cultivation, in order to achieve higher final VCDs with the same amount of medium. As a result, the CSPR dropped to 29 pL cell^{-1} d^{-1} towards the end, but a maximum VCD of 238 $\times$ 10^6 cells mL^{-1} with a viability of 98.8% was achieved after 8.2 d. Despite the limitation of the perfusion rate on the last day of cultivation, the cells continued to grow exponentially until the end of cultivation; over the whole period, the growth rate was 0.0265 $\pm$ 0.0065 h^{-1}, comparable to 0.0259 $\pm$ 0.0096 h^{-1} in perfusion cultivation P03 (Section 3.2).

4. Discussion

The intensification of production processes with regard to the cryopreserved cell banks has so far been limited to high volumes through the use of cryobags and maximum freezing VCDs of up to 150 $\times$ 10^6 cells mL^{-1} [10,13–19]. Regarding N−1 perfusion processes, the production of cells in the perfusion mode has already been achieved up to >200 $\times$ 10^6 cells mL^{-1} [11,12], but the cells have so far only been used with cell densities of up to 100 $\times$ 10^6 cells mL^{-1} for inoculation in subsequent experiments [7–10]. In this work, both approaches, the intensification of the seed train by freezing UHCD and the use of perfusion processes for cell production, were pursued. In the first stage of the study, the use of direct inoculation from cryovials was investigated. If cryovials with 15 $\times$ 10^6 cells mL^{-1} and 10% DMSO are used for inoculation with a VCD of 1 $\times$ 10^6 cells mL^{-1}, it must be noted that almost 0.7% DMSO remain in the medium. Direct inoculation from cryovials with 15 $\times$ 10^6 cells mL^{-1} was found to have a lag phase compared to a seven-day inoculum production, but, nevertheless, comparable maximum VCDs and product titers were found. Kleman et al. described that up to 1% DMSO did not affect the growth of a HEK cell line, but greater than 0.3% did for a CHO-S cell line [25]. In this work, no growth inhibition was observed for the ExpiCHO-S cell line used. At higher freezing VCDs, the consideration of the remaining DMSO is negligible due to the large dilution effect. When using cryovials containing up to 250 $\times$ 10^6 cells mL^{-1} for direct inoculation, higher freezing VCDs resulted in an increased lag phase. However, this growth shift by a few hours had no influence on the maximum VCDs and IgG titers. The viability remained >99% until the beginning of the death phase. In previous publications, a viability drop often was reported in addition to a lag phase in the first days after inoculation [14,16,17].

A robust perfusion process was established, and it was shown that VCDs >200 $\times$ 10^6 cells mL^{-1}, as well as very high perfusion rates (9.4 vvd), are realizable with the wave-mixed perfusion bags with an internal filter. When using cells from a perfusion process for subsequent batch experiments, it was found that growth and product formation in the batch experiments were fastest between VCDs of 36 $\times$ 10^6 cells mL^{-1} (d6 of the perfusion process) and 102 $\times$ 10^6 cells mL^{-1} (d8) in the perfusion process, with a peak on day 7 (62 $\times$ 10^6 cells mL^{-1}). At lower as well as higher VCDs, the growth rates were slightly lower; nevertheless, the batch experiment inoculated at the highest VCD of 170 $\times$ 10^6 cells mL^{-1} also achieved a comparable IgG titer and peak VCD.

Another perfusion cultivation was performed, harvested at 90 $\times$ 10^6 cells mL^{-1} and frozen as UHCD-WCB with 260 $\times$ 10^6 cells mL^{-1}. Concentration of the cell density and media exchange with fresh medium containing DMSO was carried out in two centrifugation steps, as the viscosity also increases with increasing freezing cell density, making handling and centrifugation more difficult. Due to the pre-cooling of the bag, the transfer of the cell suspension to the centrifugation tubes, and the two-step medium exchange, the freezing process from a wave-mixed bag with 1 L working volume was more time-consuming than freezing from shake flasks, but Heidemann et al. described a time window of up to 2.5 h for the preparation of cryovials [26]. For the process described in this study, cells were transferred to the −80 °C freezer within 30 min of being removed from the controlled conditions of the wave-mixed bioreactor, compared to 15 min for freezing of cells grown in shake flasks.

