# Intensification of Cell Clarification in Monoclonal Antibody Manufacturing

Von der Naturwissenschaftlichen Fakultät der

Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation

von

Martin Saballus, Dipl.-Ing.

2022

*„The most common reaction of the human mind to achievement*

*is not satisfaction, but craving for more.”*

Yuval Noah Harari
in Homo Deus: A History of Tomorrow

# Danksagung

An dieser Stelle möchte ich mich recht herzlich bei allen bedanken, die mich während der Promotion und der Anfertigung dieser Arbeit unterstützt haben.

Meinen Dank gilt Prof. Dr. Thomas Scheper für die Übernahme des Erstgutachtens am Institut für Technische Chemie der Leibniz Universität Hannover sowie Dr. Gerhard Greller für die Möglichkeit meine Dissertation in der Abteilung BioProcessing Downstream bei Sartorius, Corporate Research anzufertigen.

Meinen besonderen Dank gilt Dr. Markus Kampmann für seine Ratschläge und Anregungen sowie die stetige Unterstützung während meiner gesamten Promotionszeit in seiner Arbeitsgruppe.

Prof. Dr. Sascha Beutel danke ich sehr für die Übernahme des Korreferats und Herrn PD Dr. Ulrich Krings für den Prüfungsvorsitz.

Allen Kollegen bei Sartorius möchte ich vielmals für die angenehme Zusammenarbeit, die Hilfsbereitschaft sowie die unzähligen wissenschaftlichen Gespräche danken, die wesentlich zum erfolgreichen Abschluss dieser Arbeit beigetragen haben. Ebenso danke ich Willi Büttner, Daniel Schorn und Kristin Koenen für ihre tatkräftige Mitarbeit an meinem Promotionsthema während ihrer von mir betreuten Abschlussarbeiten.

Meiner Familie, besonders meinen Eltern Andrea und Frank, meiner Schwester Anja, sowie meinem Opa Heinz, danke ich von Herzen, da sie mich stets ermutigt haben und immer ein offenes Ohr für mich hatten.

Der größte Dank gebührt meiner Frau Jessika für ihre fortwährende Geduld mit mir und ihrem bedingungslosen Rückhalt während der gesamten Zeit.

# Zusammenfassung

Monoklonale Antikörper (mAb) ermöglichen die Behandlung einer Vielzahl schwerer, chronischer Erkrankungen, wie bestimmte Formen von Krebs und Rheuma, weshalb stetig neuartige Therapieansätze entwickelt werden und der weltweite Bedarf rasant ansteigt. Um die Produktivität bei der mAb-Herstellung zu steigern, werden vermehrt Hochzelldichte (HCD) Kultivierungen von Säugetier Suspensionszelllinien verwendet, die das Produkt in das Umgebungsmedium segregieren. Jedoch stellt am Ende dieser ohnehin kostenintensiven Herstellungsprozesse die Abtrennung des mAb´s von den partikulären Verunreinigungen, primär von den Wirtszellen sowie Zellbruchstücken, eine Herausforderung dar. Herkömmliche Klärungsverfahren auf Basis von Edelstahl Teller-Separatoren und Einweg Tiefenfiltern haben die Nachteile geringer Partikelbeladungskapazitäten und hoher Produktverluste, wodurch sie ungeeignet für die Klärung von HCD-Prozessen sind.

Im ersten Teil der Arbeit wurde daher ein alternativer Klärungsansatz mittels einer neuartigen Gegenstromzentrifuge gefolgt von einem Filtrationsschritt entwickelt und für die HCD-Klärung optimiert. Im zweiten Teil wurde die Eignung dieses Ansatzes für verschiedene Zellsuspensionen untersucht sowie der Klärungsprozess in Abhängigkeit der Zellkonzentration modelliert. Es konnte gezeigt werden, dass der komplett auf Einwegmaterialen basierende Ansatz eine robuste Klärung sowohl von moderaten Zellkonzentrationen als auch von HCD-Kultivierungen mit mehr als 100 Millionen Zellen/mL ermöglicht. Dabei konnte der entsprechende Prozessdurchsatz vorher bestimmt und zuverlässig hohe Produktausbeuten von etwa 95 % erreicht werden.

Im dritten Teil dieser Arbeit lag der Fokus auf der Intensivierung der Klärung mittels verschiedener Vorbehandlungsmethoden der Zellsuspension. Es wurden dabei zwei Methoden mit unterschiedlicher Wirkungsweise, Flockung und Präzipitation, identifiziert, die einen geringen Einfluss auf die Gegenstrom-Zentrifugation haben, jedoch den nachfolgenden Filtrationsschritt durch eine bis zu vierfach höhere Filterkapazität verbesserten. Zusätzlich konnten mit beiden Ansätzen mehr als 90 % der gelösten DNA-Verunreinigungen bei gleichbleibender mAb-Qualität entfernt werden.

Im vierten Teil dieser Arbeit wurde der entwickelte Ansatz zur Zellklärung rationalisiert, indem die Filtration in den Zentrifugationsschritt integriert wurde. Durch eine direkte, sterile Verbindung beider Prozessschritte konnte der gesamte Prozess verschlankt sowie die Prozesszeit verkürzt werden. Der neuartige Aufbau sowie die entwickelte Methode diesen zu betreiben, wurden als Patent angemeldet und in einer Konzeptstudie untersucht. Dabei konnte erfolgreich die Intensivierung der Klärung von HCD-Prozessen demonstriert werden.

**Schlagwörter:** Monoklonale Antikörper, Zellklärung, Downstream-Processing, Bioprozess-Intensivierung, Gegenstrom-Zentrifugation

# Abstract

Monoclonal antibodies (mAb) enable the treatment of many serious, chronic diseases, such as various types of cancer and immunological disorders, which is why novel therapeutic approaches are being continuously developed and global demand is growing rapidly. To increase productivity in mAb production, high cell density (HCD) cultivations of mammalian suspension cell lines that segregate the product into the culture medium are increasingly used. However, at the end of these already cost-intensive manufacturing processes, clarification of the mAb from particulate contaminants, primarily high amounts of host cells as well as cell fragments, presents a challenge. Conventional clarification processes based on stainless steel disc stack centrifuges and single-use (SU) depth filters have at high particle loads the drawbacks of low loading capacities and high product losses, making them not suitable for clarification of HCD processes.

In the first part of the work, an alternative clarification approach using a novel fluidized bed centrifuge followed by a filtration step was therefore developed and optimized for HCD clarification. In the second part, the suitability of this approach for different cell suspensions was investigated and the clarification process was modeled as a function of the cell concentration. It was shown that the approach, which is based entirely on SU materials, enables robust clarification of both, moderate cell concentrations and concentrations of more than 100 million cells/mL from HCD cultivations. The corresponding process throughput could be predicted and high product yields of about 95 % could be consistently achieved. In the third part of this work, the focus was on intensifying clarification by applying various pretreatment methods to the cell suspension. Two methods with different modes of action, flocculation and precipitation, were identified that had a minor effect on fluidized bed centrifugation but improved the subsequent filtration step by increasing filter capacities up to fourfold. In addition, both approaches were able to remove more than 90 % of the dissolved DNA impurities while maintaining mAb quality.

In the fourth part of this work, the developed approach for cell clarification was rationalized by process integration of the filtration step into the centrifugation step. As a result of the direct, sterile connection of both steps, the entire process could be streamlined and the process time shortened. The novel setup and the method developed to operate it were submitted as a patent and investigated in a concept study. Overall, the intensification of the clarification of HCD processes could be successfully demonstrated.

**Key words:** Monoclonal Antibodies, Cell Clarification, Downstream Processing, Bioprocess Intensification, Fluidized Bed Centrifugation

# Table of Contents

# 1 Introduction

Therapeutic monoclonal antibodies (mAb) enable improved health care for millions of people by treating many severe diseases such as various types of cancer, virus infections and immunological disorders [1,2]. In recent years, efforts to develop new mAb-based treatments have resulted in the approval of approximately 10 drugs per year [3]. In 2021, the United States Food and Drug Administration (FDA) has approved the 100th mAb product [4]. As the number of novel therapies continues to grow, more patients can be treated, increasing the demand for large quantities of mAb. Therefore, the global market value of mAb is expected to reach $300 billion by 2025 [5]. An additional boost in both demand and economic terms could be the widespread use of mAb for pandemic control [6]. To prevent severe progression of COVID-19, four of the six treatments authorized by the European Medicines Agency (EMA) are based on mAb [7].

Due to their complex structure, antibodies are produced by mammalian cells, like Chinese Hamster Ovary (CHO) cell lines, which segregate the product into the medium [8]. For industrial mAb production, cells are usually cultivated in fed-batch (FB) processes using a cascade of stirred bioreactors in the upstream processing (UPS) [9]. To harvest the product, the cells are separated from the mAb containing media by filtration, centrifugation, or both [10]. In the subsequent downstream processing (DSP), the mAb is purified from contaminants and host cell impurities by a multitude of filtration and chromatographic steps to achieve a desired quality and to ensure the safety of products [11].

The elaborate manufacturing and the low USP product concentrations in the order of 3 g/L in commercial processes resulting in high production costs of on average 300 $/g and thus limit the accessibility to affordable treatments for patients [12]. Therefore, intensification of biopharmaceutical manufacturing is needed to decrease costs and increase productivity [13]. Promising approaches are high cell density (HCD) cultivations, where concentrations of 100 million cells/mL and higher have already been reached [14,15]. However, these improvements in USP shift the bottleneck to the cell clarification, where established methods are not capable or not economical to operate, due to low biomass loading capacities of filters and low product recoveries of conventional centrifuges [16,17]. An additional challenge of HCD processes is the also higher concentration of dissolved impurities, which increases the pressure on the already cost-intensive DSP [18].

For the clarification of HCD suspensions, novel and flexibly applicable single-use (SU) technologies like fluidized bed centrifugation could be an opportunity [19]. Additional removal of dissolved impurities and intensification of the clarification could be reached by cell broth pretreatments [20]. However, the use of such approaches as a HCD clarification platform requires robust clarification of various cell broth characteristics, scalability of the setup, preservation of product quality, and high mAb recovery.

## 2 Objective

The goal of this dissertation was to overcome the limitations in the clarification of biopharmaceutical manufacturing processes. A CHO cell line producing an industry relevant mAb was used to model different process broths. The focus was placed on HCD processes with cell concentrations on the order of 100 million cells/mL due to the lack of appropriated clarification technologies. An additional goal was the intensification of the clarification process in terms of increasing product recovery and purity.

First, an approach needs to be developed that is capable to completely remove cells and cell debris from HCD broth and can be integrated into a SU production process. For a potential process solution, optimization of process parameters is required to achieve efficient clarification with a high recovery of mAb. Subsequently, the effect of various cell broth characteristics, like different cell viabilities and concentrations, needs to be determined to investigate robustness and scalability of the approach. To study the clarification process performance, both the process variables, such as throughput, and the product parameters, such as recovery, have to be considered. In addition, flocculation or precipitation pretreatment methods of cell broth could have the potential to intensify the clarification and the subsequent DSP by simplifying the removal of aggregated host cell impurities. Therefore, their impact on the developed clarification approach should be examined. To finally evaluate the applicability of treatment methods, the mAb quality attributes such as level of aggregation and N-linked glycan patterns must also be considered. For an even more intensified process, clarification substeps could be connected and synchronized to allow improved process automation and to streamline the setup.

The results of this work are intended to develop a novel SU process platform for intensified clarification of HCD processes and thus enable the production of affordable mAb.

# 3 Theoretical Background

## 3.1 Antibodies

Antibodies, also known as immunoglobulins, are proteins with the ability to bind highly specifically to molecular structures, the epitopes of antigens. The binding can trigger an immune response directed against the antigen. Thus, antibodies play a crucial role in the adaptive immune system. They are produced and segregated by B-cells in the blood serum, where immunoglobulin G (IgG) is the most abundant out of five mammalian antibody classes [21].

The IgG molecule consists of four subunits: two heavy chains, each consisting of three constant regions (CH1, CH2, and CH3) and one variable region VC, and two light chains, each consisting of one constant light (CL) and one variable light (VL) region (Figure 1). The chains are connected by disulfide bridges, resulting in the characteristic Y-shaped structure. A common antibody (IgG1 subclass) has a hydrodynamic diameter in the order of 10 nm and a molecular weight of 146 kDa. The flexible hinge region in the middle divides the molecule into two superordinate domains: the fragment antigen binding (Fab) and the fragment crystallizable (Fc). In the Fab domain, the variable regions on each side form the antigen-binding sites, also called paratopes, which are specific for the respective antibody. The constant Fc domain is recognized by the host immune cell receptors and induces an immune response. Glycans which are covalently bound to the CH2 region by posttranslational modification affect the molecule´s conformation [22], solubility [23], and lifetime in blood circulation [24]. Therefore, the cell type specific glycosylation pattern has a major impact on cytotoxicity and biological activity [25,26].
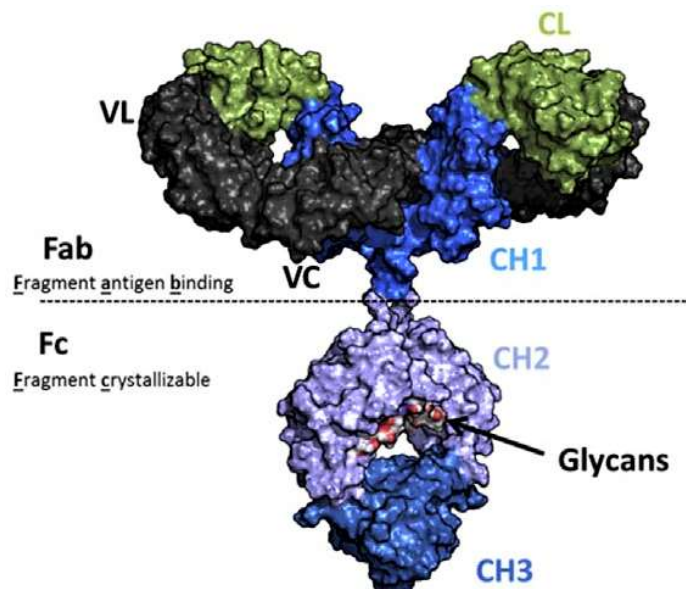


**Figure 1.** Structure of an IgG antibody with highlighted constant regions (CL, CH1, CH2, and CH3), variable regions (VC and VL), and glycosylation at CH2 (adapted from [27]).

The invention of the hybridoma technology in 1975 by César Milstein, Georges Köhler and Niels Jerne paved the way for the production of monoclonal antibodies (mAb) [28]. Due to the enormous potential of this technology, they were awarded the Nobel Prize in Medicine even before the first therapeutic mAb, used to prevent rejection in kidney transplants, was approved in 1986.

In contrast to the natural immune response, in which a mixture of polyclonal antibodies binds to different epitopes of an antigen, mAb target a single epitope. However, the murine production system for the first mAb therapeutics caused immunogenicity in human due to non-human glycan patterns and was therefore not suitable for chronic therapies [29]. To decrease immunogenicity of mAb, chimeric, humanized, and fully human antibodies were developed using glyco-engineered cell lines such as CHO [30]. Moreover, the glycan pattern is considered as a critical quality attribute (CQA) for therapeutic use due to its considerable impact on safety and function [29].

Beside the in-vivo application, antibodies can also be used in a variety of laboratory diagnostic applications. For qualitative or quantitative analysis, they are usually applied in combination with a dye, such as in the antigen tests for SARS-CoV-2 diagnosis [31] or in enzyme-linked immunosorbent assays (ELISA) [32]. However, the majority of the antibodies produced are used for therapeutic purposes. Their numerous mechanisms of actions, such as ligand or receptor antagonism, triggering cell death of certain cells or activating the endogenous immune system, provide powerful tools for successful treatment of numerous diseases.

A large proportion of mab-based therapies are directed against specific tumors, like nivolumab for lung cancer indication or trastuzumab for breast or gastric cancer indication [33]. Another field of application is the treatment of chronic diseases, which is necessary, for example, in various autoimmune disorders. Furthermore, some infection diseases can also be treated, like HIV infection using ibalizumab [34].

As a result, mAb´s are the fastest growing class of biopharmaceuticals, with strong growth in total annual sales. Blockbusters of the past, such as Humira® (adalimumab), which is used to treat some chronic inflammatory diseases like rheumatoid arthritis, reached a global market volume of $19.9 billion dollar in 2018 [33]. However, some next generation mAb product candidates currently being developed such as for the treatment of COVID-19 and Alzheimer diseases have the potential to generate even higher sales by reaching a much larger patient population [35,36].

## 3.2 Biopharmaceutical Manufacturing

The process stream in biopharmaceutical manufacturing is divided into the upstream processing (USP), in which a biological product is generated using microorganisms, and the downstream processing (DSP), in which the product is purified to achieve a desired quality (Figure 2). This chapter provides an overview of the unit operations (UO) involved in the steps of industrial production of mAb and the recent developments for their intensification.

Depending on the perspective on the overall process, the cell clarification UO is sometimes considered part of the USP [37] and sometimes considered part of the DSP [38]. Since the focus of this work is on the clarification, more details on the requirements and the technical realizations are presented in the chapter *3.3 Cell Clarification Strategies*.
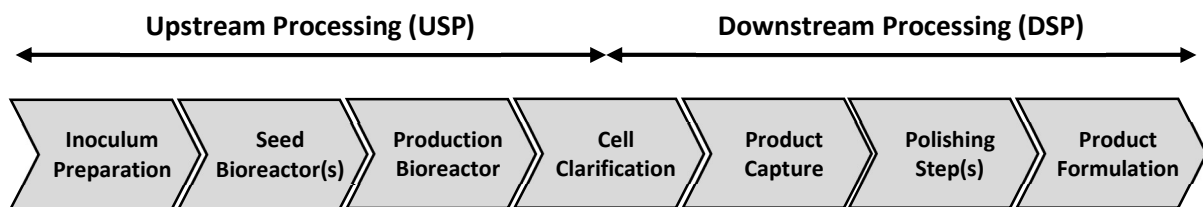


**Figure 2.** Overview of the process stream for production of monoclonal antibodies

### 3.2.1 Upstream Processing

For mAb production, the gene sequence encoding the light and heavy chain of the target mAb is integrated into a host cell to use their cellular protein synthesis apparatus [39]. As heterologous expression systems, mammalian cell lines dominate over non-mammalian cell lines because of the ability of post-translational modifications, like glycosylation of the mAb. Therefore, CHO (approximately 60 %) and murine myeloma (over 30 %) cell lines are commonly used for industrial production [40]. Cultivated in chemically defined media, they achieve cell specific production rates of 20 pg/cell/day and higher [41], which is equivalent to approximately 100 million mAb molecules released into the medium by a single cell per day.

The USP starts with the inoculum preparation by transferring thawed cells from a cell bank into shake flasks to initiate cell growth (Figure *2*). After a series of batch-wise passages, the cells are transferred to a stirred or rocking motion seed bioreactor that provides a highly controlled environment for a successful proliferation of the cells [42]. Depending on the number of cells required for the inoculation of the production bioreactor, a cascade of seed reactors with increasing volumes is used.

For seed and production, cultivation can be accomplished in batch, perfusion or fed-batch (FB) process mode. In the batch mode, the entire process volume consisting of cells and media is loaded into the

bioreactor at the beginning of the cultivation. This mode is not particularly efficient but is commonly used in the seed train due to its simplicity [43]. Another process mode is perfusion, where a cell retention device keeps the cells in the bioreactor while the media is continuously exchanged, providing fresh nutrients to the cells and removing potentially cytotoxic metabolites. Therefore, perfusion processes enables an increased cell growth and thus the generation of high cell concentrations which can be used as alternative seed train [44]. Although it has been shown that also increased mAb concentrations of more than 25 g/L can be achieved [45,46], this process has rarely been applied for industrial production so far. Limitations of the applicability are, for example, the consumption of large amounts of expensive media, large membrane devices for cell retention, and a complex process control [47].

In the FB mode, the cells are supplied with nutrients during the cultivation process by feeding concentrated solutions to the bioreactor. Depending on the needs of the cells, the feeding rate can be adjusted, resulting in enhanced growth and production, while the reactor volume is filled over the process time. Therefore, most of industrial processes typically are operated in the FB mode [48]. In large scale biomanufacturing, production bioreactors can have volumes of more than 10 m³ [49] to generate batch sizes of up to 100 kg mAb [50]. In such FB processes, cell concentrations in the order of 20 million cells/mL are usually achieved [51], allowing production of average mAb concentrations of 3 g/L [12]. The appropriate harvest time point of the bioreactor is a trade-off between maximizing the use of cells for product formation and increasing release of impurities in the cell death phase at the end of the FB cultivation.

### 3.2.2 Downstream Processing

After the removal of cells and debris in the clarification step, the mAb is purified from soluble product and process related impurities in the DSP to preserve activity of the therapeutic and ensure safety for the patient. Product related impurities consist of undesired aggregates, fragments, and charge variants of mAb [52]. Process related impurities released from host cells or by the applied steps of USP and DSP consist of DNA, host cell proteins (HCP), virus contaminates, leached protein A, and media components [13].

Since therapeutic mAb are administered intravenously, they have to meet high quality and purity standards. The World Health Organization (WHO) recommends a residual amount of DNA of less than 10 ng in the final product [53]. In addition, HCP residues that may cause immunogenic patient reactions are usually limited by the regulatory authorities to a final level of less than 100 ppm per dose, which is assessed using a risk-based approach for the particular product [54,55]. Moreover, two orthogonal

virus inactivation steps are mandated by the FDA because virus-contaminated host cell lines pose a serious risk to patient health [56].

To fulfill these high purity demands, a multitude of UO´s are necessary, starting with the product capture using protein A affinity chromatography. This commonly used platform approach uses the binding between the Fc region of the antibody and the immobilized protein A. Due to the high selectivity, most impurities flow through the column or are subsequently washed out, resulting in consistent high purities of more than 95 % [57]. Elution of the mAb is performed with low pH buffer and additionally cause concentration of the product in the harvest pool. In the following step, the already low pH of the eluate facilitates further acidification for a first virus inactivation, which requires a pH-value of 3.8 or lower for a sufficient inactivation of retroviruses [58]. As most mAb´s are only stable for a short time at low pH conditions, a neutral titration is conducted after the process for stabilization [59].

The subsequent polishing steps target the removal of the residual impurities to meet the specifications of the authorities. Generic process platform uses at least two chromatographic steps based on the separation by molecular net surface charge or hydrophobicity. Depending on the isoelectric point of mAb, the pH value and conductivity of the environment determine the operation windows of the chromatography process [60]. The isoelectric point is the pH where the mAb has a net surface charge of zero; a pH below this point results in a positive charge and above this point results in a negative charge of the molecule. A commonly used chromatography combination is cation exchange chromatography (CEX) executed in the bind and elute mode followed by anion exchange chromatography (AEX) executed in flow through mode [61,62]. During CEX, the positively charged mAb adsorbs on the negatively charged column resin while the negatively charged impurities, such as a large group of HCP, flow through. During AEX, the mAb flows through the column while impurities, mainly the negatively charged DNA, leached protein A, and viruses, are adsorbed [63]. As an alternative or in addition, hydrophobic interaction chromatography or mixed mode chromatography are applied which are particularly suitable for the removal of mAb aggregates [64]. After or during polishing, a second virus removal step is incorporated using virus filtration.

In the final product formulation, the buffer is exchanged and the mAb is concentrated using ultrafiltration/diafiltration [38]. Membranes with a molecular weight cutoff below the mAb are applied, usually 100 kDa or 30 kDa, allowing the retention of product. The formulation buffer, which is finally filled with the therapeutic product in a medical dosage, aims to ensure the stability and optimal bioavailability of the drug [65].

### 3.2.3    Process Intensification

Intensification of biopharmaceutical manufacturing is the key to satisfy the increasing demand for affordable therapeutics [13]. A variety of approaches is used to intensify UO´s, which consider throughput, recovery, robustness, scalability, time to market, product purity, and costs of goods [38]. By improving the cell line characteristics, the productivity of USP has been steadily enhanced. Due to selection of highly productive clones, improved cultivation media, and advanced feeding strategies, the specific growth rates and the cell specific production rates were increased [37]. As a result, average product titers increased from 0.1 g/L in initial commercial production to 3 g/L in current processes [12]. In some studies, mAb titers of more than 10 g/L have been achieved with FB processes [66,67].

For these and higher titers in USP, cultivation of high cell densities (HCD) is essential, where cell concentration of 100 million cells/mL have already been reached [14,44]. Alternative HCD process scenarios are provided by perfusion and concentrated FB processes using cell retention devices for continuous exchange of cultivation media while the cells are retained in the bioreactor [68]. The supply of cells with fresh nutrients and the flush out of metabolites favors proliferation and increases productivity. In perfusion processes, microfiltration cell retention devices, such as alternating tangential flow filtration, allow continuous harvest of product and therefore also require a continuous DSP [69]. Concentrated FB processes additionally retain the product by applying ultrafiltration devices, which requires clarification of the HCD broth at the end of the cultivation [70]. Both approaches can be applied to increase USP productivity, however, so far most industrial platforms are based on FB processes due to their high flexibility, lower investment costs, lower consumption of expensive media and simplified process control [9,48].

For the intensification of DSP, continuous UO´s such as multicolumn chromatography offer high potential for increasing throughput, reducing the use of expensive chromatography resin and thus saving costs [71,72]. Hybrid approaches that combine batchwise USP plus clarification UO with continuous DSP are also promising for that purpose [73,74].

Another driver of process intensification is the application of single-use (SU) based technologies. Compared to conventional processes using stainless steel equipment, disposable process equipment is exchanged after each process run, eliminating the need for cleaning, sterilization, and validation. This prevent cross-contamination between batches, reduce setup times and thus reduce production costs [75]. Furthermore, SU allows efficient utilization of plant capacities by increasing the flexibility of UO´s [76]. In recent years, the share of SU-equipment in commercial production has increased sharply [77]. In USP, stirred SU bioreactors are widely established for small- and intermediate-scale production up to a volume of 2,000 L [78]. The DSP follows by transferring more complex steps into SU, such as chromatography, centrifugation or tangential flow filtration, and by implementing new technologies

[79]. For specific process scenarios and scales, the feasibility of a complete SU-based production stream was already demonstrated [80]. However, for larger process scales and certain UO´s the applicability of SU equipment often limited, for example, by low biomass loading capacities of filters at the clarification of HCD broths [17].

To develop intensified platform concepts suitable for industrial mAb production, combinations of HCD cultivation, SU technologies, and integrated continuous processing are considered the most promising [15,79,81].

## 3.3   Cell Clarification Strategies

Cell clarification aims to remove all particles, such as cells and cell debris, from the cell culture suspension while recovering the mAb in the clarified liquid. A cascade of clarification steps is used for harvesting the production reactor, except for perfusion processes, where the cells are retained. Since the clarification operation connects USP with DSP, it is challenged by both: On the one hand, the UO needs to be suitable for the respective characteristic of the feed stream, such as biomass concentrations, cell viability and particle size distributions. On the other hand, it needs to meet the high requirements of a subsequent DSP, such as clarity, product quality, and throughput. This chapter reviews the existing clarification strategies, focusing on their limitations and advantages in clarifying mammalian cell broths.

### 3.3.1   Cell Broth Pretreatment

Prior to the primary clarification, pretreatments of the cell broth using additives target the aggregation of impurities to facilitate their removal (Figure 3). Various pretreatment approaches have been described in the scientific literature, which are usually classified according to their aggregation mechanism: precipitation or flocculation [20,82].

In precipitation approaches, the solubility limit of dissolved impurities such as certain HCP´s is exceeded to allow their separation as a particulate system in the subsequent clarification UO. For this purpose, the cell broth conditions such as pH value or ionic strength are changed [13]. A well-known approach is the lowering of the pH value to 5 in combination with subsequent filtration [82,83] or centrifugation [84]. In flocculation approaches, the size of particles already present in a suspension is increased using charged additives that cover or bridge the repulsive forces between similarly charged particle surfaces while dissolved impurities can additionally be bound to the aggregates [85,86]. Since most of the impurities are negatively charged under neutral pH conditions, cationic polymers such as polyethylenimine or polymer poly(diallyldimethylammonium chloride) (pDADMAC) are widely used [13,87,88]. With both pretreatment approaches, improved the filterability [89] and an additional reduction of DNA impurity by several log-levels [51] can be achieved. Some pretreatments may provide further advantages for the DSP, such as the inactivation of viruses [90].

However, the application of pretreatments is limited by their often cytotoxic nature and the risk to alter the mAb [20]. Cytotoxicity may result in the release of even more impurities through cell lysis in the broth, and additionally requires complete removal of the additives in the subsequent DSP to ensure patient safety of the final product [91,92]. In addition, the harsh environmental conditions in pretreated cell broth pose risks that mAb is lost through co-aggregation or degradation. Moreover, there is also the risk of a loss in mAb activity due to an additive-induced change in glycan structure

[93]. Therefore, the stability of mAb and host cells as well as regulatory quality requirements must be considered for the selection of suitable additives.
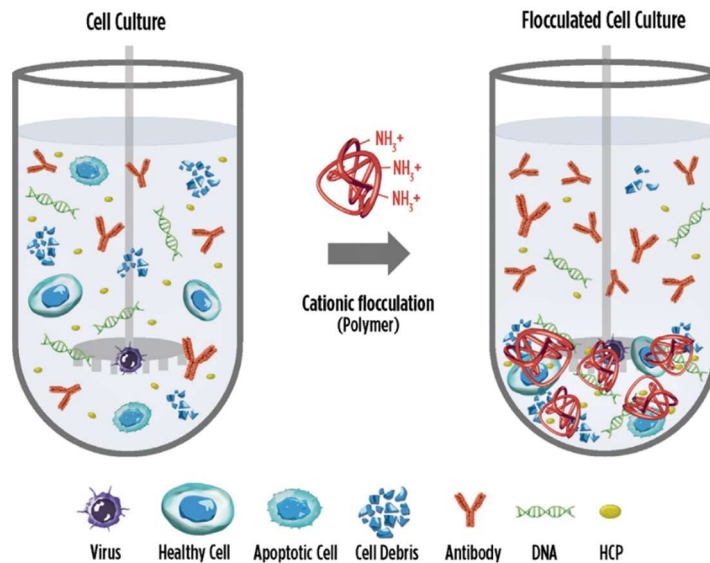


**Figure 3.** Pretreatment mechanisms using cationic flocculants to aggregate negatively charged virus, cells, cell debris, DNA, and host cell proteins (HCP), maintaining the antibody in solution (adapted from [13]).

### 3.3.2    Filtration

To remove the entire biomass by filtration, at least two subsequent filter stages are required. In the primary clarification stage, cells and larger debris are removed. In the secondary filtration, a dead-end sterile filter for the removal of sub-micron debris and colloids is used to protect the chromatographic columns of the subsequent DSP from fouling [10,13].

As a primary filtration technology, tangential flow filtration enables reduced filter cake formation and thus high throughputs [94,95]. However, this approach is rarely used in industrial processes because of low recovery rates and the risk of damaging cells and releasing impurities due to shear forces [61].

A widely used method for primary cell clarification is depth filtration [96]. The approach has a long history of success, as it has been used for water purification for more than 4,000 years [97]. In biopharmaceutical production, depth filters consist of synthetic or cellulose fibers and a filter aid that increases the surface area and provide a porous structure, such as diatomaceous earth [98]. Some depth filters are additionally charged using a binder chemistry [99]. Particles are retained by their size on the surface and, unlike membrane filters, in the depth of the porous structure [100]. In addition, smaller particles and soluble impurities, such as DNA or HCP, can be adsorbed onto the depth filter material through electrostatic and hydrophobic interactions [101,102]. Depending on the amount and particle size distribution, a proper selection of the filter grade is required, where often multilayer depth

filters as well as two successive depth filter stages are used [96]. Another method is dynamic body feed filtration using diatomaceous earth or other filter aids which are added directly to the cell broth to increase the retention capacity of the filters [103].

However, since the biomass loading capacities of dead-end filters remain limited, clarification of large volumes and high cell concentrations is challenged by high filter costs and technological feasibility, such as high plant footprint, long setup times, and large amounts of waste that need to be treated.

### 3.3.3 Centrifugation

Centrifugation-based separation uses the differences in the density between culture media and biomass particles. The centrifugation step is usually applied for the primary clarification, followed by a small depth and sterile filter stage [13].

Disc stack centrifuges (DSC) are widely used in industrial processes for 2,000 L batch scales and above, providing low cost operation at large scale and a scalable continuous process platform [10,38,104]. The separation is achieved by pumping the cell broth through a rotating stack of discs. Due to the centrifugal force, the cells are accelerated towards the outside of the bowl and collected in the solids holding space, while the supernatant leaves the centrifuge using an axial outlet [10]. The biomass in the solid holding space is continuously or frequently pushed out by a specific bowl periphery. However, the required high shear forces can cause cell disruption, thus releasing impurities, which reduces clarification efficiency and increases stress on the DSP [96,105]. In addition, a high biomass content in HCD cultures can result in a loss of recovery due to the need to remove solids more frequently combined with a poor dewatering capability of the system [104]. Furthermore, the stainless steel system requires pre-sterilization, evaluation, and cleaning for each batch.

Therefore, there are efforts to manufacture centrifuges and all parts that come into contact with the product as SU equipment [79]. The designs are based either on the classical DSC technology [106] or on an alternative technology that allows the capture of cells in a fluidized bed [107].

The working principle of a fluidized bed centrifuge (FBC) was first described in 1948 [108], however, only the recent transfer to a SU concept made the technology attractive for biopharmaceutical cell clarification applications [107]. Separation of cells is achieved by pumping a cell broth through a conically shaped chamber against the centrifugal force which results from the radial rotation of the chambers. While the supernatant flows through the chamber, the cells are captured in it at the point where the drag force of the fluid flow and the opposing centrifugal force are equal [109]. The FBC is operated semi-continuously in repeating cycles, consisting of loading of the chambers with cells, washing of the cells, and discharging the chambers, which enables low shear rates on the cells [19]. So far there are no reports on the use of SU centrifuges in commercial production.

### 3.3.4 Alternative Clarification

In addition to the commonly applied mechanical separation methods based on density or size, there are some alternative technologies. The following examples have already been demonstrated to be capable for cell clarification in laboratory or pilot scale mAb processes.

Aqueous two-phase extraction is an integrated approach that combines cell clarification, product capture, and initial polishing [110]. To obtain a suitable biphasic system, two hydrophilic components whose concentration are above a certain threshold are added to the cell broth and mixed [111]. The mAb is enriched in the light phase due to its selective solubility, while some impurities are soluble in the heavy phase and the biomass accumulates in the interphase [112,113]. However, subsequent separation of the phases, adaption of the following DSP, and careful selection of the phase system for the respective mAb is required [113].

Acoustic cell separation is another approach for primary clarification that uses the density and different mechanical properties of cells to capture them in the knots of a three-dimensional standing wave [114]. The captured cells inside the flow channel aggregate and sediment, resulting in a biomass reduction in the cell broth. Systems with SU flow path were developed, but most applications focus on microfluidic cell processing [115].

Microfluidic spiral separation was shown to be suitable for continuous removal of CHO cells with high efficiency [116]. Due to internal fluidic shear characteristics of a flow in spiral channel geometry, cells are concentrated on the inner sidewall and can be removed at the outlet [117]. Since the effect is limited to microfluidic channels, so far there are only applications for research and in small-scale perfusion processes.

A further approach was demonstrated by a concept study aiming for direct capture and purification of mAb from the cell broth by magnetic beads to circumvent the cell clarification UO [118]. However, it needs to be investigated whether such approaches are scalable, sufficiently robust, and economically feasible to operate.

# 4 Experimental Results

Aim of this PhD thesis was to establish an intensified clarification strategy for biopharmaceutical manufacturing of mAb´s due to the lack of appropriated HCD clarification technologies and the need to increase efficiency of established processes. The experimental work for this purpose was divided into four closely related parts: development of a fluidized bed centrifugation approach to enable HCD clarification (Chapter 4.1), investigation of robustness for various cell suspension and modeling of the developed approach (Chapter 4.2), intensification of this approach using cell broth pretreatments (Chapter 4.3), and implementation of a rationalized setup to streamline the clarification process (Chapter 4.4). The results of the first three parts were published in separate research articles in different peer-reviewed journals. The developed setup of the last part was published as an international patent application due to its high industrial relevance.

In a preliminary study of this work, different SU clarification technologies were investigated to identify a suitable approach for the clarification of high CHO cell concentrations. For this purpose, depth filtration, dynamic body feed (DBF) filtration, and fluidized bed centrifugation were examined for clarification of HCD broths with concentrations in the range of 100 million cells/mL. As a result of the proof-of-concept experiments, the tested filtration technologies showed relatively low biomass loading capacities and thus were hardly applicable for HCD clarification. Interestingly, the investigated SU fluidized bed centrifuge (FBC) was capable to separate cells in HCD broth from the mAb containing supernatant which suggests a high potential of the approach to be used for HCD clarification. This results were presented on a poster with the title "High Cell Density Clarification Using Single-Use Technologies" at the 26th European Society for Animal Cell Technology (ESACT) Meeting 2019 and published in the conference proceedings [119].

Due to these findings, a FBC-based HCD clarification approach was developed in the first part of this work. To enable efficient separation of biomass with simultaneous high recovery of mAb, the FBC process parameters for loading of the centrifuge chambers with cell broth, washing out of mAb from the captured cells, and discharging of biomass were optimized. The investigation of cell amount and viability during the processes suggested mild separation of almost all cells and confirmed a maximum FBC chamber loading capacity of approximately 10 billion cells per 100 mL chamber volume [120]. In contrast to the conventional applied disc stack centrifuge, where high particle loads results in reduced product recoveries [10], a high mAb recovery of 95 % was achieved due to the adaption of the washing strategy of cells which were captured in the fluidized bed.

After the FBC process step, small amounts of residual biomass in the supernatant needed to be removed by an appropriated subsequent filtration step. Therefore, a filter screening was conducted to

identify a filter setup with high filter throughput and high filtrate clarity. As a result of the screening, a double layer depth filter with nominal pore sizes of 8 µm and 0.8 µm, followed by a 0.2 µm membrane filter, was found to be best suited to clarify the FBC supernatant and thus was used to complete the HCD clarification approach. The results of the developed approach using a FBC as first and a small filtration step as a second clarification step were published, entitled "Clarification of intensified mAb processes with up to 100 million cells/mL using a single-use fluidized bed centrifuge" (Biochemical Engineering Journal, 2021) [121].

In order to investigate the applicability and the robustness of the approach for various cell broth characteristics a further study was conducted, which is presented in the second part of this work. The cell broths with concentrations ranging from 20 million cells/mL to 110 million cells/mL were successfully clarified. The achieved consistent mAb recoveries of approximately 95 % and harvest turbidities below 13 NTU again demonstrates that FBC followed by filtration is a suitable alternative clarification approach. Furthermore, a process model was developed to predict the process throughput and product pool dilution in dependency of the cell concentration. The experimental results confirmed the calculated values, showing an increase in throughput and a decrease in dilution being associated with a decrease of cell concentration in the cell broth to be clarified. These results were published as a part (chapter 3.7) of an industrial review highlighting different approaches for process intensification, entitled "Process Intensification in the Biopharma Industry: Improving Efficiency of Protein Manufacturing Processes from Development to Production Scale Using Synergistic Approaches" (Chemical Engineering and Processing, 2022) [122].

In addition, based on these results, another application was identified where the viable cells could be re-used in a second production cycle after their mild and sterile separation by the FBC. This enables the development of novel USP modalities to further intensify mAb production. The results of a first proof-of-concept study indicated a high potential to more than double the productivity in FB processes which was presented at the ECCE&ECAB 2021 conference with the title "Boosting monoclonal antibody productivity of fed-batch processes by recovering viable cells.".

The third part of this PhD thesis represents a feasibility study to intensify the developed clarification approach by treatments prior to the FBC step using flocculation and precipitation of cell broth. The primary goal of these pretreatments was to facilitate the removal of cell debris as well as to additionally remove process related impurities. Therefore, model CHO cell cultures with a low viability and thus a high content of impurities were used. A screening of potential cell broth additives was accomplished, identifying low pH precipitation by acetic acid and cationic flocculation by pDADMAC as promising pretreatments. However, due to a significant change in cell broth conditions by the pretreatment additives, their application can lead to the release of impurities from damaged cells and alter the

product. This effect can be additionally exacerbated by the subsequent clarification process. Therefore, the applicability of these two different pretreatments in combination with the FBC approach was investigated. In this study, focus was placed on the aggregate formation and stability of cells as well as on the impact of the pretreatments on selected critical product quality attributes. Overall, it was found that pretreatments tailored to the cell cultures can be successfully used to intensify FBC clarification. The results of this feasibility study were published as a research article, entitled "Fluidized bed centrifugation of precipitated and flocculated cell cultures: An intensified clarification approach for monoclonal antibodies" (Journal of Biotechnology, 2022) [123].

In the fourth part of this work, a novel clarification setup with a corresponding method of operation was developed and filed as an international patent application with the title "Clarification setup of a bioprocessing installation" (WO 2022/008536 Al) [124]. This SU approach based on the clarification process developed in the preceding parts of this PhD thesis consisting of a cyclical FBC step and a depth and sterile filtration step, which have so far been operated independently of each other. The novelty of the invention is that both process steps have been combined in a single unit operation and implemented into the biomanufacturing process, aiming for a closed flow path from the bioreactor to the sterile harvest vessel, which was so far not possible with any other centrifugation approach. In the streamlined setup no intermediate surge vessels and only the pumps of the FBC system were used, resulting in a reduction of peripheral devices, SU materials and process time.

A proof-of-concept study was conducted to evaluate the applicability of the intensified setup as a platform approach. The robustness of the approach was assessed by the clarification of different cell broth characteristics including HCD broth with 110 million cells/mL. To conclude, the scalability of the approach was successfully demonstrated by clarifying various volumes of cell broth up to 200 L bioreactor pilot scale. The results were published as a conference poster at the 5th European BioProcessing Summit 2022 [125]. Overall, a scalable and robust clarification process was developed that can be used for different cell broth characteristics, supporting the efforts of bioprocess intensification.

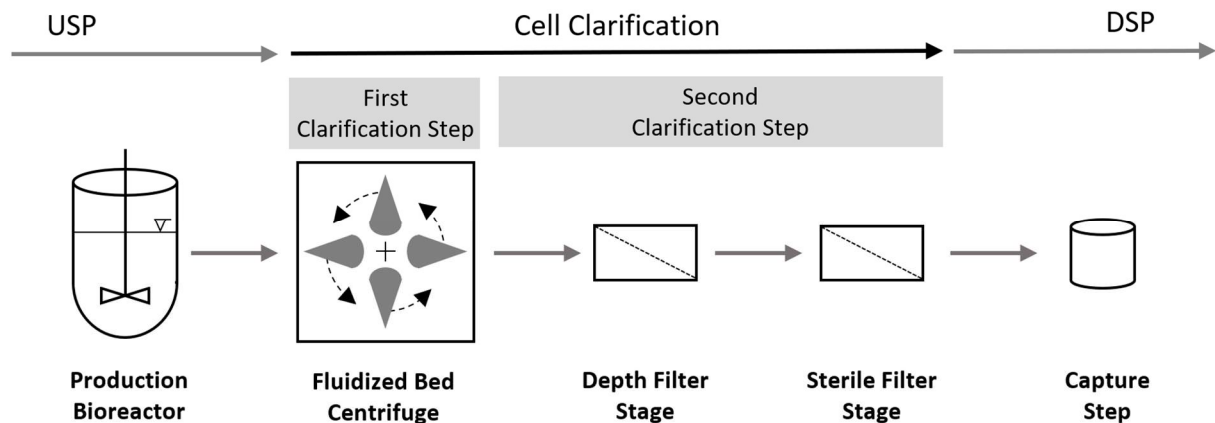## 4.1 Development of a High Cell Density Clarification Approach



**Figure 4.** Overview of the developed high cell density (HCD) clarification approach consisting of a fluidized bed centrifugation as a first clarification step, and a depth filter stage followed by a sterile filter stage as a second clarification step to connect HCD upstream processing (USP) with downstream processing (DSP).

Biopharmaceutical manufacturing using high cell concentration poses a challenge for conventional cell clarification UO´s due to low product recoveries of stainless-steel disk stack centrifuges and low biomass loading capacities of filters [17]. Therefore, cell clarification represents a technological bottleneck in intensified mAb production processes. In addition, there is an ongoing trend to apply SU-based UO from research up to production scale due to higher flexibility, lower hardware costs and reduced setup times [79].

Therefore, the first publication focuses on development and optimization of a novel, SU-based clarification approach, which overcomes the limitations of conventional clarification UO´s. The approach is based on fluidized bed centrifugation as a first clarification step (Figure 4). This technology enables the capture and concentration of mammalian cells in a fluidized bed by balancing the drag force of a continuous flow and an opposing centrifugal force which is generated inside a rotating chamber. As the cells undergo zero-net-force in the fluidized bed, mild process conditions were expected. As a result of this study, this effect was confirmed by determination of high cell recoveries and high viabilities of processed cells, additionally suggesting a low release of host cell impurities.

Furthermore, the publication focuses on the parameter optimization for HCD broth with 100 million CHO cells/mL, since several FBC process parameters, such as flow rates and void volumes, are critical for efficient cell capture and supernatant washout. Suitable flow rates were identified that enables almost complete removal of cells and almost complete recovery of mAb. In order to achieve acceptable dilution of product and short process times, process volumes were adjusted to obtain mAb recoveries of 95 %, which was validated in a 2 L scale. It was found that the complete HCD broth was

clarified within 30 minutes and successfully achieved the predefined high mAb recovery of 95 ± 2 %. In addition, a high turbidity reduction from 8,000 NTU in the HCD broth to 170 NTU in the clarified supernatant was obtained.

However, for protection of the expensive column material in the DSP product capture step, the turbidity of the obtained supernatant must be further reduced by removing the remaining small particles, such as cell debris. Therefore, a small depth filter followed by a sterile filter was applied as a second clarification step. A screening of six different depth filters with different nominal pore sizes followed by a 0.2 µm sterile filter was conducted. The most promising filter combination using as depth filter stage a double layer filter with nominal pore sizes of 8 µm and 0.8 µm achieved high filter capacity of 157 L/m² in combination with a filtrate turbidity below 5 NTU.

