Qualification of a Single-Use Disk Stack Separator for Cell Separation in Mammalian Cell-Based Antibody Production

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Single-use technology is now an indispensable part of biopharmaceutical production processes. This not only applies to research and development, but also to commercial processes. The manufacturers of single-use systems are working on improving them for use in intensified processes as well as on new developments. One of the bottlenecks was the availability of single-use disk stack separators whose stainless steel variants are widely used in industry. In this study, the suitability of the new single-use disk stack separator kytero 500 was investigated for the first time for cell separation in pilot-scale Chinese hamster ovary cell-based antibody production. The cells were completely separated over a wide range of separator settings with nearly no release of intracellular substances and no loss of the antibody.

Keywords: Cell separation, Chinese hamster ovary cells, Clarification efficiency, Harvest, Monoclonal antibodies

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1 Introduction

Nowadays, diseases such as cancer or autoimmune diseases can be treated with biopharmaceuticals. These are produced with genetically modified organisms and include monoclonal antibodies (mAbs), enzymes, hormones, vaccines, and blood clotting factors. More than 90 % of mammalian cultivation capacities are attributed to the production of mAbs, which are primarily produced with Chinese hamster ovary (CHO) cells [1]. The mAb production process resulting in the active pharmaceutical ingredient (API) includes upstream (USP) and downstream processing (DSP), which are connected by the cell separation step [2].

Common cell separation methods are depth filtration, microfiltration, or centrifugation. Tangential flow filtration (TFF)-based microfiltration has long been the method of choice in CHO cell-derived mAb productions. Due to the ongoing development of cell lines and processes, cell numbers and accordingly the amount of cell debris and therefore membrane fouling has increased, pushing TFF-based cell separation technologies into the background [3]. Centrifugation-based disk stack separators are capable of separating this high solids content [4]. However, a complete separation of all particles is not possible; mainly small cell debris remain in the API-containing phase and so a second clarification step is potentially necessary [3, 5]. By use of depth filters, separation is achieved by size exclusion and adsorptive effects, which means that the process can be used for both primary and secondary clarification. However, the large dead volume in the depth filter can significantly reduce the product yield [3]. In conventional industrial mAb production in stainless steel bioreactors at double-digit cubic meter scale, clarification is carried out in two standard steps. In the first step, the cell suspension is separated from the API-containing phase using a disk stack separator. The remaining solid particles are removed in a second clarification step with depth filtration [4, 6].

Cell damage can occur in all cell separation processes, in the case of centrifugation systems due to shear forces or high centrifugal forces or, when using depth filters or microfiltration systems, due to high pressures [3, 6–8]. If cell damage occurs during the process, intracellular substances such as host cell protein (HCP), DNA, and lipids are released which can negatively influence the DSP [9, 10].

The increasing interest in implementing single-use systems in mAb production processes also led to new

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equipment for cell separation. The Centritech from Pneumatic Scale Angelus has the longest tradition. It was preferred as the cell retention system in perfusion processes [11, 12]. The Centritech consists of a bag that is placed on a rotor and several tubes that are needed for the transfer of the cell suspension and the separated phases. High mechanical forces act on the tubing system. Due to the frequent sale of the technology to other companies and quality problems, the system took a back seat [11]. The Unifuge and the U2k are also distributed by Pneumatic Scale Angelus and are tubular centrifuges. With the U2k, the concentrate is discharged continuously, and with the Unifuge discontinuously. While the U2k can be used for harvesting, the focus of the Unifuge is again on perfusion cultivations [5]. With the introduction of the single-use fluidized bed centrifuges kSep from Sartorius and Rotea from Thermo Fisher Scientific, new single-use cell harvest devices emerged [5, 13]. It is worth mentioning that due to the very small volumes that can be processed, the Rotea is primarily used in cell therapy processes [5]. With the kSep, several 1000 L cell suspension can be separated depending on process conditions [5, 14]. However, both systems allow washing of the cell pellet within the centrifuge, which, e.g., enables the cells to be transferred to another medium [5, 15]. In mAb production processes, the washing process can increase the product recovery rate [20]. Despite the importance of disk stack separators as cell separation devices in conventional facilities and often in single-use facilities as well, the first single-use disk stack centrifuges, namely, the GEA kytero 500 and the CultureOne from AlfaLaval were launched within the last 2 years. As with its stainless-steel counterparts, the CultureOne hermetically separates the process chamber from the environment using mechanical seals [16]. The kytero 500 is driven entirely without mechanical bearings and mechanical seals, using magnetic technology. While mechanical seals have a limited lifetime, the completely bearingless drive technology in the kytero 500 allows the rotating parts to last for many days, depending on the bowl speed [17]. Both companies offer their single-use disk stack centrifuges in two versions; the kytero 500 and the CultureOne Primo are recommended for cell harvesting of bioreactor sizes up to 500 L and are thus used on a pilot-scale [19, 20]. The kytero 2000, which was launched in October 2022, and the CultureOne Maxi are suitable for production scale and can process cell suspension volumes of up to 2000 L [16]. Both disk stack centrifuge types are suitable for washing processes, too. In this case, the concentrate needs to be diluted with fresh media and clarified by the same centrifuge again.