The established UHCD-WCB was used for direct inoculation of batch experiments as well as a wave-mixed perfusion bioreactor. A low viability drop after inoculation was observed, as well as a 9% lower peak VCD in the batch experiments compared to the control group, with a comparable IgG titer. The perfusion process was comparable to previous experiments. Cells grew exponentially after a short lag phase on the first day to a maximum VCD of 238 $\times$ 10^6 cells mL^{-1} and a viability of 98.8% reached after 8.2 d.

This study demonstrates that high freezing cell densities of up to 260 $\times$ 10^6 cells mL^{-1} with cells taken from perfusion processes are suitable for establishing a UHCD-WCB with high viability and that only short lag phases occur after thawing. The UHCD-WCB is intended to be used to inoculate either the final inoculum production step (step N−1) as perfusion or even the production bioreactor (step N) directly from the cryovial. The successful perfusion cultivation with inoculation from a UHCD cryovial lays the foundation for further experiments. Direct inoculation of a 1 L bioreactor bag as N−1 perfusion would produce a sufficient number of cells within one week to inoculate a high-seed fed-batch at a 50 L scale with a starting VCD of 5 $\times$ 10^6 cells mL^{-1}. This would shorten inoculum production by more than 35% compared to the standard process (7 d shake flasks + 4 d wave-mixed bag, low-seed fed-batch process). In addition, the high-seed approach can shorten the production process for the ExpiCHO-S cell line and achieve more than 25% higher IgG titers within the same time (research article in preparation). Since a cryovial with 4.5 mL cell suspension is sufficient to inoculate the largest available wave-mixed perfusion bioreactor with an internal filter (max. working volume 25 L, available from Cytiva and Sartorius) at a minimum working volume of 5 L with about 0.25 $\times$ 10^6 cells mL^{-1}, even one-step inoculum production for bioreactors at production scale can be achieved. Here, a cubic meter scale bioreactor could be inoculated from the wave-mixed perfusion bag.

During the experiments of this study, the growth behavior, viability, and production performance of the cells were investigated as quality parameters of the cell suspensions. In further experiments for the development of IgG production processes based on the established UHCD-WCB, additional investigations, for example, of the IgG quality, can be carried out.

Author Contributions: Conceptualization, J.M., V.O., D.E. and R.E.; methodology, J.M. and V.O.; validation, J.M. and V.O.; formal analysis, J.M. and V.O.; investigation, J.M. and V.O.; resources, D.E. and R.E.; data curation, J.M.; writing—original draft preparation, J.M. and V.O.; writing—review and editing, R.E. and D.E.; visualization, J.M. and V.O.; supervision, D.E. and R.E.; project administration, D.E. and R.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

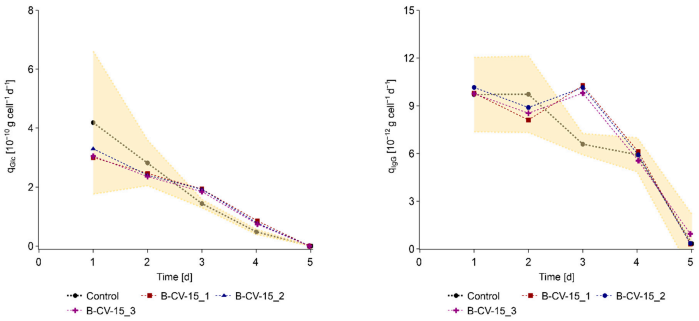
Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to thank Deniz Türkcan and Noémi Weiss for the support during the experimental part.

Conflicts of Interest: The authors declare no conflict of interest.