To conclude, it was possible to develop a novel method that enable the clarification of HCD broth with 100 million cells/mL. In contrast to the commonly applied methods, the FBC followed by a small filter step provides a complete SU-based approach. The optimization of process parameters achieved short process times and high recovery of product, offering great potential for process intensification.

# A novel clarification approach for intensified monoclonal antibody processes with 100 million cells/mL using a single-use fluidized bed centrifuge

Martin Saballus *, Lucy Nisser, Markus Kampmann, Gerhard Greller

*Sartorius Stedim Biotech GmbH, Corporate Research, August-Spindler-Straße 11, 37079 Göttingen, Germany*

ABSTRACT

Intensified high cell density (HCD) processes for the production of therapeutic proteins have already reached cell concentrations up to 100 million cells/mL (> 300 g/L wet cell weight, WCW). The clarification of such HCD processes using technologies established for conventional fed-batch processes (< 100 g/L WCW) is faced with low biomass loading capacities and relatively low product recoveries, which limit their applicability. This and the growing demand for single-use (SU) technologies increases the pressure to develop scalable, robust and cost-effective SU based HCD clarification solutions.

Therefore, in this study a SU fluidized bed centrifuge (FBC) system was investigated to clarify HCD broths with 100 million cells/mL from a monoclonal antibody production process. FBC parameters including process volumes and flow rates during the FBC operating steps were optimized so that an almost complete removal of biomass and product recoveries of 95 % were achieved. In addition, the mild centrifugation conditions were demonstrated by the superior cell viability for the separated cells while minimizing release of host cell impurities. Furthermore, a post-centrifugal filter screening showed acceptable turbidities below 5 NTU of the sterile filtrate. This concept of FBC with subsequent filtration offers excellent potential to achieve a robust HCD clarification process solution.

## 1. Introduction

The increasing number of approved therapeutic proteins, especially of monoclonal antibodies (mAb) [1], has boosted the development of intensified biopharmaceutical processes to reduce costs and increase production quantities for a growing patient population [2,3]. As a result, mAb titers have been increased a hundredfold from less than 0.1 g/L in the 1990's to 10 g/L in modern fed-batch cultivations [4] by the use of high producing clones, optimized media and increased mammalian cell concentrations [5]. By 2026, novel concentrated fed-batch processes with cell concentrations of 60–100 million cells/mL and product titers in the range of 30–50 g/L are expected by the BioPhorum group, a collaboration of global biopharmaceutical industries [6]. Such high cell density (HCD) process scenarios are based on parallel operation of small and highly flexible single-use (SU) bioreactors with working volumes up to a maximum scale of 2 m³ [7]. It has already been demonstrated that HCD upstream processes using cell concentrations of 100 million cells/mL are capable to increase to more than double the manufacturing output compared to a standard fed-batch process [8].

However, HCD upstream processing (USP) requires the removal of large quantities of biomass as well as high levels of dissolved impurities in the downstream processing (DSP) [9]. Established stainless steel production processes with cell concentrations in a typical range of 10–20 million cells/mL (< 100 g/L wet cell weight, WCW) and volumes of 15 m³ are clarified by disc stack centrifugation followed by a depth and membrane filter stage [10,11]. However, the application of disc stack centrifuges for clarifying novel HCD processes is restricted due to their high investment costs and long turnaround times of up to 8 h [12] as well as due to their lower product yields with increasing cell concentrations [13]. Other established technologies based only on

*M. Saballus et al.*

filtration, like depth filters, are also hardly applicable for HCD clarification due to their limited biomass loading capacities [14]. This shifts the bottleneck from USP to the clarification and the further DSP steps [15], where established technologies are not capable or too expensive to clarify and purify products from intensified USP [16].

Therefore, novel and SU based clarification technologies such as acoustic cell settling [17], alternating tangential flow filtration [18] and aqueous two-phase extraction [19] are getting more strongly in the center of interest. SU based unit operations are usually less expensive and easier to handle, without the risk of cross-contamination, and have shorter setup times as cleaning and sterilization are eliminated [20]. Despite these advantages, the industry is lacking in feasible SU clarification of HCD processes with cell concentration in the order of magnitude of 100 million cells/mL ($\geq$ 300 g/L WCW).

A challenge in all the above mentioned clarification technologies is that biomass and product containing liquid can usually not be completely separated. Therefore, a significant amount of product is discharged in HCD processes with the high amounts of separated biomass with adverse reduction in mAb recovery. This could be overcome by an additional washing of the separated biomass to flush out remaining product. In addition to assessing the technical feasibility of novel clarification approaches, the quality of the harvested material has to be investigated, since the release of impurities due to cell damage during the clarification represents an additional burden for the subsequent purification process.

Fluidized bed centrifuges (FBC), also termed counter flow centrifuges, represent a novel SU clarification approach for the removal of cells from biopharmaceutical processes and offer an integrated option to wash separated cells during the clarification process [21]. Furthermore, FBC promise mild process conditions compared to disk stack centrifugation [22], as they were developed to separate cells, such as different blood cell types [23]. Therefore, FBC could be a potent clarification technology for HCD broths to increase product recovery and to minimize the release of impurities. So far, however, there are no reports in the scientific literature about HCD clarification approaches using FBC.

Unlike the established disc stack centrifuges, FBC have closed disposable separation modules and automatically work in a cyclic mode of cell loading, washing and discharging [24]. Cells and cell debris are retained in the chambers in a fluidized bed when the particle sedimentation velocity equals the velocity of a counter flow through the chamber [25]. Therefore, the centrifugal parameters such as flow rates, process volumes and cell retaining capability of each cycle step have to be adapted for the cell suspension to be clarified to achieve optimal biomass removal and product recovery. To ensure a biomass free and sterile intermediate fluid for further purification, the FBC harvest fluid requires to be filtered in a subsequent small filter stage. Therefore it is necessary to determine a filter setup that achieves both a robust reduction of turbidity and a high filter capacity.

In this study, the clarification of HCD suspensions from intensified mAb processes was examined using a FBC. For this investigation a Chinese hamster ovary (CHO) cell broth with 100 million cells/mL producing a mAb was used as a model system of a concentrated fed-batch. With the objective of a high biomass reduction, a high chamber utilization and thus a fast cell clarification, the centrifugal parameters of cell broth loading and washing of the fluidized bed were optimized. Furthermore, the mAb throughput was characterized during HCD processing to optimize buffer removal and washing volumes aiming high product recovery with minimal product dilution. In addition, the impact of centrifugation conditions on the cells and process related impurities was examined to evaluate the harvest quality. The optimized FBC parameter settings were validated in three independent HCD clarification trials to verify the robustness of this approach. Furthermore, post-centrifugal filtration was investigated and a suitable filter setup was validated to complete the HCD clarification unit operation.

## 2. Materials and methods

### 2.1. High cell density suspension

A mAb (immunoglobulin type G) producing CHO-DG44 cell line was used to generate a HCD model suspension for clarification. The cells were cultivated in a perfusion SU bioreactor (BIOSTAT® RM 20, Sartorius, Germany) using a commercial serum-free medium. During the cultivation time of 7 days a cell specific perfusion rate of 50 pL*cell$^{-1}$*d$^{-1}$ based on the online measured biomass value was applied. A pO$_2$ value of 60 % was maintained by a rocking cascade starting at 30 rpm with a rocker angle of 10°. The temperature was set to 36.8 °C and the pH value was controlled at 6.95.

At harvest time point the viable cell concentration (VCC) was in the range of 79–110 million cells/mL corresponding to a wet cell weight (WCW) of at least 320 g/L. The cells had a viability of > 93 % and an average diameter in the range of 16–18 μm. The concentration of mAb was approximately 1 g/L due to permeation of product during the perfusion process.

In some cases the cell broth was spiked with mAb to increase the titer of the HCD suspension to at least 2 g/L and to dilute the VCC to 100 million cells/mL. The concentrate for the spike was prepared by 30 kDa ultra filtration (Sartocon® Slice 200 Hydrosart® Cassette, Sartorius, Germany) of clarified cell broth to obtain enriched mAb solutions with concentrations of 10–15 g/L.

### 2.2. Fluidized bed centrifugation

To evaluate FBC for HCD clarification, a kSep® 400 System with associated SU harvest clarification consumable kits (Sartorius, Germany) was used. The consumable kits consisted of a set of four radially installed conical centrifuge chambers (Ch) with a volume of 100 mL each (Fig. 1) and a tubing set.

The FBC clarification process was started by priming the tubing set and the chambers with buffer fluid as well as by ramp-up the centrifugal force. In all experiments, pH 7.4 phosphate buffered saline (PBS, chemicals supplied by Carl Roth, Germany) was used as priming and washing buffer. In order to retain as small particles as possible in the chambers, the maximum centrifugal force of the FBC system of 1000×$g$ was applied during the entire process.

In the first cycle step the chambers were loaded with cell broth (Fig. 2, A) using loading flow rates in the range of 50–300 mL/min/Ch for the application of different cell loads ($7 \times 10^9$ – $11 \times 10^9$ cells/Ch). PBS buffer liquid, which was initially in the chambers due to priming or a previous clarification cycle, was replaced by the cell suspension and
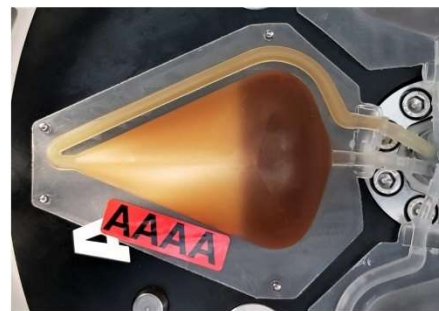


**Fig. 1.** Image of a filled single-use centrifuge chamber, partly loaded with cells (opaque yellow, left) and the separated supernatant fluid (clear orange, right) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).
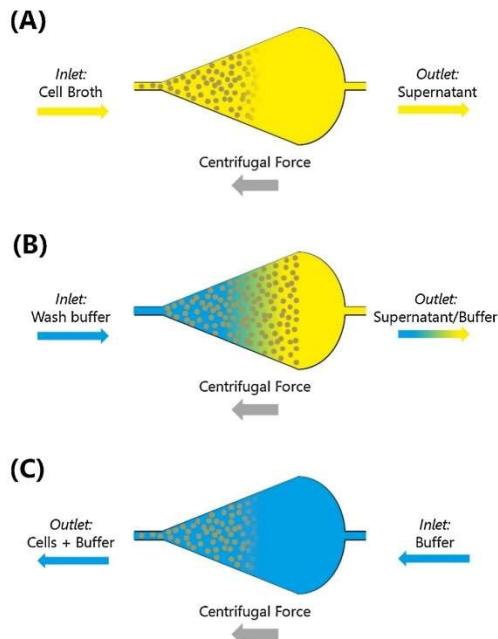
2

**(A)**

Inlet: Cell Broth

Outlet: Supernatant

Centrifugal Force

**(B)**

Inlet: Wash buffer

Outlet: Supernatant/Buffer

Centrifugal Force

**(C)**

Outlet: Cells + Buffer

Inlet: Buffer

Centrifugal Force

**Fig. 2.** Sequence of cycle steps during fluid bed centrifugation: (A) Loading of cell broth where the supernatant (yellow) passes the conical chamber and the cells (brown dots) are retained inside to generate a fluidized bed; (B) Washing of the fluidized bed with buffer (blue) to flush out the product containing supernatant; (C) Discharging buffer and cells by switching the flow direction of buffer (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

the supernatant was collected in a harvest pool vessel. In some experiments, an initial dump volume (IDV) of 100 mL/Ch was directed into the FBC waste pool during the loading.

After the chamber was loaded with a certain amount of biomass the residual supernatant of the fluidized bed was washed out into the harvest pool (Fig. 2, B) by washing the fluidized bed with 250–350 mL/Ch PBS buffer liquid using wash flow rates of 50–250 mL/min/Ch.

In the last cycle step the chambers were discharged into a waste vessel by switching the flow direction in the chambers (Fig. 2, C). A discharge flow rate of 300 mL/min/Ch and a discharge volume of 150 mL/Ch was used in all experiments. This semi-continuous centrifugation procedure consisting of sequential loading, washing and discharging steps was repeated until a specific volume was clarified. The performance of the HCD clarification was assessed by the determination of biomass content, cell concentration, turbidity and mAb amount in cell broth, harvest and waste pools.

### 2.3. Post-centrifugal filter screening

The screening setup comprised a 25 cm$^2$ filter device for the first stage followed by a smaller sterilizing grade filter of 4.5 cm$^2$ for the second filter stage. As a first filter stage the depth filter types DL60, S400 + S020, DL20 and DL75 (Sartoclear®, Sartorius, Germany) with various retention rates as well as the 1.2 μm membrane filter types GF+ and PP3 (Sartopure®, Sartorius, Germany) with different membrane materials were tested. A Sartopore® 2 XLG sterile filter (Sartorius, Germany) was used as second stage filter in all setups. SU pressure sensors (Type

17525SP-10, Sartorius, Germany) were installed in front of each filter inlet port. A peristaltic pump with pressure recording (SciLog®, Parker, US) was used for process control.

Duplicate runs were performed for each filter setup. Initially, a pre-flush of depth filters with 50 L/m$^2$ was carried out using pH 7.4 PBS. Then the sterile filter was connected to the outlet of the flushed depth filter stage. The tested membrane filters were not pre-flushed. One centrifuged HCD harvest pool was aliquoted and used as feed in order to provide the same starting conditions for all filtration experiments. The prepared assemblies were tested using a constant volumetric flux of 120 L/m$^2$/h based on the area of the first filter stage. The filtration was stopped when the overall pressure drop of both filters reached the pressure limit of 1.5 bar. No post-flush or blow out was performed to simplify the screening. To compare filter performance, the filtrate volume and turbidity were measured after the filtration and the filter capacity of the setups were determined by dividing the volume of the filtrate by the area of the first filter stage.

The setup with the highest capacity and a sufficient turbidity reduction was evaluated regarding robustness and product recovery in four filtration runs using different FBC harvests. Process conditions were identical to those in the previous screening trials, with the exception of an additional filter post-flush of 20 L/m$^2$ with PBS (pH 7.4) to recover residual product from the filter stages.

### 2.4. Analytics

Viable and Trypan Blue stained non-viable cells were analyzed using an automatic cell counter (Cedex HiRes Analyzer, Roche, Germany) to determine total cell concentration (TCC), VCC and cell viability. In addition, the instrument was used to determine cell diameter.

For the determination of the WCW 10 mL cell suspension were transferred in a 15 mL centrifuge tube and centrifuged at $5000 \times g$ for 10 min. Then supernatant was completely removed by gentle tapping of the centrifuged tube on a tissue cloth and the remaining pellet was weighed. Pellet mass determination was performed at least in duplicates and results were averaged. WCW was calculated by dividing the pellet mass by the suspension volume.

Turbidity was measured by a nephelometer (TL2350, Hach, Germany) which was calibrated with standard solutions provided by the manufacturer.

The density of particle free solutions was measured by an oscillating U-tube density meter (DMA 38, Anton Paar, Austria) at a constant temperature of 20.0 °C.

The quantification of mAb was performed by size exclusion chromatography (SEC) using a high performance liquid chromatography (HPLC) system (Dionex UltiMate 3000, ThermoFisher Scientific, USA). In preparation for the SEC analysis, samples were filtered (Minisart RC4 0.2 μm syringe filter, Sartorius, Germany) and, if necessary, diluted with SEC buffer. The SEC buffer with a pH of 6.6 comprised of 100 mM Na$_2$SO$_4$, 5 mM NaH$_2$PO$_4$ and 50 mM Na$_2$HPO$_4$ (chemicals supplied by Carl Roth, Germany). The samples were loaded onto a gel column (Yarra 3 μm SEC-3000, Phenomenex, USA) at a flow rate of 1 mL/min. A standard stock solution was used to determine the calibration curve. The concentration of mAb in the samples was calculated by linear regression of the corresponding mAb peak areas at 280 nm. In order to ensure that mAb peaks are not interfered by impurities of the same size, SEC quantification was previously verified by analytical protein A HPLC (data not shown).

Product recovery was calculated taking into account the volume of biomass in the process volume as already described for other HCD processes [26]. Therefore, the volume of biomass was subtracted from the corresponding total volume where the density of the biomass ($\rho_{BM}$), mainly cells, was assumed to be similar to that of the liquid. The product containing liquid volume ($V$) was defined as

$$V = V_{total} - V_{total} \times \frac{WCW}{\rho_{BM}} \qquad (1)$$

Furthermore, the supplied total mAb mass of the feed was calculated after the clarification by sum up the mAb amount in waste and harvest pool. A direct determination of the mAb amount in the feed at process start was not satisfactory for the calculation of the recovery due to the observation that mAb concentrations in the HCD broth continued to increase within the (short) clarification process resulting in over-determined recoveries of $> 100$ %. Therefore, the mAb *recovery* was calculated according to

$$recovery = \frac{V(H) \times c(H)}{V(W) \times c(W) + V(H) \times c(H)} \times 100 \qquad (2)$$

where $c(H)$ is the mAb concentration in the harvest volume $V(H)$ and $c(W)$ is the mAb concentration in the waste volume $V(W)$.

The concentration of host cell proteins (HCP) was determined in HCD supernatant, FBC harvest and FBC waste pool as an indication for cell damage. Measurement of HCP concentration was performed in triplicates using a HCP-ELISA kit (CYG-F550, Cygnus Technologies, USA). Samples were diluted in ELISA buffer (20 mM TRIS, 50 mM NaCl, all chemicals purchased from Carl Roth, Germany) to reach the detection range of 1–100 ng/mL according to the manufacturer instructions. Absorption values were measured by a plate reader (infinite® 200, Tecan, Switzerland).

All indicated deviations of the presented results refer to their standard deviations for multiple measurements.

## 3. Results and discussion

### 3.1. Biomass removal by applying FBC

#### 3.1.1. Examination and optimization of flow rates

To achieve a high clarification throughput, loading and washing flow rates were investigated to determine the highest feasible flow rates where cells are retained in the FBC chamber. In each experiment, one flow rate was varied while the other was kept constant at 100 mL/min/Ch. The chambers were loaded with approximately $10 \times 10^9$ cells/Ch in all trials.

The tested loading flow rates showed a constant TCC in the harvest pool below $0.5 \times 10^6$ cells/mL, indicating an almost complete cell retention during chamber loading (Fig. 3). Therefore, the maximum loading flow rate of 300 mL/min/Ch was applied in all following experiments to achieve fast clarification without cell breakthrough.

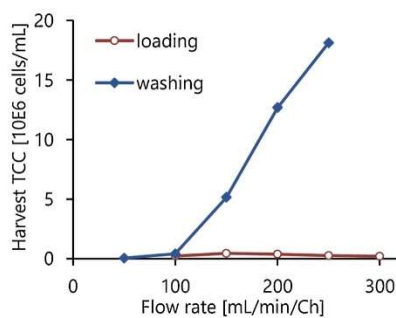However, the subsequent washing of the fluidized bed with wash

flow rates higher than 100 mL/min/Ch showed a steep and almost linear increase in TCC of the harvest pool (Fig. 3). A reason for the washing out of the cells from the chambers was most likely a disruption of the fluidized bed during the entry of wash buffer into the chambers, induced by differences in liquid density between wash buffer (1.005 kg/L) and supernatant (1.007–1.011 kg/L). The observed density variation of the HCD supernatant was probably caused by slight batch to batch variations in the supernatant composition, such as different protein, glucose and lactate concentrations. Since an adjustment of the wash buffer density to the supernatant density is not practicable for each clarification, a wash flow rate of 100 mL/min/Ch was applied in all further trials to enable robust cell retention with hardly any cell breakthrough into the harvest pool.

#### 3.1.2. Cell loading capacity of FBC chambers

As an indicator for the loading of the chambers with cells, the filling level of the fluidized bed was visually monitored during the entire FBC cycle. It was observed that a filling level of approximately 90 % at the end of the loading step results in an almost cell-free FBC harvest (Fig. 4).

For a more precise determination of the filling level and for an enhanced utilization of the FBC chambers, the maximum number of cells retained per chamber, also termed maximum chamber capacity ($C_{max}$), was examined. Therefore, loading test were performed by loading a chamber in a centrifugation cycle with a known number of cells using different HCD broth volumes with a TCC of $79 \times 10^6$ cells/mL. The cell breakthrough as an indication of chamber overloading was assessed by measuring turbidity and TCC in the harvest pool after each tested cell load.

As shown in Fig. 5, harvest turbidities below 100 NTU with hardly any cell breakthrough were achieved for chamber loads up to $9.5 \times 10^9$ cells/Ch. At a loading of $10.4 \times 10^9$ cells/Ch, a strong increase of turbidity to 340 NTU and also of TCC in the harvest pool was observed (Fig. 5), indicating a chamber overloading by exceeding $C_{max}$. Therefore, a $C_{max}$ of $10 \times 10^9$ cells/Ch was determined for the HCD clarification, which is consistent with the previously published capacities for the clarification of moderate CHO concentrations of $5 \times 10^6$ cells/mL [25].

A calculation approach was chosen for the volume of cell broth that can be clarified per chamber and cycle (CV) without exceeding $C_{max}$. The total number of retained cells depends on g-force, flow rate, cell volume as well as media density and viscosity [22]. If these conditions are kept constant, the CV only depends on the maximum chamber capacity ($C_{max}$) and the TCC of the feed as shown in Eq. (3):



**Fig. 3.** Total cell concentration (TCC) of harvest pool as a function of centrifuge loading and washing flow rates. Each data point indicates a separate experiment of a completed centrifugation cycle using four chambers (Ch) and a loading of approximately 10 × 10E9 cells/Ch using the same high cell density broth as feed.
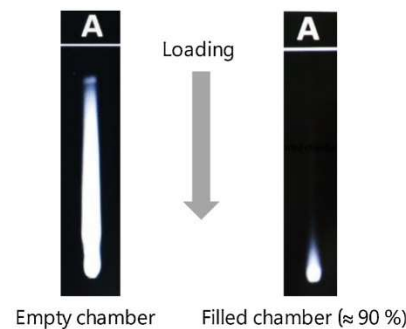


**Fig. 4.** Progress of chamber loading as displayed by the kSep® 400 centrifuge system, where the biomass, mainly consisting of CHO cells, are retained in a fluidized bed. A chamber utilization of approximately 90 % is used as an indicator for the maximum chamber capacity where hardly any cells were observed in the harvest pool.
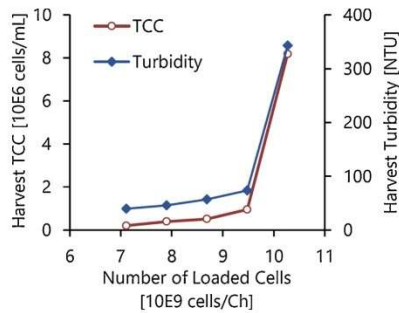
4

**Fig. 5.** Total cell concentration (TCC) and turbidity of the harvest pool as a function of chamber load with CHO cells. Each data point represents a centrifugation trial using the same cell broth as feed.

$$CV = \frac{C_{max}}{TCC} \qquad (3)$$

In all further studies, CV was calculated on the basis of the fixed $C_{max}$ of $10 \times 10^9$ cells/Ch for different TCC according to Eq. (3).

### 3.1.3. Evaluation of flow rates and chamber loading

In order to evaluate the determined flow rates of 300 mL/min/Ch for loading and 100 mL/min/Ch for washing as well as the determined $C_{max}$ of $10 \times 10^9$ cells/Ch, the removal of biomass was examined. Therefore, 2 L HCD broth containing a TCC of $109.7 \times 10^6$ cells/mL with a viability of 93.6 % was clarified.

The cell broth was processed within 18 min in six centrifugation cycles using four FBC chambers. During clarification, the total biomass amount of 808 g in the feed was reduced to 69 g in the FBC harvest pool, as also shown by a low FBC harvest turbidity of 158 NTU (Fig. 6). This high biomass reduction of 92 % showed that the optimized parameters were sufficient to achieve a separation of the major part of the biomass in the FBC harvest pool.

### 3.1.4. Characterization of mAb throughput

To examine the mAb throughput of the FBC, two experiments were performed using different HCD broths with a TCC of $100 \times 10^6$ cells/mL and mAb concentrations of 1.4 g/L and 2.7 g/L. The total throughput of

mAb at the FBC harvest outlet was determined by harvesting the complete outlet stream during chamber loading (IDV = 0 mL/Ch) and by using a high wash volume (WV) of 350 mL/Ch. In addition, the FBC outlet stream was collected in 40 mL fractions and mAb concentrations were measured to optimize FBC parameters of IDV and WV.

As shown in Fig. 7 (A) similar concentration trends were observed in both trials. During the loading of the chambers, hardly any mAb was detected in the harvest outlet. In the subsequent washing step the mAb concentrations increased strongly. The maxima were achieved with a total harvest volume of approximately 0.65 L. During continued washing, the concentrations slowly decreased until, at total harvest volumes of approximately 1.6 L, hardly any mAb was detected in the outlet stream.

This characteristic mAb throughput can be explained as follows: Chamber loading with feed displaces the buffer solution of the pre-filled chambers and the tubing, resulting in a delay of mAb concentration in the harvest outlet. After the chambers are fully loaded, the fluidized bed is washed until the product-containing medium is completely washed out.

Based on the observation that at the beginning and end of the FBC cycle there was hardly any product in the harvest outlet stream, the parameters IDV and WV were selected to ensure a high mAb recovery in the harvest fraction with minimal product dilution. Therefore, the ratio of mAb reaching the harvest pool during the centrifugation cycle was calculated by cumulating mAb masses of the fractions divided by the total mAb mass processed during the complete cycle, which is shown in Fig. 7 (B). An IDV of 100 mL/Ch was determined to discard the pre-filled buffer to avoid dilution of the harvest pool. After 260 mL buffer were subsequently flushed through each chamber, approximately 95 % of the processed mAb reached the harvest pool. Further washing of the fluidized bed would result in a high dilution of the harvest due to the strong flattening of the cumulative mAb curve. Therefore, a WV of 260 mL/Ch was applied in all further trials to achieve mAb recoveries of approximately 95 %.

The similar mAb throughput curves for both tested concentrations additionally suggest that the throughput is independent of the mAb concentration in the feed and thus promise the applicability of the clarification parameters also for other processes such as concentrated fed-batches.



**HCD Broth**

808 g Biomass; 7473 NTU

**FBC Harvest**

69 g Biomass; 158 NTU

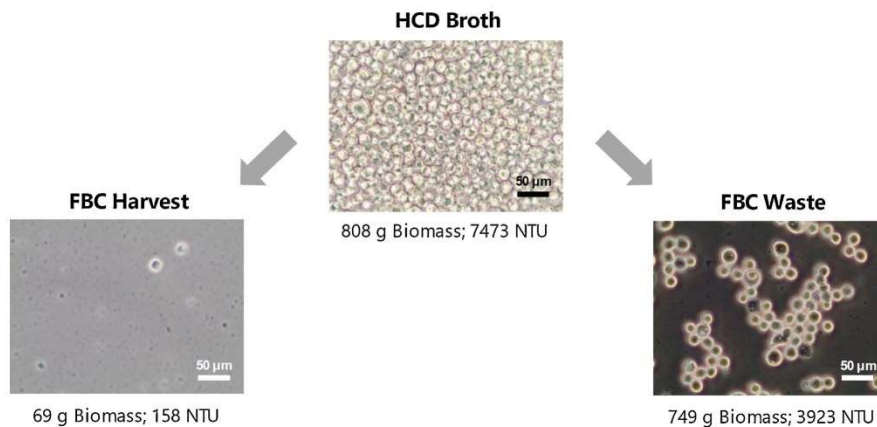**FBC Waste**

749 g Biomass; 3923 NTU

**Fig. 6.** Total biomass and turbidity of unclarified high cell desity broth and the obtained fluidized bed centrifuge (FBC) clarified harvest pool containing low biomass as well as the FBC waste pool containing the bulk of the biomass.
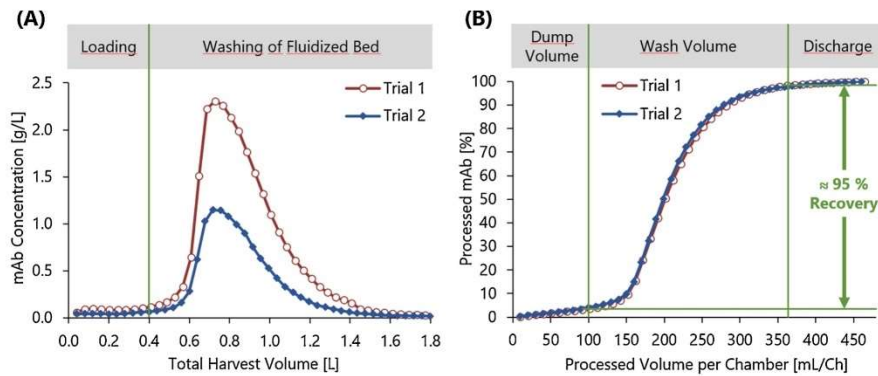
5

**Fig. 7.** Monoclonal antibody (mAb) throughput curves of the fluidized bed centrifuge harvest stream using four chambers to clarify high cell density (HCD) suspensions with two different mAb concentrations of 2.7 g/L (trial 1) and 1.4 g/L (trial 2). In both trials 0.4 L HCD culture volume was loaded (100 mL/Ch) with a flow rate of 100 mL/min/Ch before the fluidized bed was washed with 1.4 L buffer (350 mL/Ch). (A) mAb concentrations in the processed volume which passed the harvest line. (B) Normalized cumulative amount of mAb plotted against the processed volume per chamber. The vertical lines (green) enclose the volume fraction, which contains ≈ 95 % of the recovered mAb (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

### 3.2. Validation and application of FBC clarification

The optimized HCD clarification parameters for the investigated FBC system (Table 1) were validated by three independent clarification runs. In all trials, 2 L broth was processed in less than 30 min with an average HCD broth throughput of 6.0 ± 0.1 L/h. The initial feed turbidities of almost 8000 NTU were reduced to 170 ± 28 NTU in the harvest suggesting a successful biomass removal (Table 2). Due to the washing (Fig. 7) harvest volumes of approximately 5.2 L were obtained. The impact of this product dilution has to be evaluated for subsequent DSP steps. However, as calculated in Section 3.2, high mAb recoveries of 95 ± 2 % were obtained, enabled by the optimized washout of product from the fluidized bed (Table 2). The observed small standard deviation of the recovery indicates a high robustness of the process.

Applying the optimized parameters, a FBC process scenario for HCD clarification could be for example the clarification of a 10 L HCD batch with 100 million cells/mL within 100 min. However, it should be considered that significantly higher throughputs can be achieved at lower cell concentrations, since a larger volume per cycle can be clarified. In addition, increased throughputs for a fast clarification of industrial production processes, like the clarification of 2 m³ SU bioreactors, can also be reached with larger FBC systems [25].

It should also be noted that the primary clarification of HCD broth with 100 million cells/mL by depth filtration using the procedure described in Section 2.3 resulted in an immediate filter blocking and thus in very low filter capacities (data not shown). This indicates a very limited applicability of filtration due to the high biomass content of HCD broths and emphasizes the high potential of the FBC approach.

**Table 1**
Overview of optimized fluidized bed centrifuge parameters for the clarification of 100 × 10E6 CHO cells/mL.

| Centrifuge Parameter | Value |
|---|---|
| Load Flow Rate | 300 mL/min/Ch |
| Wash Flow Rate | 100 mL/min/Ch |
| Loading Capacity | 10 × 10E9 cells/Ch |
| Initial Dump Volume | 100 mL/Ch |
| Wash Volume | 260 mL/Ch |

**Table 2**
Validation results of the optimized clarification parameters for high cell density (HCD) processes using a fluidized bed centrifuge (FBC). Different HCD broths, characterized by their turbidity, concentration of viable cells (VCC), and viability, were used as FBC feed. Turbidity and mAb recovery of the FBC harvest were determined as an indication of FBC performance.

| HCD Broth | | | FBC Harvest | |
|---|---|---|---|---|
| HCD Broth Turbidity [NTU] | VCC [10E6 cells/mL] | Viability [%] | Harvest Turbidity [NTU] | mAb Recovery of FBC Step [%] |
| 7950 | 100.8 | 93.8 | 151 | 97.0 |
| 7473 | 102.7 | 93.6 | 158 | 95.8 |
| 7955 | 97.1 | 94.0 | 202 | 93.2 |

### 3.3. Impact of FBC on cells and harvest quality

To investigate potential damage of cells during the FBC clarification process, the viability and the amount of cells were analyzed before and after centrifugation. It was observed that a high cell viability of 89 ± 3 % was maintained after the centrifugal separation. Compared to the initial viability, only a slight decrease of approximately 5 % was detected. Furthermore, a total cell amount of 93 ± 8 % in waste and harvest pool was found. This high recovery of cells was probably achieved due to low shear stress for the cells during FBC separation, which was also predicted by a published simulation of shear rates for cells in the fluidized bed using the same chamber type as in the FBC system investigated here [27].

However, the flow of cell broth through the branched tubing set, the extensive pumping and the change from cell culture media to phosphate buffer during cell load and wash were not considered in the simulation. These influences of the clarification process may cause cell damage as well as a slight cell loss due to lysis, resulting in a release of impurities such as HCP, thus impairing the quality of the harvest [16].

Therefore, HCP concentrations and total HCP amounts were determined as a key attribute of the harvest quality. An initial HCP concentration of 400 ± 75 mg/L was measured in the unclarified HCD broth. After the clarification process, a slightly increased total HCP amount of 105 ± 11 % in harvest and waste pool was observed, suggesting no significant release of host cell components into the process medium. The harvest pool accounted for 85 ± 12 % of this HCP amount, which was in

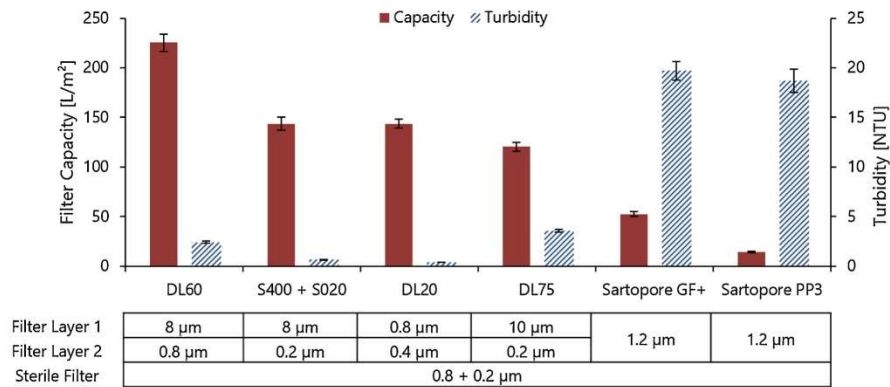| | DL60 | S400 + S020 | DL20 | DL75 | Sartopore GF+ | Sartopore PP3 |
|---|---|---|---|---|---|---|
| Filter Layer 1 | 8 μm | 8 μm | 0.8 μm | 10 μm | 1.2 μm | 1.2 μm |
| Filter Layer 2 | 0.8 μm | 0.2 μm | 0.4 μm | 0.2 μm | | |
| Sterile Filter | 0.8 + 0.2 μm | | | | | |

**Fig. 8.** Filter capacities and filtrate turbidities of different depth and membrane filter combinations after filtration of a pre-clarified high cell density broth from a fluidized bed centrifuge (FBC). Standard deviations result from duplicate trials using the same FBC harvest pool with an initial turbidity of 147 NTU for all trials.

a similar range as the mAb recovery.

These results proved mild process conditions by using the optimized FBC parameters as well as no negative impact of the HCD clarification approach on the quality of the harvest.

### 3.4. Post-centrifugal filtration

In order to remove residual biomass of pre-clarified HCD broth after FBC processing, a screening of different two-stage filter setups was performed. As a first filter stage, various membrane and depth filters were applied with the aim of removing small particle amounts of residual cells and cell fragments. As a second filter stage a sterile filter with a 5.6 times smaller filter area was used in all setups to ensure the removal of all particles and contaminants > 0.2 μm.

Fig. 8 shows that both tested 1.2 μm membrane filters achieved the lowest filter capacities and the lowest turbidity reductions to approximately 20 NTU. All four tested depth filter setups achieved filtrate turbidities below 5 NTU, indicating significant particle reduction and efficient clarification by depth filtration. The setup comprising of a DL60 depth filter followed by sterile filter showed the highest filter capacity of 225 L/m². The high filter capacity of the DL60 can be explained by the average retention rates of 8 μm for the first and 0.8 μm for the second filter layer, which are apparently highly efficient for the retention of small cells and cell fragments from the FBC harvest pool (Fig. 6).

Therefore, the combination of DL60 depth filter with sterile filter was selected for further evaluation using four different FBC harvests as filtration feeds (Table 3). Average depth filter capacities of 157 ± 45 L/m² were achieved with this setup. Significant variations in filter capacity were probably caused by differences in the initial HCD broths where slight batch to batch deviations in viability and WCW resulted in turbidity variations of 121–158 NTU in the FBC harvest. However, low final turbidities < 5 NTU of the sterile filtrates showed that the selected setup is suitable for post-centrifugal filtration. High mAb recoveries of ≥ 96 %

were determined in all filtration trials. Therefore, total mAb recoveries of > 90 % for the combination of FBC with subsequent filtration were achieved, suggesting a robust and suitable clarification approach for HCD broths.

### 4. Conclusion

In this study, a SU based clarification approach for intensified mAb production processes with 100 million cells/mL using a FBC was developed with particular focus on the product recovery. The HCD clarification process was characterized and a robust removal of biomass from HCD cultures was demonstrated. Based on the results, a maximum chamber loading capacity of $10 \times 10^9$ cells/Ch was determined and the FBC parameters of flow rates for the loading and washing step of the clarification cycle were optimized. Washing the separated cells inside the centrifuge chamber was identified as an advantageous process step of FBC clarification in order to flush out entrapped supernatant with residual product from the fluidized bed. Therefore, dump and washing volumes were adapted to decrease dilution of harvest and increase mAb recovery.

The validation of the determined FBC parameters in experiments on a 2 L scale showed an almost complete removal of biomass from HCD suspensions and high mAb recoveries of 95 ± 2 %. Furthermore, it was shown that the mild process conditions of the optimized process parameters maintain a great viability of cells, resulting in hardly any release of HCP impurities and thus in a high quality of the harvest.

Additionally, a filter screening study for a post-centrifugal clarification was performed. A filter combination consisting of a depth filter followed by a sterilizing grade filter with a high filter capacity of 157 ± 45 L/m² was selected. The combination of the two successive clarification steps, fluidized bed centrifugation and post-centrifugal filtration, was capable of reducing the turbidity of HCD broths from almost 8000 NTU to < 5 NTU in the sterile filtrate, achieving a total mAb recovery of > 90 %. This excellent HCD clarification performance has not been described with any other SU process so far.

In summary, the developed HCD clarification approach presented in this article is able to efficiently remove cell concentrations of 100 million cells/mL with a high recovery of mAb, offering high potential to be used as a platform approach for a broad range of intensified biopharmaceutical manufacturing processes.

**CRediT authorship contribution statement**

**Martin Saballus:** Conceptualization, Investigation, Writing -

**Table 3**
Filtration results of different fluidized bed centrifuge (FBC) harvests using a filter setup consisting of a DL60 depth filter followed by sterile filter stage.

| Filtration Trial | FBC Harvest Turbidity [NTU] | Filtrate Turbidity [NTU] | Filter Capacity [L/m²] | mAb Recovery of Filtration Step [%] |
|---|---|---|---|---|
| 1 | 121 | 1.89 | 223 | 98 |
| 2 | 132 | 2.44 | 142 | 97 |
| 3 | 151 | 3.09 | 130 | 96 |
| 4 | 158 | 2.27 | 131 | 98 |

7

original draft, Visualization. **Lucy Nisser:** Investigation, Writing - review & editing. **Markus Kampmann:** Validation, Writing - review & editing. **Gerhard Greller:** Supervision.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Appendix A. Supplementary data**

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2020.107887.

**References**

[1] P. Mayrhofer, R. Kunert, Nomenclature of humanized mAbs: early concepts, current challenges and future perspectives, Hum. Antibodies 27 (2019) 37–51, https://doi.org/10.3233/HAB-180347.

[2] D. Pollard, M. Brower, Y. Abe, A. Lopes, A. Sinclair, Standardized economic cost modeling for next-generation mAb production, Bioprocess Int. 14 (8) (2016) 14–23.

[3] S. Klutz, L. Holtmann, M. Lobedann, G. Schembecker, Cost evaluation of antibody production processes in different operation modes, Chem. Eng. Sci. 141 (2016) 63–74, https://doi.org/10.1016/j.ces.2015.10.029.

[4] B. Kiss, U. Gottschalk, M. Pohlscheidt (Eds.), New Bioprocessing Strategies: Development and Manufacturing of Recombinant Antibodies and Proteins, Springer, Cham, Switzerland, 2018.

[5] Y. Takagi, T. Kikuchi, R. Wada, T. Omasa, The enhancement of antibody concentration and achievement of high cell density CHO cell cultivation by adding nucleoside, Cytotechnology 69 (2017) 511–521, https://doi.org/10.1007/s10616-017-0066-7.

[6] BioPhorum Operations Group Ltd, Biomanufacturing Technology Roadmap: Process Technologies, 2017 (accessed 24 July 2020), https://www.biophorum.com/wp-content/uploads/bp_downloads/ProcessTechs.pdf.

[7] B. Boedeker, A. Goldstein, E. Mahajan, Fully disposable manufacturing concepts for clinical and commercial manufacturing and ballroom concepts, Adv. Biochem. Eng. Biotechnol. 165 (2018) 179–210, https://doi.org/10.1007/10_2017_19.

[8] W.C. Yang, D.F. Minkler, R. Kshirsagar, T. Ryll, Y.-M. Huang, Concentrated fed-batch cell culture increases manufacturing capacity without additional volumetric capacity, J. Biotechnol. 217 (2016) 1–11, https://doi.org/10.1016/j.jbiotec.2015.10.009.

[9] N. Singh, A. Arunkumar, S. Chollangi, Z.G. Tan, M. Borys, Z.J. Li, Clarification technologies for monoclonal antibody manufacturing processes: current state and future perspectives, Biotechnol. Bioeng. 113 (2016) 698–716, https://doi.org/10.1002/bit.25810.

[10] M. Iammarino, J. Nti-Gyabaah, M. Chandler, D. Roush, K. Göklen, Impact of cell density and viability on primary clarification of mammalian cell broth: an analysis using disc-stack centrifugation and charged depth filtration, Bioprocess Int. (2007) 38–50.

[11] A.A. Shukla, B. Hubbard, T. Tressel, S. Guhan, D. Low, Downstream processing of monoclonal antibodies-application of platform approaches, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 848 (2007) 28–39, https://doi.org/10.1016/j.jchromb.2006.09.026.

[12] M. Brower, Y. Hou, D. Pollard, Monoclonal antibody continuous processing enabled by single use, in: G. Subramanian (Ed.), Continuous Processing in Pharmaceutical Manufacturing, Wiley-VCH, Weinheim, 2014, pp. 255–296, https://doi.org/10.1002/9783527673681.ch11.

[13] J.P. Pieracci, J.W. Armando, M. Westoby, J. Thommes, Industry review of cell separation and product harvesting methods, in: G. Jagschies, E. Lindskog, K. Łącki, P. Galliher (Eds.), Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes, Elsevier, Amsterdam, 2018, pp. 165–206, https://doi.org/10.1016/B978-0-08-100623-8.00009-8.

[14] N. Singh, K. Pizzelli, J.K. Romero, J. Chrostowski, G. Evangelist, J. Hamzik, N. Soice, K.S. Cheng, Clarification of recombinant proteins from high cell density mammalian cell culture systems using new improved depth filters, Biotechnol. Bioeng. 110 (2013) 1964–1972, https://doi.org/10.1002/bit.24848.

[15] D.J. Roush, Y. Lu, Advances in primary recovery: centrifugation and membrane technology, Biotechnol. Prog. 24 (2008) 488–495, https://doi.org/10.1021/bp070414x.

[16] P. Gronemeyer, R. Ditz, J. Strube, Trends in upstream and downstream process development for antibody manufacturing, Bioeng. Basel (2014) 188–212, https://doi.org/10.3390/bioengineering1040188.

[17] J. Hummel, M. Pagkaliwangan, X. Gjoka, T. Davidovits, R. Stock, T. Ransohoff, R. Gantier, M. Schofield, Modeling the downstream processing of monoclonal antibodies reveals cost advantages for continuous methods for a broad range of manufacturing scales, Biotechnol. J. 14 (2019) https://doi.org/10.1002/biot.201700665 e1700665.

[18] S.R. Hadpe, A.K. Sharma, V.V. Mohite, A.S. Rathore, ATF for cell culture harvest clarification: mechanistic modelling and comparison with TFF, J. Chem. Technol. Biotechnol. 92 (2017) 732–740, https://doi.org/10.1002/jctb.5165.

[19] T. Kruse, A. Schmidt, M. Kampmann, J. Strube, Integrated clarification and purification of monoclonal antibodies by membrane based separation of aqueous two-phase systems, Antibodies (Basel) 8 (2019), https://doi.org/10.3390/antib8030040.

[20] R. Eibl, Gd. Abreu (Eds.), Disposable Bioreactors, Springer, Heidelberg, 2009.

[21] S. Mehta, Automated single-use centrifugation solution for diverse biomanufacturing processes, in: G. Subramanian (Ed.), Continuous Processing in Pharmaceutical Manufacturing, Wiley-VCH, Weinheim, 2014, pp. 385–400, https://doi.org/10.1002/9783527673681.ch15.

[22] G. Subramanian (Ed.), Continuous Processing in Pharmaceutical Manufacturing, Wiley-VCH, Weinheim, 2015.

[23] R.J. Sanderson, K.E. Bird, Cell separations by counterflow centrifugation. Methods in Cell Biology 15, Elsevier, Academic Press, Amsterdam, 1977, pp. 1–14.