The aim of this paper was to investigate the suitability of the single-use disk stack separator kytero 500 (Fig. 1) for the separation of mAb expressing CHO cells. For this purpose, different separation settings were tested. In addition to studying the separation efficiency based on turbidity, the concentrations of HCP, DNA, and lactate dehydrogenase (LDH) were examined as quality parameters to evaluate cell damage during separation.

2 Material and Methods

2.1 Cell Line, Media, and Cell Production at Pilot Scale

An immunoglobulin G (IgG)-producing ExpiCHO-S cell line (Gibco, Waltham, MA, USA) was used for the experiments. The cell suspension was produced in a Flexsafe STR 200-L bag (Sartorius, Göttingen, Germany) in combination with a stirred bioreactor (BIOSTAT STR) from Sartorius. Inoculum production took place in N–1 perfusion mode in a wave-mixed bioreactor with internal membrane (Cytiva, Sweden) and is based on the ultra-high cell density N–1 perfusion process described by Müller et al. [18]. Since this 200-L process focused on cell expansion, the cells were separated with a viable cell density of 16.8×10^6 cells mL^{-1}, an average cell diameter of 14.2 µm, and a viability of 98.9 %. Cultivation was performed at 37 °C, pH ≤ 7.2, dissolved oxygen ≥ 40 %, 10 L min^{-1} overlay gassing, and a specific power input of 20 W m^{-3}. For inoculum production, High-Intensity Perfusion CHO medium (Gibco) was used. In the case of the 200-L fed-batch cultivation, Efficient-Pro Medium (Gibco) was used as the basal medium and Efficient-Pro Feed 2 (Gibco) as the feed medium.

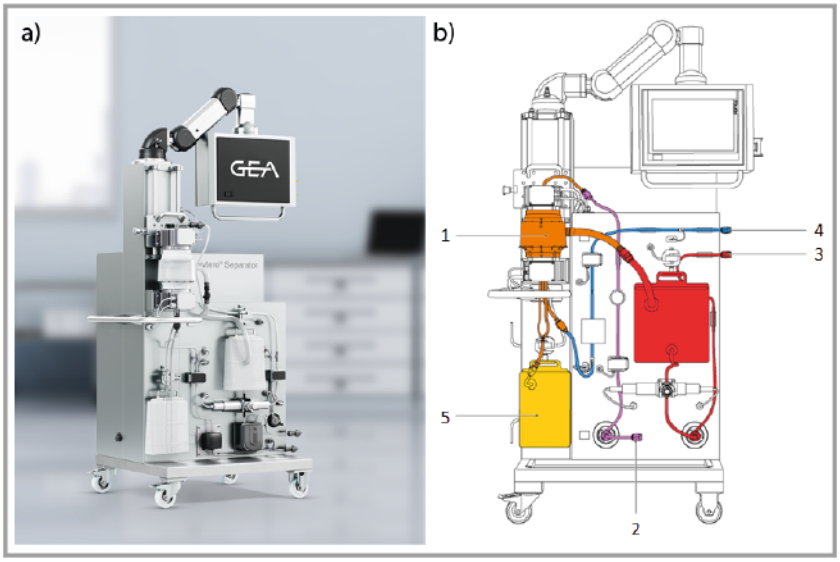

Figure 1. a) The kytero 500 disk stack separator and b) scheme of the single-use kit inserted in the kytero 500. (1) Separation unit with disk stack, (2) feed pathway, (3) centrate pathway, (4) concentrate pathway, (5) drain bag.