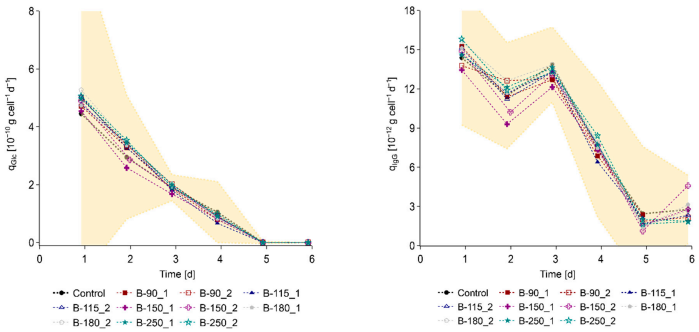
Appendix A

The glucose and IgG concentration courses of the batch experiments are shown and described in the results section. The influences of the different experimental approaches on the course of the glucose consumption rate and IgG production rate can be seen in the following Figures A1–A4.



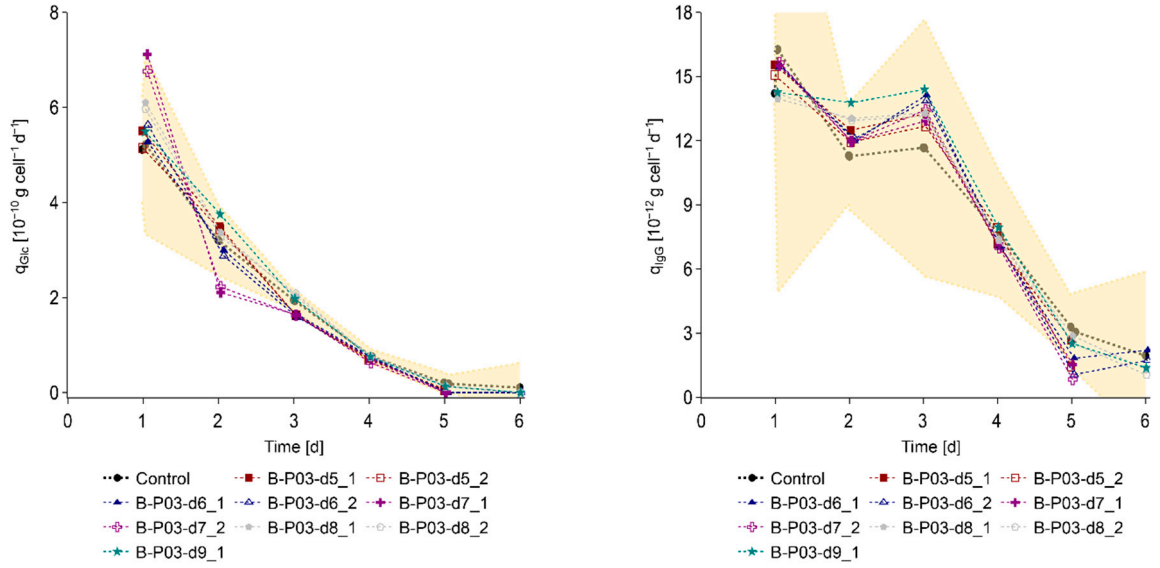
(**a**) (**b**)

Figure A1. Course of (**a**) glucose consumption rate, and (**b**) IgG production rate during the batch experiments inoculated directly from cryovials (nomenclature: B = Batch, CV = inoculated out of cryovial, 15 = VCD in cryovial ($\times 10^6$ cells mL^{-1})). Control: batch experiments with standard inoculum production (n = 3).