[24] R. Turner, A. Joseph, N. Titchener-Hooker, J. Bender, Manufacturing of proteins and antibodies: chapter downstream processing technologies, in: T. Scheper (Ed.), Advances in Biochemical Engineering/Biotechnology 165, Springer, Heidelberg, 2017.

[25] H.-F. Ko, R. Bhatia, Evaluation of single-use fluidized bed centrifuge system for mammalian cell harvesting, BioPharm Int. 25 (2012) 34–40.

[26] T. McNerney, A. Thomas, A. Senczuk, K. Petty, X. Zhao, R. Piper, J. Carvalho, M. Hammond, S. Sawant, J. Bussiere, PDADMAC flocculation of Chinese hamster ovary cells: enabling a centrifuge-less harvest process for monoclonal antibodies, MAbs 7 (2015) 413–428, https://doi.org/10.1080/19420862.2015.1007824.

[27] W. Kelly, J. Rubin, J. Scully, H. Kamaraju, P. Wnukowski, R. Bhatia, Understanding and modeling retention of mammalian cells in fluidized bed centrifuges, Biotechnol. Prog. 32 (2016) 1520–1530, https://doi.org/10.1002/btpr.2365.

## 4.2 Validation and Modeling of Clarification for Various Cell Suspensions

As demonstrated in the previous section, fluidized bed centrifugation enables the clarification of HCD broth with 100 million cells/mL. However, to use the FBC setup as a platform approach for cell clarification, its robustness and adaptability for a wide variety of USP modalities must be ensured. Indications for robustness of the clarification UO are the overall mAb recovery as well as the harvest turbidity. Furthermore, important parameters to predict process performance are the average cell broth throughput and the harvest dilution rate. These parameters enable the calculation of process time and wash buffer consumption and thus enable adaption of the UO to different process volumes. Furthermore, these parameters are also an indication of the process efficiency.

In order to investigate the impact of different cell broth characteristics, suspensions with cell concentrations ranging from $20 \times 10^6$ cells/mL to $110 \times 10^6$ cells/mL and viabilities variating from 60 % to 97 % were clarified. The optimized FBC parameters of the previous study were used as a basis and adapted to the cell concentration. It was found that the maximum FBC loading capacity of approximately $10^{10}$ CHO cells per 100 mL chamber volume is independent of the cell concentration in the feed. Therefore, the maximum cell broth volume loaded into a FBC chamber mainly depends on cell concentration, indicated by lower load volumes with increasing cell concentrations. Consequently, higher cell concentrations increase the number of FBC cycles to clarify a specific volume and thus also increase the process time and buffer consumption. This effect on the clarification process was modeled using the adapted process parameters for calculation. It was shown that the experimental results correspond to the calculated throughput and dilution rates for all tested cell concentrations. The model is therefore suitable to predict process times and buffer consumption for various process scenarios.

After completing the clarification UO by the subsequent filtration step, overall high mAb recoveries of approximately 95 % were achieved. Furthermore, the initial cell broth turbidities, which ranged from 2,300 NTU to 8,000 NTU, were reduced to below 13 NTU in all sterile harvest pools, also confirming consistent clarification results.

Overall, this study was the first to successfully demonstrate the capability of a clarification approach over a wide range of cell concentrations. Despite a lower throughput and higher buffer consumption at higher cell concentrations, the FBC approach is particularly suitable for high cell densities clarification due to the consistently high recovery rates, which have not been reported from any other approach to date. As shown in the industrial review where the results were published, process intensification in biomanufacturing requires multiple efforts and holistic approaches. In this context, the developed robust clarification UO promises to be an efficient platform approach suitable for connecting intensified upstream processes with intensified downstream processes.

# Process intensification in the biopharma industry: Improving efficiency of protein manufacturing processes from development to production scale using synergistic approaches

Dirk Müller [a,*], Lukas Klein [a], Johannes Lemke [b], Markus Schulze [b], Thomas Kruse [b], Martin Saballus [b], Jens Matuszczyk [b,c], Markus Kampmann [b], Gerben Zijlstra [b]

[a] Sartorius Stedim Cellca GmbH, Marie-Goeppert-Mayer-Str. 9, d-89081 Ulm, Germany
[b] Corporate Research, Sartorius Stedim Biotech GmbH, August-Spindler-Str. 11, d-37079 Göttingen, Germany
[c] Advanced Therapies, Sartorius Stedim Biotech GmbH, August-Spindler-Str. 11, d-37079 Göttingen, Germany

## ARTICLE INFO

## ABSTRACT

Process intensification strives for more efficient conversion of raw materials into products while minimizing resource usage. In the biopharma industry, typical intensification gains include increased plant capacities, reduced raw material costs, smaller facilities and improved sustainability.

Rapid growth combined with increasingly diverse and challenging molecule formats in the biopharma sector necessitate strategies for fast development of highly productive and cost-efficient processes. Using monoclonal antibody production in CHO cells as example, we present intensification techniques and process sequences that deliver synergistic benefits like increasing space-time yields up to 10-fold, shortening production runs by 30%, or saving numerous days in cell expansion. Combining highly productive perfusion with continuous downstream purification promises improved yields and production economics, especially for advanced, often labile molecules.

Predictive scale-down models demonstrated here for selecting intensification-ready cell lines and for rapid setup of intensified processes are essential to realize such benefits broadly in the industry. They enable testing and quick tailoring of intensification alternatives like high-inoculation fed-batch or perfusion to suit molecule needs, facility space, and financial conditions. Since many techniques also readily extend to other protein biotherapeutics, intensification can be regarded as key pillar enabling fast, cost-efficient development and production of biopharma products in the coming years.

## 1. Introduction

Process intensification is a key market trend in biomanufacturing and a logical next step in the evolution towards increased process efficiency and lower cost of goods (COGs). Recent market reports show that cost pressures on the biopharma industry are mounting [1,2] because average peak sales of biologicals have halved, while development costs have increased by 30% since 2010 [3]. This has led to the establishment of manufacturing platform technologies that allow for fast product development with reduced efforts and enable manufacturing at low cost of goods [4]. Furthermore, the number of low volume products is increasing, due to the advent of more potent molecules and smaller target patient groups [3]. Interestingly, at the same time the ultimate

market demands are more unpredictable than before, in part due to increased competition of pipelines with multiple molecules for the same indication as for biosimilars [2,5]. This has resulted in the establishment of flexible, multi-product manufacturing facilities with Single Use (SU) technologies for easy, low risk product change-over. Finally, besides the traditional monoclonal antibodies, there is a growing percentage of more complicated protein modalities, such as Bi-specifics or Fc-fusion proteins, which may not, or not efficiently, be produced using traditional fed-batch (FB) technology. The latter has led to the renewed interest in perfusion technologies [5].

Interestingly, perfusion technology also allows to intensify mammalian cell culture profoundly [6,7] and can be used to shorten process times in the main bioreactor for more flexibility in the plant, and/or increase the bioreactor titer for higher facility output, and/or

**Notation & Abbreviations**

| | | | |
|---|---|---|---|
| | | FB | fed-batch |
| | | FBC | fluidized bed centrifuge |
| ATF | alternating tangential flow filtration | HCP | host cell protein |
| BPOG | biophorum operations group | HIFB | high-inoculation fed-batch |
| CCF | cell culture fluid | IVCC | integral of viable cell concentration |
| CHO | chinese hamster ovary cells | MCC | multi column chromatography |
| CIP | cleaning in place | N-1 Perfusion | perfusion process for cell expansion at the last seed |
| CSPR | cell-specific perfusion rate | | train step(N-1) before the main production bioreactor |
| COGs | cost of goods | | (stage N) |
| ChVar | charge variants of a biomolecule | PR | perfusion rate |
| CV | column volumes | SEC | size exclusion chromatography |
| DO | dissolved oxygen tension | STY | space-time yield |
| DoE | design of experiments | SU | single use |
| DNA | desoxyribonucleic acid | USP | upstream process |
| DSP | downstream process | TCC | total cell concentrations |
| EMA | European medicines agency | VCC | viable cell concentration |
| FDA | U.S. Food and drug administration | VVD | vessel volume changes per day |
| HCD | high cell density | | |
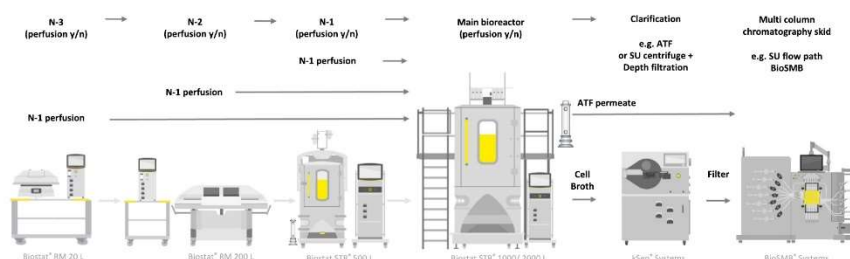


**Fig. 1.** Overview of a representative bioproduction process workflow featuring different elements of process intensification.

reduce the equipment and facility footprint. More interestingly process intensification can also lead to lower COGs [8,9].

Different from the past, process intensification is nowadays seen as standard tool in establishing biotherapeutic cell culture processes right from the start, also for advanced therapeutic application areas ranging from viral vector applications to DNA vaccines or exosomes [10–12]. Despite the wide range of applications, these processes generally share similar intensification approaches for different unit operations, e.g., cell retention methods for generating and maintaining high cell numbers or for enabling continuous downstream processing and several excellent articles and reviews on the topic exist [13–18]. For this reason, the present article will focus on showcasing concrete implementation examples and development tools using intensified processes for monoclonal antibody production, but many of the applications discussed can also be applied to other molecule types with appropriate modifications.

Major recent upstream process intensification successes underscoring this upstream process intensification trend have been published by, amongst others DSM Biologics using XD® Technology, a concentrated FB approach, where very high cell densities (> $200 \cdot 10^6$ cells·mL$^{-1}$) were obtained while still retaining high cell viability at the end of the batch, resulting in monoclonal antibody titers of 27 g·L$^{-1}$ [19].

Another example featuring N-1 perfusion and high inoculation FB (HIFB) was published by Biogen Idec researchers, who used the high cell density seed culture ($40 \cdot 10^6$ cells·mL$^{-1}$) to inoculate the production bioreactor at a much higher concentration ($10 \cdot 10^6$ cells·mL$^{-1}$). They showed that with two CHO cell lines 5 g·L$^{-1}$ product titers were

achieved in 12 rather than 17 days at good product quality [20]. A group at Merck was even able to reduce the culture duration to 8 days [21].

More recently (2019) WuXi announced that their WuXi-Up technology achieved a breakthrough in cell culture productivity for an Fc-fusion protein, achieving productivity equating to 51 g·L$^{-1}$ over a 20-day perfusion process [22].

Latest reports from Bristol-Myers Squibb show that with HIFB, not only a reduction of the process duration whilst reaching similar titers can be achieved, but that it is also feasible to keep process duration equal while doubling titers [23]. The changes in the subsequent harvest, i. e. higher titer, potentially more biomass and increased host cell impurities that come along with these achievements have then to be handled by the subsequent downstream processing. Ideally, this happens also in a continuous fashion to further reduce COG in GMP biomanufacturing [24].

In view of this industry trend towards process intensification, the BioPhorum Operations Group (BPOG) has published a joint process technology roadmap describing common biomanufacturer needs for different biotherapeutic application areas with the purpose to share them openly with supply partners, academics, regulators and government agencies so that directions can be aligned and collaboration enabled [20–22,25–27].

Key elements of the roadmap are process intensification (Fig. 1), including the establishment of high-performing (perfusion enabled) cell lines, high-density cell banks in e.g. cryobags to reduce seed train length and allow fully closed operation, seed train intensification, and different

2

intensification strategies for the main bioreactors, such as: N-1 perfusion followed by HIFB, concentrated FB, dynamic Perfusion or N-stage perfusion. Depending on the N-stage operational mode, the product may need to be harvested from the high cell density bioreactor, requiring high cell density compatible clarification technologies such as Ksep® centrifugation or the product is harvested continuously through the retention filter and needs to be continuously captured by e.g. multi column chromatography such as BioSMB. Such fully integrated continuous solutions have garnered increased interest in recent years in biopharma with the aim to reduce capital expenditures, operational cost, and enhance process portability – akin to the drivers for continuous manufacturing in other industries [27–30].

In this paper, we have evaluated the different process intensification approaches as outlined in the BPOG process technologies roadmap [31] in the context of research examples, with commercially available products and technologies, to assess the potential and remaining challenges of these approaches and the feasibility of performing them using the available technologies. In addition, we have assessed the importance of scale-down models for realizing synergistic intensification benefits by enabling reliable selection of production clones suitable for intensification, rapid setup of intensified process variants, and media optimization already at early development stages.

## 2. Material and methods

### 2.1. Upstream: cell culture and bioreactors

Except where indicated otherwise, Sartorius 4Cell XtraCHO SAM was used as seed medium (XtraCHO SAM) for cell passaging and Sartorius 4Cell XtraCHO and for FB and HIFB cultivations. Sartorius 4Cell Xtra-CHO production medium (PM) was used as basal medium of N-stage cultivations along with the corresponding feed media A (FMA) and B (FMB). For perfusion cultivations, a proprietary media formulation was employed.

### 2.1.1. Clone selection in Ambr® 15

For the present study, seven different Cellca CHO DG44 production clones were assayed, five of which expressed IgG1-type molecules and one each expressed an IgG2 and a Fc-fusion protein, respectively. Ambr® 15 vessels (Sartorius, Germany) were inoculated with a density of $2.5 \cdot 10^6$ cells·mL$^{-1}$. Density was calculated for a working volume of 10 mL. pH set-point was set to 7.0 with an upper limit of 7.15. Dissolved oxygen tension (DO) was set to 60% and temperature to 36.8 °C. For DO and pH control, $CO_2$ and $O_2$ were applied to the system and a basic flow of 0.15 mL·min$^{-1}$ air was maintained throughout the whole process. Furthermore, a downward stirring speed of 1200 rpm was applied.

In a daily procedure, viable cell concentration (VCC) counts via a coupled Vi-Cell XR (Beckman Coulter, USA) as well as pH measurements with the internal analysis module were performed automatically by the Ambr® 15 system. Subsequently, the following semi-perfusion approach was conducted on each vessel: after stopping stirring, temperature control and gas flow, vessels were removed from the Ambr® 15 culture stations and centrifuged using dedicated centrifuge inserts (500 × g, 5 min). Supernatant was discarded and the pellet re-suspended with 10 mL fresh perfusion medium. To save the cells from further stress, stirring speed was ramped up step-wise from 700 rpm to 1200 rpm. After ramping, antifoam was added to each vessel. Glucose and lactate measurements were done off-line with an EKF BIOSEN S-Line device (EKF-Diagnostic GmbH, Germany) according to manufacturer's protocol. Additional glucose feeding was performed manually if required.

As the cultures reached their bleeding VCC of $20 \cdot 10^6$ cells·mL$^{-1}$, a calculated amount of cell suspension was removed prior centrifugation automatically by the liquid handler of the Ambr® 15 system.

### 2.1.2. Seed train with N-1 perfusion

For N-1 perfusion seed cultures and following standard or high inoculation fed-batch cultures, a Cellca CHO DG44 cell line expressing an IgG1 molecule was used. After cryo vial thaw from a cell bank, cells were passaged and incubated (36.8 °C, 7.5% $pCO_2$, 85% humidity, 120 rpm, 50 mm diameter) four times every 3 to 4 days in 4Cell XtraCHO SAM media at $0.2 \cdot 10^6$ cells·mL$^{-1}$ in non-baffled shake flasks prior inoculation and onset of N-1 perfusion. The first two passages after thawing contained 15 nM methotrexate hydrate (Sigma, USA).

Perfusion cultivations were performed using 2 to 50 L perfusion bioreactor bags (Flexsafe®, Sartorius, Germany) at their respective maximum working volume (1 to 25 L) with a 2-dimensional rocking motion bioreactor (BIOSTAT® RM 20|50, Sartorius, Germany). The bioreactors were inoculated at $0.2 \cdot 10^6$ cells·mL$^{-1}$ in 4Cell XtraCHO SAM medium in batch mode (36.8 °C, 7.1 pH, 60% DO, rocking rate of 30 rpm with 10° angle). The pH-level was controlled by $CO_2$ gassing. When a VCC of $2.5 \cdot 10^6$ cells·mL$^{-1}$ was reached, perfusion was started with a perfusion rate of one vessel volume exchange of medium per day (VVD). Simultaneously, pH was shifted to 6.95, and control was extended by addition of 1 M $Na_2CO_3$. Perfusion rate was either adjusted daily based on expected cell growth for the following day (step-wise increase) or continuously adjusted based on in-line biomass measurement (BioPAT® ViaMass, Sartorius, Germany) to maintain a constant cell-specific perfusion rate (CSPR). Offline samples were taken manually from all cultivations. Cell growth (VCC, viability, and average diameter) was measured with a CedexHiRes Cell Counter (Roche, Switzerland). The pH, DO, glucose, and lactate levels were measured using a blood gas analyzer (ABL800 Basic, Radiometer, Denmark).

### 2.1.3. Intensified N-stage cultivations

#### 2.1.3.1. Media optimization in Ambr® 15.
For media optimization in a Ambr® 15 perfusion mimic process (cf. section 0), a CHO-S cell line expressing an IgG1 molecule was used. As for clone evaluation, Ambr® 15 vessels were inoculated with a density of $2.5 \cdot 10^6$ cells·mL$^{-1}$. pH set point was either 7.10 or 6.95 (depending on DoE setting) with an upper level of 7.20 or 7.15, respectively. DO was set to 50% and cultivation temperature to 37.0 °C. For DO and pH control, $CO_2$ and $O_2$ were applied to the system and a basic flow of 0.15 mL·min$^{-1}$ air was maintained throughout the whole process. One day before the cells reached their bleeding phase, the temperature was shifted to 34.0 °C. From this day on, the cells were bled each day to $40 \cdot 10^6$ cells·mL$^{-1}$ after cell count reading. Stirring speed was adjusted to 1200 rpm in a down-stirring manner with the possibility to increase to 1500 rpm. The culture was maintained at a perfusion rate of 1 VVD and daily procedure was performed as described in section 2.1.1. Media composition of a proprietary, chemically-defined perfusion medium was varied by preparing different media mixes with modified content of selected amino acids and metal ions that were filled into the respective reactors. Per media setting, duplicate runs were performed.

#### 2.1.3.2. STR200 FB.
Two FB cultivations were performed in Biostat STR® 200 L bioreactors (Sartorius, Germany) to evaluate the effect of intensified seed trains on N-stage performance. Both processes were run identical according to earlier publications [32,33] with temperature control at 36.8 °C, pH of 7.1, DO 60% and feed start at day 3. The cultures were inoculated at $0.3 \cdot 10^6$ cells·mL$^{-1}$ either from a standard batch or N-1 perfusion seed culture (see 2.1.2).

#### 2.1.3.3. Ambr®250 HIFB.
Based on N-1 perfusion, two HIFBs (2.5 and $5 \cdot 10^6$ cells·mL$^{-1}$) and one standard FB as reference were conducted in single-use multi-parallel 250 mL bioreactors (Ambr® 250 High Throughput, Sartorius). For all approaches, identical process parameters were used and the same volumetric feeding strategy was applied starting at day 3 for the standard FB [34], but earlier for the HIFBs. The bioreactors were automatically sampled and analyzed for VCC, viability and metabolites with an integrated BioProfile® FLEX2 (Nova

Biomedical).

#### 2.1.3.4. Univessel 2 L N-stage perfusion.
2 L Univessel® Glass bio-reactors (Sartorius, Germany) were inoculated at 0.2·10⁶ cells·mL⁻¹ from standard batch seed cultures. Processes were controlled at 36.8 °C, pH 7.1 and DO 60%. After a three-day batch phase, pH was shifted to 6.95 and perfusion was started at 1 VVD using an ATF2 device (Repligen, USA) for cell retention. The perfusion rate was increased as required to maintain a CSPR of 50 pL·cell⁻¹·day⁻¹. The process control strategy is demonstrated in Fig. 10. In summary, perfusion rate was controlled using a gravimetric harvest controller in combination with fresh media addition to maintain a constant working volume of 2 L. After an initial growth phase to evaluate the systems suitability to support HCD cell cultures, automatic cell bleed to maintain the target VCC was started. This was achieved using a PID controller in BioPAT® MFCS (Sartorius, Germany) to control the bleed pump speed based on in-line biomass measurement (BioPAT® ViaMass, Sartorius, Germany).

### 2.2. Downstream

#### 2.2.1. Cell clarification
For primary clarification of cell culture fluids (CCF) from intensified processes, a fluidized bed centrifuge (FBC) approach using a Ksep® 400 System (Sartorius, Germany) was investigated. The FBC was operated in a cyclic mode consisting of the steps of loading CCF in the rotating chambers, washing the IgG out of the fluidized bed, and discharging the biomass [35]. These cycle steps were repeated until 3 to 7 liters CCF were clarified for each experiment.

As a second clarification step, a depth filter (Sartoclear® DL60 Midscale Cassette, Sartorius, Germany) followed by a sterile filter (Sartopore® 2 XLG MidiCap, Sartorius, Germany) was used.

To examine the robustness of the clarification setup, CCF with different total cell concentrations (TCC) in a range of 20·10⁶ cells·mL⁻¹ (2300 NTU) up to 110·10⁶ cells·mL⁻¹ (8000 NTU) were used. Viabilities varied from 60% to 97% and IgG titers ranged from 1.6 g·L⁻¹ up to 21.6 g·L⁻¹.

For the different CCF, FBC process parameters optimized for CHO cell removal were applied as described by Saballus et al. [35], with exception of a reduced CCF loading flow rate of 400 mL·min⁻¹ to improve removal of cell debris from the low viable CCF.

The subsequent filtration was performed according to the manufacturer's instructions until a pressure limit of 1.3 bar was reached or the complete volume was processed. Thereafter, the filter setup was post-flushed (20 L·m⁻²) to achieve high recoveries.

#### 2.2.2. Continuous capture by multi column chromatography
The IgG from the permeate stream of the perfusion bioreactor was continuously captured by multi column chromatography (MCC). Either a BioSC Lab (Novasep, France) or a BioSMB PD (Sartorius, Germany) chromatography system was used as indicated with 5 mL MabSelect SuRe™ pre-packed HiTrap columns (2.5 cm column height, 1.6 cm column diameter, Cytiva, USA). The basic recipe was: 5 column volumes (CV) equilibration, loading (perfusion permeate), 8.5 CV wash, 0.35 CV elution (waste), 4 CV elution (product), 5 CV elution (waste), 3 CV equilibration, 5 CV cleaning in place (CIP). Buffer chemicals for equilibration and wash (1xPBS), elution (50 mM C₂H₃NaO₂, pH 3.0) as well as CIP (0.1 M NaOH) were purchased from Carl Roth (Germany). The flow rate, except for the loading step, was kept constant at 2.5 mL/min. The load volume as well as the number and interconnection of the columns were chosen based on previously performed breakthrough curves at different velocities [36]. The final continuous capture recipe and productivity predictions were generated with the Predict software (Novasep, France) for the BioSC Lab [37] and according to Bisschops et al. [36, 38] for the BioSMB PD. The chromatographic yield values represent the ratio of IgG mass in the product fraction relative to the total mass of IgG

recovered in all outlet fractions.

### 2.3. Analytics

#### 2.3.1. Product quantification and quality attributes
IgG concentrations in samples were quantified by size exclusion chromatography as described by Saballus et al. [35]. For the comparison of the productivity of different process strategies, the space-time yield (STY) according to Bausch et al. [39] was used, except in cases of non-negligible titer carryover from the seed culture or previous process step. Here, a slightly modified equation set was employed to calculate STY values for fed batch-type cultivations according to

$$STY_{FB} = \frac{c_{IgG,final} \cdot V_R\left(t_{final}\right) - c_{IgG,0} \cdot V_R(t_0)}{\left(t_{final} - t_0\right) \cdot \overline{V_R}} \tag{1}$$

where $t_{final}$ (d) and $c_{IgG,final}$ (g·L⁻¹) are the time of harvest and the product concentration at harvest, $t_0$ and $c_{IgG,0}$ the corresponding values at process start, respectively. $\overline{V_R}$ denotes the arithmetic mean of the working volume during the fed batch process.

STY for perfusion processes was determined as time-weighted average of volumetric productivity values in the culture

$$STY_{Perfusion} = \frac{\sum_{i=1}^{n} Q_P(t_i - t_{i-1}) \cdot (t_i - t_{i-1})}{\left(t_{final} - t_0\right)} \tag{2}$$

where $Q_P(t_i - t_{i-1})$ denotes the instantaneous volumetric productivity in the time interval between $t_{i-1}$ and $t_i$ (defined as in Eq. (3) and Eq. (4) below) and $n$ is the number of time intervals for which productivities are calculated.

For downstream applications, product recoveries were calculated by dividing the IgG mass of the filtrate pool by the IgG mass of the supernatant in the CCF prior to the clarification process [35].

In clone selection and media optimization experiments, product concentration was determined using the Octet QKe device (Sartorius FortéBio, USA) according to manufacturer's protocol.

In order to assess product quality attributes of the protein of interest, harvested cultures were first purified using Protein A purification plates (PreDictor MabSelect SuRe®, Cytiva, USA) or Protein A HP SpinTrap columns (Cytiva, USA). For glycan analysis and assessment of charge variants, these samples had to be desalted with membranes having a molecular weight cut-off of 50 kDa to prevent interferences during analysis. Both assessments were done using LabChipGXII Touch24 device (Perkin Elmer Inc., USA) and according to the respective manufacturer's protocols.

Purified (but not desalted) samples were used for detection of aggregates and monomers of the protein of interest. The analysis was carried out using an ÄKTAmicro HPLC (Cytiva, USA) and respective Yarra 3 u column (Phenomenex, USA).

#### 2.3.2. Process related impurities
DNA concentration was measured by the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher Scientific, USA) with salmon sperm dsDNA as standard (Biomol, Germany). Samples were diluted in TE buffer (10 mM TRIS, 1 mM EDTA, 0.1% SDS; all chemicals purchased from Karl Roth, Germany) if necessary.

Host cell protein (HCP) concentration was measured by the CHO HCP-ELISA (F550, Cygnus Technologies, Southport, USA) according to the manufacturer's instructions. Samples were diluted in ELISA buffer (20 mM TRIS, 50 mM NaCl; all chemicals purchased from Karl Roth, Germany) if necessary.

DNA and HCP analytics were performed as analytical duplicates and measurements were performed with an Infinite® 200 PRO plate reader (Tecan, Switzerland).

For turbidity measurement, a nephelometer (TL2350, Hach, Germany) was used.

## 2.4. Theory/calculation

### 2.4.1. Product titer correction for carry-over

For calculation of volumetric productivity $Q_P$ in semi-perfusion systems between two consecutive time points $t_{i-1}$ and $t_i$ of media exchange, product titer values were corrected for product carryover due to residual product content in the cell pellet after centrifuging the reactions vessels. Daily productivity was then obtained by multiplying by the number of media exchanges per day $VVD$ (based on feeding rates) resulting in:

$$Q_P(t_i, t_{i-1}) = VVD \cdot \frac{(m_P(t_i) - m_P(t_{i-1}'))}{V_R} \tag{3}$$

with

$$m_P(t_i) = V_R \cdot c_P(t_i) \tag{4}$$

as the product mass in the (constant) reaction volume $V_R$ at product concentration $c_P$ in the reaction vessel. The residual product mass in the cell pellet after centrifugation can be described by

$$m_P(t_{i-1}') = V_{P,pore} \cdot c_P(t_{i-1}') \tag{5}$$

Where $V_{P,pore}$ denotes the pellet pore volume and $t_{i-1}'$ the time point after removing spent media and before adding fresh media to the reaction vessel. The pore volume in the cell pellet was inferred using experimentally determined values for cell pellet porosity $\varepsilon_{Pellet}$ and an empirical linear correlation relating cell pellet volume to the viable cell concentration $VCC$.

$$V_{P, pore} = \varepsilon_{Pellet} \cdot V_R \cdot \alpha \cdot VCC(t_{i-1}') \tag{6}$$

The parameter $\alpha$ of this relation had been determined for multiple CHO DG44 clones in the scale-down model under the given process and media conditions using data for cell count, media density, and measurements of cell wet weight and cell dry weight, respectively. For the CHO cell lines used we determined an average $\varepsilon_{Pellet} = 0.61$ mL pore volume/mL pellet volume and $\alpha = 0.00666$ mL pellet volume per mL culture volume and per viable cell concentration of $10^6$ cells$\cdot$mL$^{-1}$. To this end, we determined the pellet dry mass contained in a cell broth sample of defined volume and viable cell concentration. Two samples of 2 mL of the same cell broth were collected and dispensed into 2 mL pre-weighed Eppendorf cups. The first sample was first centrifuged at 500 g for 5 min, washed once with PBS, and centrifuged again. Subsequently, the supernatant was removed and the pellet wet weight determined by weighing and subtracting the weight of the empty cup. The second broth sample was filtered using a syringe with 0.2 μm PES filters membranes in metal holders. Filter membranes were weighed prior to and after filtration following drying at 80 °C for 24 h. A separate filter membrane flushed with 2 mL PBS and dried as above served as blank. Cell dry weight was then determined by subtracting the weight difference (after drying – before drying) for the blank filter from the weight difference of the filter containing the broth filter cake. Entering the resulting values into the above equations enabled performing a correction for product carry-over effects without having to perform more than one product titer measurement per media exchange cycle.

### 2.4.2. Product quality tuning approach in perfusion

For the experiments described in section 3.4, a reduced combinatorial design consisting of 43 runs plus 3 replicate runs (46 in total) was designed, carried out in an Ambr® 15–48 and evaluated using the MODDE® (v12) design of experiments (DoE) software. The design comprised 5 factors including media mix type (4 mixes) as qualitative factor and multilevel concentration settings for three different supplements and pH, respectively. Responses tracked covered variables characterizing cell growth (e.g., IVCC, viability), culture stability (process duration, number of bleeding days), glucose and lactate levels, cellular productivity (e.g., volumetric productivity) and product quality

attributes like main glycan groups (mannosylation, galactosylation, fucosylation), percentage of aggregates, and charge variants (basic, acidic, main), respectively.

For an integral optimization of product quality while simultaneously optimizing the perfusion process the following approach was employed: average volumetric productivity in the bleeding phase was used as integral measure of growth, culture longevity and cellular productivity. For characterizing the total absolute deviation of the biosimilar product quality from that of the originator the following type of formula was applied, here illustrated for charge variants (ChVar):

$$\Delta ChVar_{total} = |\Delta\%Acidic| + |\Delta\%Basic| + |\Delta\%Main| \tag{7}$$

Similar expressions were derived for glycan patterns or aggregate content. For the simultaneous overall optimization of factors characterizing cell performance and product quality, a weighted objective function value was assigned to each experimental run $j$ of the DoE as follows:

$$f_{obj}^j = \sum_{i=1}^{n_{Factors}} w_i \cdot (-1)^{k_i} \cdot \frac{Factor_i^j - Factor_i^{min}}{Factor_i^{max} - Factor_i^{min}} \tag{8}$$

Here, $n_{Factors}$ denotes the total number of response factors considered in the optimization and $w_i$ represents the weighting factor assigned to a given response factor. The exponent $k_i$ was set to 0 in case a maximization was desired and to 1 for minimization, respectively. $Factor_i^{min/max}$ denote the minimum or maximum value of a factor across the whole DoE data set, respectively, whereas $Factor_i^j$ stands for the factor value in a particular experimental run $j$. This approach ensured a normalization of the different factors to the range [0,1] to enable a fair comparison of factors with qualitatively different content. Weightings $w_i$ of the individual objective function contributions could be adapted to the problem at hand as required.

### 2.4.3. Modeling of FBC clarification

To determine the effect of the TCC on primary clarification, the FBC process was modeled. The maximum CCF volume ($V_{CCF}$) that can be clarified in one FBC cycle depends on the maximum FBC chamber capacity ($C_{FBC}$), number of chambers used ($n$) and the TCC of the feed, as shown in Eq. (9). Based on previous studies, a $C_{FBC}$ of $10 \cdot 10^9$ cells was assumed for the CCF with the applied FBC chambers [35,40].

$$V_{CCF} = \frac{C_{FBC} \cdot n}{TCC} \tag{9}$$

The time for a FBC clarification cycle ($t_{FBC\_cycle}$) is the total time for all cycle steps, that can be described as a function of the volumes of $V_{CCF}$, washing ($V_{wash}$) and discharging ($V_{dc}$) divided by their corresponding flow rates for clarification ($\dot{V}_{CCF}$), washing ($\dot{V}_{wash}$), and discharging ($\dot{V}_{dc}$), according to Eq. (10) and controlled by the FBC system (section 2.2.1).

$$t_{FBC\_cycle} = \frac{V_{CCF}}{\dot{V}_{CCF}} + \frac{V_{wash}}{\dot{V}_{wash}} + \frac{V_{dc}}{\dot{V}_{dc}} \tag{10}$$

The average CCF throughput ($TP_{FBC}$) takes into account the CCF volume ($V_{CCF}$) and the time ($t_{FBC\_cycle}$) of a FBC clarification cycle, as shown in Eq. 11.

$$TP_{FBC} = \frac{V_{CCF}}{t_{FBC\_cycle}} \tag{11}$$

The harvest dilution rate ($D$) is defined as the ratio of the obtained harvest volume considering $V_{CCF}$, $V_{wash}$ and an initial dump volume ($V_{ID}$) to the clarified $V_{CCF}$, according to Eq. (12). $V_{ID}$ describes the void volume without IgG set by the FBC process parameters to be directed into waste at the beginning of each FBC cycle.

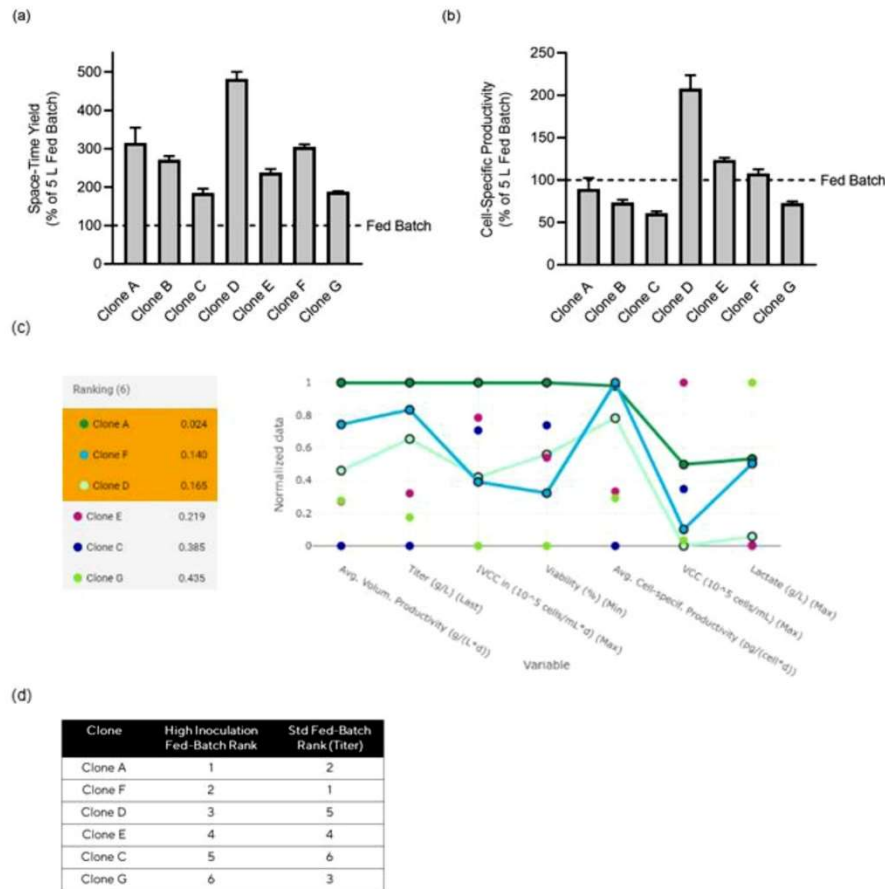$$D = \frac{V_{CCF} + V_{wash} - V_{ID}}{V_{CCF}} \tag{12}$$

5

**Fig. 2.** Comparison of (a) volumetric productivity and (b) cell-specific productivity for different CHO DG44 production clones in the Ambr® 15 semi-perfusion ($n = 3$) relative to the value observed in a 5 L FB. Each of the clones expresses a different protein product: Clone D: Fc-fusion protein, Clone E IgG2, others: different IgG1 mAbs. Cultivations were performed as outlined in section 2.1.1. Product carryover effects due to the semi-perfusion approach were corrected for when calculating productivity values. The TOP 3 clones according to this ranking are indicated by the orange box in the table and the solid lines in the plot, respectively. (c) Multi-parameter clone ranking of HIFB clones using the Ambr clone selection software and (d) comparison of ranking result with that according to standard FB titer.

Since CCF throughput and dilution rate represent the key variables of the FBC performance, Eq. (9) to Eq. (12) were used to calculate them as a function of TCC.

### 3. Results and discussion

In the following paragraphs, we present an overview of different applications of intensified process elements, which have been implemented and evaluated in the context of feasibility studies for CHO cultures in a research setting.

#### 3.1. Selection of production clones for intensified processes

Among the many factors influencing process economics, cell culture productivity typically has the largest overall impact [26]. Volumetric cell culture productivity in gram product produced per liter reaction volume can be maximized by ensuring high cell-specific productivity

and high viable cell numbers. In addition, robust re-growth and high cell viability as well as low cell lysis are key to ensure good harvestability of the cell culture fluid, long process duration and maintenance of low profiles of process-related impurities like HCP and DNA [15,41]. Low cell-specific media consumption as expressed by CSPR is a desirable trait, too, as media demand constitutes a key cost factor, for example, in perfusion processes [8]. This complex profile of competing requirements illustrates that the choice of production clone for a given product will have a large impact on achievable process economics at production scale. This is why investing effort in proper cell line development pays off and clone evaluation for an intensified process is best conducted in scale-down systems mimicking the actual target system, e.g., a HIFB or perfusion for intensified processes [15,42].

Different systems for these purposes have been suggested such as deep-well plates [15], vented tube spin systems [43] or different perfusion mimic options performed in Ambr® 15 microbioreactors with full control of pH and dissolved oxygen levels ([44], this work). Fig. 2(a)
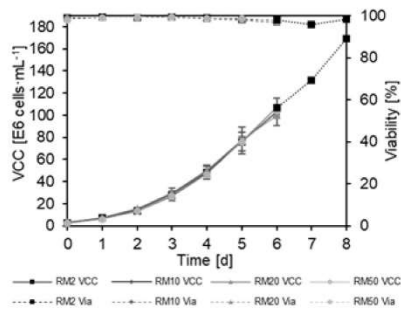
6

D. Müller et al.



**Fig. 3.** Cell growth and viability in N-1 perfusion process using 2D rocking motion bioreactor bags with integrated filter membrane. Cultivations were performed at maximum working volume which equals 50% of total bag volume for each size, e.g. 1 L working volume for RM 2 L. Error bars represent standard deviation (RM2: $n = 6$; RM10: $n = 4$; RM20: $n = 2$; RM50: $n = 1$). For one RM 2 L cultivation, process duration was prolonged until day 8 to test the maximal cell concentration.
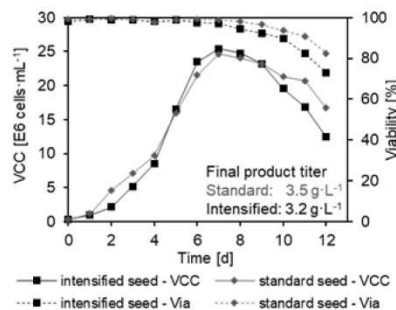


**Fig. 4.** Comparison of STR200 fed-batch cultivations inoculated at identical starting VCC either from standard batch or intensified N-1 perfusion seed trains.
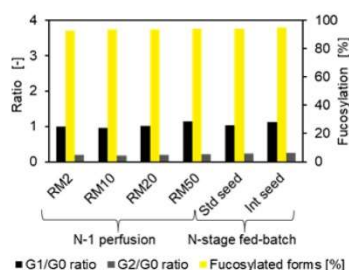


**Fig. 5.** Glycosylation profile of IgG produced in different N-1 perfusion scales and N-stage FB cultivations inoculated from standard batch or intensified perfusion seed cultures.

and (b) illustrate the productivity assessment of CHO DG44 clones expressing different mAb-type protein products (IgG1, IgG2, Fc-fusion protein) in an Ambr® 15 semi-perfusion setup, where media was exchanged daily employing a centrifugation procedure (cf. section 2.1.1 for details). Each of the clones had been identified as top performer in a corresponding FB evaluation of the respective cell line development program. For all seven clones tested, marked increases in volumetric

productivity even up to over 400% were registered when switching to perfusion mode. In some cases, higher volumetric productivity was solely achieved by increased cell numbers, whereas for other clones (e.g. Clone D) also cell-specific productivity increased under these conditions.

New software tools enable weighted multi-parametric assessment of clone performance beyond mere product titer and are, therefore, well suited for objectively balancing performance tradeoffs in productivity, cell growth, and byproduct formation, e.g., during clone screening in a scale-down intensified process like a HIFB (Fig. 2(c)). Notably, the assessment demonstrates that the resulting clone ranking can differ significantly from that considering standard FB titers of the corresponding clones (Fig. 2(d)). This highlights that different clones will be preferred for the intensified process application. A similar approach has been applied previously by Popp et al. [45] for clone selection in FB processes. Such software tools support smooth integration of comprehensive data on cell physiology and process demands, which is routinely generated during clone evaluation, into the scoring process. In this way, they can support more reliable choices of production clones for a given process scenario, which is a notoriously difficult challenge in cell line development [46].

### 3.2. N-1 perfusion seed cultures using rocking motion bioreactors

Robust and reliable seed trains are essential for efficient bio-manufacturing processes. Historically a sequence of stirred tank bioreactors in batch mode, increasing culture volumes and low seed ratios of 1:2 to 1:10 [47] have been the industrial standard. By applying perfusion technology to seed culture processes, much higher cell concentrations can be achieved which allows for the reduction of seed culture steps, thus minimizing seed train equipment and effort. While stirred tank bioreactors with external cell retention devices may be used, perfusion-ready cultivation bags for 2D rocking motion (RM) bioreactors with an integrated perfusion membrane enable convenient handling and scaling of perfusion seed cultures as shown in Fig. 3. Cultivations were inoculated at default seed VCC of $0.2 \cdot 10^6$ cells·mL$^{-1}$ and perfusion was started after a three-day batch phase at about $2.5 \cdot 10^6$ cells·mL$^{-1}$. High cell densities of $100 \cdot 10^6$ cells·mL$^{-1}$ could be achieved after 6 days perfusion while maintaining high cell viabilities above 95% with very consistent cell growth for all evaluated scales from 2 L to 50 L cultivation bags. The systems suitability for high cell density processes was further challenged in a 2 L cultivation, reaching $170 \cdot 10^6$ cells·mL$^{-1}$ on day 8. The process setpoints, e.g. DO and pH, could be well controlled even at high cell densities as shown exemplary for the 50 L scale (see Fig. A3).

Cell growth was successfully monitored using a bio-capacitance probe, which showed linear correlation of online permittivity and reference VCC even at high cell concentrations and independent of cultivation scale. (see Fig. A1). Implementation of an automatic perfusion rate control based on online VCC resulted in a constant CSPR, which ensures sufficient nutrient supply while also reducing overall media usage by 15% compared to manual PR adjustment (see Fig. A2 and Fig. A4).

To evaluate the effect of intensified seed trains on N-stage performance, two STR200 FB cultivations were performed, either inoculated from a standard batch seed or N-1 perfusion culture. In total 0.4 L of intensified seed and 14 L of standard batch seed were used for inoculation, demonstrating the power of N-1 perfusion processes to reduce seed train scales. Both processes showed very similar performance, reaching peak VCC of about $25 \cdot 10^6$ cells·mL$^{-1}$ on day 7 and a cell viability of $> 70\%$ on harvest day 12 (see Fig. 4). Final product titer for the process inoculated from N-1 perfusion was slightly lower compared to standard seed at 3.2 and 3.5 g·L$^{-1}$, respectively, but within range of historical processes (data not shown). The seed train intensification did not affect product glycosylation as shown in Fig. 5, neither by carryover from seed nor in production culture. These results demonstrate how N-1 perfusion seed cultures can be utilized to intensify established
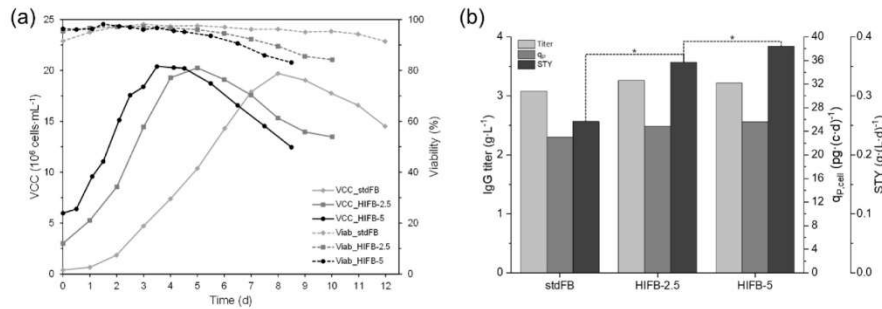
**Fig. 6.** Implications of process intensification at the N-stage demonstrated by comparing one standard ($0.3 \cdot 10^6$ cells·mL$^{-1}$) and two high inoculated fed-batches (2.5 and $5 \cdot 10^6$ cells·mL$^{-1}$) inoculated from a conventional seed train and N-1 perfusion, respectively. Experiments ($n = 2$) were conducted in Ambr® 250 ht bioreactors, of which the means are depicted: a) Viable cell concentration and viability and b) IgG titer, cell-specific productivity and STY. '*': significant difference compared to stdFB acc. to $t$-test ($p < 0.05$).
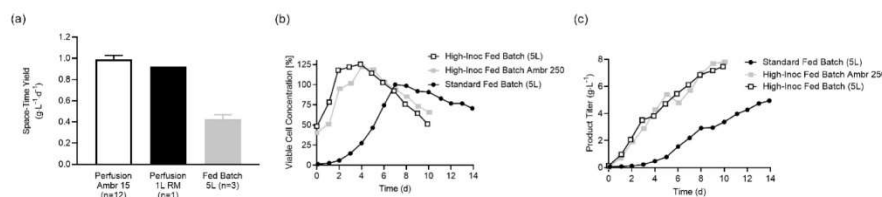


**Fig. 7.** Scaling of process performance between scale-down models and benchtop process. (a) Space-time yield in different perfusion setups comparing 2 week processes of an IgG1-expressing CHO cell culture; RM: Rocking Motion Reactor, n values indicate number of independent runs, error bars denote standard deviations. High Inoculation Fed Batch in Ambr 250®ht and 5 L scale of a second CHO DG44 clone expressing another IgG1: (b) viable cell concentration and (c) product titer.

production processes.