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2.2 Cell Separation with the Kytero 500

The 200 L CHO cell suspension was separated with a kytero 500 from GEA Westfalia (Oelde, Germany). The main volume flows $\dot{V}_{Centrate}$ and $\dot{V}_{Concentrate}$ resulting from the separation were subjected to the different analytical methods shown in Fig. 2. To qualify the separation process of CHO cells with the kytero 500, two separator speeds (3500 and 4750 rpm), four feed flow rates $\dot{V}_{Feed}$ (30, 60, 90, and 120 L h^{-1}) and three targeted packed cell volumes (PCV_{Target}) in the concentrate (50, 65, and 80 vol %) were used. In total, this resulted in eight different separation experiments (Tab. 1).

2.3 Determination of the Packed Cell Volume

The PCV was determined at each separation condition in the centrate, also known as supernatant, and concentrate as well as for the bioreactor. For this purpose, 10 mL of the sample was centrifuged in a discontinuous centrifuge for 2 min at 2500*g* and the resulting PCV was determined visually. If the PCV concentration was ≥ 80 vol %, the sample was diluted 1:2 with phosphate-buffered saline (PBS) beforehand.

Table 1. Overview of separation conditions and sample names.

Name	Separator speed [rpm]	$\dot{V}_{Feed}$ [L h^{-1}]	PCV_{Target} [%]
3500rpm-30L/h-50%	3500	30	50
3500rpm-60L/h-50%	3500	60	50
3500rpm-60L/h-65%	3500	60	65
3500rpm-60L/h-80%	3500	60	80
3500rpm-90L/h-80%	3500	90	80
3500rpm-120L/h-80%	3500	120	80
4750rpm-60L/h-80%	4750	60	80
4750rpm-90L/h-80%	4750	90	80

2.4 Clarification Efficiency by Turbidity Measurement

The turbidity-based clarification efficiency (*CE*) is a quantitative parameter that is used to evaluate the separation efficiency. For this purpose, the turbidity in the centrate was determined for each condition using a TB1 turbidimeter (VELP Scientifica, Usmate Velate, MB, Italy). As a reference value for determining the *CE*, the cell suspension from the bioreactor was centrifuged for 2 min at 2500*g* and the turbidity in the supernatant was determined. The *CE* results from the ratio of the turbidity *T* in the centrate $T_{Centrate}$ and the supernatant turbidity of the cell suspension to be separated T_{Feed} (Eq. (1)).

$$CE[\%] = \left(1 - \frac{T_{Centrate}}{T_{Feed}}\right) \times 100 \quad (1)$$

2.5 Analytics

To evaluate the separation process, the IgG, DNA, HCP, and LDH concentrations were determined in the cell suspension before separation and in the centrate. All measurements were performed in triplicate for DNA and HCP measurements. The IgG and LDH measurement was determined in a single determination with a Cedex Bio from Roche (Basel, Switzerland). For the DNA measurement, the Quant-iT PicoGreen kit (Invitrogen, Waltham, MA, USA) was used according to the manual. The fluorescence signal of the samples was determined at an excitation of 490 nm and an emission wavelength of 560 nm with the fluores-

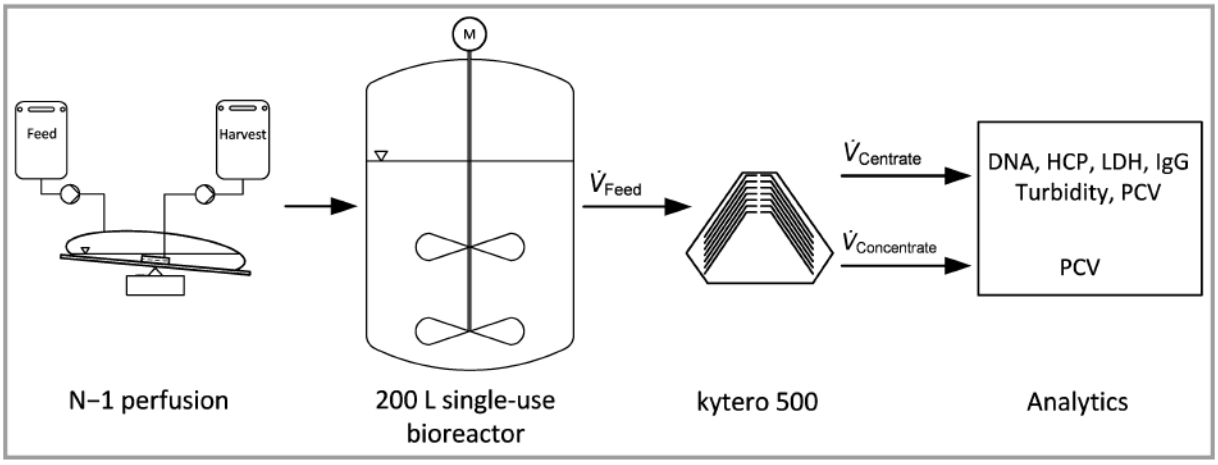

Figure 2. Schematic representation of the CHO cell production and separation experiments with the kytero 500 and the performed analytical methods.