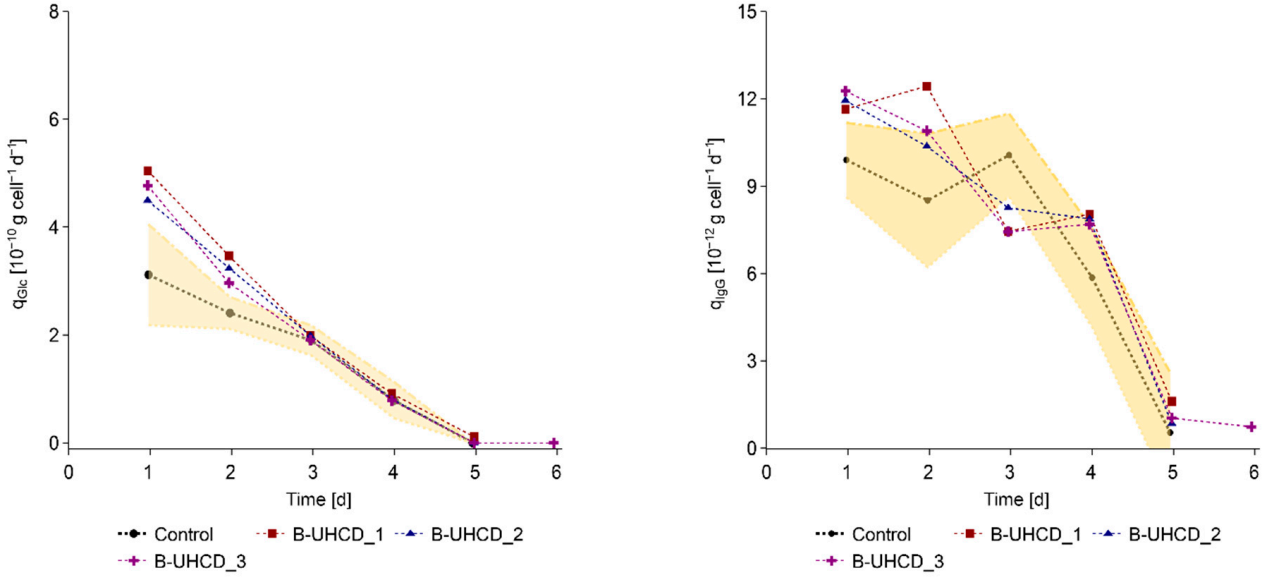
(**a**) (**b**)

Figure A2. Course of (**a**) glucose consumption rate, and (**b**) IgG production rate during the batch experiments inoculated directly from cryovials containing high VCDs from 90 to 250 $\times$ 10^6 cells mL^{-1} (nomenclature: B = Batch, 90/115/150/180/250 = VCD in cryovial ($\times 10^6$ cells mL^{-1})). Control: batch experiments inoculated directly from cryovials containing lower VCDs (15–40 $\times$ 10^6 cells mL^{-1}, n = 3).



(**a**) (**b**)

Figure A3. Course of (**a**) glucose consumption rate, and (**b**) IgG production rate during the batch experiments inoculated from perfusion cultivation (nomenclature: B = Batch, P03 = Source of inoculum, d5–d9 = day of cell harvest from P03). Control: batch experiments inoculated from P03 on days 3 and 4 (n = 4).



(**a**) (**b**)

Figure A4. Course of (**a**) glucose consumption rate, and (**b**) IgG production rate during the batch experiments inoculated directly from the UHCD-WCB (nomenclature: B = Batch, UHCD = inoculated from UHCD-WCB). Control: batch experiments inoculated directly from cryovials containing lower VCDs (15×10^6 cells mL^{-1}, n = 3).