Similar findings were reported also for other mAb-producing CHO cell lines where the impact of N-1 cultivation mode on N-stage cell growth, titer and product quality were compared [23,48]. In addition to the N-1 perfusion concept already discussed, also the use of enriched batch or fed-batch processes in N-1 stage could be used to achieve high seed cell concentrations. In combination with an increased inoculation cell concentration in the N-stage fed-batch process, the final product titer could be increased by up to 100% at 500 and 1000 L scale [23,24], further demonstrating the potential of seed intensification.

### 3.3. Rapid prototyping of intensified processes in scale-down models

Intensified process formats like HIFB or perfusion often require careful tuning of process parameters like inoculation cell density, temperature shifts or adaptation of feeding profiles to maximize process productivity while maintaining product quality. For this task, parallel scale-down systems are needed that reliably mimic the larger-scale process and enable rapid testing of different process variants in a short time frame.

For example, different inoculation cell densities were tested in a HIFB setup in an research setting to assess their impact on cell growth performance (Fig. 6a)) and STY (Fig. 6b) and compared with a standard FB. Clearly, the initial phase of the standard FB was skipped with higher inoculation density. This phase is usually characterized by relatively high biomass but low product increase. Thus, the initial phase of HIFBs directly started with more net IgG production. Similar peak cell concentrations of about $20 \cdot 10^6$ cells·mL$^{-1}$ were reached in all approaches, but earlier in time for the HIFBs. Both viabilities and the VCC declined subsequently, so that the processes were terminated earlier in time, but all with final viabilities of $> 80\%$. All approaches yielded similar IgG

titers of about 3.2 g·L$^{-1}$ and while, from a biological aspect, the cell-specific productivity was not affected ($\sim$ 24 pg·(cell·d)$^{-1}$), the STY was significantly improved with increased inoculation density by up to 50% since the length of bioreactor 'idle' stages was reduced by 3.5 days [49]. Such promising experiments can be the starting positions for further process improvements, e.g. with media enrichments to account for an enhanced nutrient demand of the increased initial number of cells in the bioreactor [23] or by further increasing the cell-specific productivity [9].

Using such parallel scale-down models is attractive because in many cases optimized process settings with significant productivity gains can be achieved in a short timeframe of one or two iterations. This is especially relevant since early process development is on the critical path when speeding a new drug to the clinic. Unless pre-established process platforms are already in place, such development tools for fast process setup and optimization can, thus, be key enablers of implementing intensified processes already before the first clinical phase. Such application, of course, necessitates that scale-down models are indeed reliable predictors of cultivation behavior at larger scales. Fig. 7 demonstrates that such scalability can indeed be achieved for a perfusion type setup. Here, a first screen for promising process settings was conducted in the semi-perfusion system as described in section 2.1.1 (Ambr® 15) before scaling up to the benchtop scale. Notably, the space-time yield was comparable between the scale-down system and a rocking motion type reactor with integrated cell retention membrane and was increased about two-fold relative to the reference setup in a 5 L FB process.

Fig. 7(b) and (c) illustrate intensification of a 5 L FB reference process, which was implemented and optimized as HIFB variant in an Ambr 250® ht scale-down system. The graphs demonstrate that very comparable growth and product titer profiles were achieved when transferring
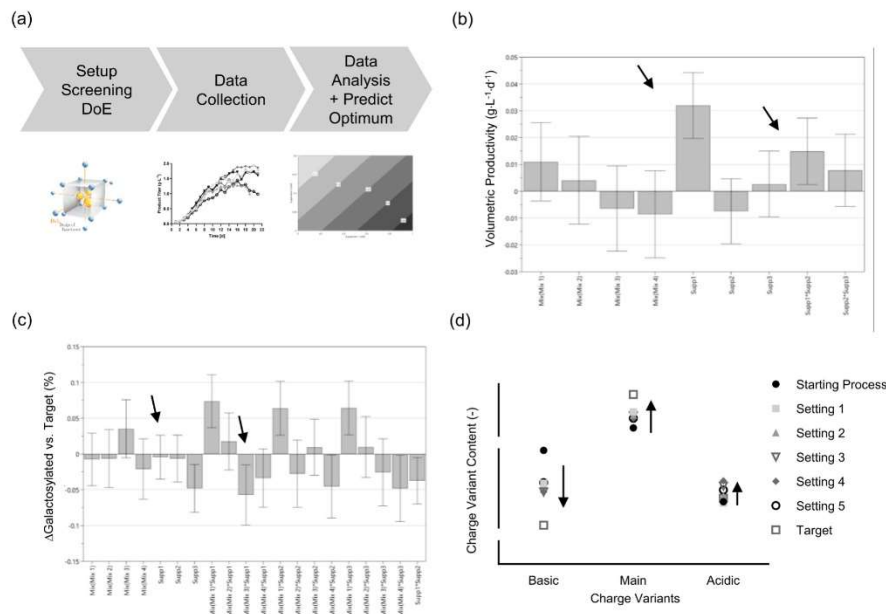
8

**Fig. 8.** Product quality optimization in a perfusion process for biosimilar production. (a) Experimental design in MODDE and data collection in Ambr® 15 perfusion mimic. DoE evaluation and identification of sensitive factor combinations regarding (b) volumetric productivity (calculated as described in section 0) and (c) galactosylation difference to originator target value. Arrows highlight that certain factors exert little effect on their own (Supplement 1), but much larger in synergy with another one (Supplement 1 and Medium 3); (d) Improvements in biosimilar charge variant profile for the Top 5 settings in the Ambr® 15 screening to approach the target profile of the originator molecule.

the optimized process back to the 5 L scale. Again, a marked increase in STY by about two-fold from 0.35 to 0.73 $g \cdot L^{-1} \cdot d^{-1}$ (calculated using Eq. (1)) could be achieved resulting from a combination of titer increase (+50%) and a shortening of the process from 14 down to 10 days, respectively. In this way, process intensification can support saving costly time in GMP facilities while at the same time increasing product yield per batch performed. Similar findings have recently been reported by Xu et al. [23], which have further demonstrated that such high increases in performance can be successfully scaled to the 1000 L level. They underlined the importance of establishing intensified processes as platforms to curb process optimization times – in their case from 9 down to 3 months including media rebalancing and lab-scale bioreactor runs. Additional savings in time and effort can be generated if multiplexed mini-bioreactor systems like the Ambr 250® ht can be used instead of the 1 - 10 L bioreactors, which are typically considered predictive for scale-up to commercial manufacturing [15]. In the present case, only a single 2 week run in an Ambr® 250 ht system was performed to optimize seeding density, feeding profile, and temperature downshift to achieve the process shown in Fig. 7(b) and (c). In this way, early process development can be markedly accelerated and further potential for process improvement seems likely since media composition was not even optimized.

### 3.4. Product quality tuning in perfusion processes

Continuous intensified process operation modes like perfusion are not only interesting because of their potential to increase volumetric productivity, but also because perfusion approaches have long been used for biotherapeutics where a consistent product quality is key. For example, perfusion has been applied to production of blood clotting

factors, labile molecules like enzymes or new complex modalities [15, 17].

This property renders perfusion processes also interesting candidates for biosimilar applications where matching the product quality profile of the originator has high priority [50]. Typical product quality attributes of interest include the N-glycosylation spectrum or the profile of charge variants.

In the example outlined below, the goal was to optimize a perfusion process for biosimilar production with the main aim to improve the match of the product charge variant profile with the originator molecule. To this end, the reference process was first transferred into an Ambr® 15 perfusion mimic system. A statistical screening design was implemented using the MODDE® software that combined different media mixes, multilevel supplement additions as well as different pH conditions. Altogether, 46 perfusion cultivations were performed in the scale-down system over a period of 3 weeks and with a perfusion rate of 1 VVD (cf. sections 2.1.3 and 2.4.2 for details). Daily media exchange and cell retention were performed using a centrifugation approach with dedicated centrifuge inserts to hold the Ambr® 15 bioreactor vessels. Key parameters tracked during the cultivation included viable cell count, viability, and product titer as well as *N*-glycan and charge variant profiles. Evaluation of the DoE setup demonstrated that different factors and factor combinations affected volumetric productivity and product galactosylation offset from the originator profile (Fig. 8).

Applying a multi-objective approach for balancing tradeoffs between competing objectives (cf. section 2.4.2) enabled identification of several process and media conditions that provided an improved match to the product quality profile: charge variant differences to the originator were reduced by 44% overall and summed N-glycan distribution differences were lowered by 17% while at the same time a modest increase in
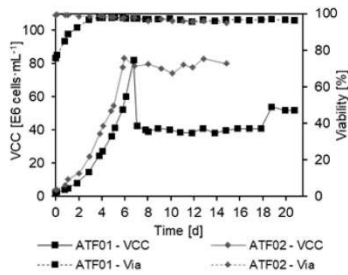
9

**Fig. 9.** Cell growth and viability of perfusion cultivations in 2 L bioreactors. After an initial growth phase up to $80 \cdot 10^6$ cells·mL$^{-1}$ to check the systems performance at high cell densities, different target cell concentrations were maintained by implementation of automated cell bleed using an in-line bio-capacitance probe.

process productivity ($> 10\%$) was achieved. Further refined process settings were then predicted in MODDE® using an optimization based on the mathematical DoE model identified. Implementation of the optimized conditions at larger scale subsequently confirmed that a significantly improved product quality match with the originator was successfully attained for the biosimilar with this approach (G. Zijlstra, personal communication).

Similar results have also been described in the literature by Brühl-mann and colleagues [51] and Weng et al. [52]. In this way, combining highly parallel microbioreactor systems with advanced data analytics and high throughput methods for product quality analytics provide an attractive approach to identify promising intensified process setups for biosimilar production. Following a screening phase, these can then be optimized further in mini-bioreactor systems like the Ambr® 250 ht, which yield an even better representation of the target process, e.g., regarding vessel geometry or complexity of parameter control.

### 3.5. N-stage perfusion processes for continuous product harvesting

N-stage perfusion cultivations with continuous product removal are especially useful for production of labile products such as factor-VIII, but can also be advantageous for the production of stable proteins such as monoclonal antibodies by increased manufacturing flexibility and volumetric productivity. In this work, ATF perfusion cultivations in 2 L stirred tank bioreactors at different target VCCs with an integrated IgG capture by multi-column-chromatography were performed. A schematic representation of the integrated process and cultivation control strategies is shown in Fig. 10. The perfusion permeate was collected in a small

surge tank to account for slight variations between bioreactor and MCC flow rates and was continuously loaded to the MCC. A steady permeate flow was achieved by implementing a flow controller maintaining constant weight of the surge tank. To achieve a stable operation, continuous bleeding was implemented using an in-line single-frequency bio-capacitance probe. The correlation of the single-frequency signal to VCC can be challenging for FB processes with large cell diameter changes, especially in the stationary growth and death phase [34]. In contrast, N-stage perfusion processes aim to reach a steady-state-like operation with constant cellular properties, whereby permittivity and VCC show a linear correlation throughout the whole process. To maintain a constant CSPR, the PR was set based on target VCC and controlled by a gravimetric harvest flow controller while taking the additional bleed flow into account. Fresh media was added to maintain a constant bioreactor working volume. The constant harvest flow rate through the perfusion membrane can help preventing filter blocking and eases the integration of following downstream operations while the level-controlled feed addition eliminates the risk of overfilling the bioreactor.

The VCC and cell viability of two perfusion cultivations are shown in Fig. 9. After an initial batch phase in the same bioreactor, the cell viability in the first cultivation ATF01 dropped to 75% after starting the perfusion due to insufficient preconditioning of the hollow fiber module. This handling error was recognized and considered for the second cultivation ATF02, where the initial viability drop could be prevented. For cultivation ATF01, cell viability recovered and the system's suitability to support HCD cultivations was evaluated during the growth phase with up to $80 \cdot 10^6$ cells·mL$^{-1}$ on day 7. Afterwards, the VCC was decreased and maintained at $40 \cdot 10^6$ cells·mL$^{-1}$ for 11 days. The continuously controlled bleed resulted in a very consistent VCC. For the last three days, target VCC was increased to $50 \cdot 10^6$ cells·mL$^{-1}$. A high cell viability above 95% could be maintained throughout the two-week steady-state-like operation. At $40 \cdot 10^6$ cells·mL$^{-1}$, a harvest rate of 2.5 VVD was applied which resulted in a stable product concentration of $0.51 \pm 0.04$ g·L$^{-1}$ in the perfusion harvest. This translates to a volumetric productivity of 1.27 g·L$^{-1}$·d$^{-1}$. The average cell-specific productivity during the 11 days steady-state operation was at $32 \pm 3$ pg·L$^{-1}$·d$^{-1}$ and therefore increased by one-third compared to the fed-batch process of this cell line (see Fig. 6).

To increase the volumetric productivity, target VCC was further increased to $80 \cdot 10^6$ cells·mL$^{-1}$ in the second cultivation ATF02. Without the viability drop at perfusion start, the initial cell growth was improved and target VCC was reached on day 6, one day earlier as for ATF01. Even at these high cell concentrations, constant VCC could be maintained for 9 days by bleed control based on in-line permittivity measurement. To ensure sufficient nutrient supply, a PR of 4 to 4.5 VVD was applied, which resulted in a stable product concentration of $0.69 \pm 0.03$ g·L$^{-1}$ in
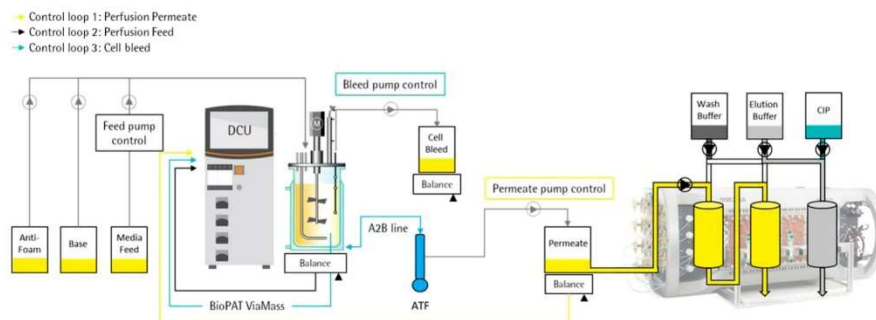


**Fig. 10.** Schematic representation of the integrated USP-DSP approach highlighting the USP perfusion control strategies and the connection to the subsequent DSP. Perfusion permeate was collected in a small surge tank to account for slight deviations between the USP and DSP flow rate and continuously loaded to the MCC.
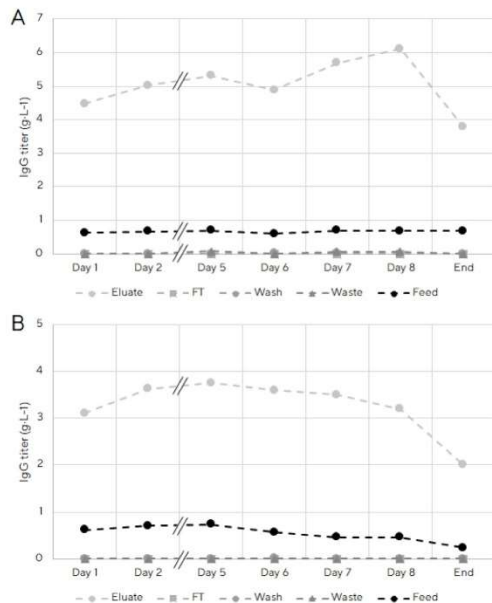
**Fig. 11.** IgG titer of the continuous capture process with the BioSC Lab for 8 days. (A) Results for the 3-column process (VCC of $50 \cdot 10^6$ cells·mL$^{-1}$). (B) Results for the 5-column process (VCC of $80 \cdot 10^6$ cells·mL$^{-1}$). The IgG titer is given for the eluate, flow-through (FT), wash, waste and feed. Pooled fractions of the different outlets were analyzed once a day except day 5 which consists of the pooled fractions of days 3–5.
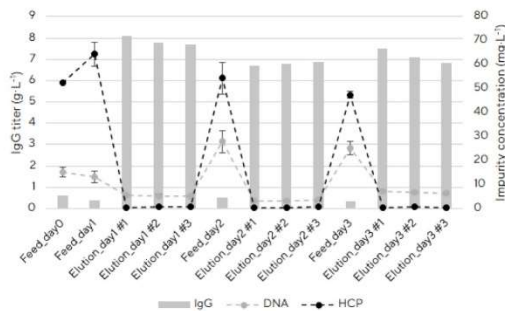


**Fig. 13.** Cell culture fluid (CCF) throughputs and harvest dilution rates for the primary clarification of cell culture from intensified processes with different total cell concentrations using a fluidized bed centrifuge system. The lines show the calculated values according to Eq. (11) and Eq. (12), whereas the symbols represent experimental results.



**Fig. 14.** IgG recoveries and harvest turbidities after the clarification of cell culture fluids with different total cell concentrations and different IgG concentrations (1.6 g·L$^{-1}$ to 21.6 g·L$^{-1}$) using a fluidized bed centrifuge with subsequent depth and sterile filtration.



**Fig. 12.** IgG titer as well as DNA and HCP concentration of the continuous capture process with the BioSMB PD for 3 days. The elution fractions of the individual columns were analyzed separately.

the perfusion harvest. Cell-specific productivity was slightly increased compared to the first perfusion cultivation at $39 \pm 2$ pg·L$^{-1}$·d$^{-1}$, which could have resulted from tighter process understanding and control. These results are in agreement with previously published data on semi-perfusion processes using this cell line and media platform [53]. Volumetric productivity was at 2.77 g·L$^{-1}$·d$^{-1}$ and therefore more than doubled compared to the perfusion cultivation at $40 \cdot 10^6$ cells·mL$^{-1}$ and corresponds to a 10-fold increase relative to the original standard FB setup (Fig. 6).
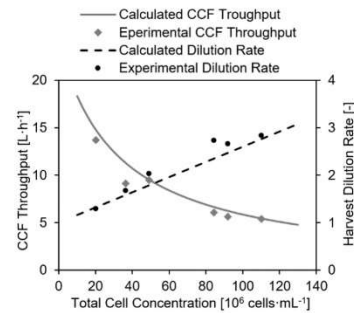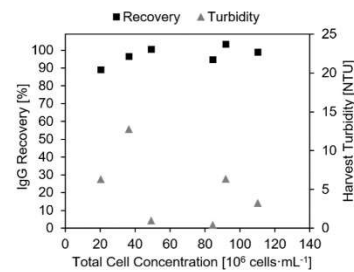
These results demonstrate the power of N-stage perfusion

cultivations to increase volumetric productivity. To increase the amount of IgG produced, the process can be scaled up to larger bioreactors, the process duration can be increased or a higher target VCC can be applied. With increased target VCC, the requirements on the cultivation system with regards to oxygen supply and mixing as well as the risk for biomass buildup and filter blocking increases. To reduce the costs associated with high media demand in perfusion processes, the minimum required CSPR should be identified by either push-to-low or push-to-high strategy [54, 55]. It was also shown that by decreasing the CSPR, cellular growth rates can be decreased, resulting in lower bleed rates and thus lower amounts of product lost [55,56]. Other approaches for decreased growth rate while also increasing specific productivity can be the implementation of a temperature shift [57] or supplementation of chemical additives such as valeric acid [58].

### 3.6. MCC for continuous IgG capture

In order to maintain and expand the advantages of continuous perfusion cultivations from the USP into the DSP, a continuous capture of the IgG from the permeate stream was performed by a MCC approach. The BioSC Lab chromatography system was directly connected to a small surge tank where the perfusion permeate was collected (Fig. 10). In this study, two perfusion processes with a constant VCC of $50 \cdot 10^6$ cells·mL$^{-1}$ (3.4 mL·min$^{-1}$ perfusion rate) and $80 \cdot 10^6$ cells·mL$^{-1}$ (6.1 mL·min$^{-1}$ perfusion rate) were continuously processed by a 3- and 5-column MCC setup respectively (Fig. 11).

It was shown that the MCC is capable of successfully processing different USP process scenarios with different VCC by making appropriate adjustments to the loading flow rate and volume as well as to the number of columns. Both capture processes were operated for 8 days without any interruption, demonstrating a successful and robust continuous processing of the perfusion permeate with an IgG titer of approximately 0.7 $g \cdot L^{-1}$. No IgG was observed in the flow-through during the loading phase. Small variations of the feed titer during the cultivation resulted in slight differences in the IgG concentration in the eluate. For both processes, a start and end of production recipe was used at the beginning and after the 8 days to ensure a sufficient start-up and shutdown, resulting in reduced IgG concentrations in the eluate (Fig. 11). A lower IgG concentration (approximately 3.5 $g \cdot L^{-1}$, Fig. 11B) was obtained for the 5-column process, due to a lower feed titer than expected and consequently less optimal resin utilization, compared to the 3-column process (approximately 5 $g \cdot L^{-1}$, Fig. 11A).

For the 3-column process, 33.1 g product were obtained with a high yield of 97.2% throughout the entire process time. The specific productivity of the process was 272.3 $g_{IgG}^{-1} \cdot L_{resin}^{-1} \cdot d^{-1}$, which was in good agreement with the estimated productivity of the prediction software (291.7 $g_{IgG} \cdot L_{resin}^{-1} \cdot d^{-1}$).

For the 5-column process an even higher IgG yield of 99.7% was obtained and in total 33.9 g IgG were recovered in the eluate. A specific productivity of 174.6 $g_{IgG}^{-1} \cdot L_{resin}^{-1} \cdot d^{-1}$ was obtained due to the higher column number at a fixed permeate flow rate. This value was again similar to the predicted productivity (198.3 $g_{IgG}^{-1} \cdot L_{resin}^{-1} \cdot d^{-1}$). For both processes, the slight deviations are likely due to some uncertainties of the feed titer estimation at the process setup.

In addition to the BioSC Lab, the BioSMB PD was utilized as MCC system for a continuous, integrated IgG capture. The same setup as shown in Fig. 10 with the same VCC, perfusion rate and chromatography process parameters like the 3-column BioSC Lab experiment was used, resulting in a similar high yield of IgG ($> 97\%$) and specific productivity (approximately 270 $g_{IgG}^{-1} \cdot L_{resin}^{-1} \cdot d^{-1}$). However, a higher IgG titer of approximately 7 $g \cdot L^{-1}$ in the eluate was obtained (Fig. 12) due to optimization of the loading step.

In addition, the IgG purity with regards to the removal of process related impurities such as DNA and HCP, was evaluated. Both impurities were significantly removed compared to the perfusion permeate (Fig. 12). For all three process days a HCP removal greater than 2-log values was observed resulting in a concentration in the eluate below 1 $mg \cdot L^{-1}$ in all fractions ($< 140$ ppm). The low concentration of HCP, which are seen as one of the most critical impurities [32], is a result of the lower initial impurity load from the USP perfusion process, compared to fed-batch cultivations, combined with the purification by protein A affinity chromatography. In addition, the DNA concentration was reduced to approximately 5 $mg \cdot L^{-1}$ corresponding to approximately 700 ppm throughout the continuous capture.

MCC offers several advantages for process intensification, like an increased overall productivity due to parallel processing of the USP and capture step as well as optimized resin utilization due to the sequential interconnection of columns [59,60]. However, the specific productivity in the presented work was limited due to the preceding USP, resulting in a fixed permeate flow rate as well as the usage of prepacked columns with a fixed resin volume. The application of smaller columns with only 1 cm bed height at the same diameter would increase the estimated specific productivity for the 3-column MCC process by 30% to 378 $g_{IgG}^{-1} \cdot L_{resin}^{-1} \cdot d^{-1}$.

In this study, a feasible approach to directly connect a perfusion bioreactor to the MCC with an easy to adopt control strategy (see section 3.5) was demonstrated for different VCC, perfusion rates and MCC systems. Further process optimization could be achieved by a dynamic loading of the chromatography unit to ensure optimal resin utilization, yield and robustness of the process. Such adaptive control strategies are for instance implemented in the BioSMB software. For this, an external sensor (such as UV, NIR or Raman) is used to determine product

breakthrough, resulting in an appropriate column switch during the MCC capture.

The high purity achieved by the MCC in combination with the perfusion process could offer the possibility of eliminating the need for a second polishing step, as it is often performed in platform DSP processes for IgG manufacturing [61], for further process intensification.

### 3.7. FBC approach for cell clarification

As an alternative scenario to perfusion processes with continuous product harvesting, other intensified processes, such as concentrated fed-batch processes, are harvested after the cultivation phase, offering the advantages of simplified process control and reduced media consumption. However, such processes require the clarification of the CCF from large amounts of biomass.

In contrast to other clarification technologies, FBC systems have been shown to be suitable for biomass removal from CCF with $100 \cdot 10^6$ $cells \cdot mL^{-1}$, achieving particular high product recoveries [35]. Therefore, the CCF throughput and the harvest dilution rate were modeled as well as experimentally determined to investigate the effect of varying cell concentrations on the clarification process.

With an increased TCC in the feed, the calculated CCF throughput (Eq. (11)) decreased from 14.8 $L \cdot h^{-1}$ at $20 \cdot 10^6$ $cells \cdot mL^{-1}$ to 5.4 $L \cdot h^{-1}$ at $110 \cdot 10^6$ $cells \cdot mL^{-1}$, while the calculated harvest dilution rates (Eq. (12)) increased linearly from 1.3 to 2.8 (Fig. 13). A similar trend was confirmed by the experimental results.

The dependence of the results on the TCC can be explained by the fact that a higher TCC requires more FBC cycles to process a defined CCF volume, since the FBC chambers have a fixed cell loading capacity. In each cycle, the fluidized bed is washed with buffer, which on the one hand increases harvest dilution and decreases CCF throughput (Fig. 13), on the other hand leads to high recoveries of IgG independent of the TCC (Fig. 14).

The dilution of harvest might be compensated by a higher throughput in the further purification process steps, for example by using MCC as described in section 3.6. In addition, for CCF with a high viability and therefore less cell fragments, higher throughputs can be achieved by increasing the applied CCF loading flow rate of 400 $mL \cdot min^{-1}$ up to 1200 $mL \cdot min^{-1}$ [35].

All tested CCF with a broad TCC range from $20 \cdot 10^6$ to $110 \cdot 10^6$ $cells \cdot mL^{-1}$ were clarified with the FBC using the same parameter setting confirming a high robustness of the approach. Furthermore, the process performance of the FBC system was successfully modeled for different TCC. Therefore, this model can be used to harmonize the clarification process with the subsequent downstream processing.

After FBC clarification, a small filter step was used to generate a sterile IgG harvest pool. The obtained turbidities were slightly different what is likely due to the different cell viabilities (60 to 97%) of the CCF and thus various compositions of host cell impurities, such as cell debris (Fig. 14). However, in all final harvest pools, low turbidities ($< 13$ NTU) were achieved, demonstrating the suitability of the clarification approach for various intensified process streams.

Moreover, for the entire clarification operation, high IgG recoveries of $97 \pm 5\%$ were achieved (Fig. 14). In some cases, the recovery was slightly higher than 100%, due to an ongoing release of IgG by the cells during the clarification process. To date, there are no reports of other technologies that are capable of clarifying CCF with TCC of more than $100 \cdot 10^6$ $cells \cdot mL^{-1}$ and achieve such high recoveries.

In addition, the complete equipment in contact with IgG consisted of single-use components that can be connected under sterile conditions. This saves time and costs for cleaning as well as validation [62] and thus contributes to an intensification of the clarification process.

Therefore, the presented clarification approach using a FBC followed by a small filter step has a high potential to be used as a clarification platform for intensified processes.

## 4. Conclusions

Process intensification is the natural next step for the biopharma industry as it transitions from a focus on global blockbuster drugs to a diversified portfolio of specialized biopharmaceuticals with more focused markets and dynamic demand. This maturation requires robust and flexible manufacturing strategies that fit variable market demand, reduce production costs and support increasingly regionalized supply needs.

The BPOG industry roadmap [26] has outlined several areas where changes are needed to achieve this goal, including switching to intensified process variants, developing improved cell lines, shorter seed trains, and robust HCD harvest technologies for the USP part. For the DSP, moving towards continuous operation, improved resin usage, or facile inline dilution need to be addressed – promising to ideally curb product purification timelines from several days to one. All these improvements target implementation of large-scale processes. In addition, tools are needed that facilitate fast and reliable setup of robust intensified processes if these more economic processes are to become the new norm.

The examples presented in this work and elsewhere illustrate that such tools are available now – be it for selecting the right production clones for intensified processes or for quickly testing different media and process conditions for alternatives like perfusion or HIFB. By using parallel mini-bioreactor systems like the Ambr®, this can be achieved quickly and at early stages of the development process without the risk of adding lengthy optimization phases that impair speed to clinic for a new molecule. The latter is, of course, only possible if the scale-down models used for process setup and optimization are predictive and the data reported here and by others demonstrate that this is achievable.

Significant advances have been made in critical steps like the robust coupling of upstream process and in harvesting technologies for high cell densities, e.g., the Ksep® centrifuge application described above. Multi-column chromatography can operate robustly with perfusion processes, supporting improved resin usage and cost reductions. Moreover, widespread use of single-use technologies in the bioprocess in general provide more flexibility in process setups and facility expansion, reduce the risk of batch failures due to contamination, simplify multi-product operation in the same facility, and reduce required capital spend and environmental impact [4,8,63].

Still, several challenges remain: more work is needed, e.g., on demonstrating scalability of new harvest technologies, on concentrated media formulations supporting HCD cultures, or on frozen high cell density bags to further curb seed train duration. On the downstream side, more experience with reliable inline dilution solutions, efficient viral clearance and continuous DSP trains is desirable. Plus, major single-use vendors have recently initiated programs to address the topic of SU waste reduction and improving sustainability [63].

Last not least, process intensification not only comes with technical challenges, but also requires adaptations in drug regulatory frameworks, for example, concerning seemingly simple concepts like batch definition for continuous processes. The regulatory agencies have recognized this need and special task forces like the Emerging Technologies Team at the FDA or the innovation task force at EMA [28] have been formed to address this topic and actively support this transition.

Attractively, different elements of process intensification like optimized clone selection, N-1 perfusion or an intensified N stage can be combined and offer synergistic gains like the ten-fold rise in volumetric productivity achieved by switching from FB to perfusion operation as demonstrated in this work. Also, simple intensification steps like HIFB can be implemented with limited financial and organizational effort in existing facilities. In contrast, perfusion setups provide higher potential for COGs reduction, reduced facility footprints, and required capital spend, but demand careful planning and come with higher operational cost, e.g. for media consumption [8]. In this way, the best intensification approach can be chosen for the application scenario at hand.

With these changes underway, process intensification provides increased momentum to advance the biopharma industry to the next level of process maturity and economical manufacturing. As it looks, process intensification is set to play an important role in delivering flexible and cost-effective biopharma processes, which help to meet the diversifying medical needs and improve drug accessibility for patients in the coming years.

## 6. Publishing model

Open Access.

## CRediT authorship contribution statement

**Dirk Müller:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Lukas Klein:** Data curation, Software, Supervision, Methodology, Investigation, Visualization. **Johannes Lemke:** Data curation, Software, Supervision, Methodology, Investigation, Visualization, Writing – review & editing. **Markus Schulze:** Data curation, Software, Supervision, Methodology, Investigation, Visualization, Writing – review & editing. **Thomas Kruse:** Data curation, Software, Supervision, Methodology, Investigation, Visualization, Writing – review & editing, Formal analysis. **Martin Saballus:** Data curation, Software, Supervision, Methodology, Investigation, Visualization, Writing – review & editing, Formal analysis, Writing – review & editing. **Jens Matuszczyk:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. **Markus Kampmann:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing. **Gerben Zijlstra:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

## Declaration of Competing Interest

All authors are employees of Sartorius, a company selling equipment and developing solutions for the biopharma industry, which is active in the field of process intensification.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cep.2021.108727.

## References

[1] S.M. Paul, D.S. Mytelka, C.T. Dunwiddie, C.C. Persinger, B.H. Munos, S. R. Lindborg, A.L. Schacht, How to improve r&d productivity: the pharmaceutical industry's grand challenge, Nat. Rev. Drug Discov. 9 (2010) 203–214, https://doi. org/10.1038/nrd3078.

[2] S.S. Farid, M. Baron, C. Stamatis, W. Nie, J. Coffman, Benchmarking biopharmaceutical process development and manufacturing cost contributions to R&D, MAbs 12 (2020), 1754999, https://doi.org/10.1080/ 19420862.2020.1754999.

[3] Deloitte L.L.P., A new future for R&D?: Measuring the return from pharmaceutical innovation 2017, 2017. https://www2.deloitte.com/content/dam/Deloitte/globa

l/Documents/Life-Sciences-Health-Care/deloitte-uk-rnd-pharmaceuticals-roi
-methodology-2017.pdf.

[4] P. Gupta, M. Monge, A. Boulais, N. Chopra, N. Hutchinson, Single-Use process platforms for responsive and cost-effective manufacturing, in: R. Eibl, D. Eibl (Eds.), Single-Use Technology in Biopharmaceutical Manufacture, John Wiley & Sons, Inc., 2019 first ed.

[5] D.M. Ecker, J. Crawford, P. Seymour, The therapeutic monoclonal antibody product market, 2020. https://bioprocessintl.com/business/economics/the-market-for-therapeutic-mab-products/.

[6] M.-F. Clincke, C. Mölleryd, Y. Zhang, E. Lindskog, K. Walsh, V. Chotteau, Very high density of cho cells in perfusion by atf or tff in wave bioreactor™. part I. effect of the cell density on the process, Biotechnol. Prog. 29 (2013) 754–767, https://doi.org/10.1002/btpr.1704.

[7] M.-F. Clincke, C. Mölleryd, P.K. Samani, E. Lindskog, E. Fäldt, K. Walsh, V. Chotteau, Very high density of chinese hamster ovary cells in perfusion by alternating tangential flow or tangential flow filtration in wave bioreactor™—Part II: applications for antibody production and cryopreservation, Biotechnol. Prog. 29 (2013) 768–777, https://doi.org/10.1002/btpr.1703.

[8] D. Pollard, M. Brower, Y. Abe, A. Lopes, A. Sinclair, Standardized economic cost modeling for next-generation MAB production, Bioprocess Int. (2016).

[9] L. Stepper, F.A. Filser, S. Fischer, J. Schaub, I. Gorr, R. Voges, Pre-stage perfusion and ultra-high seeding cell density in cho fed-batch culture: a case study for process intensification guided by systems biotechnology, Bioprocess Biosyst. Eng. (2020). https://doi.org/10.1007/s00449-020-02337-1.

[10] B. Whitford, Bioprocess intensification: aspirations and achievements, BioTechniques 69 (2020) 84–87, https://doi.org/10.2144/btn-2020-0072.

[11] T. Williams, O. Goodyear, L. Davies, C. Knevelman, M. Bransby, J. Miskin, K. Mitrophanous, Lentiviral vector manufacturing process enhancement utilizing TFDF™ technology, Cell and Gene Therapy Insights 6 (2020) 455–467, https://doi.org/10.18609/cgti.2020.053.

[12] P.R. Joshi, L. Cervera, I. Ahmed, O. Kondratov, S. Zolotukhin, J. Schrag, P. S. Chahal, A.A. Kamen, Achieving high-yield production of functional AAV5 gene delivery vectors in a platform cell-one baculovirus system, Mol. Ther. - Methods Clin. Dev. 13 (2019) 279–289, https://doi.org/10.1016/j.omtm.2019.02.003.

[13] F. Masri, E. Cheeseman, S. Ansorge, Viral vector manufacturing: how to address current and future demands? Cell & Gene Therapy Insights (2019) 949–970, https://doi.org/10.18609/cgti.2019.104.

[14] O.-W. Merten, Present situation of viral vector manufacturing and ways to overcome potential barriers in view of the routine large scale production and use of viral vectors, CTBEB 7 (2017), https://doi.org/10.19080/CTBEB.2017.07.555704.

[15] J.-M. Bielser, M. Wolf, J. Souquet, H. Broly, M. Morbidelli, Perfusion mammalian cell culture for recombinant protein manufacturing - a critical review, Biotechnol. Adv. 36 (2018) 1328–1340, https://doi.org/10.1016/j.biotechadv.2018.04.011.

[16] D. Voisard, F. Meuwly, P.-A. Ruffieux, G. Baer, A. Kadouri, Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells, Biotechnol. Bioeng. 82 (2003) 751–765, https://doi.org/10.1002/bit.10629.

[17] M.S. Croughan, K.B. Konstantinov, C. Cooney, The future of industrial bioprocessing: batch or continuous? Biotechnol. Bioeng. 112 (2015) 648–651, https://doi.org/10.1002/bit.25529.

[18] F. Steinebach, T. Müller-Späth, M. Morbidelli, Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production, Biotechnol. J. 11 (2016) 1126–1141, https://doi.org/10.1002/biot.201500354.

[19] R. Douwenga, XD® technology, 2011. https://bioprocessintl.com/2011/xd-technology-320208/(accessed 29 March 2021).

[20] W.C. Yang, J. Lu, C. Kwiatkowski, H. Yuan, R. Kshirsagar, T. Ryll, Y.-M. Huang, Perfusion seed cultures improve biopharmaceutical fed-batch production capacity and product quality, Biotechnol. Prog. 30 (2014) 616–625, https://doi.org/10.1002/btpr.1884.

[21] I. Padawer, W.L.W. Ling, Y. Bai, Case study: an accelerated 8-day monoclonal antibody production process based on high seeding densities, Biotechnol. Prog. 29 (2013) 829–832, https://doi.org/10.1002/btpr.1719.

[22] D. Stanton, Up titer: wuXi breaks 50 g/L with continuous cho process, 2019. https://bioprocessintl.com/bioprocess-insider/upstream-downstream-processing/up-titer-wuxi-breaks-50g-l-with-continuous-cho-process/ (accessed 29 March 2021).

[23] J. Xu, M. Rehmann, M. Xu, S. Zheng, C. Hill, Q. He, M. Borys, Z.J. Li, Development of an intensified fed-batch production platform with doubled titers using N-1 perfusion seed for cell culture manufacturing, Bioresour. 7 (2020), https://doi.org/10.1186/s40643-020-00304-y.

[24] J. Xu, X. Xu, C. Huang, J. Angelo, C.L. Oliveira, M. Xu, X. Xu, D. Temel, J. Ding, S. Ghose, M.C. Borys, Z.J. Li, Biomanufacturing evolution from conventional to intensified processes for productivity improvement: a case study, MAbs 12 (2020), 1770669, https://doi.org/10.1080/19420862.2020.1770669.

[25] J.M. Woodgate, Chapter 37 - Perfusion N-1 culture: opportunities for process intensification, in: G. Jagschies, E. Lindskog, K. Łącki, P. Galliher (Eds.), Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes, Elsevier, 2018.

[26] D. Sawyer, K. Sanderson, R. Lu, T. Daszkowski, E. Clark, P. McDuff, J. Astrom, C. Heffemann, L. Duffy, S. Poole, T. Ryll, P. Sheehy, D. Strachan, J. Souquet, D. Beattie, D. Pollard, O. Stauch, P. Bezy, T. Sauer, L. Boettcher, C. Simpson, J. Dakin, S. Pitt, A. Boyle, BioPhorum Operations Group: Biomanufacturing Technology Roadmap (2017) 1–48, https://www.biophorum.com/wp-content/uploads/bp_downloads/Overview-1.pdf.

[27] R. Orozco, S. Godfrey, J. Koury, A. Kwong, C. Capron, Z. Kyser, M. Brown, E. Zimmerman, D. Ogawa, J. Coffman, J. Salm, M. Jankowski, R. Kottmeier,

M. Stork, R. Fahrner, iSKID: from integrated pilot scale runs to GMP implementation approach, in: V. Warikoo, A. Jungbauer, J. Coffman, J. Walther (Eds.), Engineering Conferences International: Continuous Biomanufacturing IV, 2019. October 6-10, 2019.

[28] A.C. Fisher, M.-H. Kamga, C. Agarabi, K. Brorson, S.L. Lee, S. Yoon, The current scientific and regulatory landscape in advancing integrated continuous biopharmaceutical manufacturing, Trends Biotechnol. (Trends in Biotechnology) 37 (2019) 253–267, https://doi.org/10.1016/j.tibtech.2018.08.008.

[29] V. Warikoo, R. Godawat, K. Brower, S. Jain, D. Cummings, E. Simons, T. Johnson, J. Walther, M. Yu, B. Wright, J. McLarty, K.P. Karey, C. Hwang, W. Zhou, F. Riske, K. Konstantinov, Integrated continuous production of recombinant therapeutic proteins, Biotechnol. Bioeng. 109 (2012) 3018–3029, https://doi.org/10.1002/bit.24584.

[30] R. Godawat, K. Konstantinov, M. Rohani, V. Warikoo, End-to-end integrated fully continuous production of recombinant monoclonal antibodies, J. Biotechnol. 213 (2015) 13–19, https://doi.org/10.1016/j.jbiotec.2015.06.393.

[31] J.M. Woodgate, Chapter 37 - Perfusion N-1 culture—opportunities for process intensification. Biopharmaceutical Processing, 2018, pp. 755–768.

[32] S. Ruhl, A. Stacey, E. Lam, C. Villemant, A. Rathod, A. Stefan, C. Zahnow, G. Bremer, A. Tait, J. Matuszczyk, U. Husemann, G. Greller, Bioreactor scaling thought new - from 5 to 2000 L with utility functions, 2017. https://bmcproc.biomedcentral.com/track/pdf/10.1186/s12919-018-0097-x.pdf (accessed 16 May 2021).

[33] S. Ruhl, N. de Almeida, M. Carpio, J. Rupprecht, G. Greller, J.-C. Matuszczyk, A rapid, low-risk approach for process transfer of biologics from development to manufacturing scale, 2020. https://bioprocessintl.com/sponsored-content/biostat-str-bioreactors-a-rapid-low-risk-approach-process-transfer-of-biologics-from-development-to-manufacturing-scale/.

[34] S. Metze, S. Ruhl, G. Greller, C. Grimm, J. Scholz, Monitoring online biomass with a capacitance sensor during scale-up of industrially relevant cho cell culture fed-batch processes in single-use bioreactors, Bioprocess Biosyst. Eng. (2019) 43, https://doi.org/10.1007/s00449-019-02216-4.

[35] M. Saballus, L. Nisser, M. Kampmann, G. Greller, A novel clarification approach for intensified monoclonal antibody processes with 100 million cells/mL using a single-use fluidized bed centrifuge, Biochem. Eng. J. 167 (2021), 107887, https://doi.org/10.1016/j.bej.2020.107887.

[36] M. Bisschops, M. Brower, The impact of continuous multicolumn chromatography on biomanufacturing efficiency, Pharmaceutical Bioprocessing 1 (2013) 361–372, https://doi.org/10.4155/pbp.13.46.

[37] V. Girard, N.-J. Hilbold, C.K. Ng, L. Pegon, W. Chahim, F. Rousset, V. Monchois, Large-scale monoclonal antibody purification by continuous chromatography, from process design to scale-up, J. Biotechnol. 213 (2015) 65–73, https://doi.org/10.1016/j.jbiotec.2015.04.026.

[38] Marc Bisschops, BioSMBTM Technology: Continuous Countercurrent Chromatography Enabling a Fully Disposable Process, in: G. Subramanian (Ed.), Biopharmaceutical Production Technology Volume 1 & Volume 2, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, 2012, pp. 769–791.

[39] M. Bausch, C. Schultheiss, J.B. Sieck, Recommendations for comparison of productivity between fed-batch and perfusion processes, Biotechnol. J. 14 (2019), 1700721, https://doi.org/10.1002/biot.201700721.

[40] S. Mehta, Automated single-use centrifugation solution for diverse biomanufacturing process, in: G. Subramanian (Ed.), Continuous Processing in Pharmaceutical Manufacturing, Wiley-VCH Verlag GmbH & Co, KGaA, Weinheim, 2015, pp. 385–400.

[41] C.E. Hogwood, A.S. Tait, N. Koloteva-Levine, D.G. Bracewell, C.M. Smales, The dynamics of the cho host cell protein profile during clarification and protein a capture in a platform antibody purification process, Biotechnol. Bioeng. 110 (2013) 240–251, https://doi.org/10.1002/bit.24607.

[42] A. Castan, P. Schulz, T. Wenger, S. Fischer, Cell line development, in: G. Jagschies, E. Lindskog, K. Łącki, P. Galliher (Eds.), Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes, Elsevier, 2018, pp. 131–146.

[43] M.J. de Jesus, P. Girard, M. Bourgeois, G. Baumgartner, B. Jacko, H. Amstutz, F. M. Wurm, TubeSpin satellites: a fast track approach for process development with animal cells using shaking technology, Biochem. Eng. J. 17 (2004) 217–223, https://doi.org/10.1016/S1369-703X(03)00180-3.

[44] S. Kreyhe, R. Stahn, K. Nawrath, A. Danielczyk, S. Goletz, GlycoExpress: a toolbox for the high yield production of glycooptimized fully human biopharmaceuticals in perfusion bioreactors atdifferent scales, in: C. Goudar, S. Farid, C. Hwang, K. Lacki (Eds.), Engineering Conferences International: Integrated Continuous Biomanufacturing II, 2015, p. 110.