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cence spectrometer Gemini from Molecular Devices (San Jose, CA, USA). For the HCP concentration determination, the immunoenzymetric HCP measurement kit from Cygnus Technologies (Southport, NC, USA) was used. The samples were measured with a Multiskan Spectrum (Thermo Fisher Scientific, Waltham, MA, USA) plate reader.

2.6 Cell Disintegration by Sonication

As negative control with the maximum possible concentration of DNA, HCP, and LDH of the disintegrated cell suspension in the bioreactor, 10 mL of the cell suspension was sonicated with a sonotrode (UP200ST, Hielscher Ultrasonics GmbH, Teltow, Germany) for 4 min. To remove the cell debris, the sample was centrifuged at 20 000*g* for 5 min and the supernatant was used to determine the concentration of LDH, HCP, and DNA.

3 Results and Discussion

An important parameter for cell separation processes is the turbidity in the centrate, as this influences the further DSP. To keep the effort required in the DSP as low as possible, the aim is to achieve the lowest possible turbidity values after cell harvest. The turbidity of the cell suspension to be separated with the kytero 500, further referred to as positive control, was 472 nephelometric turbidity units (NTU). This could be reduced to a turbidity of 13 NTU by discontinuous centrifugation (Fig. 3a). The turbidity-based *CE* in this case was 97.3 %. The turbidity across all separation settings was 12 to 16 NTU, resulting in similar *CE* values of 96.6–97.5 %. The lowest turbidity value of 12 NTU was achieved at a separation speed of 3500 rpm, $\dot{V}_{Feed} = 30\,L\,h^{-1}$ and a PCV_{Target} content of 50 vol % in the concentrate. When the separation speed and the PCV_{Target} in the concentrate were kept constant at 3500 rpm and 80 vol % and the feed low rate $\dot{V}_{Feed}$ was varied, a slight increase in turbidity was observed from 13 NTU at $60\,L\,h^{-1}$ to 15 NTU at $90\,L\,h^{-1}$ and 16 NTU at $120\,L\,h^{-1}$. The turbidity-based *CE* in the case of separation at $120\,L\,h^{-1}$ was 96.6 %, which is 0.7 % lower than the positive control. The slight increase in turbidity and the associated decrease in *CE* may be due to the shorter residence time of the cell suspension and therefore a reduction in sedimented particles [7]. Nevertheless, the *CE* values of 96.6–97.5 % achieved are very high and in agreement with other cell separation methods described in the literature [6, 14, 19–21]. Minow et al. [6] achieved a turbidity-based *CE* of 96.4–98.0 % when clarifying a CHO cell suspension with depth filters with or without diatomaceous body-feed filters. When separated with the single-use fluidized bed centrifuge kSep, *CE* values of 90.2–93.4 and 97.5–98.1 %, respectively, were described [14, 20]. Regardless of the separation parameters used and the slightly decreasing turbidity-based *CE* depending on the separation setting, no cells or cell debris in the form of a solid pellet could be detected in the centrate, even after a further discontinuous centrifugation step. The PCV-based *CE* achieved by discontinuous centrifugation was accordingly 100 % for all separations performed.

Another important parameter monitored during separation with a disk stack separator is the PCV_{Real} in the concentrate. In this work, the aim was to increase the PCV from 2.7 vol % in the suspension to 50, 65, or 80 vol % during separation. The PCV_{Real} in the concentrate varied