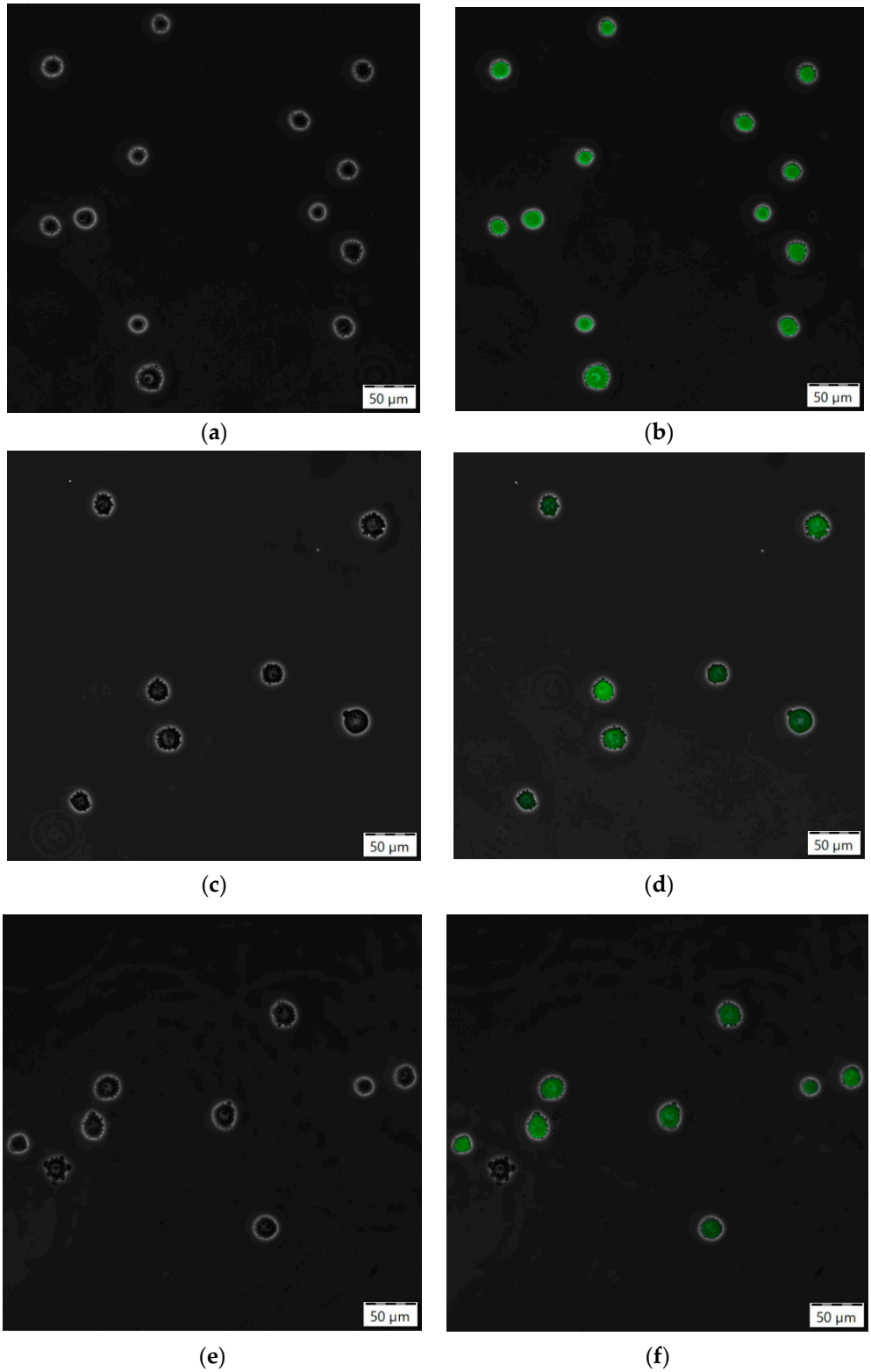


Figure A5. Microscopic images (200×) of (**a**,**b**) cells after one-week standard inoculum production, (**c**,**d**) cells from a cryovial containing 15 × 10^6 cells mL^{-1}, 1 d after thawing, and (**e**,**f**) cells from a cryovial containing 260 × 10^6 cells mL^{-1}, 1 d after thawing. (**a**,**c**,**e**) phase contrast images, (**b**,**d**,**f**) phase contrast and fluorescence images (fluorescein diacetate staining for cells with intact cell membrane).

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