[45] O. Popp, D. Müller, K. Didzus, W. Paul, F. Lipsmeier, F. Kirchner, J. Niklas, K. Mauch, N. Beaucamp, A hybrid approach identifies metabolic signatures of high-producers for chinese hamster ovary clone selection and process optimization, Biotechnol. Bioeng. 113 (2016) 2005–2019, https://doi.org/10.1002/bit.25958.

[46] A.J. Porter, A.J. Racher, R. Preziosi, A.J. Dickson, Strategies for selecting recombinant cho cell lines for cGMP manufacturing: improving the efficiency of cell line generation, Biotechnol. Prog. 26 (2010) 1455–1464, https://doi.org/10.1002/btpr.443.

[47] H.-P. Meyer, W. Minas, D. Schmidhalter, Industrial-Scale fermentation, in: C. Wittmann, J.C. Liao (Eds.), Industrial Biotechnology: Products and Processes, Wiley-VCH Verlag GmbH & Co. KGaA, 2016, p. 25.

[48] A. Yongky, J. Xu, J. Tian, C. Oliveira, J. Zhao, K. McFarland, M.C. Borys, Z.J. Li, Process intensification in fed-batch production bioreactors using non-perfusion seed cultures, MAbs 11 (2019) 1502–1514, https://doi.org/10.1080/19420862.2019.1652075.

[49] C. Chen, H.E. Wong, C.T. Goudar, Upstream process intensification and continuous manufacturing, Curr. Opin. Chem. Eng. 22 (2018) 191–198, https://doi.org/10.1016/j.coche.2018.10.006.

[50] A.G. Vulto, O.A. Jaquez, The process defines the product: what really matters in biosimilar design and production? Rheumatology (Oxford) 56 (2017) https://doi.org/10.1093/rheumatology/kex278 iv14-iv29.

[51] D. Brühlmann, M. Sokolov, A. Butté, M. Sauer, J. Hemberger, J. Souquet, H. Broly, M. Jordan, Parallel experimental design and multivariate analysis provides efficient screening of cell culture media supplements to improve biosimilar product quality, Biotechnol. Bioeng. 114 (2017) 1448–1458, https://doi.org/10.1002/bit.26269.

[52] Z. Weng, J. Jin, C. Shao, H. Li, Reduction of charge variants by cho cell culture process optimization, Cytotechnology 72 (2020) 259–269, https://doi.org/10.1007/s10616-020-00375-x.

[53] S. Janoschek, M. Schulze, G. Zijlstra, G. Greller, J. Matuszczyk, A protocol to transfer a fed-batch platform process into semi-perfusion mode: the benefit of automated small-scale bioreactors compared to shake flasks as scale-down model, Biotechnol. Prog. (2018), https://doi.org/10.1002/btpr.2757.

[54] K. Konstantinov, C. Goudar, M. Ng, R. Meneses, J. Thrift, S. Chuppa, C. Matanguihan, J. Michaels, D. Naveh, The "Push-to-Low" approach for optimization of high-density perfusion cultures of animal cells, in: W.-S. Hu (Ed.), Cell Culture Engineering, Springer Berlin Heidelberg, Berlin, Heidelberg, 2006, pp. 75–98.

[55] M.K. Wolf, A. Pechlaner, V. Lorenz, D.J. Karst, J. Souquet, H. Broly, M. Morbidelli, A two-step procedure for the design of perfusion bioreactors, Biochem. Eng. J. 151 (2019), 107295, https://doi.org/10.1016/j.bej.2019.107295.

[56] M. Gagnon, S. Nagre, W. Wang, G.W. Hiller, Shift to high-intensity, low-volume perfusion cell culture enabling a continuous, integrated bioprocess, Biotechnol. Prog. 34 (2018) 1472–1481, https://doi.org/10.1002/btpr.2723.

[57] S. Chuppa, Y.-S. Tsai, S. Yoon, S. Shackleford, C. Rozales, R. Bhat, G. Tsay, C. Matanguihan, K. Konstantinov, D. Naveh, Fermentor temperature as a tool for control of high-density perfusion cultures of mammalian cells, Biotechnol. Bioeng. 55 (1997) 328–338, https://doi.org/10.1002/(SICI)1097-0290(19970720)55:2<328:AID-BIT10>3.0.CO;2-D.

[58] M.K.F. Wolf, A. Closet, M. Bzowska, J.-.M. Bielser, J. Souquet, H. Broly, M. Morbidelli, Improved performance in mammalian cell perfusion cultures by growth inhibition, Biotechnol. J. 14 (2019), e1700722, https://doi.org/10.1002/biot.201700722.

[59] D. Baur, M. Angarita, T. Müller-Späth, F. Steinebach, M. Morbidelli, Comparison of batch and continuous multi-column protein a capture processes by optimal design, Biotechnol. J. 11 (2016) 920–931, https://doi.org/10.1002/biot.201500481.

[60] M. Pagkaliwangan, J. Hummel, X. Gjoka, M. Bisschops, M. Schofield, Optimized continuous multicolumn chromatography enables increased productivities and cost savings by employing more columns, Biotechnol. J. 14 (2019), 1800179, https://doi.org/10.1002/biot.201800179.

[61] A.A. Shukla, L.S. Wolfe, S.S. Mostafa, C. Norman, Evolving trends in mAb production processes, Bioeng. Transl. Med. (Bioengineering and Translational Medicine) 2 (2017) 58–69, https://doi.org/10.1002/btm2.10061.

[62] B. Manser, M. Glenz, M. Bisschops, Single-Use downstream processing for biopharmaceuticals, in: R. Eibl, D. Eibl (Eds.), Single-Use Technology in Biopharmaceutical Manufacture, John Wiley & Sons, Inc., 2019, pp. 117–126, first ed.

[63] J. Markarian, sustainability in disposal of single-use systems, BioPharm International 32 (2019) 19.

42

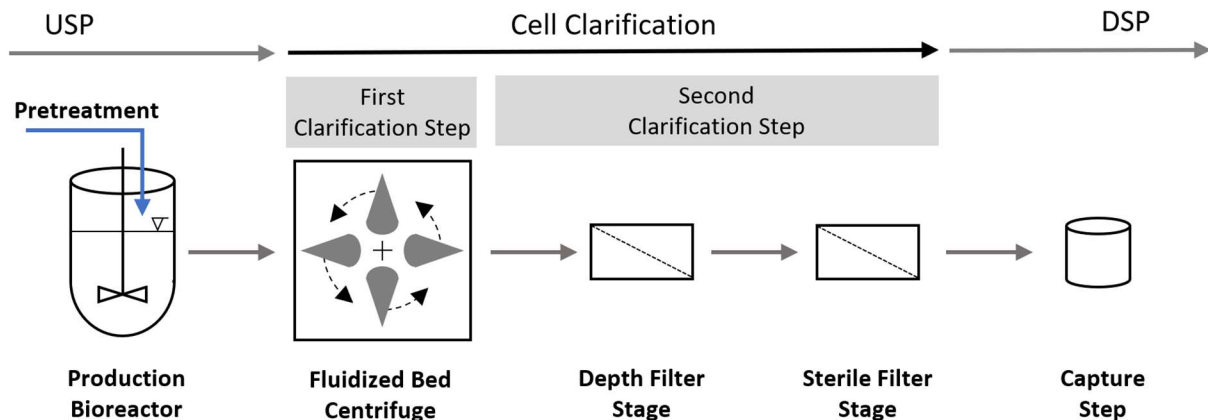## 4.3 Intensification of Clarification by Pretreatments



**Figure 5.** Overview of the clarification process using cell culture pretreatment approaches to intensify the subsequent fluidized bed centrifugation and filtration steps

In the previous chapter, the suitability of an FBC-based platform approach for cell clarification was demonstrated. However, it was also found that cell broths with low viability (< 90 %), in some cases achieved lower filter capacities in the second clarification step compared to capacities of ≈ 150 L/m² at viabilities > 90 % (chapter 4.1). One explanation for this effect could be that low cell viabilities are accompanied by a higher proportion of debris from already apoptotic and lysed cells. These cell debris might probably not be retained in the fluidized bed of the centrifuge and thus are removed by the subsequent filter stages which increases the ability to block the filters. In addition, these cell cultures tend to have higher levels of dissolved impurities, which place an additional burden on the subsequent DSP.

To overcome the limitations in the clarification of low viable cell cultures, flocculation and precipitation pretreatment approaches were investigated for their potential to intensify the developed cell clarification process platform (Figure 5). For this purpose, the effect of different treatments on the particle size distribution and on the stability of the low viable cell broth was examined. Furthermore, the possibility of additionally remove dissolved process related impurities by the pretreatments was determined. Another criterion that was also taken into account for selection of suitable additives was the effect on the product quality indicated by the aggregation level and the glycan profile of mAb.

As a result of an additive screening, low pH precipitation by 2 M acetic acid and cationic flocculation by 0.75 g/L pDADMAC were identified as promising pretreatments. As observed by a particle size analysis, the pH treatment caused formation of small impurity aggregates in a sub-micrometer diameter range whereas the cells were not aggregated. In contrast, the flocculation treatment caused formation of large cell aggregates with an average diameter of approximately 170 μm compared to an

average particle size of 21 µm of an untreated reference. In both approaches, the treated cell broths were stable for at least one hour, what allows their application in combination with FBC clarification. However, both pretreatments showed hardly any effect on the FBC step, but enhanced the subsequent filtration step, resulting in an increase in filter capacities by precipitation to more than 200 L/m² and by flocculation to more than 400 L/m². Further advantages of both approaches were, that more than 90 % of the dissolved DNA impurities were removed and the harvest turbidites were reduced below 3.1 NTU compared to approximately 6.5 NTU achieved in an untreated reference process. In addition, mAb glycosylation pattern and aggregate formation were studied in which only minor effects of the treatments on these critical quality attributes were found. All tested cell clarification approaches achieved overall mAb recoveries of ≈ 90 % and above.

To conclude, this part of the thesis again proves that fluidized bed centrifugation followed by a small filtration step is suitable to clarify various cell broth characteristics. Additional pretreatment of the cell broth with suitable flocculants or precipitation additives can improve the clarification process by requiring less filter area and lowering the obtained harvest turbidity, which is both particularly beneficial for cell cultures with low viability. The additional removal of DNA host cell impurities through the treatments offers high potential to simplify and reduce the size of subsequent DSP steps as well as to increase the lifetime of expensive chromatography materials in the capture step. Even though the positive effects of pretreatments beyond the cell clarification still need to be proven, the results of this chapter provide a basis for intensification of the clarification operation and the subsequent DSP of mAb.
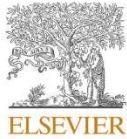
# Fluidized bed centrifugation of precipitated and flocculated cell cultures: An intensified clarification approach for monoclonal antibodies

Martin Saballus [*], Markus Kampmann

*Corporate Research, Sartorius Stedim Biotech GmbH, August-Spindler-Str. 11, 37079 Göttingen, Germany*

ABSTRACT

Precipitation and flocculation pretreatments promise improved clarification of cell culture fluids (CCF) to intensify the production of monoclonal antibodies (mAb). However, such pretreatments pose the risks to alter the mAb and damage cells. This can be additionally exacerbated by the subsequent clarification process, for example by high shear forces during disk stack centrifugation, resulting in a release of host cell impurities. To overcome these limitations and enhance the clarification particularly of cell cultures with low viability and thus a high level of impurities, this study investigated low-pH precipitation and cationic polymer flocculation, each in combination with a mild fluidized bed centrifuge (FBC) separation and a subsequent filtration step. Therefore, low-viable CCFs were pretreated and characterized to investigate the effects of additives on CCF composition and stability. In clarification experiments, both pretreatments achieved similar FBC throughput compared to an untreated reference but increased the maximum filter throughput up to four times. Furthermore, high mAb recoveries ($>$ 91%), low turbidities ($<$ 3.1 NTU) and high DNA removals ($>$ 91%) were achieved. Similar glycan profiles and dimer ratios suggest consistent mAb quality. These findings have a great potential to intensify mAb downstream processing with both CCF pretreatments using a FBC clarification approach.

## 1. Introduction

Intensified manufacturing of monoclonal antibodies (mAb) is driven by an increasing number of approved mAb-based biopharmaceuticals and the need to provide safe and less-expensive treatments for a growing patient population (Kelley, 2020; Lu et al., 2020). Although there are efforts to implement continuous manufacturing (Yang et al., 2019), the majority of industrial mAb manufacturing platforms use established fed-batch (FB) cultivation processes due to their high flexibility, lower investment costs and simplified process control (Papathanasiou and Kontoravdi, 2020; Chen et al., 2018).

For an efficient production of extracellular mAb, FB processes have been enhanced over the last decade by improved media and mammalian production cell lines, like Chinese hamster ovary (CHO) cells, to cultivate them in high cell density (HCD) broths with peak cell concentrations higher than 20 million cells/mL (Kshirsagar and Ryll, 2018). To achieve high mAb titers of more than 10 g/L in modern FB processes

(Pohlscheidt et al., 2018), the bioreactor with the cell culture fluid (CCF) is usually harvested late in the cell death phase where the viability and the total amount of cells already have started to decrease (Pieracci et al., 2018). In this phase, the cells become more sensitive to shear stress, what further increases shear-induced rupture of cells during gassing and mixing in the bioreactor (Singh and Chollangi, 2017), before they disintegrate into apoptotic bodies. As a result, impurities, like the most abundant DNA and host cell protein (HCP) impurities as well as cell debris, are released and accumulated in the CCF (Singh and Chollangi, 2017).

Therefore, downstream processing (DSP) of mAb includes the removal of cells and cell debris in the primary clarification step as well as the purification from dissolved impurities in several subsequent chromatographic steps to ensure product safety. Established clarification processes for FB cultivations apply two serial steps: For the first step, depth filtration or disc stack centrifugation is used. In the second clarification step, a depth filter followed by a sterile filter is applied for a

complete removal of solids in both scenarios (Pieracci et al., 2018).

However, it has been shown that the clarification of CCF with increased cell concentrations challenges both established steps due to a limited biomass removal capacity of filters and a low mAb recovery at disc stack centrifugation (Tomic et al., 2015; Iammarino et al., 2007). In addition, high shear rates during the separation process can damage the cells and release impurities (Wilson et al., 2019). As a result, released cell debris in a submicron range cause faster blocking of filters (Iammarino et al., 2007), whereas high levels of dissolved impurities increase the pressure on the subsequent chromatographic purification steps (Wilson et al., 2019). Therefore, the primary clarification step has been identified as one of the major bottlenecks in intensified FB processes (Roush and Lu, 2008).

A promising approach to overcome these challenges is a fluidized bed centrifuge (FBC), which can be used as an alternative first clarification step (Ko and Bhatia, 2012). Compared to processes using stainless steel disc stack centrifuges, all FBC components that come into contact with the liquid streams are single-use (SU) materials, eliminating the need for cleaning and validation to reduce set-up times and prevent cross-contamination between batches (Mehta, 2014). Therefore, FBC systems are particularly suitable for SU-based biomanufacturing processes where SU bioreactors with typical volumes of 2000 L are used. Here, FBC clarification can be used for example to connect discontinuous FB processes and continuous DSP, which represents a promising combination from an economic and ecological perspective (Cataldo et al., 2020). In contrast to approaches using filtration in the first clarification step, the FBC can be flexibly adapted to various CCF characteristics including HCD suspensions with more than 100 million cells/mL without the need for additional consumables (Müller et al., 2022). In addition, FBC processes enable a mild separation of cells from CCF and an almost complete recovery of product due to the ability to wash retained biomass in the fluidized bed (Saballus et al., 2021).

However, the retention of biomass in the fluidized bed depends on the process parameters of the centrifuge as well as on the sedimentation properties of biomass particles, which are affected by their density and size (Kelly et al., 2016). Large particles, such as intact CHO cells, are retained with a high efficiency in a fluidized bed, whereas small particles, such as submicron cell debris, can pass and be removed in the second clarification step using a fine grade depth filter followed by a sterile filter (Kelly et al., 2016). The capacities of these filters decrease with a higher content of small particles passing the FBC stage, as is the case with low-viable CCF, so larger filter areas have to be utilized. A promising approach to enhance FBC clarification and streamline the subsequent filtration step could be a pretreatment of the CCF, prior to the clarification.

A large number of different CCF pretreatments have been described in the scientific literature (Singh and Chollangi, 2017). Some pretreatment approaches are based on precipitation that aim for exceeding the solubility limit of dissolved impurities to enable their separation as a particulate system in the primary clarification step. By changing environmental conditions, such as the pH value and the ionic strength, entire molecule classes can be precipitated (Singh et al., 2016). For example, lowering the pH value to 5 is used in some CHO clarification processes in combination with subsequent diatomaceous earth filtration (Minow et al., 2014) or disk stack centrifugation (Richardson and Walker, 2021). Other pretreatment approaches are based on flocculation to increase size of the particles already present in a suspension, whereas dissolved impurities can also be bound on the aggregates. This can be achieved by the addition of charged additives, such as the well characterized cationic polymer poly(diallyldimethylammonium chloride) (pDADMAC) (Carvalho, 2019; Kang et al., 2013), which cover or bridge the repulsive forces between similarly charged particle surfaces, like negatively charged cell fragments (Senczuk et al., 2016).

It has been shown that both pretreatment approaches are able to improve the filterability of CCF (Burgstaller et al., 2018). Furthermore, these approaches can achieve additional impurity reduction, like the removal of DNA by several log-levels (Westoby et al., 2011), which helps to reduce the size of the subsequent purification facility. Some pretreatments may provide further advantages for the DSP, such as the inactivation of viruses (Trapp et al., 2018).

However, the harsh environmental conditions in pretreated CCF pose the risk that mAb is lost through co-aggregation or degradation and even more impurities are released through cell lysis (Singh and Chollangi, 2017). In addition, there is also the risk to alter the N-linked glycan profile of the mAb by the pretreatment additives (Gómez et al., 2018). This can lead to a loss of mAb activity as the glycan profile regulates specific binding on the constant (Fc) domain of the mAb (Irvine and Alter, 2020). Therefore, the glycan profile is one of the most important quality attributes (Papathanasiou and Kontoravdi, 2020). Another aspect that must be considered when selecting additives for CCF pretreatment is to ensure the removal of these additives in the subsequent purification process due to their often cytotoxic nature (Tomic et al., 2015).

To date, however, there are no reports about the application of pretreatments in FBC processes. Therefore, this study investigated the effect of precipitation and flocculation pretreatment in combination with a FBC and a subsequent small filter stage to develop an intensified clarification approach for CCF with low viability and thus a high contaminant load (Fig. 1). As a model of such a CCF, a CHO cell broth from a FB process for mAb production was used. For precipitation pretreatment, the pH value was reduced to 5 using acetic acid. Flocculation was induced by the addition of pDADMAC. The amount of this flocculant was optimized to generate large and compact flocs. In addition, size distributions of biomass particles were determined to assess particle formation and stability for both pretreatments.

In clarification trials the influence of both pretreatments on throughputs of the FBC step as well as the subsequent filtration step were investigated. Furthermore, product recovery, turbidity as well as DNA and HCP impurity changes were assessed. To evaluate the impact of pretreatments on the critical quality attributes, glycan profiles and dimer ratios of the clarified mAb were determined.

## 2. Materials and methods

### 2.1. Cell culture

A CHO-DG44 cell line expressing a mAb (immunoglobulin type G, IgG; isoelectric point of 8.4) was cultivated in a FB process using a SU bioreactor (Biostat STR® 200, Sartorius, Germany) and commercially available production medium with two different feeds. During the 12 days cultivation, the cell broth was stirred at 120 rpm and the temperature setpoint was 36.8 °C. The pH value was controlled to 7.1 by $CO_2$-sparging and the level of dissolved oxygen was controlled to 60% using nitrogen, air and oxygen gassing. A glucose concentration of 5 g/L was maintained by the feeding strategy. In all experiments performed, total cell concentrations (TCC) at harvest point were between 16 and 18 million cells/mL. Viabilities ranged from 67% to 91%. DNA
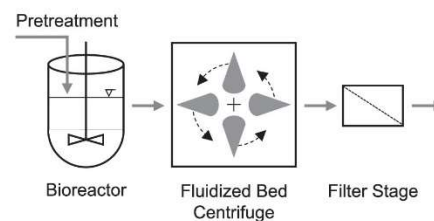


**Fig. 1.** Schematic process flow of an intensified clarification approach with pretreatment of the cell culture fluid prior to fluidized bed centrifugation followed by a filtration step.

17

concentration was in a range of 0.26–0.84 g/L and HCP concentration ranged from 0.33 g/L to 0.53 g/L. The achieved mAb concentrations were above 3 g/L.

## 2.2. Cell culture pretreatments

Prior to pretreatment of the cell broth, a required volume was transferred to a vessel and thoroughly mixed with a magnetic stirrer to avoid local overconcentration of the additives and thus cell lysis during addition. Precipitation of CCF was accomplished by acidification to pH 5 using 2 M acetic acid (Carl Roth, Germany). For flocculation, a defined pDADMAC concentration was adjusted by adding a 10% pDADMAC solution (Merck, Germany).

## 2.3. Characterization of pretreatments

To investigate aggregation and stability of the pretreated FB cell culture broths, the precipitation and flocculation approaches were initially characterized in a 100 mL beaker scale. In addition, pDADMAC flocculant concentrations of 0.25, 0.75, and 1.5 g/L were tested to determine a suitable concentration for the formation of large and stable flocs. The concentrations were chosen based on values that have previously been shown to be effective for clarifying mammalian cell cultures (Tomic et al., 2015; McNerney et al., 2015).

Samples from all tested approaches were taken and compared to the untreated CCF after 10 min and 60 min of pretreatment. The particle size distribution of the samples was determined as well as visually evaluated. As an indication of cell damage, cell viability and diameter were measured, except for flocculated CCF to prevent blocking of the micro-channels in the cell counter. Moreover, the reduction of DNA and HCP impurities was assessed. All analytical samples were measured at least in duplicates.

## 2.4. Clarification

In order to investigate the influence of pretreatments on the clarification process and the harvest quality, precipitated, flocculated and untreated CCF were clarified using a FBC system followed by a filtration step. To examine mAb recovery, turbidity reduction as well as removal of DNA and HCP impurities by the clarification operations, samples were taken before and after each process step.

To investigate the robustness, two independent series of measurements were performed with all three approaches, using CCF with a viability of 91% as well as with a low viability of 67%. The results of each clarification approach were averaged, and their standard deviation determined. For each trial, 5 L CCF was pretreated (according to section 2.2) and incubated for 10 min before starting the clarification process.

As a first clarification step, a Ksep® 400 FBC System (Sartorius, Germany) with a SU harvest clarification consumable kit was applied. Optimized centrifugal parameters for CHO cell clarification (Saballus et al., 2021) were applied with two exceptions: A lower loading flow rate of chambers (400 mL/min) was used to reduce shear stress on aggregates and to increase the capability of the system to retain small particles. Furthermore, the chamber loading volume was manually controlled due to unknown chamber capacities of the pretreated CCF's. For untreated and flocculated CCF pH 7.4 phosphate buffered saline (PBS, chemicals supplied by Carl Roth, Germany) was used as wash buffer. In the precipitation approach the fluidized bed was washed using a pH 5 citric acid buffer (48.5 mM citric acid, 103 mM $Na_2HPO_4$; chemicals purchased from Carl Roth, Germany) to ensure that aggregates were not resolved by a changed pH value. In addition, this buffer showed a similar osmolality as the CCF and had no effect on the viability of CHO cells (data not shown).

Some of the harvested liquid was used to examine the capacity of a filter arrangement as a second clarification step. The filter arrangement, selected by a screening (Saballus et al., 2021), consisted of a double layer depth filter with retention rates of 8 μm for the first and 0.8 μm for the second filter layer (DL60, 25 cm²; SartoClear® Cap, Sartorius, Germany) and a 0.2 μm sterile filter (Sartopore® 2 XLG, 4.5 cm²; SartoScale 25, Sartorius, Germany). A peristaltic pump (SciLog®, Parker, US) with inline SU pressure sensors (17525SP-10, Sartorius, Germany) in front of each filter step was used for process control. Depth filters were pre-flushed with FBC wash buffer (PBS or citric acid buffer, 50 L/m²) using a volumetric flux of 150 L/m²/h. The filtration was performed in a constant flow mode at 5 mL/min (120 L/m²/h) until a pressure of 1.3 bar was reached. To increase mAb recovery, a buffer post-flush (20 L/m²) was performed in constant pressure mode with a pressure limit of 1.5 bar. Total filter throughputs were calculated dividing the filtered volume of harvested liquid by the depth filter area. The filtrate of the pH 5 FBC harvest was neutralized to pH 7 using 1 M sodium hydroxide (Carl Roth, Germany) before samples were taken.

## 2.5. Analytics

### 2.5.1. Characterization of cell broth

Viable cell concentrations, TCC, and average cell diameters were analyzed using a Trypan Blue assay-based cell counter system (Cedex HiRes Analyzer, Roche, Germany). For turbidity measurement a nephelometer (TL2350, Hach, Germany) was used. A microscope (BX43 Upright, Olympus, Germany) was used to evaluate structure and size of flocs.

To determine volume-weighted particle size distribution and De Brouckere mean diameter, a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Panalytical, UK) was applied. Prior to the measurements, a part of the CCF was centrifuged and filtered (Sartopore2 XLM 0.1 μm, Sartorius, Germany). The particle-free liquid was used in the particle size analyzer to blank the background signal and to provide a suitable matrix for sample dilution without interfering with aggregate formation, aiming for obscuration values in range of 10–15%. During particle size measurement (20 s) diluted samples were recirculated through the measurement cell to avoid sedimentation. Sample data evaluation and Mie theory fitting was performed using the Mastersizer software (v5.60, Malvern Panalytical, UK). For the Mie scattering model refractive indices of the particle-free liquids were determined using a refractometer (Abbemat 550, Anton Paar, Germany). In addition, an absorption constant of 0.01 and a refractive index value of 1.39 for CHO cells were chosen from literature (Takagi et al., 2007).

### 2.5.2. Antibody quality and quantity

The concentration of mAb and its aggregated forms were measured using a Dionex UltiMate 3000 HPLC system (ThermoFisher Scientific, USA) with a gel column (Yarra 3 μm SEC-3000, Phenomenex, USA) for size exclusion chromatography (SEC). The running buffer contained 100 mM $Na_2SO_4$, 50 mM $Na_2HPO_4$, and 50 mM $NaH_2PO_4$ (all chemicals supplied by Carl Roth, Germany) at a pH of 6.6. Samples were diluted, filtered through a 0.2 μm syringe filter (Minisart RC4, Sartorius, Germany), and then separated in the SEC column at a flow rate of 1 mL/min. For the calibration of mAb monomer concentration, an internal standard solution of the purified mAb was used to correlate its concentration in the range between 25 mg/L and 2 g/L with the UV peak areas at 280 nm. This method was previously verified by analytical protein A HPLC with filtered CCF samples to ensure that corresponding peak areas were not interfered by impurities of the same size (data not shown). Dimers and other high molecular weight aggregates of mAb were identified in the SEC chromatograms by correlating their retention times with those of authentic samples as already shown in other publications (Carvalho, 2019; Stein et al., 2020). The proportion of mAb dimers was calculated dividing the peak area by the sum of mAb monomer and mAb dimer peak areas.

The mAb glycan profiles were analyzed using the LabChip GXII Touch instrument with ProfilerPro® Glycan Profiling assay (PerkinElmer, USA). For sample preparation, supernatants were purified by

Protein A columns (Protein A HP Spintrap™, Cytiva, USA) as well as desalted and concentrated by ultrafiltration (Vivaspin® Turbo 4, 10 kDa, Sartorius, Germany). Enzymatic splitting of N-linked glycans from the mAb, fluorescent labeling, and capillary electrophoretic separation was performed according to the manufacturer's instructions. To assess mAb glycan profiles, ratios of the major glycan types and the degree of fucosylation were determined.

### 2.5.3. Host cell impurities

To quantify DNA, a Quant-iT™ PicoGreen™ DNA assay (Thermo-Fisher Scientific, USA) with lyophilized salmon sperm DNA standards (Biomol, Germany) was used. Samples were diluted with TRIS-EDTA buffer (10 mM TRIS, 1 mM EDTA, 0.1% SDS; chemicals purchased from Carl Roth, Germany) to reach the detection range of 31.25–2000 ng/mL. For the determination of HCP content, a HCP-ELISA kit (CYG-F550, Cygnus Technologies, USA) was used according to the manufacturer's instructions. A plate reader (infinite® 200, Tecan, Switzerland) was applied in both assays. All samples were analyzed at least in duplicates.

### 2.5.4. Calculation of recovery and impurity reduction

To determine the biomass concentration in the samples, they were centrifuged at 5000 × g for 10 min (Centrisart A-14 C, Sartorius, Germany), pellets were weighed, and their mass was divided by the weight of centrifuged sample. All samples of initial CCF, pretreated CCF, post-FBC, and post-filtration were prepared the same way to ensure comparability. The densities of biomass and fluid were assumed to be similar due to their minor difference. The total masses ($m_s$) of soluble mAb, DNA, and HCP in a process volume ($V$) was calculated according to Eq. (1), taking into account the volume of biomass ($BM$) and the concentration of the soluble component ($c_s$).

$$m_s = c_s * (V - BM) \tag{1}$$

Recovery of mAb (*recovery*) was calculated according to Eq. (2), where $m_{mAb,begin}$ is the total mass of mAb at the beginning of each clarification step and $m_{mAb,end}$ is the mass of mAb in the harvest pool at the end of each clarification step.

$$recovery = \frac{m_{mAb,\ end}}{m_{mAb,\ begin}} * 100 \quad \% \tag{2}$$

Impurity reduction of DNA and HCP (*reduction*) were calculated according to Eq. (3), using the total mass of the respective impurity in the feed pool at the beginning ($m_{imp,begin}$) and in the harvest pool at the end ($m_{imp,end}$) of each clarification step.

$$reduction = \left(1 - \frac{m_{imp,\ end}}{m_{imp,\ begin}}\right) * 100 \quad \% \tag{3}$$

## 3. Results and discussion

### 3.1. Characterization of pretreatments

#### 3.1.1. Size distribution of particles

After 10 min of acid precipitation and flocculation treatment, the formation and aggregation of particles in a low viable CCF (16 million cells/mL TCC, 77% viability) were investigated based on the change in the particle size distributions (Fig. 2).

In the untreated CCF, the bulk of the particles consisted of intact single cells with a volume-weighted mean particle diameter of approximately 21 μm. An additional portion of smaller particles in a size range of 2–8 μm most likely consisted of apoptotic bodies and vesicles of ruptured cells (Kakarla et al., 2020), suggesting a high amount of impurities that are more difficult to remove compared to the cells. The measurement of the same CCF with a cell counter showed a slightly smaller cell diameter of approximately 18 μm. The deviation can be explained by different measuring methods as well as by few cell aggregates that were counted as one particle in the particle size analyzer.

After acid precipitation the fraction of smaller particles slightly decreased due to their aggregation while the diameter of cells was kept constant. These findings were confirmed by the microscopic investigation, where clusters of attached particles with sizes below the cell diameter were observed, probably consisting of aggregated cellular debris and precipitated impurities.

The flocculation treatment of CCF with a pDADMAC concentration of 0.75 g/L increased the particle sizes to a range of 50–300 μm with a mean diameter of approximately 170 μm. Microscopic examination of the flocs showed a high compactness of aggregated cells, suggesting stability during homogenization and thus also during the separation process.

Despite different aggregation mechanisms (Singh and Chollangi, 2017), both approaches shifted particle size distribution to higher diameters and were therefore further investigated as promising candidates to improve FBC clarification.
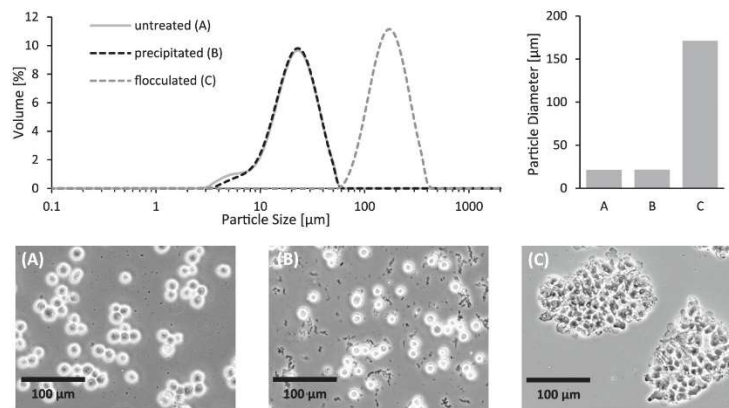


**Fig. 2.** Volume-weighted particle size distribution (top, left) of cell culture fluids that were untreated (A), precipitated by acidification to pH 5 (B), or flocculated by addition of pDADMAC (0.75 g/L). Volume-weighted (De Brouckere) particle mean diameter (top, right) and representative microscopic images (bottom) of these cell culture fluids (A, B, C).

19

### 3.1.2. Stability of precipitated cell culture fluid

The initial viability of 79% in the untreated CCF was slightly reduced by pH 5 precipitation treatment to 74% after 10 min and to 69% after 60 min (Fig. 3, A). This and an almost constant cell diameter in the range of 17.0–17.8 μm suggest that the pretreatment had only minor effect on cell stability.

Compared to the untreated reference the amount of DNA in the supernatant was 70% or 73% lower after 10 min or 60 min, respectively (Fig. 3, B). In addition, up to 30% of the HCP amount was reduced after 60 min of pretreatment.

These observations suggest that the dissolved impurities were precipitated without significant lysis of the cells by the pH 5 pretreatment for at least 60 min.

### 3.1.3. Stability of flocculated cell culture fluid

In order to determine a sufficient amount of flocculant for the generation of large and compact flocs, the effect of pDADMAC concentrations on floc size was assessed. The largest flocs with a mean diameter of approximately 170 μm were found when a pDADMAC concentration of 0.75 g/L was used (Fig. 4, A). Lower (0.25 g/L) as well as higher (1.5 g/L) flocculant concentrations resulted in smaller flocs (< 120 μm). The underlying effect for this is that, on the one hand, only small aggregates are formed if the cationic flocculant concentration is too low to cover the negative charge of the cell surfaces completely. On the other hand, too
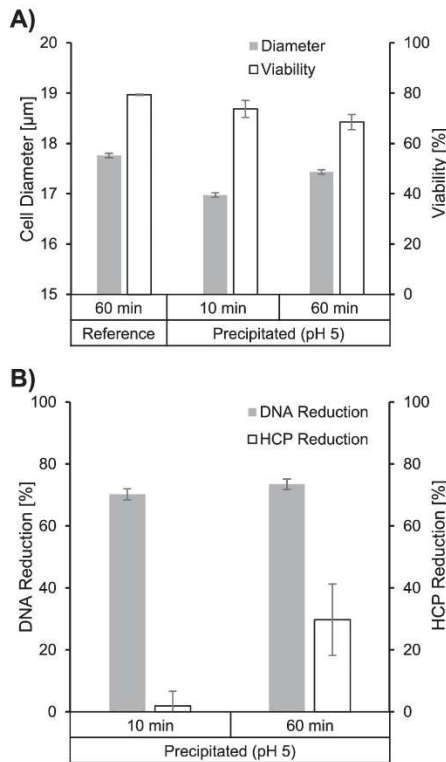


**Fig. 3.** Properties of a fed-batch cell culture fluid after 10 min and 60 min of pH 5 pretreatment compared to an untreated reference after 60 min. A) Averaged diameter and viability of cells. B) DNA and host cell protein (HCP) reduction in the supernatant compared to the untreated reference.
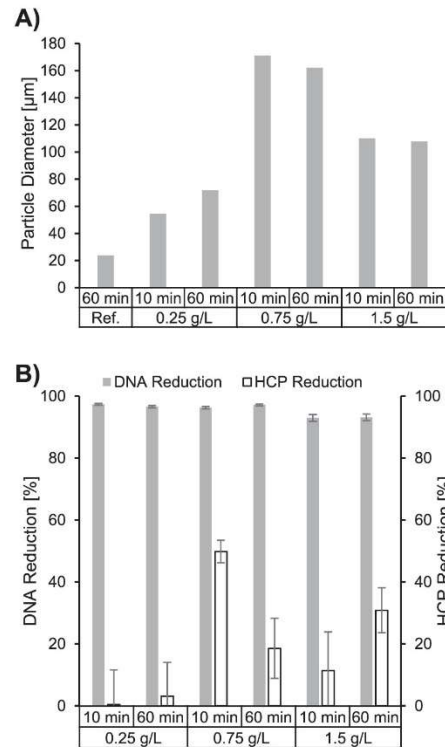


**Fig. 4.** Properties of a fed-batch cell culture fluid after 10 and 60 min of flocculation using different pDADMAC flocculant concentrations (0.25, 0.75 and 1.5 g/L) as well as after 60 min of an untreated reference. A) Volume-weighted (De Brouckere) mean diameter of flocs. B) DNA and host cell protein (HCP) reduction in the supernatant compared to the untreated reference.

high flocculant concentrations can cause reduced floc diameters by a too high coverage of surfaces with the flocculant, resulting in increased repulsive forces between positively charged particles (Burgstaller et al., 2018).

For all tested concentrations, almost constant floc sizes after 10 min and 60 min indicate a fast floc formation and a good stability of the flocs during mixing (Fig. 4, A), allowing a fast start of the clarification process after pretreatment.

Moreover, the ability to reduce dissolved DNA and HCP was examined. A high DNA reduction of 93– 97% was achieved independent of flocculant amount and process time (Fig. 4, B), probably due to strong ionic interaction of negatively charged DNA with the cationic flocculant (Peram et al., 2010).

At a pDADMAC concentration of 0.25 g/L, the HCP content did not change significantly, whereas at 1.5 g/L, the amount of HCP was reduced by 31% after 60 min (Fig. 4, B). Application of a pDADMAC concentration of 0.75 g/L resulted in a HCP reduction of 50% after 10 min and of 19% after 60 min. This could be an indication of cell rupture in the flocs due to the cytotoxicity of the applied flocculant, starvation of cells inside the flocs and hydrodynamic forces (Aunins and Wang, 1989). However, due to a large diameter of flocs and a high DNA removal, CCF was treated with a pDADMAC concentration of 0.75 g/L in all further flocculation trials.

### 3.2. Clarification of pretreated cell culture fluids

#### 3.2.1. Influence on processability

To investigate the influence of pretreatments on the FBC process, the volume of CCF that can be loaded per chamber before reaching the maximum chamber capacity was determined. The chamber loading volume depends on the number of particles, the particle size distribution, and the density of particles in the CCF, in addition to the FBC process parameters.

Therefore, two counteracting effects are relevant for FBC clarification of pretreated CCF: The more compact the aggregates, the more biomass in total can be retained in the fluidized bed of the chamber, increasing the loading volume until the maximum chamber capacity is reached. The more dissolved impurities and cell debris are aggregated by a pretreatment, the higher the particle load in the FBC feed stream and the lower the chamber loading volume.

Compared to the untreated reference the processing of precipitated CCF resulted in a reduced chamber loading volume (Fig. 5), suggesting that the observed precipitated particles (Fig. 2, B) were retained in the FBC chamber. Flocculation of CCF also caused a slight reduction in chamber loading volume that could be explained by co-precipitation of dissolved impurities as well as by a similar particle density of the generated flocs compared to the untreated cells (Fig. 5). However, the slightly reduced chamber loading volumes in both CCF pretreatments had hardly any effect on the average CCF throughput of $12.1 \pm 0.8$ L/h.

After the primary FBC clarification step, the harvest pools were filtered through a combination of a double layer depth filter with average retention rates of 8 µm and 0.8 µm followed by a 0.2 µm sterile filter to determine filter throughputs.

For untreated CCF, a strong pressure increase of more than 1.2 bar over the sterile filter stage (P2) was observed (Fig. 6). This indicates the blocking of the sterile filter by small particles that have passed the FBC and the 0.8 µm depth filter stage. Therefore, relatively low maximum filter throughputs of $101 \pm 36$ L/m$^2$ at the depth filter stage were obtained for the untreated CCF (Fig. 7). Due to the high pressure drop over the sterile filter stage, post-flushing was hardly possible despite a reduced flow rate. Based on these observations, the ratio of sterile filter to depth filter area needs to be increased to improve the utilization of the depth filter stage and facilitate post-flushing after the filtration of untreated FBC harvest pools from low viable CCF.

In contrast, the pressure increase during the filtration of pretreated CCF resulted from blocking of both filter stages, as indicated by the significantly lower pressure drop over the sterile filter stages (P2) compared to the respective depth filter stages (P1) (Fig. 6). As a result, higher utilization of both filter stages and thus increased filter throughputs were achieved.
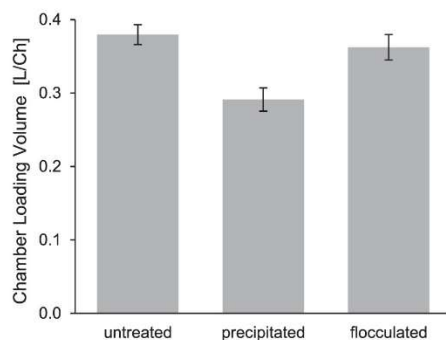
In the precipitation approach a high maximum throughput of $246 \pm 70$ L/m$^2$ was achieved (Fig. 7). This was likely caused by an aggregation of cell debris, which were partially removed by the FBC and partially removed by the subsequent filter stage. In contrast, the larger aggregates consisting of cells and cell debris obtained from flocculation were almost completely removed in the FBC step, resulting in the highest filter throughput of $432 \pm 48$ L/m$^2$.

Compared to the throughput of the untreated FBC harvest, the precipitated CCF achieved a more than double and the flocculated CCF a more than 4x higher maximum filter throughput. This fourfold increase in throughput by CCF flocculation was in the same ratio as reported in another study using two multilayer depth filters in series without previous FBC (Burgstaller et al., 2018). However, it should be noted that total filter throughputs of the studies cannot be compared as the CCFs used in this study had significantly higher cell concentrations and lower viabilities. For comparison and evaluation of different clarification technologies, further studies using CCF with the same characteristics are required. However, the increase of filter throughputs by both pretreatments in this study suggests high potential to streamline the post-centrifugal processing.

#### 3.2.2. Reduction of impurities and recovery of product

In order to investigate the influence of the clarification steps on the harvest clarity, the turbidity was measured after each step (Fig. 8). Initial turbidities of the untreated CCF were 1700 – 2200 NTU in both fed-batches with TCCs in the range of $17.9 \times 10^6 - 20.5 \times 10^6$ cells/mL. The pretreatments of CCF increased turbidity values up to $6400 \pm 1000$ NTU at low-pH precipitation due to the formation and aggregation of particles.

After FBC processing, turbidities were reduced by separating most of the biomass. The flocculated CCF achieved the highest clearance ($53 \pm 17$ NTU) compared to the untreated FBC harvest pool ($91 \pm 71$ NTU), where high variation was observed. The turbidity in the precipitated harvest pool was relatively high ($270 \pm 153$ NTU), indicating a high level of remained particles that were probably too small to be removed by the FBC and passed the FBC chamber. However, in the final filtration step a high clarity of the precipitated CCF was achieved, showing successful removal of the precipitated impurities. Adjusting the pH in the filtrate to 7.0 had no effect on turbidity (data not shown).

Finally, turbidity levels in the harvest pools were below 3.1 NTU for both pretreatments and $6.5 \pm 1.2$ NTU in the untreated reference, demonstrating high clarity for all three approaches tested.

Recovery of mAb as well as reduction of DNA and HCP amounts were determined for different pretreated CCF after each clarification unit operation (Fig. 9).

In the untreated reference process, a slightly higher recovery of $101.0 \pm 0.2$% compared to the expected maximum of 100% was observed (Fig. 9, A), likely due to the continued release of mAb during the clarification process. After pH 5 precipitation a slightly reduced mAb recovery of $96.4 \pm 2.0$% was determined, suggesting co-precipitation of mAb, which was also reported for pH 4 pretreatments (Hadpe et al., 2020). The flocculation pretreatment achieved a recovery of $98.5 \pm 2.0$%, indicating hardly any loss of mAb. After the FBC unit operation, obtained recoveries ranged from 100.9% in the reference to 94.0% in the precipitated CCF. This lower recovery at pH 5 pretreatment could be due to progressive co-precipitation of mAb during the FBC step. Furthermore, the application of citric acid pH 5 buffer for washing of the fluidized bed could have increased mAb precipitation. However, in previous screening experiments (data not shown) citric acid buffer was selected due to its low interference with the precipitated CCF and hardly any effect on cell viability. In the filtration step of the untreated reference, a low recovery of 88.7% was obtained, due to an insufficient post-flush of the filters (see chapter 3.2.1). Therefore, a modified filter setup with finer depth filter retention rates or an increased sterile filter area will be investigated in further studies to ensure complete flushing of
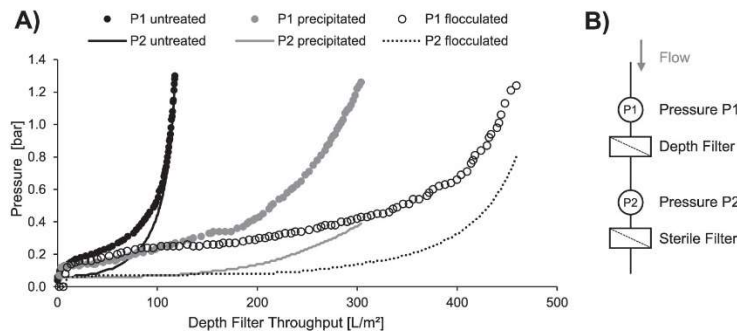


**Fig. 5.** Chamber loading volumes of a fluidized bed centrifuge during clarification of the same starting cell culture fluid that was untreated, precipitated and flocculated.