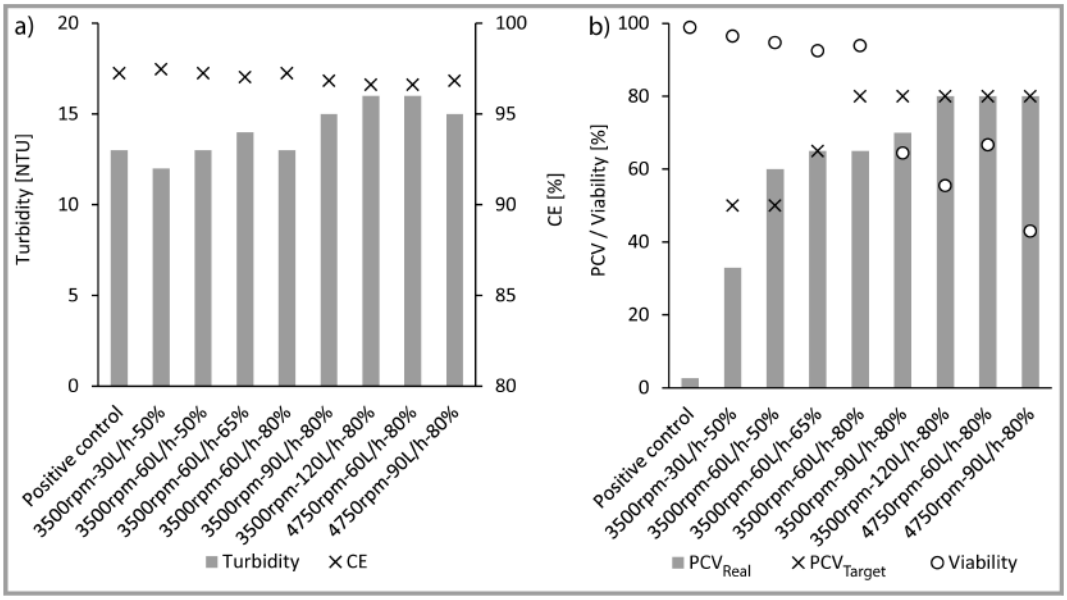

Figure 3. a) Turbidity and turbidity-based *CE* in the centrate and b) PCV_{Real} and PCV_{Target} concentrations and cell viability after separation in the concentrate. Nomenclature: separator speed-$\dot{V}_{Feed}$-PCV_{Target}; positive control: bioreactor cell suspension.

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between 33 and 80 vol %, depending on the PCV_{Target} value (Fig. 3b). The minimum value of 33 vol % was obtained with the first separation carried out with a separation speed of 3500 rpm, $\dot{V}_{Feed}$ = 30 L h^{-1} and a PCV_{Target} of 50 vol %. When the PCV_{Target} in the concentrate was increased to 80 vol %, the PCV_{Real} value gradually increased from 65 vol % (60 L h^{-1}) to 70 vol % (90 L h^{-1}) and then reached a PCV_{Real} of 80 vol % in the third separation carried out with a feed flow rate $\dot{V}_{Feed}$ of 120 L h^{-1}. The fact that the PCV_{Real} value reached the PCV_{Target} with a time delay after an adjustment of the PCV_{Target} can be attributed to the balance adjustment in the separator bowl and the transfer of the concentrate into the waste container.

The viability of the cells in the concentrate decreased when the separation speed and $\dot{V}_{Feed}$ were increased (Fig. 3b). When the separation speed and PCV_{Target} were kept constant at 3500 rpm and 80 vol % and $\dot{V}_{Feed}$ was varied, the viability of the cell suspension decreased from 93.9 % at 60 L h^{-1} to 64.4 % at 90 L h^{-1} and reached a minimum value of 55.5 % at 120 L h^{-1}. The same effect can be seen when increasing the separation speed to 4750 rpm. There, the viability decreased from 66.7 % at 60 L h^{-1} to 43.0 % at 90 L h^{-1}. The decrease in viability with an increased $\dot{V}_{Feed}$ may be due to damage incurred during the separation process and during the transfer of the concentrate through the peristaltic pump into the concentrate container. Varying only the separation speed and keeping $\dot{V}_{Feed}$ and PCV_{Target} constant at 60 L h^{-1} and 80 vol %, a decrease in viability from 93.9 % (3500 rpm) to 64.4 % (4750 rpm) is observed. This drop indicates cell damage, e.g., due to excessive *g* forces or shear forces in the separator bowl. This effect was also visible with $\dot{V}_{Feed}$ = 90 L h^{-1}.