**Fig. 6.** Post-centrifuge filtration of untreated, precipitated and flocculated cell culture fluids. A) Filtration pressures as a function of the depth filter throughput. B) Arrangement of inline pressure sensors (P1 and P2) installed in front of each filter stage.
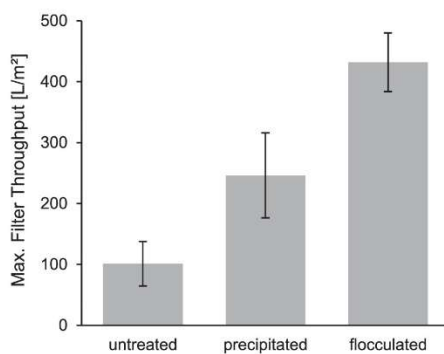


**Fig. 7.** Maximum depth filter throughputs of untreated, precipitated, and flocculated cell culture fluids pre-clarified using a fluidized bed centrifuge.
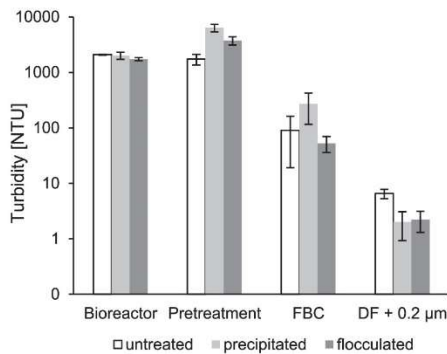


**Fig. 8.** Change in turbidities of untreated, precipitated, and flocculated cell culture fluids starting from the bioreactor, followed by the treatments, then clarified with a fluidized bed centrifuge (FBC) and finally filtered using a depth filter (DF) followed by a 0.2 µm sterile filter stage.

post-centrifugal filters for untreated CCF with low viability.

For both pretreatments, high recoveries of more than 97% were obtained in the filtration step due to the performed post-flushing (20 L/

$m^2$) of the filter setup. Furthermore, in the precipitation approach, the recovered amount of mAb was maintained during the additional pH neutralization step of the filtrate, which thus did not negatively influence the process. However, relatively low overall recoveries below 92% were determined for the reference process due to a sharp recovery decrease in the filtration step as well as for the precipitation pretreatment due to a progressive decline in recovery after pretreatment (Fig. 9, A). The highest recoveries of the overall clarification process were achieved with the flocculation pretreatment (97.5 ± 0.8%), as a result of enhanced processability of the CCF without co-precipitating the mAb (Table 1).

The amount of dissolved DNA was reduced by 84% in the precipitation step and by almost 98% in the flocculation step (Fig. 9, B). The DNA amount of the untreated reference remained almost constant during the pretreatment time. In all approaches, the subsequent FBC step hardly changed the DNA content, whereas an additional DNA reduction was observed in the subsequent filtration step. Probably due to adsorption on the filter material, the amount of DNA was reduced by almost 50% in untreated reference and precipitated CCF as well as by 20% in the flocculated CCF. Additionally, a part of this high reduction in the untreated reference could have resulted from insufficient post-flushing. However, after the entire clarification process, the highest DNA reductions of 91% using precipitation and more than 98% using flocculation were achieved by the pretreatments (Table 1).

The amount of HCP increased by 38% in the untreated and by 24% in the flocculated CCF, probably due to a release of HCP during the mixing of CCF for the time of pretreatment (Fig. 9, C). The FBC step showed no significant change in the HCP level for all approaches. In the filtration step, HCP reduction was achieved only for the untreated reference (33%). This observation suggests adsorption of HCP onto the depth filter material, what has also been observed in other filtration studies (Khanal et al., 2018). However, the HCP amount over all steps was only slightly reduced by the precipitation pretreatment (31 ± 22%) (Table 1). Due to intrinsically relatively high standard deviations of up to 10% in the ELISA assay (Cygnus Technologies, 2021), the resolution of small changes in the HCP amount is limited. Therefore, the pretreatment methods showed no significant effect on the overall reduction of the HCP amount, as shown by other studies (Singh and Chollangi, 2017).

Taking into account the mAb recovery and the impurity reductions, the flocculation pretreatment showed the best clarification results due to the combination of a high overall recovery of 97.5% with the highest reduction in the DNA amount of 98% (Table 1). The lower impurity levels may contribute to reduce the burden on the subsequent purification steps, such as protein A chromatography, which need to be investigated in further studies to facilitate implementation of intensified FBC clarification in DSP.
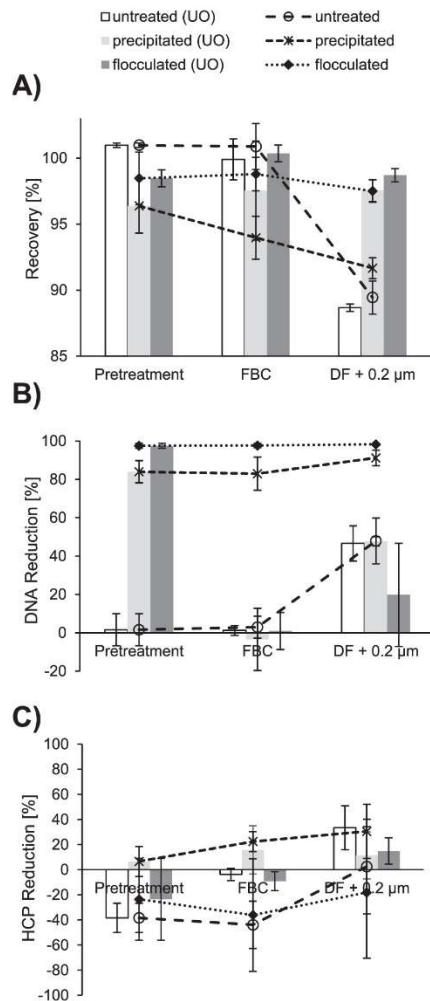
**Fig. 9.** Recovery of mAb A), reduction of DNA amount B), and reduction of host cell protein (HCP) amount C) of untreated (reference), precipitated, and flocculated cell culture fluids after the respective steps of the clarification process. Bar charts represent changes in the amount by each clarification step of pretreatment, fluidized bed centrifuge (FBC), and depth filtration followed by a sterile filter (DF + 0.2 μm). The overall change of the amount during the clarification process is represented by dotted lines. Standard deviations indicate the variation between two independent test series.

### 3.2.3. Harvest quality

To evaluate the influence of pretreatments on mAb quality, the glycan profiles and the ratio of dimers were examined.

The N-glycan profiles were characterized by the ratios of structures with one (G1) and two (G2) galactose molecules per structures without galactose (G0) as well as by the percentage of fucosylated glycans. Similar characteristic glycan profiles with a G1/G0-ratio in a range of 1.23–1.24 and a G2/G0-ratio in a range of 0.27–0.29 as well as an amount of 90.1 ± 0.6% fucosylated glycans were observed in all

**Table 1**
Overview of final recoveries as well as the reduction of DNA and HCP amounts after completed clarification using low-pH precipitation and pDADMAC flocculation pretreatments and an untreated reference. Standard deviations indicate the variation between two independent test series.

|  | Recovery [%] | DNA Reduction [%] | HCP Reduction [%] |
|---|---|---|---|
| untreated | 89.4 ± 1.3 | 48 ± 12 | 3 ± 38 |
| precipitated | 91.7 ± 0.8 | 91 ± 4 | 31 ± 22 |
| flocculated | 97.5 ± 0.8 | 98 ± 1 | -18 ± 52 |

investigated clarification approaches (Fig. 10).

In addition, dimers were investigated as an indication of unwanted mAb aggregates that can be formed by challenging conditions in the CCF due to the pretreatments (Joubert et al., 2011).

The mAb used in this study had an average initial dimer ratio of 0.26 ± 0.02% prior to clarification. After clarification, dimer ratios of the reference (0.18 ± 0.14%) and precipitation (0.23 ± 0.06%) approaches remained constant whereas a slightly increased ratio of 1.07 ± 0.06% was detected in the flocculation approach. This mAb aggregation by the pDADMAC treatment of CCF is consistent with observations of another study (Carvalho, 2019). However, this slight increase in mAb aggregates could be acceptable in the early DSP process as aggregates can be removed in the subsequent purification process, for example by a cation exchange chromatography step (Stone et al., 2019).

The marginal changes observed in dimer ratio and glycan profile suggest that both pretreatments had no significant effect on product quality.

For flocculation pretreatments an additional critical quality attribute is the amount of residual flocculant, which can be assessed by commercially available assays or surface plasmon resonance spectroscopy (Tomic et al., 2015). Due to the often cytotoxic nature of flocculants, their absence in the final formulated product needs to be ensured (McNerney et al., 2015). For the developed FBC approach, pDADMAC removal has to be evaluated in further studies. However, previous studies have shown that dissolved pDADMAC is bound on depth filter materials with a very high efficiency and can also be removed in the subsequent polishing steps (Singh and Chollangi, 2017). Complete removal of additives with established DSP steps would facilitate the use of pretreatment methods in biopharmaceutical processes.

### 4. Conclusion

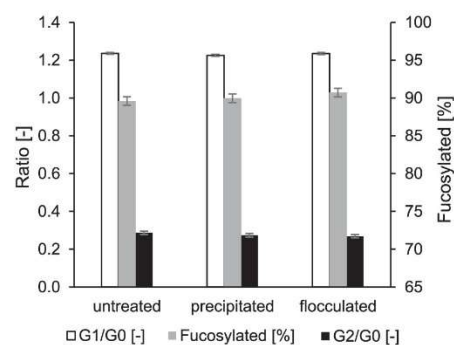Precipitation and flocculation pretreatments of low-viable CCF in



**Fig. 10.** N-glycan profiles showing the G1/G0- and G2/G0-ratio as well as the content of fucosylated glycans of harvested monoclonal antibodies from different pretreatment approaches (untreated, precipitated, and flocculated) of the cell culture fluid.

23

mAb production were investigated targeting the intensification of FBC-based clarification processes. The applied pretreatment approaches showed significant changes in particle size distribution of impurities through their aggregation: Acidification to pH 5 resulted in aggregation of cell fragments and precipitation of dissolved DNA, whereas most of the intact cells with an average diameter of approximately 21 µm remained non-aggregated. In contrast, flocculation using a pDADMAC concentration of 0.75 g/L significantly increased the average particle size up to 170 µm due to aggregation of cells, cell debris and dissolved DNA. In both approaches almost constant particle diameters and host cell protein concentrations indicated stable properties of the pretreated CCF for at least 60 min.

Therefore, clarification within this timeframe was examined using a FBC and a subsequent small depth and sterile filter step for both pretreatment approaches. Primary clarification of pretreated cell cultures was successfully performed without interfering with the FBC process. As indicated by a significant reduction of turbidity, most of the biomass, including cells and large aggregates, was removed by the FBC. In the subsequent filtration step, both pretreated CCF showed a significant increase in the maximum filter throughputs of up to $432 \pm 48$ L/m$^2$ in the flocculation approach compared to $101 \pm 36$ L/m$^2$ in the untreated reference. The improved filterability resulted from the increase in particle sizes, facilitating the removal of submicron impurities, such as degraded cells, which are present particularly in CCF with low viability. Therefore, both pretreatments have a high potential to reduce the post-centrifugal filter area. In addition, an excellent removal of particles was shown by turbidities below 3.1 NTU for the pretreated and 6.5 NTU for the untreated CCF in the final harvest pools.

Throughout the clarification process the amount of DNA was reduced by 91% at low-pH precipitation and by 98% at pDADMAC flocculation. Both values were significantly higher than the DNA reduction of 48% obtained in the untreated reference process. The additional DNA reduction could increase robustness or streamline subsequent polishing steps, which need to be investigated in further studies. The HCP level was not significantly changed by either the pretreatments or the FBC separation process. The overall mAb recoveries ranged from 89.4% to 97.5% for the clarification approaches, which are all in an acceptable range. The highest recoveries were achieved with the flocculated CCF. This in combination with the increased throughput of the post-centrifugal filters and a high reduction in the amount of DNA, make pDADMAC flocculation a promising approach for intensification of the clarification process.

The concentration of mAb dimers remained unchanged in the precipitated CCF and increased only slightly at pDADMAC flocculation. In addition, similar mAb glycan profiles were observed for all approaches, suggesting consistent mAb quality. However, it should be noted that the flocculant and mAb aggregates need to be removed from the product in subsequent purification steps. To apply the pretreatments for other mAbs, the suitability of the pretreatment should be verified with the particular mAb and the host cell line used. Such verification could be performed at an early stage with a few milliliters of CCF, since no effects of a subsequent FBC-based clarification on mAb quality are expected.

In summary, the developed low-pH precipitation and flocculation approaches presented in this study were capable to improve the removal of particles and dissolved host cell impurities while maintaining a high recovery and quality of mAb. Therefore, FBC-based clarification in combination with both investigated pretreatments has a high potential to intensify the clarification of low viable CCF and to streamline the subsequent product purification.

## CRediT authorship contribution statement

**Martin Saballus:** Conceptualization, Investigation, Writing – original draft, Visualization. **Markus Kampmann:** Validation, Writing – review & editing.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors are employees of Sartorius Stedim Biotech GmbH and used some, but not exclusively, products of this company for this study, what might be seen as a potential conflict.

## Acknowledgement

## References

Aunins, J.G., Wang, D.I., 1989. Induced flocculation of animal cells in suspension culture. Biotechnol. Bioeng. 34 (5), 629–638. https://doi.org/10.1002/bit.260340507.

Burgstaller, D., Krepper, W., Haas, J., Maszelin, M., Mohoric, J., Pajnic, K., Jungbauer, A., Satzer, P., 2018. Continuous cell flocculation for recombinant antibody harvesting. J. Chem. Technol. Biotechnol. 93 (7), 1881–1890. https://doi.org/10.1002/jctb.5500.

Cataldo, A.L., Burgstaller, D., Hribar, G., Jungbauer, A., Satzer, P., 2020. Economics and ecology: modelling of continuous primary recovery and capture scenarios for recombinant antibody production. J. Biotechnol. 308, 87–95. https://doi.org/10.1016/j.jbiotec.2019.12.001.

Carvalho, R.J., 2019. Comparison of cationic flocculants for the clarification of CHO-derived monoclonal antibodies. Biotechnol. Bioprocess Eng. 24 (5), 754–760. https://doi.org/10.1007/s12257-019-0158-7.

Chen, C., Wong, H.E., Goudar, C.T., 2018. Upstream process intensification and continuous manufacturing. Curr. Opin. Chem. Eng. 22, 191–198. https://doi.org/10.1016/j.coche.2018.10.006.

Cygnus Technologies (2021, November 08). CHO HCP 3G ELISA Product Insert: Immunoenzymetric Assay for the Measurement of CHO Host Cell Proteins, 2018 [PDF file]. Retrieved from ⟨https://www.cygnustechnologies.com/media/folio3/productattachments/product_inserts/F550%20PI_CHO%203G%20ELISA_Rev%204.pdf⟩.

Gómez, I.A., Hernández, V., Palomares, L.A., Ramírez, O.T., 2018. Flocculation of CHO cells for primary separation of recombinant glycoproteins: Effect on glycosylation profiles. Biochem. Eng. J. 132, 244–254. https://doi.org/10.1016/j.bej.2018.01.027.

Hadpe, S.R., Mohite, V., Alva, S., Rathore, A.S., 2020. Pretreatments for enhancing clarification efficiency of depth filtration during production of monoclonal antibody therapeutics. Biotechnol. Prog., e2996 https://doi.org/10.1002/btpr.2996.

Iammarino, M., Nti-Gyabaah, J., Chandler, M., Roush, D., Göklen, K., 2007. Impact of cell density and viability on primary clarification of mammalian cell broth: an analysis using disc-stack centrifugation and charged depth filtration. Bioprocess Int., 2007 38–50. In: ⟨https://bioprocessintl.com/downstream-processing/filtration/impact-of-cell-density-and-viability-on-primary-clarification-of-mammalian-cell-broth-110120075/⟩.

Irvine, E.B., Alter, G., 2020. Understanding the role of antibody glycosylation through the lens of severe viral and bacterial diseases. Glycobiology 30 (4), 241–253. https://doi.org/10.1093/glycob/cwaa018.

Joubert, M.K., Luo, Q., Nashed-Samuel, Y., Wypych, J., Narhi, L.O., 2011. Classification and characterization of therapeutic antibody aggregates. J. Biol. Chem. 286 (28), 25118–25133. https://doi.org/10.1074/jbc.M110.160457.

Kakarla, R., Hur, J., Kim, Y.J., Kim, J., Chwae, Y.-J., 2020. Apoptotic cell-derived exosomes: messages from dying cells. Exp. Mol. Med. 52 (1), 1–6. https://doi.org/10.1038/s12276-019-0362-8.

Kang, Y.K., Hamzik, J., Felo, M., Qi, B., Lee, J., Ng, S., Liebisch, G., Shanehsaz, B., Singh, N., Persaud, K., Ludwig, D.L., Balderes, P., 2013. Development of a novel and efficient cell culture flocculation process using a stimulus responsive polymer to streamline antibody purification processes. Biotechnol. Bioeng. 110 (11), 2928–2937. https://doi.org/10.1002/bit.24969.

Kelley, B., 2020. Developing therapeutic monoclonal antibodies at pandemic pace. Nat. Biotechnol. 38 (5), 540–545. https://doi.org/10.1038/s41587-020-0512-5.

Kelly, W., Rubin, J., Scully, J., Kamaraju, H., Wnukowski, P., Bhatia, R., 2016. Understanding and modeling retention of mammalian cells in fluidized bed centrifuges. Biotechnol. Prog. 32 (6), 1520–1530. https://doi.org/10.1002/btpr.2365.

Khanal, O., Singh, N., Traylor, S.J., Xu, X., Ghose, S., Li, Z.J., Lenhoff, A.M., 2018. Contributions of depth filter components to protein adsorption in protein bioprocessing (Advance online publication). Biotechnol. Bioeng.. https://doi.org/10.1002/bit.26607.

Ko, H.-F., Bhatia, R., 2012. Evaluation of single-use fluidized bed centrifuge system for mammalian cell harvesting. BioPharm. Int. 25, 34–40. ⟨http://www.biopharminternational.com/evaluation-single-use-fluidized-bed-centrifuge-system-mammalian-cell-harvesting⟩.

Kshirsagar, R., Ryll, T., 2018. Innovation in cell banking, expansion, and production culture. Adv. Biochem Eng. Biotechnol. 165, 51–74. https://doi.org/10.1007/10_2016_56.

Lu, R.-M., Hwang, Y.-C., Liu, I.-J., Lee, C.-C., Tsai, H.-Z., Li, H.-J., Wu, H.-C., 2020. Development of therapeutic antibodies for the treatment of diseases. J. Biomed. Sci. 27 (1), 1. https://doi.org/10.1186/s12929-019-0592-z.

McNerney, T., Thomas, A., Senczuk, A., Petty, K., Zhao, X., Piper, R., Carvalho, J., Hammond, M., Sawant, S., Bussiere, J., 2015. Pdadmac flocculation of Chinese hamster ovary cells: enabling a centrifuge-less harvest process for monoclonal antibodies. MAbs 7 (2), 413–428. https://doi.org/10.1080/19420862.2015.1007824.

Mehta, S., 2014. Automated single-use centrifugation solution for diverse biomanufacturing process, 385–400. In: Subramanian, G. (Ed.), Continuous Processing in Pharmaceutical Manufacturing. Wiley-VCH. https://doi.org/10.1002/9783527673681.ch15.

Minow, B., Egner, F., Jonas, F., Lagrange, B., 2014. High-cell-density clarification by single-use diatomaceous earth filtration. Bioprocess Int. 36–47. In: ⟨https://bioprocessintl.com/2014/highcell-density-clarification-by-single-use-diatomaceous-earth-filtration-351074⟩.

Müller, D., Klein, L., Lemke, J., Schulze, M., Kruse, T., Saballus, M., Matuszczyk, J., Kampmann, M., Zijlstra, G., 2022. Process intensification in the biopharma industry: improving efficiency of protein manufacturing processes from development to production scale using synergistic approaches. Chem. Eng. Process. - Process. Intensif. 171, 108727 https://doi.org/10.1016/j.cep.2021.108727.

Papathanasiou, M.M., Kontoravdi, C., 2020. Engineering challenges in therapeutic protein product and process design. Curr. Opin. Chem. Eng. 27, 81–88. https://doi.org/10.1016/j.coche.2019.11.010.

Peram, T., McDonald, P., Carter-Franklin, J., Fahrner, R., 2010. Monoclonal antibody purification using cationic polyelectrolytes: an alternative to column chromatography. Biotechnol. Prog. 26 (5), 1322–1331. https://doi.org/10.1002/btpr.437.

Pieracci, J.P., Armando, J.W., Westoby, M., Thommes, J., 2018. Industry review of cell separation and product harvesting methods. In: Jagschies, G., Lindskog, E., Łącki, K., Galliher, P. (Eds.), Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes. Elsevier, pp. 165–206. https://doi.org/10.1016/B978-0-08-100623-8.00009-8.

Pohlscheidt, M., Kiss, R., Gottschalk, U., 2018. An introduction to recent trends in the biotechnology industry: development and manufacturing of recombinant antibodies and proteins. Adv. Biochem. Eng. /Biotechnol. 165, 1–8. https://doi.org/10.1007/10_2017_39.

Richardson, A., & Walker, J. (2021, November 08). Continuous Solids-Discharging Centrifugation: A Solution to the Challenges of Clarifying High–Cell-Density Mammalian Cell Cultures, 2018 [PDF file]. Retrieved from ⟨https://bioprocessintl.com/downstream-processing/separation-purification/continuous-solids-discharging-centrifugation-a-solution-to-the-challenges-of-clarifying-high-cell-density-mammalian-cell-cultures/⟩.

Roush, D.J., Lu, Y., 2008. Advances in primary recovery: centrifugation and membrane technology. Biotechnol. Prog. 24 (3), 488–495. https://doi.org/10.1021/bp070414x.

Saballus, M., Nisser, L., Kampmann, M., Greller, G., 2021. A novel clarification approach for intensified monoclonal antibody processes with 100 million cells/mL using a single-use fluidized bed centrifuge. Biochem. Eng. J. 167, 107887 https://doi.org/10.1016/j.bej.2020.107887.

Senczuk, A., Petty, K., Thomas, A., McNerney, T., Moscariello, J., Yigzaw, Y., 2016. Evaluation of predictive tools for cell culture clarification performance. Biotechnol. Bioeng. 113 (3), 568–575. https://doi.org/10.1002/bit.25819.

Singh, N., Arunkumar, A., Chollangi, S., Tan, Z.G., Borys, M., Li, Z.J., 2016. Clarification technologies for monoclonal antibody manufacturing processes: Current state and future perspectives. Biotechnol. Bioeng. 113 (4), 698–716. https://doi.org/10.1002/bit.25810.

Singh, N., Chollangi, S., 2017. Next-generation clarification technologies for the downstream processing of antibodies. In: Gottschalk, U. (Ed.), Process Scale Purification of Antibodies, second edition, Vol. 19. Wiley, pp. 82–112. https://doi.org/10.1002/9781119126942.ch4.

Stein, D., Thom, V., Hubbuch, J., 2020. High throughput screening setup of a scale-down device for membrane chromatography-aggregate removal of monoclonal antibodies. Biotechnol. Prog., e3055 https://doi.org/10.1002/btpr.3055.

Stone, M.T., Cotoni, K.A., Stoner, J.L., 2019. Cation exchange frontal chromatography for the removal of monoclonal antibody aggregates. J. Chromatogr. A 1599, 152–160. https://doi.org/10.1016/j.chroma.2019.04.020.

Takagi, M., Kitabayashi, T., Ito, S., Fujiwara, M., Tokuda, A., 2007. Noninvasive measurement of three-dimensional morphology of adhered animal cells employing phase-shifting laser microscope. J. Biomed. Opt. 12 (5), 54010. https://doi.org/10.1117/1.2779350.

Tomic, S., Besnard, L., Fürst, B., Reithmeier, R., Wichmann, R., Schelling, P., Hakemeyer, C., 2015. Complete clarification solution for processing high density cell culture harvests. Sep. Purif. Technol. 141, 269–275. https://doi.org/10.1016/j.seppur.2014.12.002.

Trapp, A., Faude, A., Hörold, N., Schubert, S., Faust, S., Grob, T., Schmidt, S., 2018. Multiple functions of caprylic acid-induced impurity precipitation for process intensification in monoclonal antibody purification. J. Biotechnol. 279, 13–21. https://doi.org/10.1016/j.jbiotec.2018.05.001.

Westoby, M., Chrostowski, J., Vilmorin, P., de, Smelko, J.P., Romero, J.K., 2011. Effects of solution environment on mammalian cell fermentation broth properties: enhanced impurity removal and clarification performance. Biotechnol. Bioeng. 108 (1), 50–58. https://doi.org/10.1002/bit.22923.

Wilson, L.J., Lewis, W., Kucia-Tran, R., Bracewell, D.G., 2019. Identification of upstream culture conditions and harvest time parameters that affect host cell protein clearance. Biotechnol. Prog. 35 (3), e2805 https://doi.org/10.1002/btpr.2805.

Yang, O., Prabhu, S., Ierapetritou, M., 2019. Comparison between batch and continuous monoclonal antibody production and economic analysis. Ind. Eng. Chem. Res. 58 (15), 5851–5863. https://doi.org/10.1021/acs.iecr.8b04717.

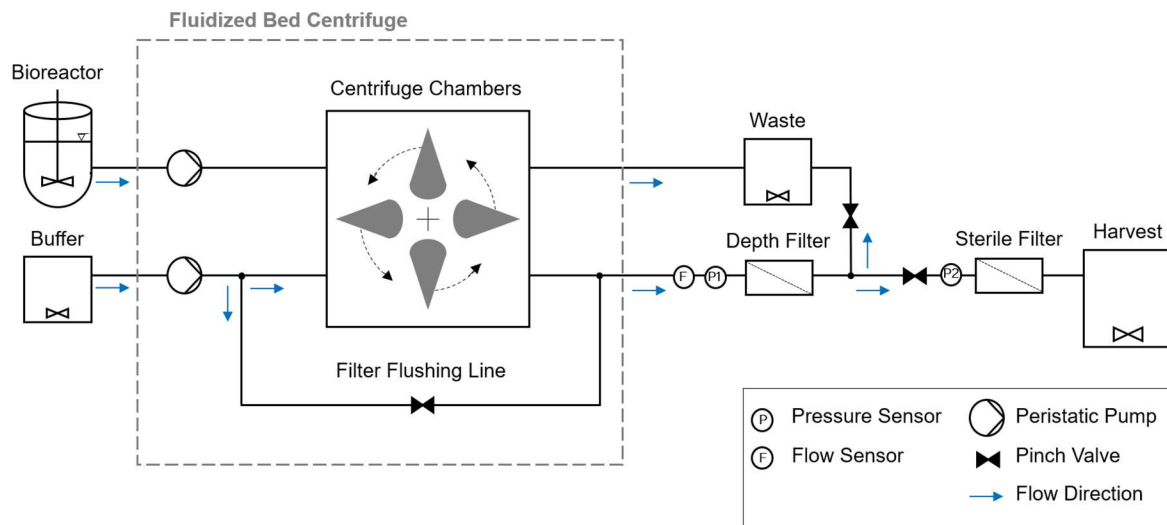## 4.4 Implementation of a Streamlined Single-Use Clarification Setup



**Figure 6.** Schematic process flow of a streamlined clarification unit operation with a direct connection of bioreactor, fluidized bed centrifuge as a first clarification step, depth and sterile filter as a second clarification step, and harvest vessel. Inline single-use pressure sensors (P1, P2) and flow sensor (F) are installed for process monitoring. Pinch valves indicate certain lines that are activated and deactivated for filter flushing.

In the previous chapters, the suitability of a novel clarification approach for robust processing of cell broths even at HCD or low viability conditions was demonstrated. The two clarification steps applied, FBC and two-stage filtration, were operated one after another in this approach, as it is also the case in conventional processes with disc stack centrifugation and subsequent filtration. Performing both steps separately is usually accomplished to reduce the complexity of each process step and to adapt the steps more flexible to different conditions of the cell broth. However, such stepwise operation requires separate sets of equipment for controlling and pumping as well as surge vessels for intermediate product storage. This increases capital expenditure for equipment, labor costs for operation, footprint of the manufacturing plant and thus decreases the cost efficiency of the UO. Another challenge can be the relatively low overall product throughput and thus long process time, which increases the risk of product degradation or impairment of the mAb quality. A way to overcome the limitations of stepwise clarification could be a direct connection of both process steps.

To enable the connection in the developed FBC-based clarification approach, a basis is provided by the work of the previous chapters. Due to the ability to model the FBC throughput in dependency of the cell concentration in the production bioreactor (chapter 4.2) as well as to predict (or even to influence) the maximum filter capacities for post-FBC processing (chapter 4.3), appropriated filter sizes can be preselected depending on the respective FBC flow rates as well as the cell broth volume and the cell concentration to clarify. In addition, the automated FBC system with its presterilized SU equipment

allows flexible connection of all inlets and outlets by sterile welding of the thermoplastic tubes. On that basis, the clarification approach was modified to integrate the filtration step into the preceding fluidized bed centrifugation step aiming for an intensification of the clarification platform.

The general concept underlying the integrated clarification setup is based on a liquid tight connection between the FBC harvest outlet and the filter setup inlet (Figure 6). This allows the operation without any surge vessel for intermediate storage of the FBC supernatant between the FBC outlet and the inlet of the connected depth and sterile filters. Moreover, the FBC pumping arrangement is used to pump the supernatant through the filters. The resulting elimination of a surge vessel and a filtration pump streamlines the setup, which is not only cost efficient, but is also making the overall process faster. The accompanied reduction in the footprint of clarification setup is particularly advantageous for large-scale applications in biopharmaceutical manufacturing, where clean room spaces are limited and cost drivers of the process.

Further notable characteristics of the setup are the also liquid tight connections between the bioreactor and the FBC as well as between the sterile filter and the harvest vessel. Due to the achieved completely closed fluid path from the bioreactor to the sterile harvest, no fluid may come into contact with any components outside, which leads to a reduced risk of contamination and thus an increased process safety. In addition, it is advantageous that all components that are in direct contact with the product are SU components, which reduces the set-up times and eliminates the need for cleaning and validation between different batches. Furthermore, SU pressure and flow sensors are installed in front of the filter stages to increase the controllability of the clarification. When a predefined pressure limit is reached in the process by blocking of filters, valves can be actuated in a pressure control loop to stop the centrifugation process and start the filter post-flush or open an additional backup filter line (backup filters not shown in Figure 6).

In addition to these sensors, a supernatant sensor arrangement, preferably an optical supernatant sensor arrangement, for measuring an occurrence level of supernatant in the respective liquid line can be used to control the process. During the FBC loading and washing cycles, depending on the measured occurrence level of supernatant, the FBC process controller deactivates or activates the outlet supernatant line and the outlet waste line via the valves installed in the respective lines. The advancements in the control strategy thus achieved, was filed as an independent international patent application with the title "Automated centrifuge setup of a bioprocessing installation" (WO 2022/008543 Al), which is not further discussed in this thesis [126].

Another feature of the approach is that only one buffer vessel and one waste vessel is connected with the FBC but used for both steps, what again streamlines the clarification setup. Before the centrifugation process is started, the buffer is used for conditioning of filters by pre-flushing them. For

that purpose, the control unit, a pump, and a predefined filter flushing line of the FBC is applied. During the clarification process the cells captured inside fluidized bed of the FBC can be washed from the same buffer vessel to increase mAb recovery. After the clarification process, the FBC and the filter flushing line are again used for post-flushing of the filters to wash out the product containing liquid from the void volume of the filter assembly into the harvest vessel.

The waste vessel is connected with the FBC waste outlet to collect the separated biomass of the centrate. An additional connection between the depth filter outlet and the waste vessel was established to collect the buffer from depth filter pre-conditioning, allowing the removal of filter associated impurities and preventing unwanted dilution of the harvest pool with buffer.

The described clarification setup may be operated in different modes. A first operating mode, which is the focus of this thesis, is the clarification of any solid particles from the cell broth by centrifugation and subsequent filtration. A second operating mode, which could be realized by the present approach as well, is the separation of cells from supernatant whereas the supernatant could additionally contain a product of interest, like mAb´s, exosomes, or viruses. The obtained product in this second operating mode can therefore be both, cells and supernatant, which is not further addressed in this work. However, in both operating modes, the cyclic fluidized bed centrifugation process, consisting of loading the cells into the FBC chambers, washing out the supernatant from the fluidized bed, and discharging of the washed cells, determines the overall clarification throughput of this UO. Therefore, the FBC system is used for controlling and harmonization of the centrifugation as well as the filtration steps.

During the loading of cells from the bioreactor into the FBC chambers, the valves of the FBC outlet lines are actuated to guide the first volume fraction containing the system void volume to the waste vessel and the second volume fraction containing the cell broth supernatant with the mAb to the filter assembly. The fluid flow in the subsequent washing step of the FBC chamber is also guided to the filters. Residual particles particularly cell debris of the FBC supernatant are removed in the filter assembly resulting in a slow increase of the pressure by an increased flow resistance through the progressed blocking of filter materials. In the last FBC cycle step, the discharging of cells, the flow direction through the FBC chamber is turned in the opposite direction to transfer the biomass to the waste vessel. During this step, no fluid is pumped through the filter assembly for a short time. Due to this interruption of fluid flow, the filter pressure decreases. However, with the start of another FBC cycle and thus again pumping supernatant through the filters, the pressure increases again. This pressure monitoring in front of each filter stage can be used to activate a valve of a backup filter assembly or stop the process when a pre-defined threshold of the peak pressure is achieved.

Both, the described clarification setup as well as the methods for its operation, were filed as an international patent application with the title "Clarification setup of a bioprocessing installation" [124].

In order to evaluate the feasibility of the invention an application study was conducted. As an example, the results of two performed proof-of-concept clarification runs, which were also presented at the 5th European BioProcessing Summit 2022 [125], are shown in the following section.

For investigation of the process robustness, CHO cell broth with different characteristics were clarified. In both trials, the maximum centrifugal force of the FBC system of 1,000 × g was applied, all four chambers were loaded with its maximum capacity and washed with 260 mL buffer per chamber, as established in the FBC parameter optimization (chapter 4.1). Stop criteria of the process was a pressure limit of 1.5 bar (sensor P1 in Figure 6) or a complete clarification of the cell broth volume.

In the first run, 170 L fed-batch cell broth from a pilot-scale 200 L SU bioreactor with a moderate cell concentration of 16.7 million cells/mL (719 NTU) was processed. For depth filtration, two cassettes with an effective filter area (EFA) of 1.6 m² in total were used. A sterile filter with an EFA of 0.8 m² was applied. The FBC loading flow rate was set to 1.2 L/min in total for all four applied chambers.

In the second clarification run, approximately 3 L cell broth from an intensified process with a cell concentration of 110 million cells/mL (7970 NTU) was used. A depth filter EFA of 0.08 m² and a sterile filter EFA of 0.065 m² was applied. In order to reach the maximum specific flow rate at the depth filters of 300 L/m²/h, the FBC loading flow rate of all four chambers was set to 0.4 L/min.

The pressure measured at the filter inlets and the FBC outlet flow rate were recorded for a complete clarification run (Figure 7). During the pre-flushing of the depth filter using the FBC, fluctuations in the flow rate indicate air bubbles that were removed by venting. After a sufficient volume of buffer was flushed through the filter, the residual liquid was blown out by pumping air, which cannot be measured by the flow sensor, but results in a slight pressure increase (visible after approximately 0.3 h process time in Figure 7). Thereafter, the FBC was sterile connected to the bioreactor and the cyclic FBC clarification was started. During the loading and washing of the cells in the FBC chamber, the supernatant was directly pumped into the filter assembly, resulting in an increase in pressure. At the end of each cycle, the filtration was interrupted for 1.5 minutes while the biomass inside the chamber was discharged into the waste vessel and a new cycle was automatically started. Although the flow was interrupted, typical blocking behavior of the depth filter (pressure 1) was observed. A very low pressure drop over the sterile filter (pressure 2) observed in both runs suggests that the sterile filter device was oversized. In both runs, the complete cell broth was processed without reaching the pressure limit. After FBC clarification, post-flushing was performed with half of the flow rate, to ensure that the pressure remained low and to avoid breakthrough of impurities.

For the clarification of a 200 L bioreactor (run 1), 100 FBC cycles were performed during 8 h process time. The FBC system was capable to run the process automated without any interruption or manual adjustments, suggesting high robustness of the approach (data not shown).

For the clarification of 3 L HCD broth (run 2), 8 FBC cycles were performed in 34 min (Figure 7). Due to the applied small filter area and a probably higher level of cell debris, an increase in pressure (P1) up to 0.7 bar was observed, which was significantly below the pressure limit of 1.5 bar.
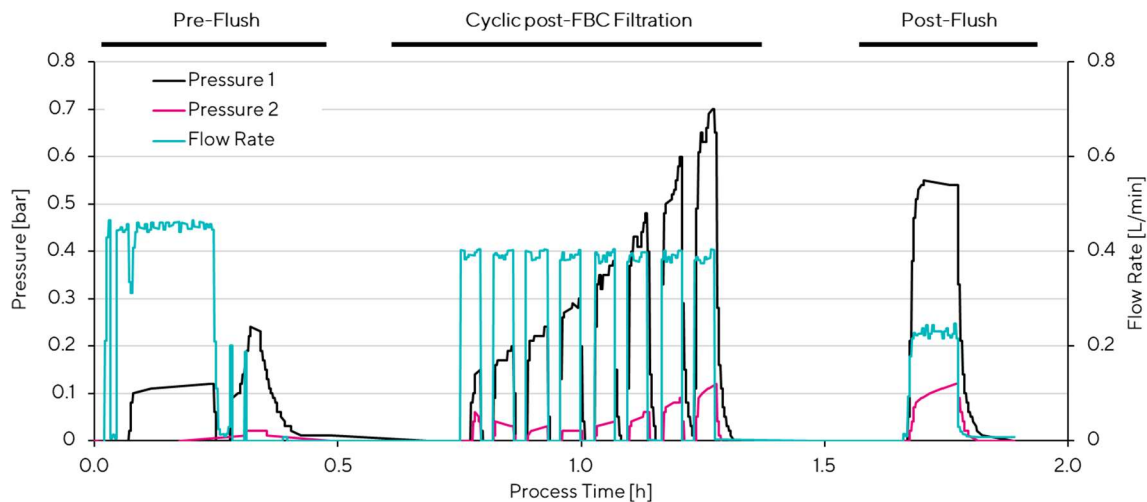


**Figure 7.** Pressures and flow rate during the clarification of a high cell density broth with 110 million cells/mL using a fluidized bed centrifuge (FBC) with integrated filtration (run 2). Prior to the clarification, the depth filter was pre-flushed with buffer including the blow out of remained liquid using the FBC. The FBC supernatant of each clarification cycle was directly filtered. After clarification, depth and sterile filter were post-flushed by the FBC.

Despite the difference in cell concentration and processed volume in both runs, turbidities in the sterile harvest pools were below 4 NTU, demonstrating high product clarity, which could further facilitate the subsequent downstream processing. In addition, a high mAb recovery above 95 % was obtained due to the sufficient washing of cells and post-flushing of the filters showing again the high efficiency.

In summary, high clarity of the harvest as well as high recoveries of mAb were achieved, confirming robustness of the approach to clarify a very broad range of cell broth characteristics from various mAb production processes. Furthermore, the limitations of stepwise clarification were bypassed through a direct connection of both clarification steps in the streamline setup. Therefore, FBC with integrated filtration can be used as a SU platform approach, which contributes to the intensification efforts in biomanufacturing. As a next step, the UO could be connected with a subsequent continuous capture step to achieve further intensification in mAb processing.

**(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)**

**(19) World Intellectual Property Organization**
International Bureau

**(43) International Publication Date**
13 January 2022 (13.01.2022)

**WIPO | PCT**

**(10) International Publication Number**
# WO 2022/008536 A1

(51) **International Patent Classification:**
*C12M 1/26* (2006.01)

(21) **International Application Number:**
PCT/EP2021/068706

(22) **International Filing Date:**
06 July 2021 (06.07.2021)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
20184330.7      06 July 2020 (06.07.2020)      EP

(71) **Applicant: SARTORIUS STEDIM BIOTECH GMBH**
[DE/DE]; August-Spindler-Str. 11, 37079 Göttingen (DE).

(72) **Inventors: SABALLUS, Martin**; Trippestraße 34, 44149 Dortmund (DE). **KAPPMANN, Markus**; Liebrecht Straße 7, 37075 Göttingen (DE).

(74) **Agent: GOTTSCHALD PATENTANWÄLTE PART- NERSCHAFT MBB**; Klaus-Bungert-Str. 1, 40468 Dues- seldorf/Deutschland (DE).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report (Art. 21(3))*

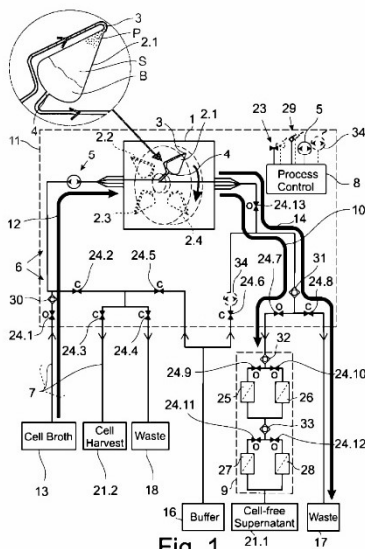(54) **Title:** CLARIFICATION SETUP OF A BIOPROCESSING INSTALLATION



Fig. 1

(57) **Abstract:** The invention is directed to a clarification setup of a bioprocessing installation for the clarification of a cell broth by centrifugation and subsequent filtration, wherein, for the centrifugation, the clarification setup comprises a centrifuge (1), wherein the centrifuge (1) comprises at least one centrifuge chamber (2) with a chamber inlet (3) and a chamber outlet (4), wherein the clarification setup comprises a liquid pumping arrangement (5) assigned to the centrifuge (1) and a liquid network (6) with a number of liquid lines (7) communicating with the liquid pumping arrangement (5), wherein the clarification setup comprises a process control (8) for controlling at least the centrifuge (1) and the liquid pumping arrangement (5). It is proposed that for filtration, the clarification setup comprises a filter arrangement (9), that the liquid network (6) provides an outlet supernatant line (10) for a liquid connection between the chamber outlet (4) and the filter arrangement (9), which is at least temporarily, preferably permanently, liquid-tight, such that due to this liquid-tight condition of the outlet supernatant line (10) the liquid pumping arrangement (5) as such may drive the supernatant from the chamber outlet (4) to the filter arrangement (9) via the outlet supernatant line (10).

WO 2022/008536 A1

- 1 -

## Clarification setup of a bioprocessing installation

The present invention relates to a clarification setup of a bioprocessing installation according to the general portion of claim 1, a bioprocess installation with such a clarification setup according to claim 15 and a method for operating such a clarification setup according to claim 16.

The bioprocessing installation in question may be applied in various fields of bioprocessing technology. High efficiency in this field has been driven by the increasing demand for biopharmaceutical drugs. The efficiency in this sense is regarding not only the cost-effectiveness of the components to be used, but also the controllability of the processes connected thereto.

The clarification setup in question serves for the clarification of a cell broth by centrifugation and subsequent filtration. The cell broth is to be understood as a suspension comprising a liquid portion including supernatant comprising soluble (bio)molecules including the product of interest and comprising a solid portion comprising cells, cell debris and other solid particles.

The clarification serves to gain the supernatant including the product of interest from the cell broth by separating solid particles such as cells and cell debris.

The known clarification setup (EP 2 310 486 B1), which is the starting point for the invention, comprises a centrifuge with a number of centrifuge chambers, which are each assigned a chamber inlet and a chamber outlet. The clarification setup also comprises a liquid pumping arrangement assigned to the centrifuge and a liquid network for the transport of the liquid, which is based on the cell broth to be clarified. Finally, the clarification setup comprises a process control for controlling at least the centrifuge and the liquid pumping arrangement.

It is also known that, depending on the application, it is necessary to filter the centrifuge solution by a filter arrangement comprising, for example, a depth filter and a sterile filter ("examining single use harvest clarification options: a case study comparing depth-filter turbidities and recoveries", Manish K. Sharma, Bioprocess International, 2017).

The known clarification setup as such is running in a highly efficient manner. The same is to be said for the known filter arrangements that may be used to filter solid particles from the centrifuged liquid, called the supernatant. However, the resulting combination is of restricted efficiency in view of redundancies in the mechanical setup as well as in the controllability of the overall process.

It is therefore the object of the invention, to improve the known clarification setup such that its combined operation with a subsequent filter arrangement is of increased efficiency.

The above noted problem is solved by a clarification setup according to the general portion of claim 1 with the features of the characterizing portion of claim 1.

The general concept underlying the invention is based on a liquid tight connection between the chamber outlet and the filter arrangement. First of all, this allows for the operation without any intermediate reservoir for the liquid between the chamber outlet and the filter arrangement. All effects with such an intermediate reservoir, which has proven to lead to undesired drawbacks, are eliminated. Second of all, the liquid pumping arrangement, which is assigned to the centrifuge, may well be used to pump the centrifuged liquid to and through the filter arrangement. This means, that the filter arrangement as such does not need a separate liquid pumping arrangement, which is not only cost efficient, but is also making the control of the overall process easier and more efficient. Furthermore, the absence of an intermediate reservoir and a separate liquid pumping arrangement for the filter arrangement significantly reduces the footprint of the clarification set-up which is especially beneficial with respect to large-scale applications in the pharmaceutical industry and limited space to run process facilities. Finally, due to the proposed closed outlet supernatant line, no fluid may come into contact with any component outside this closed system, which leads to a reduced risk of contamination and a particularly environment friendly process and increased process savety.

In detail, it is proposed, that for filtration, the clarification setup comprises a filter arrangement, that the liquid networks provides an outlet supernatant line for a

- 3 -

liquid connection between the chamber outlet and the filter arrangement, which is at least temporarily, preferably permanently, liquid-tight, such that due to this liquid-tight condition the liquid pumping arrangement as such may drive the supernatant from the chamber outlet to the filter arrangement via the outlet
5    supernatant line.

The preferred embodiment according to claim 2 regards the combination of components in a self contained unit. The expression "self contained unit" represents a unit that is physically one single unit and that as a unit provides a
10   function, that results from the combination of its components. With a structure according to claim 2, the realization of costly redundant components such as separate liquid pumping units for the centrifuge and the filter arrangement, may be prevented.

15   The process control is preferably designed to execute a number of predetermined process cycles according to claim 3, such as a loading cycle, a washing cycle and a discharging cycle. Such cycles are preferably each defined by a software module, which may be run on the process control.

20   Claims 4 to 6 are directed to the realization of the above noted, most essential process cycles, namely the loading cycle, the washing cycle and the discharging cycle. Due to the liquid tight condition of the outlet supernatant line, the liquid pressure and/or the liquid flow may easily be controlled by the liquid pumping arrangement.
25

The preferred embodiment according to claim 7 is directed to providing a valve arrangement for activating/deactivating the liquid lines of the liquid network in order to support the execution of the respective cycle. Here, deactivating means, that liquid flow through the respective liquid line may be blocked by the
30   respective valve. Activating means, accordingly, that liquid flow is allowed through the respective liquid line.

According to claim 8, activating and deactivating of the filter arrangement or at least one filter of the filter arrangement is possible by the control of the valve
35   arrangement. This allows for example a proper reaction to the blockage of a filter of the filter arrangement. In addition according to claim 9, the valve

arrangement may be used for the redirection of liquid, for example, in order to flush the filter arrangement. This makes it possible to double use certain liquid lines for different tasks.

5   Claim 10 clarifies, that the filter arrangement may comprise at least one  filter stage, peferably two filter stages comprising a depth filter stage and a steril filter stage, which may be individually laid out. The proposed clarification setup allows to realize a nearly unrestricted range of filter setups. Due to the centralized process control, it is possible to directly react to a filter blockage, for
10  example by terminating the execution of the respective process cycle.

Claims 11 to 13 are directed to a sensor arrangement for measuring properties of the liquid in at least one of the liquid lines. Such properties may be the occurrence level of biomass and/or supernatant, the liquid pressure, the liquid
15  flow or the like. The resulting sensor signals are being provided to the process control, which allows automation of the process cycles to a wide extent.

20  The term "occurrence level" in this application generally is a variable, which represents the degree of occurrence of the respective entity, here and preferably of biomass and/or supernatant, in the respective liquid line. This variable may represent a continuous range between "occurrence" and "no occurrence". This is for example the concentration of the respective entity within
25  the liquid in the liquid line. The occurrence level may also represent a binary information being either "occurrence" or "no occurrence" of the entity in the liquid line.

In order to guarantee a cost efficient process and at the same time perfectly
30  clean condition of the clarification setup, according to claim 14, at least part of the clarification setup is provided as a single use component.

A second independent teaching according to claim 15 is directed to a bioprocess installation with a proposed clarification setup and with a cell broth
35  source in the form of a storage vessel or a production vessel, such as a

- 5 -

bioreactor. All explanations given with regard to the first teaching are fully applicable to this second teaching.

A third independent teaching according to claim 16 is directed to a method for operating a proposed clarification setup, wherein the clarification setup comprises a valve arrangement controlled by the process control and wherein at least one filter of the filter arrangement is being deactivated and activated by shutting and unshutting the respective filter. Again, all explanations given with regard to the first teaching are fully applicable to this third teaching.

Preferably, the proposed method is based on a sensor arrangement for measuring properties of the liquid in at least one of the liquid lines, which sensor arrangement provides sensor signals to the process control and that at least one filter of the filter arrangement is being deactivated/activated based on the sensor signals of the sensor arrangement. Here as well, all explanations given with regard to the first teaching, in particular in view of the application of a sensor arrangement, are fully applicable.

In the following, a preferred embodiment of the invention is being described with regard to the drawings. In the drawings show

Fig. 1     a schematic view on a proposed clarification setup during the loading cycle,

Fig. 2     a schematic view on the clarification setup according to fig. 1 during the washing cycle and

Fig. 3     a schematic view on the clarification setup according to fig. 1 during the discharging cycle.

The proposed clarification setup, here and preferably, is assigned to the downstream process of a cell culture containing a storage vessel or a production vessel such as a bioreactor, processing a liquid in the form of a cell broth. Accordingly, the wording "liquid" is to be understood in a broad sense. It includes not only liquids as such, but also solutions and suspensions with particles like cells, cell debris, etc.

- 6 -

This clarification setup may be operated in different operating modes. A first operating mode, which is the focus of the present invention, is the clarification of the cell broth from any solid particles by centrifugation and subsequent
5       filtration. The goal here is to separate the cell broth from solid particles such as cells, cell debris, etc., which solid particles are considered biomass in the following. The product to be obtained in this first operating mode is the supernatant of the cell broth containing a product of interest, e.g. a recombinant protein such as an antibody.
10

A second operating mode, which may generally be realized by the present invention as well, is the clarification of cells in the cell broth from supernatant. The product to be obtained in this second operating mode are the cells in the cell broth.
15

For the centrifugation, the clarification setup comprises a centrifuge 1, wherein the centrifuge 1 comprises at least one centrifuge chamber 2.1, 2.2, 2.3, 2.4, each with a chamber inlet 3 and a chamber outlet 4. In the drawings, the altogether four centrifuge chambers 2.1, 2.2, 2.3, 2.4 are indicated. In this
20       particular case, the chamber inlets 3 of the centrifuge chambers 2.1, 2.2, 2.3, 2.4 are connected with each other and the chamber outlets 4 of the centrifuge chambers 2.1, 2.2, 2.3, 2.4 are connected with each other to act as a single chamber inlet respective a single chamber outlet from the outside.

25       Here and preferably, each chamber 2.1, 2.2, 2.3, 2.4 is located offset a centrifuge rotor axis, wherein, further preferably, the respective chamber inlet 3 is located further away from the centrifuge rotor axis than the respective chamber outlet 4.

30       The expression "chamber inlet" means that the liquid to be centrifuged enters the respective chamber 2.1, 2.2, 2.3, 2.4 via the respective chamber inlet 3. The expression "chamber outlet" means that the centrifuged liquid exits the respective chamber 2.1, 2.2, 2.3, 2.4 via the respective chamber outlet 4. This is only to be understood as a definition of the fluid interface of the respective
35       chamber 2.1, 2.2, 2.3, 2.4. As will be explained later, in certain situations, the

- 7 -

chamber inlets 3 may be used as outlets and the chamber outlets 4 may be used as inlets respectively.

In order to facilitate the explanation of the invention, in the following, the specification states only one centrifuge chamber 2.1 with a chamber inlet 3 and a chamber outlet 4. All explanations regarding this centrifuge chamber 2.1 are fully applicable to any other centrifuge chamber, which may be provided.

The clarification setup comprises a liquid pumping arrangement 5 assigned to the centrifuge 1 and a liquid network 6 with a number of liquid lines 7 communicating with the pumping arrangement 5, wherein the clarification setup comprises a process control 8 for controlling at least the centrifuge 1 and the liquid pumping arrangement 5. Here and preferably, the pumping arrangement 5 is located upstream the centrifuge chamber 2.1 during the loading cycle to be explained.

The process control 8 may be realized as a central unit controlling all or at least most of the components of the clarification setup. The process control 8 may also be realized in a decentralized structure, comprising a number of decentralized units. In any case, the process control 8 comprises at least one microprocessor, on which a software may be run.

Here and preferably, the centrifuge 1 is designed as a fluidized bed centrifuge for performing a continuous centrifugation process. The preferred setup of the centrifuge 1 is described in European Patent EP 2 485 846 B1.

The centrifuge 1 comprises a rotor, which may be rotated around the centrifuge rotor axis by a preferably electric motor, which is controlled by the process control 8 for the realization of a centrifuge revolution speed. For centrifugation, the liquid pumping arrangement 5 pumps cell broth through the centrifuge chamber 2.1.
The centrifuge revolution speed as well as the pumping rate are adjusted, preferably by the process control 8, with the aim to establish a fluidized bed of particles such as cells or cell debris in the centrifuge chamber 2.1. A fluidized bed is achieved when the centrifugal force on a particle is equal to the opposing fluid flow force so that a zero net force is exerted on the particle.

- 8 -

In order to obtain a particle free supernatant, the centrifuged cell broth is pumped through a filter arrangement 9 of the clarification setup as shown in Fig. 1. For this, the liquid network 6 provides an outlet supernatant line 10 for a liquid connection between the chamber outlet 4 and the filter arrangement 9. Downstream of the filter arrangement 9 is provided a supernatant reception 21.1, in particular a supernatant vessel.

For better understanding, in the detailled view of the centrifuge chamber 2.1 in Fig. 1, the buffer is indicated with reference "B", the supernatant is indicated with reference "S" and the particles are indicated with reference "P".

It is particularly essential for the invention, that the outlet supernatant line 10 is at least temporarily, preferably permanently, liquid-tight, such that due to this liquid-tight condition of the outlet supernatant line 10 the liquid pumping arrangement 5 as such may drive the supernatant from the chamber outlet 4 to the filter arrangement 9 via the outlet supernatant line 10. In other words, the outlet supernatant line 10 is at least temporarily, preferably permanently, liquid tight such that the liquid pressure and/or the liquid flow may be controlled by the liquid pumping arrangement 5, which is assigned to the centrifuge 1. The expression "temporarily" means, that in the installed state of the clarification setup, the liquid tightness may be canceled for a certain time, for example by switching of a valve or the like. The expression "permanently" means, that in the installed state of the clarification setup, the liquid tightness is always guaranteed. The later embodiment includes, preferably, the outlet supernatant line 10 being fluid tight also in the uninstalled state.

As a result, the liquid connection between the centrifuge 1 and the filter arrangement 9 via the outlet supernatant line 10 at least temporarily, preferably permanently, is a closed system regarding liquids in the above noted sense. In addition, preferably, the connection between the centrifuge 1 and the filter arrangement 9 via the outlet supernatant line 10 at least temporarily, preferably permanently, is a closed system regarding gaseous media.

Here and preferably, the centrifuge 1 and the liquid pumping arrangement 5 are combined in a unit 11. Preferably, this means that a unit carrier is provided,

which carries all unit components, here the centrifuge 1 and the liquid pumping arrangement 5. The unit carrier may well include a housing, which houses all unit components.

5    The above noted unit 11 is preferably a self contained unit. The expression "self contained unit" means, that this unit 11 includes all components to provide a desired function, here the function of centrifugation. Such a self contained unit provides interfaces in mechanical, electrical, electronical and liquidical view, in order to be chained into an overall process.

10

Further preferably, the filter arrangement 9 is part of the above noted unit 11. As an alternative or in addition, the process control 8 is part of this self-contained unit 11 as well. As a result, the part of the downstream process including the centrifugation and subsequent filtration may well be realized as a

15    fully integrated process in a single, preferably self contained, unit.

The process control 8 is preferably designed to periodically execute a number of predetermined process cycles sequentially by controlling at least the centrifuge 1 and the liquid pumping arrangement 5. One of the process cycles

20    is a loading cycle as indicated in Fig. 1, during which cell broth is pumped into the chamber inlet 3. Another one of the cycles may be a washing cycle as indicated in Fig. 2, during which a buffer is pumped into the chamber inlet 3. Finally, another one of the cycles may be a discharging cycle, during which a buffer is pumped to the chamber outlet 4. While those cycles of loading cycle,

25    washing cycle and discharging cycle are well known for fluidized bed centrifugation, it is interesting here that the process control 8 is automatically executing those cycles sequentially, according to a certain control strategy. In the easiest case, the control strategy includes the execution of the respective cycles according to a fixed sequence in a fixed time pattern. However, the

30    control strategy may well be based on sensor signals to be discussed later.

In the following, the above noted process cycles will be explained in detail.

Preferably, as indicated in Fig. 1, the liquid network 6 comprises an inlet feed

35    line 12 between the chamber inlet 3 and a cell broth source 13, preferably a storage vessel or production vessel such as a bioreactor. During a loading

cycle, the cell broth may then be pumped by the liquid pumping arrangement 5 from the cell broth source 13 to the chamber inlet 3 via the inlet feed line 12, while the supernatant is flowing from the chamber outlet 4 to the filter arrangement 9 via the outlet supernatant line 10. In an especially preferred embodiment, during the loading cycle, the liquid pressure and/or the liquid flow at an inlet of the filter arrangement 9 may be controlled by the liquid pumping arrangement 5. This is due to the liquid tightness of the outlet supernatant line 10. It may also be necessary, that in the beginning of the loading cycle, remaining buffer in the centrifuge chamber 2.1 may be flushed into the waste reception 17 via the outlet waste line 14, before the chamber outlet 4 is connected by valve switching to the filter arrangement 9 via the outlet supernatant line 10.

Here it is to be understood, that the outlet supernatant line 10 and the outlet waste line 14 overlap each other along a certain liquid line section. Accordingly, the outlet supernatant line 10 on the one hand and the outlet waste line 14 on the other hand do not have to be separate from each other along their complete extent. This is true for all other definitions of liquid lines presented here, which are each being provided by part of the liquid network 6.

According to Fig. 2, the liquid network 6 preferably comprises an inlet washing line 15 between the chamber inlet 3 and a buffer source 16 and an outlet waste line 14 between the chamber outlet 4 and a waste reception 17, preferably a waste vessel, which here and preferably is the same as the waste reception 17. During a washing cycle, the buffer may be pumped by the liquid pumping arrangement 5 from the buffer source 16 to the chamber inlet 3 and from the chamber outlet 4 via the outlet supernatant line 10 to the filter arrangement 9 or from the chamber outlet 4 via the outlet waste line 14 to the waste reception 17. This means, that during the washing cycle, in an embodiment, the buffer may be pumped exclusively through the outlet supernatant line 10, and in another embodiment , the buffer may be pumped exclusively through the outlet waste line 14. In still another embodiment, during the washing cycle, the buffer may be pumped firstly through the outlet supernatant line 10 and subsequently through the outlet waste line 14.

5      Also during the washing cycle, the liquid pressure and/or the liquid flow in the
outlet waste line 14 may be controlled by the liquid pumping arrangement 5. It
may also be necessary, that in the beginning of the washing cycle, buffer and
remaining supernatant in the centrifuge chamber 2.1 is being pumped to the
filter arrangement 9 via the outlet supernatant line 10, before the chamber
outlet 4 is connected by valve switching to the waste reception 17 via the outlet
waste line 10.

10     Finally, it may be provided, that during the whole washing cycle, the chamber
outlet 4 is connected by valve switching to the filter arrangement 9 via the outlet
supernatant line 10. Then, the volume of the buffer to be pumped during the
washing cycle is to be as low as possible, in order to prevent an unduly dilution
of the supernatant in the supernatant reception 21.1. Preferably, during the
15     washing cycle, buffer and remaining supernatant in the centrifuge chamber 2.1
is being pumped from the chamber outlet 4 via the outlet supernatant line 10 to
the filter arrangement 9 for a short time, after which the washing cycle is being
terminated.

20     Fig. 3 shows, that the liquid network 6 comprises an outlet buffer line 19
between the chamber outlet 4 and the buffer source 16 and an inlet cell harvest
line 20 between the chamber inlet 3 and a cell harvest reception 21.2,
preferably a cell harvest vessel. This is for the case, that the cells in the cell
broth are to be obtained as a product. During a discharging cycle, the buffer
25     may then be pumped from the buffer source 16 to the chamber outlet 4 via the
outlet buffer line 19, while the buffer including solid particles, preferably cell
harvest, is flowing from the chamber inlet 3 to the cell harvest reception 21.2 via
the inlet cell harvest line 20. During the discharging cycle, the liquid pressure
and/or the liquid flow in the outlet buffer line 19 may be controlled by the liquid
30     pumping arrangement 5.

In case, during discharging, the cells are to be dismissed, as an alternative to
the inlet cell harvest line 20, there may be provided an inlet waste line 22
between the chamber inlet 3 and another waste reception 18, which may also
35     be the waste reception 17 at the same time. In this case, during the discharging
cycle, the buffer may be pumped from the buffer source 16 to the chamber

outlet 4 via the outlet buffer line 19, while the buffer including solid particles, preferably cell harvest, is flowing from the chamber inlet 3 to the waste reception 18 via the inlet waste line 22.

5     The clarification setup preferably comprises a valve arrangement 23 with at least one valve 24, here and preferably with valves 24.1-24.13, controlled by the process control 8, that allows to deactivate and activate at least one of the liquid lines 7, preferably by closing and opening the respective valve 24. In the drawings, the valves 24.1-24.13 are assigned the status "c" for closed and "o"
10     for opened. Accordingly, the valves 24 of the valve arrangement 8 are located within or at least at one end of the respective liquid lines 7 to be activated and deactivated.

For deactivation and activation of the respective liquid line 7, at least one valve
15     24 of the valve arrangement 23 is assigned to the respective liquid line 7. In the drawings, the valve arrangement 23 comprises valves 24.1 to 24.13 as noted above.

It is generally possible, that the filters 25-28 of the filter arrangement 9 are
20     activated and deactivated manually, for example by manually controllable valves.
It is also possible, that no valve is assigned to the filter arrangement 9, in which case, instead of deactivating part of the filter arrangement, the flushing of the filter arrangement 9 is being started manually.
25

The term "flushing" means a pre-conditioning of filters by rinsing them with buffer before the centrifugation process is started. In Addition, after the centrifugation process is finished, a post-flushing of the filters is also prefered in some cases in order to flush out remaining product in the filters and filter lines
30     into the harvest reception.

Here and preferably, however, it is provided that the valve arrangement 23 is controllable by the process control 8, such that activation and deactivation of the filters 25-28 of the filter arrangement 9 may be easily automated. The term
35     "activate" and "deactivate" with regards to the filters 25-28 of the filter

- 13 -

arrangement 9 generally means, that liquid flow through the respective filter 25-28 is enabled or disabled.

5

In the shown and insofar preferred embodiment the valve arrangement 23 allows to deactivate and activate at least one filter 25-28 of the filter arrangement 9, preferably by closing and opening the respective valve 24. Further preferably, for deactivation and activation of the respective filter 25-28, at least one valve 24 of the valve arrangement 23 is assigned to the respective filter 25-28.

10

The valve arrangement 23 also may allow to connect by valve switching the buffer source 16 to the filter arrangement 9 for flushing at least one filter 25-28 of the filter arrangement 9. For flushing, another liquid pumping arrangement 34, which is preferably assigned to the unit 11, may be provided as shown in

15    the drawings.

Generally, the filter arrangement 9 may comprise only one filter stage. Here and preferably, though, the filter arrangement 9 comprises a first filter stage 25,26 and a subsequent second filter stage 27, 28, wherein, further preferably, the

20    first filter stage 25, 26 comprises at least one depth filter and wherein the second filter stage 27, 28 comprises at least one sterile filter. In a particularly simple case, the first filter stage and the second filter stage comprise only one filter. Again, as an alternative, those filters may be activated and deactivated manually.

25

As noted above, the filter arrangement 9 may also comprise only a single filter stage. For example, the filter arrangement may then comprise a multi layer membrane filter, which combines the function of the depth filter with the function of the sterile filter.

30

Further preferably, the clarification setup comprises a sensor arrangement 29 for measuring properties of the liquid in at least one of the liquid lines 7, which sensor arrangement 29 provides sensor signals to the process control 8.

35

The process control 8 may activate or deactivate at least one of the liquid lines 7 via the valve arrangement 23 based on the sensor signals of the sensor

- 14 -

arrangement 29 and according to the respective control strategy. It is preferred, that the sensor arrangement 29 comprises a biomass sensor arrangement 30, preferably an optical biomass sensor arrangement, for measuring an occurrence level of biomass, preferably the biomass concentration, in the

5    respective liquid line 7. During the loading cycle, depending on the measured occurrence level, preferably biomass concentration, the process control 8 then deactivates or activates the inlet feed line 12 and/or starts the washing cycle.

As an alternative or in addition, the sensor arrangement 29 comprises a

10   supernatant sensor arrangement 31, preferably an optical supernatant sensor arrangement, for measuring an occurrence level of supernatant in the respective liquid line 7. During the loading cycle and/or the washing cycle, depending on the measured occurrence level of supernatant, the process control 8 then deactivates or activates the outlet supernatant line 10 and the

15   outlet waste line 14 via the valve arrangement 23.

Preferably during the loading cycle, the process control 8 activates or deactivates at least one filter 25-28 of the filter arrangement 9 based on the sensor signals of the sensor arrangement 29. In a particularly preferred

20   embodiment, the sensor arrangement 29 comprises at least one pressure sensor 32, 33 assigned to a filter 25-28 of the filter arrangement 9, which pressure sensor 32, 33 measures the liquid pressure upstream of the respective filter 25-28. Further preferably, the process control 8 initiates an overpressure routine in case the measured liquid pressure exceeds a

25   predefined value. It is also preferred, that the overpressure routine includes a flush routine of the respective filter 25-28 of the filter arrangement 9, and/or, that the overpressure routine includes circumpassing the respective filter 25-28 by a backup filter of the filter arrangement 9, and/or, that the overpressure routine includes terminating the loading cycle of the clarification setup.

30

Generally it may be provided, that the centrifuge chamber 2 is designed as a single use component. As an alternative or in addition, it may be advantageous, that at least part of the liquid network 6, in particular the outlet supernatant line 10, is designed as a single use component, further preferably, that at least part

35   of the liquid filter arrangement 9 is designed as a single use component. The single use component, in particular the supernatant line 10, is preferably made

- 15 -

of a plastic material at least partly, such that it may be realized with low effort. In a particularly preferred embodiment, the single use component is at least partly made of a silicon material and/or a polymer material.

5    In particular and as indicated above, the outlet supernatant line 10 as such may be provided as a single use component. With this, it is particularly easy to guarantee the proposed liquid tightness of the outlet supernatant line 10 without the risk of compromising the liquid tightness due to user errors during installation.

10

All in all it may be advantageous to provide all components, that are in direct contact with liquid, as single use components. This would at least include all liquid lines 7, all centrifuge chambers 2.1, 2.2, 2.3, 2.4, all filter 25-28 of the filter arrangement 9. It may also include at least part of the valve arrangement

15   23 and/or at least part of the sensor arrangement 29. In particular, single use sensors may be part of the sensor arrangement 29.

According to a separate teaching, the bioprocess installation with a proposed clarification setup and with a cell broth source 13 in the form of a storage vessel

20   or production vessel, such as a bioreactor, is claimed as such. As noted above, all explanations given for the proposed clarification setup are fully applicable.

According to another separate teaching, a method for operating the proposed clarification setup is claimed as such, wherein the clarification setup comprises

25   a valve arrangement 23 controlled by the process control 8 and wherein at least one filter 25-28 of the filter arrangement 9 is being deactivated and activated by closing and opening the respective valve 24 of the valve arrangement 23. As also noted above, all explanations given for the proposed clarification setup are fully applicable.

30

## Claims

1.    Clarification setup of a bioprocessing installation for the clarification of a cell broth by centrifugation and subsequent filtration, wherein, for the centrifugation, the clarification setup comprises a centrifuge (1), wherein the centrifuge (1) comprises at least one centrifuge chamber (2.1) with a chamber inlet (3) and a chamber outlet (4), wherein the clarification setup comprises a liquid pumping arrangement (5) assigned to the centrifuge (1) and a liquid network (6) with a number of liquid lines (7) communicating with the liquid pumping arrangement (5), wherein the clarification setup comprises a process control (8) for controlling at least the centrifuge (1) and the liquid pumping arrangement (5),

**characterized in**

that for filtration, the clarification setup comprises a filter arrangement (9), that the liquid network (6) provides an outlet supernatant line (10) for a liquid connection between the chamber outlet (4) and the filter arrangement (9), which is at least temporarily, preferably permanently, liquid-tight, such that due to this liquid-tight condition of the outlet supernatant line (10) the liquid pumping arrangement (5) as such may drive the supernatant from the chamber outlet (4) to the filter arrangement (9) via the outlet supernatant line (10).

2.    Clarification setup according to claim 1, characterized in that the centrifuge (1) and the liquid pumping arrangement (5) are combined in a preferably self-contained unit (11), preferably, that the filter arrangement (9) is part of this self-contained unit (11), and/or, that the process control (8) is part of this self-contained unit (11).

3.    Clarification setup according to claim 1 or 2, characterized in that the process control (8) is designed to execute a number of predetermined process cycles sequentially by controlling at least the centrifuge (1) and the liquid pumping arrangement (5), preferably, that one of the process cycles is a loading cycle, during which cell broth is pumped into the chamber inlet (3), and/or, that one of the cycles is a washing cycle, during which a buffer is pumped into the chamber inlet (3), and/or, that one of the cycles is a discharging cycle, during which a buffer is pumped to the chamber outlet (4).

4. Clarification setup according to any one of the preceding claims, characterized in that the liquid network (6) comprises an inlet feed line (12) between the chamber inlet (3) and a cell broth source (13), preferably a storage vessel or production vessel, such as a bioreactor, and that during a loading cycle the cell broth may be pumped by the liquid pumping arrangement (5) from the cell broth source (13) to the chamber inlet (3) via the inlet feed line (12), while the supernatant is flowing from the chamber outlet (4) to the filter arrangement (9) via the outlet supernatant line (10), preferably, that during the loading cycle, the liquid pressure and/or the liquid flow at an inlet of the filter arrangement (9) may be controlled by the liquid pumping arrangement (5).

5. Clarification setup according to any one of the preceding claims, characterized in that the liquid network (6) comprises an inlet washing line (15) between the chamber inlet (3) and a buffer source (16) and an outlet waste line (14) between the chamber outlet (4) and a waste reception (17, 18), preferably a waste vessel, and that during a washing cycle the buffer may be pumped by the liquid pumping arrangement (5) from the buffer source (16) to the chamber inlet (3) and from the chamber outlet (4) via the outlet supernatant line (10) to the filter arrangement (9) or from the chamber outlet (4) via the outlet waste line (14) to the waste reception, preferably, that during the washing cycle, the liquid pressure and/or the liquid flow in the outlet waste line (14) may be controlled by the liquid pumping arrangement (5).

6. Clarification setup according to any one of the preceding claims, characterized in that the liquid network (6) comprises an outlet buffer line (19) between the chamber outlet (4) and a buffer source (16) and an inlet cell harvest line (20) between the chamber inlet (3) and a cell harvest reception (21.2), preferably a cell harvest vessel, and that during a discharging cycle the buffer may be pumped from the buffer source (16) to the chamber outlet (4) via the outlet buffer line (19), while the buffer including solid particles, preferably cell harvest, is flowing from the chamber inlet (3) to the cell harvest reception (21.2) via the inlet cell harvest line (20) or from the chamber inlet (3) to the waste reception (18) via the inlet waste line (22), preferably, that during the discharging cycle, the liquid pressure and/or the liquid flow in the outlet buffer line (19) may be controlled by the liquid pumping arrangement (5).

7. Clarification setup according to any one of the preceding claims, characterized in that the clarification setup comprises a valve arrangement (23) with valves (24), each controlled by the process control (8), which allows to deactivate and activate at least one of the liquid lines (7), preferably by closing

5    and opening the respective valves (24) of the valve arrangement (23), further preferably, that, for deactivation and activation of the respective liquid line (7), at least one valve (24) of the valve arrangement (23) is assigned to the respective liquid line (7).

10   8. Clarification setup according to claim 7, characterized in that the valve arrangement (23) allows to deactivate and activate at least one filter of the filter arrangement (9), preferably by closing and opening the respective filter (25-28), further preferably, that, for deactivation and activation of the respective filter (25-28), at least one valve (24) of the valve arrangement (23) is assigned to the

15   respective filter (25-28).

9. Clarification setup according to any one of the preceding claims, characterized in that the valve arrangement (23) allows to connect by valve switching the buffer source (16) to the filter arrangement (9) for flushing at least

20   one filter (25-28) of the filter arrangement (9).

10. Clarification setup according to any one of the preceding claims, characterized in that the filter arrangement (9) comprises at least one filter stage, more preferably a first filter stage (25, 26) and a subsequent second filter

25   stage (27, 28), preferably, that the first filter stage (25, 26) comprises at least one depth filter and that the second filter stage (27, 28) comprises at least one sterile filter.

11. Clarification setup according to any one of the preceding claims,
30   characterized in that the clarification setup comprises a sensor arrangement (29) for measuring properties of the liquid in at least one of the liquid lines (7), which sensor arrangement (29) provides sensor signals to the process control (8).

35   12. Clarification setup according to any one of the preceding claims, characterized in that the process control (8) activates or deactivates at least

- 19 -

one of the liquid lines (7) based on the sensor signals of the sensor arrangement (29), preferably, that the sensor arrangement (29) comprises a biomass sensor arrangement (30), preferably an optical biomass sensor arrangement, for measuring an occurrence level of biomass, preferably the

5    biomass concentration, in the respective liquid line (7) and that during the loading cycle, depending on the measured occurrence level of biomass, the process control (8) deactivates/activates the inlet feed line (12), and/or, that the sensor arrangement (29) comprises a supernatant sensor arrangement (31), preferably an optical supernatant sensor arrangement, for measuring an

10   occurrence level of supernatant in the respective liquid line (7) and that during the loading cycle and/or the washing cycle, depending on the measured occurrence level of supernatant, the process control (8) deactivates/activates the outlet supernatant line (10) and the outlet waste line (14).

15   13. Clarification setup according to any one of the preceding claims, characterized in that, preferably during the loading cycle, the process control (8) activates or deactivates at least one filter (25-28) of the filter arrangement (9) based on the sensor signals of the sensor arrangement (29), preferably, that the sensor arrangement (29) comprises at least one pressure sensor (32, 33)

20   assigned to a filter (25-28) of the filter arrangement (9), which pressure sensor (32, 33) measures the liquid pressure upstream of the respective filter (25-28), further preferably, that the process control (8) initiates an overpressure routine in case the measured liquid pressure exceeds a predefined value, further preferably, that the overpressure routine includes a flush routine of the

25   respective filter (25-28) of the filter arrangement (9), and/or, that the overpressure routine includes circumpassing the respective filter (25-28) by a backup filter of the filter arrangement (9), and/or, that the overpressure routine includes terminating the loading cycle of the clarification setup.

30   14. Clarification setup according to any one of the preceding claims, characterized in that the centrifuge chamber (2) is designed as a single use component, preferably, that at least part of the liquid network (6), in particular the outlet supernatant line (10), is designed as a single use component, further preferably, that at least part of the filter arrangement (9) is designed as a single

35   use component.

- 20 -

15. Bioprocess installation with a clarification setup according to any one of the preceding claims and with a cell broth source (13) in the form of a storage vessel or a production vessel such as a bioreactor.

5       16. Method for operating a clarification setup according to any one of the claims 1 to 14 or a bioprocess installation according to claim 15, wherein the clarification setup comprises a valve arrangement (23) controlled by the process control (8) and wherein at least one filter (25-28) of the filter arrangement (9) is being deactivated and activated by closing and opening the
10      respective valve (24) of the valve arrangement (23).

17. Method according to claim 16, characterized in that the clarification setup comprises a sensor arrangement (29) for measuring properties of the liquid in at least one of the liquid lines (7), which sensor arrangement (29) provides
15      sensor signals to the process control (8) and that at least one filter (25-28) of the filter arrangement (9) is being deactivated/activated based on the sensor signals of the sensor arrangement (29).
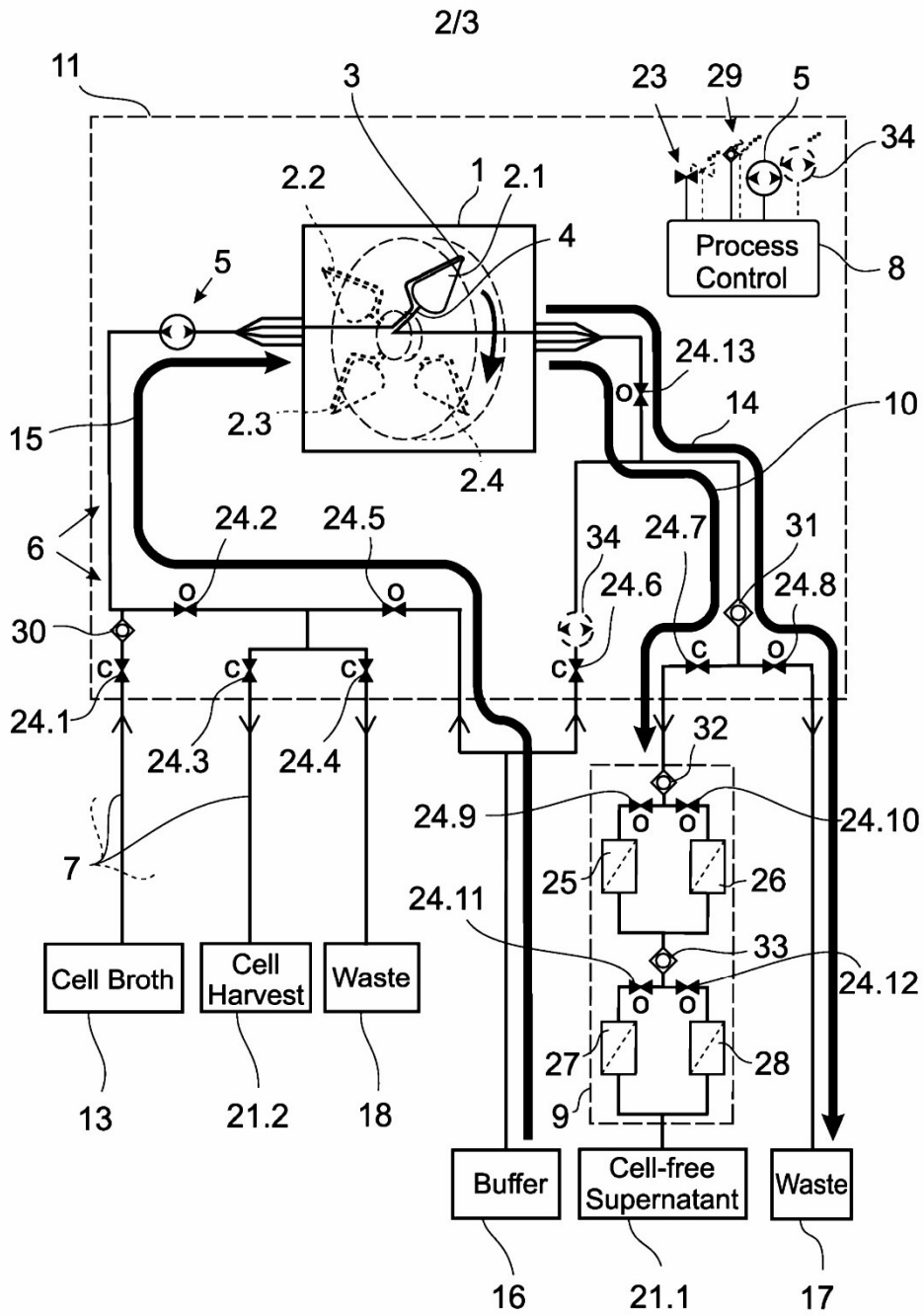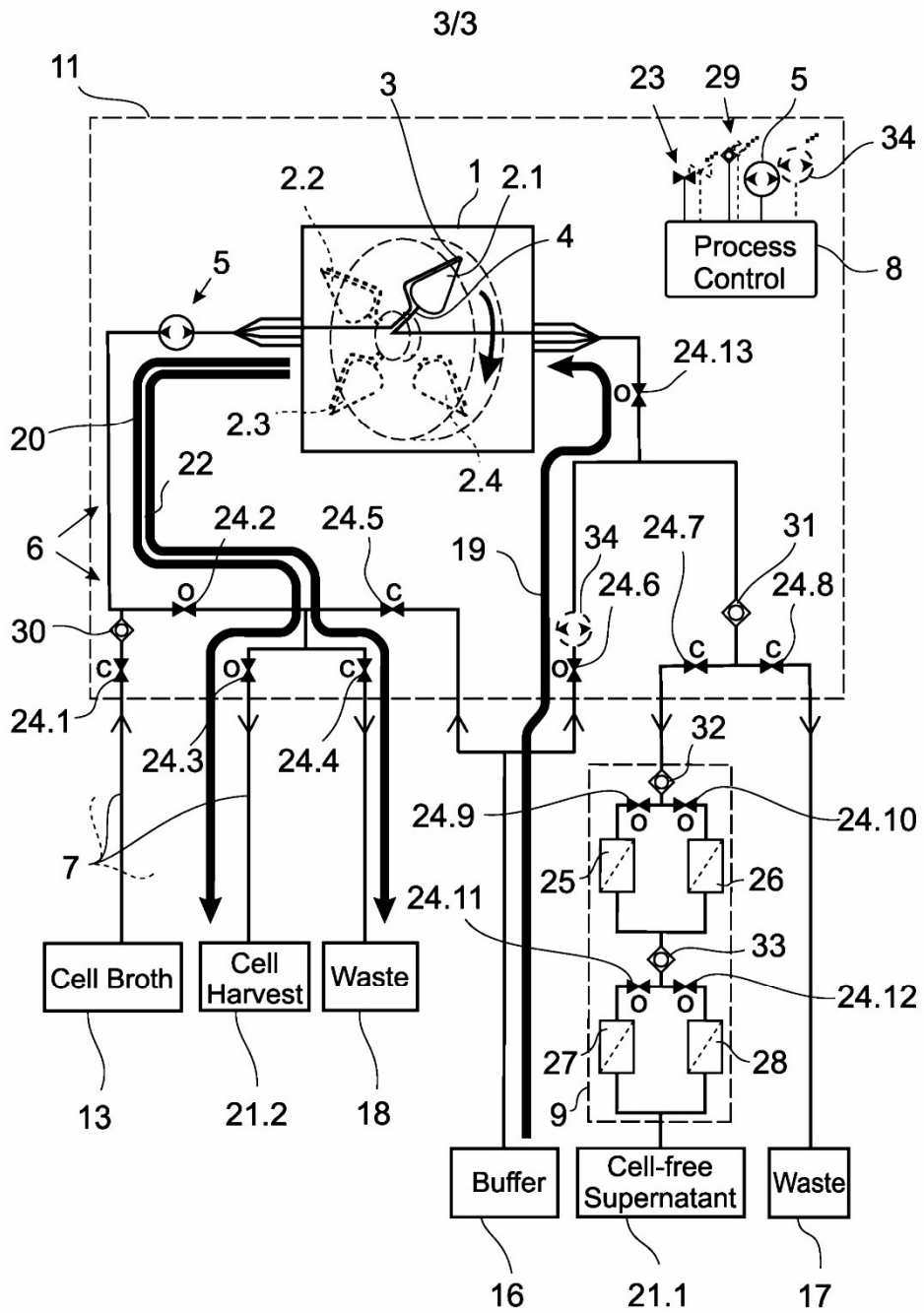
20

1/3



Fig. 1
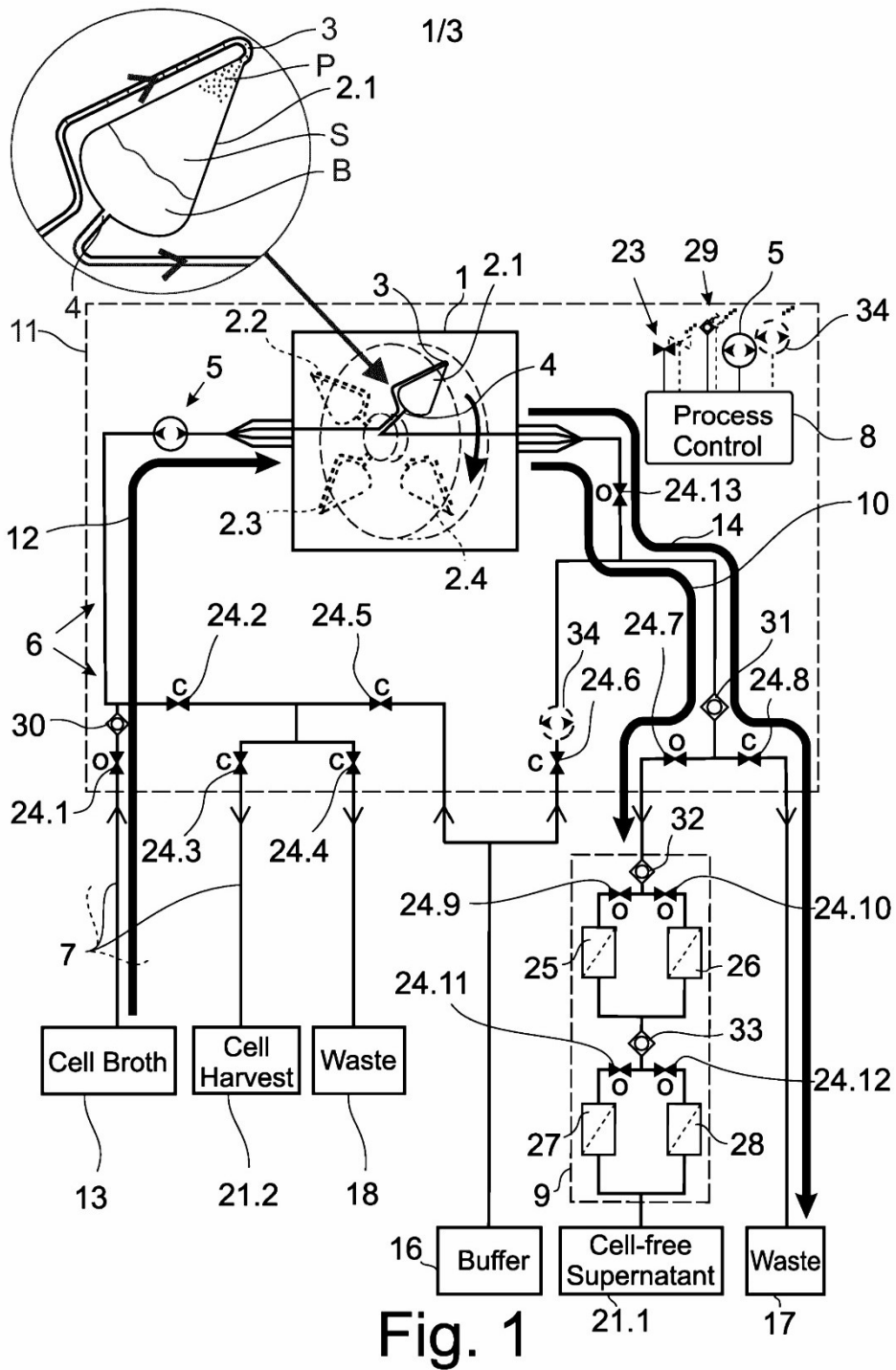
Fig. 2

Fig. 3

**INTERNATIONAL SEARCH REPORT**

| International application No |
| --- |
| PCT/EP2021/068706 |

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. C12M1/26
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| --- | --- | --- |
| Y | WO 2011/044237 A1 (KBI BIOPHARMA INC [US]; MEHTA SUNIL [US] ET AL.) 14 April 2011 (2011-04-14) claim 1 ----- | 1-17 |
| Y | WO 2006/007459 A1 (BIOGEN IDEC INC [US]; PHAM CHRISTINE Y [US]; THOEMMES JOERG [US]) 19 January 2006 (2006-01-19) claim 1 ----- | 1-17 |
| A | US 2018/330043 A1 (OPPENHEIM SHELDON F [US] ET AL) 15 November 2018 (2018-11-15) claim 1 ----- | 1-17 |

☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
| --- | --- |
| 4 October 2021 | 13/10/2021 |

| Name and mailing address of the ISA/ | Authorized officer |
| --- | --- |
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016 | Jones, Laura |

1

Form PCT/ISA/210 (second sheet) (April 2005)

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| WO 2011044237 | A1 | 14-04-2011 | AU | 2010303553 A1 | 03-05-2012 |
| | | | CA | 2776750 A1 | 14-04-2011 |
| | | | EP | 2485846 A1 | 15-08-2012 |
| | | | EP | 3391970 A1 | 24-10-2018 |
| | | | JP | 5774012 B2 | 02-09-2015 |
| | | | JP | 6468959 B2 | 13-02-2019 |
| | | | JP | 6679695 B2 | 15-04-2020 |
| | | | JP | 2013506556 A | 28-02-2013 |
| | | | JP | 2015211968 A | 26-11-2015 |
| | | | JP | 2019030876 A | 28-02-2019 |
| | | | KR | 20120102050 A | 17-09-2012 |
| | | | KR | 20180002895 A | 08-01-2018 |
| | | | SG | 10201406807Q A | 29-01-2015 |
| | | | US | 2012270717 A1 | 25-10-2012 |
| | | | US | 2018065127 A1 | 08-03-2018 |
| | | | WO | 2011044237 A1 | 14-04-2011 |
| WO 2006007459 | A1 | 19-01-2006 | AU | 2005262431 A1 | 19-01-2006 |
| | | | BR | PI0512378 A | 11-03-2008 |
| | | | CA | 2572181 A1 | 19-01-2006 |
| | | | CN | 101014402 A | 08-08-2007 |
| | | | EA | 200700094 A1 | 27-04-2007 |
| | | | EP | 1776174 A1 | 25-04-2007 |
| | | | IS | 8582 A | 19-12-2006 |
| | | | JP | 2008503218 A | 07-02-2008 |
| | | | KR | 20070046818 A | 03-05-2007 |
| | | | RS | 20060680 A | 05-06-2008 |
| | | | US | 2005037333 A1 | 17-02-2005 |
| | | | US | 2007267360 A1 | 22-11-2007 |
| | | | WO | 2006007459 A1 | 19-01-2006 |
| | | | ZA | 200700180 B | 28-05-2008 |
| US 2018330043 | A1 | 15-11-2018 | US | 2014324360 A1 | 30-10-2014 |
| | | | US | 2018330043 A1 | 15-11-2018 |
| | | | WO | 2013043445 A2 | 28-03-2013 |

Form PCT/ISA/210 (patent family annex) (April 2005)

85

# 5 Conclusion and Outlook

Cell clarification is the connective unit operation between USP and DSP. In the biomanufacturing of mAb, clarification must therefore meet both the robust product harvest from various cell culture broths after USP and the requirements of the subsequent DSP to ensure the high purity standards of the authorities for biopharmaceutical products.

In recent years, the USP have been continuously intensified to increase productivities in order to satisfy the growing global demand for affordable mAb´s. However, the high cell concentrations usually applied for this purpose increase the burden on all subsequent steps. As a result, established platform approaches for cell clarification, such as disc stack centrifugation and depth filtration, are limited by low biomass loading capacities and low product recoveries, making them unsuitable for the clarification of HCD processes.

Due to the lack of appropriated technologies, the motivation of this study was to develop and optimize a process platform enabling efficient clarification of HCD broth with up to 100 million cells/mL. A further requirement on the approach was its ability to be integrated into SU-based production processes, which is an additional strategy for process intensification. Focus of this thesis was also placed on the process robustness to reach a high mAb recovery and a high purity for a broad range of different cell broth characteristics. Beside the removal of all particulate cell and cell debris impurities, additional removal of molecular process related impurities should also be investigated with the aim of further intensifying a potential clarification operation. Finally, the impact of the clarification approach on critical mAb quality attributes should be assessed to evaluate the efficiency and safety of the process.

As one of the main findings of this PhD thesis, SU-based fluidized bed centrifugation was identified as an efficient first step for the removal of CHO cells from HCD cultures. Due to an optimization of the FBC process parameters, the cell broth throughput, supernatant purity, and mAb recovery were increased. It was shown that the ability to wash the captured cells in the fluidized bed is one of the most significant impact factors to obtain high mAb recoveries of approximately 95 %. In order to achieve complete removal of all particulate impurities, a suitable filter assembly consisting of a depth filter followed by a sterile filter was selected by a screening.

An experimental study to clarify various cell broth conditions ranging from $20 \times 10^6$ to $110 \times 10^6$ cells/mL confirmed applicability and robust process performance of the approach. This finding demonstrated that a FBC step with a small filtration step has a high potential to be used as a platform approach in industrial biomanufacturing.

In addition, a process model of the FBC step was established to determine the average throughput and dilution rate of the supernatant. The knowledge gained in this work, can be used to adapt the clarification UO depending on the bioreactor volume and the cell concentration in the cultivation broth.

In order to intensify the clarification process and additionally improve the removal of process related impurities in the subsequent DSP, the clarification approach was combined with pretreatments of the cell cultures. A flocculation and a precipitation treatment were found, that had both hardly any effect on the FBC process as well as the quality of mAb but were capable to increase the maximum depth filter capacity up to four-fold and remove approximately 90 % of DNA impurities already in the clarification UO. However, on the one hand, this study showed that due to the harsh environmental conditions caused by the pretreatments, evaluation and careful adaptation to the particular mAb is required. On the other hand, the approach promises significant improvements in the process, which is particularly advantageous for low-viability cell cultures with a high load of cell debris and process-related impurities.

A further achievement of this work is the development of an enhanced SU process platform based on the mentioned approach. Through a direct connection of both clarification steps the limitations of stepwise clarification were bypassed, resulting in improved process control, faster clarification, and significant reduction of required process equipment. Furthermore, the established connected and thus closed flow path from the bioreactor to the sterile particle free harvest vessel increases the process safety. In a proof-of-concept study, the feasibility of this streamlined clarification approach using fluidized bed centrifugation with integrated filtration was successfully demonstrated up to 200 L bioreactor pilot scale.

Another interesting field of application identified during this work could be the re-use of viable cells after the removal of the product containing supernatant by the FBC. Usually, the concentrated and washed cells are discharged after the clarification, however, it was found that the mild process conditions in combination with sterile processing keeps the cells intact during the FBC process. After transferring the cells back into fresh culture medium further cell growth and mAb production were observed, suggesting that the cells can be re-used in a second production cycle. Since this approach could significantly increase the productivity of USP, further studies are needed to evaluate this effect and use it in new USP modalities.

In further studies, additional focus should be placed on integration of the developed clarification platform in continuous production to meet the requirements of fully continuous mAb processing and connection of further DSP UO. Such operation could enable synergistic effects, like the combination of the cyclic clarification processing and the continuous product capture using multi column chromatography, and thus further intensify the overall manufacturing process.

Moreover, further studies are needed to validate the developed process platform using upscaled FBC systems to increase the throughput so that they can be used in large-scale industrial SU manufacturing, where typical bioreactor volumes of 2,000 L are applied. Additional cost-of-goods modeling is recommended to identify the largest cost drivers of the process and to compare the costs of other platform concepts with the developed approach.

In conclusion, a robust and efficient clarification UO is a key to connect USP with DSP enabling production of large quantities of affordable mAb´s. The developed SU approach using a FBC followed by a filtration step allows such intensified clarification for various process streams including HCD processes. This and the ability to perform both connected clarification steps in an automated way offers great potential to use the approach as a process platform. The outcome of this work therefore supports the holistic efforts to intensify biopharmaceutical production, which helps to provide patients with better treatments.

# 6 List of References

[1] S. Singh, N.K. Kumar, P. Dwiwedi, J. Charan, R. Kaur, P. Sidhu, V.K. Chugh, Monoclonal Antibodies: A Review, Curr. Clin. Pharmacol. 13 (2018) 85–99. https://doi.org/10.2174/1574884712666170809124728.

[2] P.C. Taylor, A.C. Adams, M.M. Hufford, I. de La Torre, K. Winthrop, R.L. Gottlieb, Neutralizing monoclonal antibodies for treatment of COVID-19, Nat. Rev. Immunol. 21 (2021) 382–393. https://doi.org/10.1038/s41577-021-00542-x.

[3] The Antibody Society, Antibody therapeutics approved or in regulatory review in the EU or US, 2022. https://www.antibodysociety.org/resources/approved-antibodies/ (accessed 24 January 2022).

[4] A. Mullard, FDA approves 100th monoclonal antibody product, Nat. Rev. Drug Discov. 20 (2021) 491–495. https://doi.org/10.1038/d41573-021-00079-7.

[5] R.-M. Lu, Y.-C. Hwang, I.-J. Liu, C.-C. Lee, H.-Z. Tsai, H.-J. Li, H.-C. Wu, Development of therapeutic antibodies for the treatment of diseases, J. Biomed. Sci. 27 (2020) 1. https://doi.org/10.1186/s12929-019-0592-z.

[6] D. Corti, L.A. Purcell, G. Snell, D. Veesler, Tackling COVID-19 with neutralizing monoclonal antibodies, Cell 184 (2021) 3086–3108. https://doi.org/10.1016/j.cell.2021.05.005.

[7] European Medicines Agency, COVID-19 treatments, 2022. https://www.ema.europa.eu/en/human-regulatory/overview/public-health-threats/coronavirus-disease-covid-19/treatments-vaccines/covid-19-treatments (accessed 24 January 2022).

[8] D. Reinhart, L. Damjanovic, C. Kaisermayer, R. Kunert, Benchmarking of commercially available CHO cell culture media for antibody production, Appl. Microbiol. Biotechnol. 99 (2015) 4645–4657. https://doi.org/10.1007/s00253-015-6514-4.

[9] C. Chen, H.E. Wong, C.T. Goudar, Upstream process intensification and continuous manufacturing, Current Opinion in Chemical Engineering 22 (2018) 191–198. https://doi.org/10.1016/j.coche.2018.10.006.

[10] J.P. Pieracci, J.W. Armando, M. Westoby, J. Thommes (Eds.), Industry Review of Cell Separation and Product Harvesting Methods. (2018) 165-206. https://doi.org/10.1016/B978-0-08-100623-8.00009-8

[11] U. Gottschalk (Hrsg.), Process Scale Purification of Antibodies. Wiley, Hoboken, NJ (2017) https://doi.org/ 10.1002/9781119126942.ch1

[12] I. BioPlan Associates, Report and Survey of Biopharmaceutical Manufacturing Capacity and Production, 2021. https://www.bioplanassociates.com/wp-content/uploads/2021/07/18th-Annual-Report-TABLE-OF-CONTENTS-07212021.pdf (accessed 24 January 2022).

[13] N. Singh, A. Arunkumar, S. Chollangi, Z.G. Tan, M. Borys, Z.J. Li, Clarification technologies for monoclonal antibody manufacturing processes: Current state and future perspectives, Biotechnol. Bioeng. 113 (2016) 698–716. https://doi.org/10.1002/bit.25810.

[14] M.-F. Clincke, C. Mölleryd, Y. Zhang, E. Lindskog, K. Walsh, V. Chotteau, Very high density of CHO cells in perfusion by ATF or TFF in WAVE bioreactor™. Part I. Effect of the cell density on the process, Biotechnol. Prog. 29 (2013) 754–767. https://doi.org/10.1002/btpr.1704.

[15] BioPhorum Operations Group, Biomanufacturing Technology Roadmap: Process Technologies 2017. https://www.biophorum. com/wp-content/uploads/bp_downloads/ProcessTechs.pdf. (accessed 24 July 2020)

[16] M. Iammarino, J. Nti-Gyabaah, M. Chandler, D. Roush, K. Göklen, Impact of Cell Density and Viability on Primary Clarification of Mammalian Cell Broth: An Analysis Using Disc-Stack Centrifugation and Charged Depth Filtration, bioprocess international 2007 (2007) 38–50.

[17] S. Tomic, L. Besnard, B. Fürst, R. Reithmeier, R. Wichmann, P. Schelling, C. Hakemeyer, Complete clarification solution for processing high density cell culture harvests, Separation and Purification Technology 141 (2015) 269–275. https://doi.org/10.1016/j.seppur.2014.12.002.

[18] L.J. Wilson, W. Lewis, R. Kucia-Tran, D.G. Bracewell, Identification of upstream culture conditions and harvest time parameters that affect host cell protein clearance, Biotechnol. Prog. 35 (2019) e2805. https://doi.org/10.1002/btpr.2805.

[19] S. Mehta, Automated Single-Use Centrifugation Solution for Diverse Biomanufacturing Process 225 (2014) 385–400. https://doi.org/10.1002/9783527673681.ch15.

[20] N. Singh, S. Chollangi (Eds.), Next-Generation Clarification Technologies for the Downstream of Antibodies, Wiley, Hoboken, NF, 2017.

[21] G. Vidarsson, G. Dekkers, T. Rispens, IgG subclasses and allotypes: from structure to effector functions, Front. Immunol. 5 (2014) 520. https://doi.org/10.3389/fimmu.2014.00520.

[22] D.J. Falconer, G.P. Subedi, A.M. Marcella, A.W. Barb, Antibody Fucosylation Lowers the FcγRIIIa/CD16a Affinity by Limiting the Conformations Sampled by the N162-Glycan, ACS Chem. Biol. 13 (2018) 2179–2189. https://doi.org/10.1021/acschembio.8b00342.

[23] S.-J. Wu, J. Luo, K.T. O'Neil, J. Kang, E.R. Lacy, G. Canziani, A. Baker, M. Huang, Q.M. Tang, T.S. Raju, S.A. Jacobs, A. Teplyakov, G.L. Gilliland, Y. Feng, Structure-based engineering of a monoclonal antibody for improved solubility, Protein Eng. Des. Sel. 23 (2010) 643–651. https://doi.org/10.1093/protein/gzq037.

[24] A.M. Goetze, Y.D. Liu, Z. Zhang, B. Shah, E. Lee, P.V. Bondarenko, G.C. Flynn, High-mannose glycans on the Fc region of therapeutic IgG antibodies increase serum clearance in humans, Glycobiology 21 (2011) 949–959. https://doi.org/10.1093/glycob/cwr027.

[25] J. Hodoniczky, Y.Z. Zheng, D.C. James, Control of recombinant monoclonal antibody effector functions by Fc N-glycan remodeling in vitro, Biotechnol Progress 21 (2005) 1644–1652. https://doi.org/10.1021/bp050228w.

[26] F. Higel, A. Seidl, F. Sörgel, W. Friess, N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins, Eur. J. Pharm. Biopharm. 100 (2016) 94–100. https://doi.org/10.1016/j.ejpb.2016.01.005.

[27] F. Cymer, H. Beck, A. Rohde, D. Reusch, Therapeutic monoclonal antibody N-glycosylation - Structure, function and therapeutic potential, Biologicals 52 (2018) 1–11. https://doi.org/10.1016/j.biologicals.2017.11.001.

[28] G. Köhler, C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256 (1975) 495–497. https://doi.org/10.1038/256495a0.

[29] R. Mastrangeli, W. Palinsky, H. Bierau, Glycoengineered antibodies: towards the next-generation of immunotherapeutics, Glycobiology 29 (2019) 199–210. https://doi.org/10.1093/glycob/cwy092.

[30] V. Tejwani, M.R. Andersen, J.H. Nam, S.T. Sharfstein, Glycoengineering in CHO Cells: Advances in Systems Biology, Biotechnol. J. 13 (2018) e1700234. https://doi.org/10.1002/biot.201700234.

[31] D. Kim, J. Lee, J. Bal, S.K. Seo, C.-K. Chong, J.H. Lee, H. Park, Development and Clinical Evaluation of an Immunochromatography-Based Rapid Antigen Test (GenBody™ COVAG025) for COVID-19 Diagnosis, Viruses 13 (2021). https://doi.org/10.3390/v13050796.

[32] S. Aydin, A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA, Peptides 72 (2015) 4–15. https://doi.org/10.1016/j.peptides.2015.04.012.

[33] R.-M. Lu, Y.-C. Hwang, I.-J. Liu, C.-C. Lee, H.-Z. Tsai, H.-J. Li, H.-C. Wu, Development of therapeutic antibodies for the treatment of diseases, J. Biomed. Sci. 27 (2020) 1. https://doi.org/10.1186/s12929-019-0592-z.

[34] M.V. Beccari, B.T. Mogle, E.F. Sidman, K.A. Mastro, E. Asiago-Reddy, W.D. Kufel, Ibalizumab, a Novel Monoclonal Antibody for the Management of Multidrug-Resistant HIV-1 Infection, Antimicrob. Agents Chemother. 63 (2019). https://doi.org/10.1128/AAC.00110-19.

[35] M.A. Mintun, A.C. Lo, C. Duggan Evans, A.M. Wessels, P.A. Ardayfio, S.W. Andersen, S. Shcherbinin, J. Sparks, J.R. Sims, M. Brys, L.G. Apostolova, S.P. Salloway, D.M. Skovronsky, Donanemab in Early Alzheimer's Disease, N. Engl. J. Med. 384 (2021) 1691–1704. https://doi.org/10.1056/NEJMoa2100708.

[36] R.A. Siemieniuk, J.J. Bartoszko, J.P. Díaz Martinez, E. Kum, A. Qasim, D. Zeraatkar, A. Izcovich, S. Mangala, L. Ge, M.A. Han, T. Agoritsas, D. Arnold, C. Ávila, D.K. Chu, R. Couban, E. Cusano, A.J. Darzi, T. Devji, F. Foroutan, M. Ghadimi, A. Khamis, F. Lamontagne, M. Loeb, A. Miroshnychenko, S. Motaghi, S. Murthy, R.A. Mustafa, G. Rada, B. Rochwerg, C. Switzer, P.O. Vandvik, R.W. Vernooij, Y. Wang, L. Yao, G.H. Guyatt, R. Brignardello-Petersen, Antibody and cellular therapies for treatment of covid-19: a living systematic review and network meta-analysis, BMJ 374 (2021) n2231. https://doi.org/10.1136/bmj.n2231.

[37] A.A. Shukla, J. Thömmes, Recent advances in large-scale production of monoclonal antibodies and related proteins, Trends Biotechnol. 28 (2010) 253–261. https://doi.org/10.1016/j.tibtech.2010.02.001.

[38] A.A. Shukla, B. Hubbard, T. Tressel, S. Guhan, D. Low, Downstream processing of monoclonal antibodies—application of platform approaches, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 848 (2007) 28–39. https://doi.org/10.1016/j.jchromb.2006.09.026.

[39] B. Voedisch, C. Menzel, E. Jordan, A. El-Ghezal, T. Schirrmann, M. Hust, T. Jostock, Heterologe Expression von rekombinanten Proteinpharmazeutika, Laborwelt 6 (2005) 31–35.

[40] A.L. Grilo, A. Mantalaris, The Increasingly Human and Profitable Monoclonal Antibody Market, Trends Biotechnol. 37 (2019) 9–16. https://doi.org/10.1016/j.tibtech.2018.05.014.

[41] F.M. Wurm, Production of recombinant protein therapeutics in cultivated mammalian cells, Nat. Biotechnol. 22 (2004) 1393–1398. https://doi.org/10.1038/nbt1026.

[42] H. Chmiel, R. Takors, D. Weuster-Botz, W. Zettlmeier (Eds.), Bioprozesstechnik, 4th ed., Springer Berlin Heidelberg, Berlin, Heidelberg, 2018.

[43] J. Xu, X. Xu, C. Huang, J. Angelo, C.L. Oliveira, M. Xu, X. Xu, D. Temel, J. Ding, S. Ghose, M.C. Borys, Z.J. Li, Biomanufacturing evolution from conventional to intensified processes for productivity improvement: a case study, MAbs 12 (2020) 1770669. https://doi.org/10.1080/19420862.2020.1770669.

[44] M. Schulze, J. Lemke, D. Pollard, R.H. Wijffels, J. Matuszczyk, D.E. Martens, Automation of high CHO cell density seed intensification via online control of the cell specific perfusion rate and its impact on the N-stage inoculum quality, J. Biotechnol. 335 (2021) 65–75. https://doi.org/10.1016/j.jbiotec.2021.06.011.

[45] J.H. Chon, G. Zarbis-Papastoitsis, Advances in the production and downstream processing of antibodies, N. Biotechnol. 28 (2011) 458–463. https://doi.org/10.1016/j.nbt.2011.03.015.

[46] M. Butler, A. Meneses-Acosta, Recent advances in technology supporting biopharmaceutical production from mammalian cells, Appl. Microbiol. Biotechnol. 96 (2012) 885–894. https://doi.org/10.1007/s00253-012-4451-z.

[47] J.-M. Bielser, M. Wolf, J. Souquet, H. Broly, M. Morbidelli, Perfusion mammalian cell culture for recombinant protein manufacturing - A critical review, Biotechnol. Adv. 36 (2018) 1328–1340. https://doi.org/10.1016/j.biotechadv.2018.04.011.

[48] M.M. Papathanasiou, C. Kontoravdi, Engineering challenges in therapeutic protein product and process design, Current Opinion in Chemical Engineering 27 (2020) 81–88. https://doi.org/10.1016/j.coche.2019.11.010.

[49] F. Li, N. Vijayasankaran, A.Y. Shen, R. Kiss, A. Amanullah, Cell culture processes for monoclonal antibody production, MAbs 2 (2010) 466–479. https://doi.org/10.4161/mabs.2.5.12720.

[50] M. Pohlscheidt, R. Kiss, U. Gottschalk, An Introduction to "Recent Trends in the Biotechnology Industry: Development and Manufacturing of Recombinant Antibodies and Proteins", Adv. Biochem. Eng. Biotechnol. 165 (2018) 1–8. https://doi.org/10.1007/10_2017_39.

[51] M. Westoby, J. Chrostowski, P. de Vilmorin, J.P. Smelko, J.K. Romero, Effects of solution environment on mammalian cell fermentation broth properties: enhanced impurity removal and clarification performance, Biotechnol. Bioeng. 108 (2011) 50–58. https://doi.org/10.1002/bit.22923.

[52] A.A. Shukla, L.S. Wolfe, S.S. Mostafa, C. Norman, Evolving trends in mAb production processes, Bioeng. Transl. Med. 2 (2017) 58–69. https://doi.org/10.1002/btm2.10061.

[53] World Health Organization, Guidelines on the quality, safety, and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (2013). WHO Technical Report Series, No. 814

[54] S. Gilgunn, H. El-Sabbahy, S. Albrecht, M. Gaikwad, K. Corrigan, L. Deakin, G. Jellum, J. Bones, Identification and tracking of problematic host cell proteins removed by a synthetic, highly functionalized nonwoven media in downstream bioprocessing of monoclonal antibodies, J. Chromatogr. A 1595 (2019) 28–38. https://doi.org/10.1016/j.chroma.2019.02.056.

[55] C. Liu, K.J. Morrow (Eds.), Biosimilars of Monoclonal Antibodies, John Wiley & Sons, Inc, Hoboken, NJ, USA, 2016.

[56] US Food and Drug Administration, Q5A Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin: Guidance for Industry (1998).

[57] P. Gronemeyer, R. Ditz, J. Strube, Trends in Upstream and Downstream Process Development for Antibody Manufacturing, Bioengineering (Basel) 1 (2014) 188–212. https://doi.org/10.3390/bioengineering1040188.

[58] K. Brorson, S. Krejci, K. Lee, E. Hamilton, K. Stein, Y. Xu, Bracketed generic inactivation of rodent retroviruses by low pH treatment for monoclonal antibodies and recombinant proteins, Biotechnol. Bioeng. 82 (2003) 321–329. https://doi.org/10.1002/bit.10574.

[59] R. Wälchli, M. Ressurreição, S. Vogg, F. Feidl, J. Angelo, X. Xu, S. Ghose, Z. Jian Li, X. Le Saoût, J. Souquet, H. Broly, M. Morbidelli, Understanding mAb aggregation during low pH viral inactivation and subsequent neutralization, Biotechnol. Bioeng. (2019). https://doi.org/10.1002/bit.27237.

[60] D. Stein, V. Thom, J. Hubbuch, High throughput screening setup of a scale-down device for membrane chromatography-aggregate removal of monoclonal antibodies, Biotechnol. Prog. (2020) e3055. https://doi.org/10.1002/btpr.3055.

[61] H.F. Liu, J. Ma, C. Winter, R. Bayer, Recovery and purification process development for monoclonal antibody production, MAbs 2 (2010) 480–499. https://doi.org/10.4161/mabs.2.5.12645.

[62] J. Weaver, S.M. Husson, L. Murphy, S.R. Wickramasinghe, Anion exchange membrane adsorbers for flow-through polishing steps: Part II. Virus, host cell protein, DNA clearance, and antibody recovery, Biotechnol. Bioeng. 110 (2013) 500–510. https://doi.org/10.1002/bit.24724.

[63] R.L. Fahrner, H.L. Knudsen, C.D. Basey, W. Galan, D. Feuerhelm, M. Vanderlaan, G.S. Blank, Industrial purification of pharmaceutical antibodies: development, operation, and validation of chromatography processes, Biotechnol. Genet. Eng. Rev. 18 (2001) 301–327. https://doi.org/10.1080/02648725.2001.10648017.

[64] S. Ghose, Y. Tao, L. Conley, D. Cecchini, Purification of monoclonal antibodies by hydrophobic interaction chromatography under no-salt conditions, MAbs 5 (2013) 795–800. https://doi.org/10.4161/mabs.25552.

[65] V.I. Razinkov, M.J. Treuheit, G.W. Becker, Accelerated formulation development of monoclonal antibodies (mAbs) and mAb-based modalities: review of methods and tools, J. Biomol. Screen. 20 (2015) 468–483. https://doi.org/10.1177/1087057114565593.

[66] P. Gronemeyer, R. Ditz, J. Strube, Trends in Upstream and Downstream Process Development for Antibody Manufacturing, Bioengineering (Basel) 1 (2014) 188–212. https://doi.org/10.3390/bioengineering1040188.

[67] Y.-M. Huang, W. Hu, E. Rustandi, K. Chang, H. Yusuf-Makagiansar, T. Ryll, Maximizing productivity of CHO cell-based fed-batch culture using chemically defined media conditions and typical manufacturing equipment, Biotechnol Progress 26 (2010) 1400–1410. https://doi.org/10.1002/btpr.436.

[68] O. Yang, S. Prabhu, M. Ierapetritou, Comparison between Batch and Continuous Monoclonal Antibody Production and Economic Analysis, Ind. Eng. Chem. Res. 58 (2019) 5851–5863. https://doi.org/10.1021/acs.iecr.8b04717.

[69] S. Särnlund, Y. Jiang, V. Chotteau, Process intensification to produce a difficult-to-express therapeutic enzyme by high cell density perfusion or enhanced fed-batch, Biotechnol. Bioeng. 118 (2021) 3533–3544. https://doi.org/10.1002/bit.27806.

[70] W.C. Yang, D.F. Minkler, R. Kshirsagar, T. Ryll, Y.-M. Huang, Concentrated fed-batch cell culture increases manufacturing capacity without additional volumetric capacity, J. Biotechnol. 217 (2016) 1–11. https://doi.org/10.1016/j.jbiotec.2015.10.009.

[71] D. Pollard, M. Brower, Y. Abe, A. Lopes, A. Sinclair, Standardized Economic Cost Modeling for Next-generation mAb Production, bioprocess international (2016).

[72] O. Yang, M. Qadan, M. Ierapetritou, Economic Analysis of Batch and Continuous Biopharmaceutical Antibody Production: A Review, J. Pharm. Innov. 14 (2019) 1–19. https://doi.org/10.1007/s12247-018-09370-4.

[73] L. David, P. Schwan, M. Lobedann, S.-O. Borchert, B. Budde, M. Temming, M. Kuerschner, F.M. Alberti Aguilo, K. Baumarth, T. Thüte, B. Maiser, A. Blank, V. Kistler, N. Weber, H. Brandt, M. Poggel, K. Kaiser, K. Geisen, F. Oehme, G. Schembecker, Side-by-side comparability of batch and continuous downstream for the production of monoclonal antibodies, Biotechnol. Bioeng. (2020). https://doi.org/10.1002/bit.27267.

[74] A.L. Cataldo, D. Burgstaller, G. Hribar, A. Jungbauer, P. Satzer, Economics and ecology: Modelling of continuous primary recovery and capture scenarios for recombinant antibody production, J. Biotechnol. 308 (2020) 87–95. https://doi.org/10.1016/j.jbiotec.2019.12.001.

[75] R. Jacquemart, M. Vandersluis, M. Zhao, K. Sukhija, N. Sidhu, J. Stout, A Single-use Strategy to Enable Manufacturing of Affordable Biologics, Comput. Struct. Biotechnol. J. 14 (2016) 309–318. https://doi.org/10.1016/j.csbj.2016.06.007.

[76] C. Gillespie, M. Kozlov, M. Phillips, A. Potty, R. Skudas, M. Stone, A. Xenopoulos, A. Dupont, J. Jaber, W. Cataldo, Integrating Continuous and Single-Use Methods to Establish a New Downstream Processing Platform for Monoclonal Antibodies, in: G. Subramanian (Ed.), Continuous Processing in Pharmaceutical Manufacturing, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2014, pp. 71–96.

[77] J. Hummel, M. Pagkaliwangan, X. Gjoka, T. Davidovits, R. Stock, T. Ransohoff, R. Gantier, M. Schofield, Modeling the Downstream Processing of Monoclonal Antibodies Reveals Cost Advantages for Continuous Methods for a Broad Range of Manufacturing Scales, Biotechnol. J. 14 (2019) e1700665. https://doi.org/10.1002/biot.201700665.

[78] R. Eibl, G.d. Abreu (Eds.), Disposable bioreactors, Springer, Berlin, Heidelberg, 2009.

[79] Regine Eibl and Dieter Eibl, Single-use technology in biopharmaceutical manufacture, Second edition, Wiley, Hoboken, NJ, 2019.

[80] Cochet, Oliver, Jean-Claude Corbiere, Andrew Sinclair, Miriam Monge, Andrew Brown, PhD and Geraldine Eschbach, A Sustainable, Single-Use Facility for Monoclonal Antibody Production, 2013. https://bioprocessintl.com/manufacturing/monoclonal-antibodies/a-sustainable-single-use-facility-for-monoclonal-antibody-production-348558/ (accessed 3 February 2022).

[81] M. Brower, Y. Hou, D. Pollard (Eds.), Monoclonal Antibody Continuous Processing Enabled by Single Use, 2014.

[82] B. Kiss, U. Gottschalk, M. Pohlscheidt (Eds.), New bioprocessing strategies: development and manufacturing of recombinant antibodies and proteins, Springer, Cham, Switzerland, 2018.

[83] B.K. Lydersen, T. Brehm-Gibson, A. Murel, Acid precipitation of mammalian cell fermentation broth, Ann. N. Y. Acad. Sci. 745 (1994) 222–231. https://doi.org/10.1111/j.1749-6632.1994.tb44375.x.

[84] A. Richardson, J. Walker, Continuous Solids-Discharging Centrifugation: A Solution to the Challenges of Clarifying High–Cell-Density Mammalian Cell Cultures 2018 (2018).

[85] A. Senczuk, K. Petty, A. Thomas, T. McNerney, J. Moscariello, Y. Yigzaw, Evaluation of predictive tools for cell culture clarification performance, Biotechnol. Bioeng. 113 (2016) 568–575. https://doi.org/10.1002/bit.25819.

[86] R. O'Kennedy, C. Murphy, T. Devine, Technology advancements in antibody purification, ANTI Volume 6 (2016) 17–32. https://doi.org/10.2147/ANTI.S64762.

[87] R.J. Carvalho, Comparison of Cationic Flocculants for the Clarification of CHO-derived Monoclonal Antibodies, Biotechnol Bioproc E 24 (2019) 754–760. https://doi.org/10.1007/s12257-019-0158-7.

[88] Y.K. Kang, J. Hamzik, M. Felo, B. Qi, J. Lee, S. Ng, G. Liebisch, B. Shanehsaz, N. Singh, K. Persaud, D.L. Ludwig, P. Balderes, Development of a novel and efficient cell culture flocculation process using a stimulus responsive polymer to streamline antibody purification processes, Biotechnol. Bioeng. 110 (2013) 2928–2937. https://doi.org/10.1002/bit.24969.

[89] D. Burgstaller, W. Krepper, J. Haas, M. Maszelin, J. Mohoric, K. Pajnic, A. Jungbauer, P. Satzer, Continuous cell flocculation for recombinant antibody harvesting, J. Chem. Technol. Biotechnol. 93 (2018) 1881–1890. https://doi.org/10.1002/jctb.5500.

[90] A. Trapp, A. Faude, N. Hörold, S. Schubert, S. Faust, T. Grob, S. Schmidt, Multiple functions of caprylic acid-induced impurity precipitation for process intensification in monoclonal antibody purification, J. Biotechnol. 279 (2018) 13–21. https://doi.org/10.1016/j.jbiotec.2018.05.001.

[91] S. Tomic, L. Besnard, B. Fürst, R. Reithmeier, R. Wichmann, P. Schelling, C. Hakemeyer, Complete clarification solution for processing high density cell culture harvests, Separation and Purification Technology 141 (2015) 269–275. https://doi.org/10.1016/j.seppur.2014.12.002.

[92] T. McNerney, A. Thomas, A. Senczuk, K. Petty, X. Zhao, R. Piper, J. Carvalho, M. Hammond, S. Sawant, J. Bussiere, PDADMAC flocculation of Chinese hamster ovary cells: enabling a centrifuge-less harvest process for monoclonal antibodies, MAbs 7 (2015) 413–428. https://doi.org/10.1080/19420862.2015.1007824.

[93] I.A. Gómez, V. Hernández, L.A. Palomares, O.T. Ramírez, Flocculation of CHO cells for primary separation of recombinant glycoproteins: Effect on glycosylation profiles, Biochemical Engineering Journal 132 (2018) 244–254. https://doi.org/10.1016/j.bej.2018.01.027.

[94] R. van Reis, A. Zydney, Bioprocess membrane technology, Journal of Membrane Science 297 (2007) 16–50. https://doi.org/10.1016/j.memsci.2007.02.045.

[95] S.R. Hadpe, A.K. Sharma, V.V. Mohite, A.S. Rathore, ATF for cell culture harvest clarification: mechanistic modelling and comparison with TFF, J. Chem. Technol. Biotechnol. 92 (2017) 732–740. https://doi.org/10.1002/jctb.5165.

[96] D.J. Roush, Y. Lu, Advances in primary recovery: centrifugation and membrane technology, Biotechnol. Prog. 24 (2008) 488–495. https://doi.org/10.1021/bp070414x.

[97] N. Nejatishahidein, A.L. Zydney, Depth filtration in bioprocessing — new opportunities for an old technology, Current Opinion in Chemical Engineering 34 (2021) 100746. https://doi.org/10.1016/j.coche.2021.100746.

[98] O. Khanal, N. Singh, S.J. Traylor, X. Xu, S. Ghose, Z.J. Li, A.M. Lenhoff, Contributions of depth filter components to protein adsorption in bioprocessing, Biotechnol. Bioeng. 115 (2018) 1938–1948. https://doi.org/10.1002/bit.26707.

[99] M.W. Jornitz (Ed.), Filtration and purification in the biopharmaceutical industry, CRC Press Taylor & Francis Group, Boca Raton, FL, London, New York, 2020.

[100] O.-W. Reif, T. Scheper, G. Antranikian (Eds.), Angewandte Mikrobiologie, Springer Berlin Heidelberg, Berlin, Heidelberg, 2006.

[101] H.R. Charlton, J.M. Relton, N.K. Slater, Characterization of a generic monoclonal antibody harvesting system for adsorption of DNA by depth filters and various membranes, Bioseparation 8 (1999) 281–291. https://doi.org/10.1023/a:1008142709419.

[102] Y. Yigzaw, R. Piper, M. Tran, A.A. Shukla, Exploitation of the adsorptive properties of depth filters for host cell protein removal during monoclonal antibody purification, Biotechnol Progress 22 (2006) 288–296. https://doi.org/10.1021/bp050274w.

[103] B. Minow, F. Egner, F. Jonas, B. Lagrange, High-Cell-Density Clarification By Single-Use Diatomaceous Earth Filtration, bioprocess international 2014 (2014) 36–47.

[104] A. Gupta, J.P. Amara, E. Gousseinov, B. Cacace, Recent advances in harvest clarification for antibodies and related products, in: Approaches to the Purification, Analysis and Characterization of Antibody-Based Therapeutics, Elsevier, 2020, pp. 117–136.

[105] J.P. Maybury, M. Hoare, P. Dunnill, The use of laboratory centrifugation studies to predict performance of industrial machines: Studies of shear-insensitive and shear-sensitive materials, Biotechnol. Bioeng. 67 (2000) 265–273. https://doi.org/10.1002/(SICI)1097-0290(20000205)67:3<265:AID-BIT2>3.0.CO;2-J.

[106] R. Hundley, S. Königsson, Development of a Single-Use Hermetic Centrifuge System, Bioprocess International 2020.

[107] R.B. Hsu-Feng Ko, 2012. Evaluation of Single-Use Fluidized Bed Centrifuge System for Mammalian Cell Harvesting: This article discusses the evaluation of a novel single-use fluidized bed centrifuge for harvesting of antibodies. BioPharm International 2012, Issue 11.

[108] Per Eric Lindahl, Principle of a counter-streaming centrifuge for the separation of particles of different sizes, Natur 1948 (1948) 648– 649.

[109] W. Kelly, J. Rubin, J. Scully, H. Kamaraju, P. Wnukowski, R. Bhatia, Understanding and modeling retention of mammalian cells in fluidized bed centrifuges, Biotechnol. Prog. 32 (2016) 1520–1530. https://doi.org/10.1002/btpr.2365.

[110] T. Kruse, M. Kampmann, I. Rüddel, G. Greller, An alternative downstream process based on aqueous two-phase extraction for the purification of monoclonal antibodies, Biochemical Engineering Journal 161 (2020) 107703. https://doi.org/10.1016/j.bej.2020.107703.

[111] M. Iqbal, Y. Tao, S. Xie, Y. Zhu, D. Chen, X. Wang, L. Huang, D. Peng, A. Sattar, M.A.B. Shabbir, H.I. Hussain, S. Ahmed, Z. Yuan, Aqueous two-phase system (ATPS): an overview and advances in its applications, Biol. Proced. Online 18 (2016) 18. https://doi.org/10.1186/s12575-016-0048-8.

[112] A. Schmidt, M. Richter, F. Rudolph, J. Strube, Integration of Aqueous Two-Phase Extraction as Cell Harvest and Capture Operation in the Manufacturing Process of Monoclonal Antibodies, Antibodies (Basel) 6 (2017). https://doi.org/10.3390/antib6040021.

[113] T. Kruse, A. Schmidt, M. Kampmann, J. Strube, Integrated Clarification and Purification of Monoclonal Antibodies by Membrane Based Separation of Aqueous Two-Phase Systems, Antibodies (Basel) 8 (2019). https://doi.org/10.3390/antib8030040.

[114] Y. Xie, Z. Mao, H. Bachman, P. Li, P. Zhang, L. Ren, M. Wu, T.J. Huang, Acoustic Cell Separation Based on Density and Mechanical Properties, J. Biomech. Eng. 142 (2020). https://doi.org/10.1115/1.4046180.

[115] A. Shamloo, M. Boodaghi, Design and simulation of a microfluidic device for acoustic cell separation, Ultrasonics 84 (2018) 234–243. https://doi.org/10.1016/j.ultras.2017.11.009.

[116] A. Enders, J.-A. Preuss, J. Bahnemann, 3D Printed Microfluidic Spiral Separation Device for Continuous, Pulsation-Free and Controllable CHO Cell Retention, Micromachines (Basel) 12 (2021). https://doi.org/10.3390/mi12091060.

[117] J. Seo, M.H. Lean, A. Kole, Membrane-free microfiltration by asymmetric inertial migration, Appl. Phys. Lett. 91 (2007) 33901. https://doi.org/10.1063/1.2756272.

[118] N.A. Brechmann, H. Schwarz, P.-O. Eriksson, K. Eriksson, A. Shokri, V. Chotteau, Antibody capture process based on magnetic beads from very high cell density suspension, Biotechnol. Bioeng. (2021). https://doi.org/10.1002/bit.27776.

[119] BMC Proceedings, Abstracts from the 26th European Society for Animal Cell Technology Meeting - Cell culture technologies: bridging academia and industry to provide solutions for patients, BMC Proc 14 (2020). https://doi.org/10.1186/s12919-020-00188-y.

[120] G. Subramanian (Ed.), Continuous Processing in Pharmaceutical Manufacturing, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2014.

[121] M. Saballus, L. Nisser, M. Kampmann, G. Greller, A novel clarification approach for intensified monoclonal antibody processes with 100 million cells/mL using a single-use fluidized bed centrifuge, Biochemical Engineering Journal 167 (2021) 107887. https://doi.org/10.1016/j.bej.2020.107887.

[122] D. Müller, L. Klein, J. Lemke, M. Schulze, T. Kruse, M. Saballus, J. Matuszczyk, M. Kampmann, G. Zijlstra, Process intensification in the biopharma industry: Improving efficiency of protein manufacturing processes from development to production scale using synergistic approaches, Chemical Engineering and Processing - Process Intensification 171 (2022) 108727. https://doi.org/10.1016/j.cep.2021.108727.

[123] M. Saballus, M. Kampmann, Fluidized bed centrifugation of precipitated and flocculated cell cultures: An intensified clarification approach for monoclonal antibodies, J. Biotechnol. 34 (2022) 629. https://doi.org/10.1016/j.jbiotec.2022.05.004.

[124] M. Saballus, M. Kampmann, Clarification setup of a bioprocessing installation, WO 2022/008536 Al, 2022.

[125] M. Saballus, T.-J. Filz, D. Pollard, M. Kampmann, Intensifying Cell Clarification by Fluidized Bed Centrifugation with Integrated Filtration, 2022. https://www.bioprocessingeurope.com/poster-titles (accessed 26 April 2022).

[126] M. Saballus, J. Scholz, Automated centrifuge setup of a bioprocessing installation, WO2022008543A1, 2022.

# List of Figures

# List of Abbreviations

| | |
|---|---|
| AEX | Anion exchange chromatography |
| CEX | Cation exchange chromatography |
| CHO | Chinese hamster ovary |
| CL | Constant light region |
| COVID | Coronavirus disease |
| CQA | Critical quality attribute |
| DBF | Dynamic body feed |
| DNA | Deoxyribonucleic acid |
| DSC | Disc stack centrifuges |
| DSP | Downstream processing |
| EFA | Effective filter area |
| ELISA | Enzyme-linked immunosorbent assays |
| EMA | European Medicines Agency |
| Fab | Fragment antigen binding |
| FB | Fed-batch |
| FBC | Fluidized bed centrifuge |
| Fc | Fragment crystallizable |
| FDA | United States Food and Drug Administration |
| HCD | High cell density |
| HCP | Host cell proteins |
| IgG | Immunoglobulin G |
| mAb | Monoclonal antibody/-ies |
| pDADMAC | poly(diallyldimethylammonium chloride) |
| SU | Single-use |
| USP | Upstream processing |
| UO | Unit Operation |
| VL | Variable light region |
| WHO | World Health Organization |

# Curriculum Vitae

**Personal Information**

| | |
|---|---|
| Name | Martin Saballus |
| Date of birth | 28 June 1989 |
| Place of birth | Schleiz |
| Nationality | German |

**Education**

| | |
|---|---|
| Since 2019 | Doctoral student (Chemistry) at the Leibniz University of Hannover, Germany |
| 2012 – 2017 | Diploma degree in bioprocess engineering at the Dresden University of Technology, Germany |
| 2009 – 2012 | Abitur (equivalent to A-levels) at the Abendgymnasium Göttingen, Germany |
| 1999 – 2005 | Realschule (Secondary school) Staatliche Regelschule Saalfeld, Germany |

**Professional Experience**

| | |
|---|---|
| Since 2018 | Scientist, Corporate Research, Downstream BioProcessing, Sartorius Stedim Biotech GmbH, Göttingen, Germany |
| 2017 | Diploma Project at the IDT Biologika GmbH, Magdeburg, Germany |
| 2015 – 2016 | Practical Semester at the Singapore Centre on Environmental Life Sciences Engineering, Nanyang Technological University, Singapore |
| 2014 – 2015 | Student Assistant at the Institute of Food Technology and Bioprocess Engineering, Dresden University of Technology, Germany |
| 2009 – 2012 | Lab Assistant, Research and Development, Pharma Process, Sartorius Stedim Biotech GmbH, Göttingen, Germany |
| 2005 – 2009 | Apprenticeship as a physics lab assistant, Sartorius AG, Göttingen, Germany |

# Publications

**List of Research Articles**

(1) **Martin Saballus (85 %)**, Lucy Nisser, Markus Kampmann and Gerhard Greller. *Clarification of Intensified mAb Processes with up to 100 Million Cells/mL using a Single-Use Fluidized Bed Centrifuge.* Biochemical Engineering Journal (2021), doi: 10.1016/j.bej.2020.107887

MS (85 %): Conceptualization, methodology, investigation, writing—original draft preparation

LN (5 %): Methodology, investigation

MK, GG (in total 10 %): Writing—review and editing, supervision

(2) Dirk Müller, Lukas Klein, Johannes Lemke, Markus Schulze, Thomas Kruse, **Martin Saballus (15 %)**, Jens Matuszczyk, Markus Kampmann and Gerben Zijlstra. *Process Intensification in the Biopharma Industry – Using Synergistic Approaches to Improve Process Efficiency from Development to Production Scale.* Chemical Engineering & Processing: Process Intensification (2021), doi: 10.1016/j.cep.2021.108727

DM (20 %): Conceptualization, methodology, investigation, writing—original draft preparation

LK, JL, MSch, TK, MS (each 15 %): Methodology, investigation, writing

JM, MK, GZ (in total 5 %): Writing—review and editing, supervision

(3) **Martin Saballus (90 %)** and Markus Kampmann. *Fluidized bed centrifugation of precipitated and flocculated cell cultures: An intensified clarification approach for monoclonal antibodies.* Journal of Biotechnology (2022), doi: 10.1016/j.jbiotec.2022.05.004

MS (90 %): Conceptualization, methodology, investigation, writing—original draft preparation

MK (10 %): Writing—review and editing, supervision

**List of Patent Applications**

(1) **Martin Saballus (50 %)** and Jochen Scholz. (Q3 2020)

*Automated centrifuge setup of a bioprocessing installation*

International Publication Number: WO 2022/008543 Al


(2) **Martin Saballus (50 %)** and Markus Kampmann. (Q3 2020)

*Clarification setup of a bioprocessing installation*

International Publication Number: WO 2022/008536 Al


(3) **Martin Saballus (40 %)**, Lucas-Nik Reger, Markus Kampmann and Jens Matuszczyk. (Q1 2021)

*Method for operating a bioprocess installation*

Patent Application Number: SM2060-EP - EP21161867.3 (publication in progress)


(4) **Martin Saballus (50 %)** and Markus Kampmann. (Q3 2021)

*Method for producing a bioproduct*

Patent Application Number: SM2104-EP (publication in progress)

**List of Conference Contributions**

(1) **Martin Saballus (85 %)**, Lucy Nisser, Markus Kampmann and Gerhard Greller. *High Cell Density Clarification Using Single-Use Technologies* (P-508). 26th ESACT Meeting 2019, Copenhagen, Denmark (Poster and Conference Article, doi: 10.1186/s12919-020-00188-y)

(2) **Martin Saballus (90 %)**, Markus Kampmann and Gerhard Greller. *Intensified cell clarification using dynamic body feed filtration with modified filter aids.* 2nd Bioprocessing Summit Europe 2019, Lisboa, Portugal (Poster)

(3) **Martin Saballus (85 %)**, Lucy Nisser, Markus Kampmann and Gerhard Greller. *High Cell Density Clarification using Continuous Single-Use Centrifugation.* ZHAW BioTech 2019, Wädenswil, Switzerland (Poster)

(4) **Martin Saballus (90 %)**, Daniel Schorn and Markus Kampmann. *Acid Precipitation vs. Flocculation: Opportunities and Risks of Intensified Cell Clarification.* DECHEMA Himmelfahrtstagung on Bioprocess Engineering 2021, online (Poster)

(5) **Martin Saballus (90 %)** and Markus Kampmann. *Single-use fluidized bed centrifugation as an intensified platform approach for clarification.* ACS Fall Meeting 2021, online (Oral Presentation, doi: 10.1021/scimeetings.1c01354)

(6) **Martin Saballus (50 %)** and Lucas-Nik Reger. *Boosting monoclonal antibody productivity of fed-batch processes by recovering viable cells.* ECCE&ECAB 2021, online (Oral Presentation)

(7) **Martin Saballus (85 %)**, Thomas-Josef Filz, David Pollard and Markus Kampmann. *Intensifying Cell Clarification by Fluidized Bed Centrifugation with Integrated Filtration.* 5th Bioprocessing Summit Europe 2022, Barcelona, Spain (Poster)