Since the cell damage that occurs during separation leads to the secretion of intracellular substances into the IgG-containing liquid phase and can thus negatively influence the DSP, the HCP, DNA, and LDH concentration in the centrate were determined as quality parameters (Fig. 4). When comparing the HCP concentration in the centrate samples with the bioreactor positive control, the HCP concentration changed by –4 to 22 % (Fig. 4a). The highest HCP concentration increase from 277±26 µg mL^{-1} in the positive control to 337±27 µg mL^{-1} was obtained at a separation speed of 4750 rpm, $\dot{V}_{Feed}$ = 90 L h^{-1} and 80 vol % PCV_{Target}. This value correlates with the low viability of 42.5 % measured in the concentrate and thus with the assumption that more HCP enters the centrate at higher separation speeds. In all other separations performed, the increase in HCP value was 7 % at maximum. This correlates with kSep-based separations, in which the HCP concentrations were 5±11 % higher than in the bioreactor cell suspension [20]. Taking the DNA concentration in the centrate into account, 90.0±1.5 µg mL^{-1} to 95.5±1.2 µg mL^{-1} DNA were detected for all separation conditions (Fig. 4a). This resulted in a 1.6 % lower to 4.4 % higher value compared to the DNA concentration in the supernatant of the bioreactor sample (positive control). The DNA concentration, unlike the HCP concentration, increased by only 1.4 % at 4750 rpm, 90 L h^{-1} and 80 vol % PCV_{Target} in the concentrate. A comparison of the LDH concentration at this separator setting also showed only an increase of 1 %, from 268 U L^{-1} in the positive control to 270 U L^{-1} (Fig. 4b). In the case of DNA and LDH concentration (Fig. 4), no significant increase in concentration was discernible in all centrates, regardless of the separation parameters selected. Apart from the highest separation setting with 4750 rpm, 90 L h^{-1}, and 80 vol % PCV_{Target}, this also applies to the HCP concentration. If the cells were damaged, an increase in DNA and LDH concentration would also be expected; in the case of the

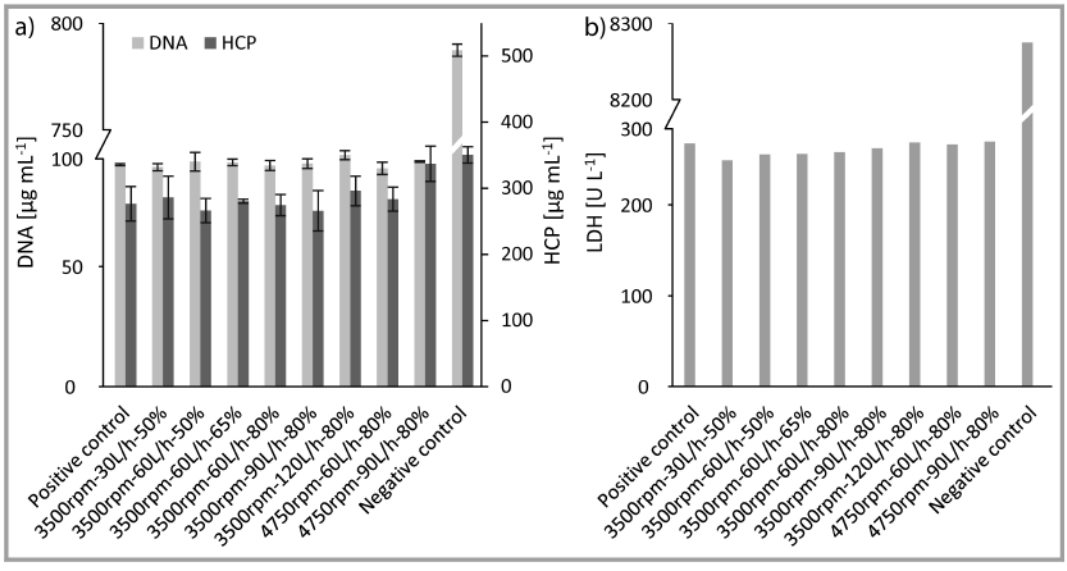

Figure 4. a) DNA and HCP concentration and b) LDH concentration in the centrate after separation. Nomenclature: separator speed-$\dot{V}_{Feed}$-PCV_{Target}; positive control: bioreactor cell suspension; negative control: disintegrated cell suspension.

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completely disintegrated cell suspension (marked as negative control), the DNA concentration increased from 91.5±0.3 µg mL^{-1} to 788.8±1.3 µg mL^{-1}. This corresponds to an 8.6-fold increase. LDH concentration was increased 31-fold by complete disintegration and the HCP concentration increased by 27 %. The results show that all separator settings investigated are suitable for the complete separation of cell cultures. If the aim is to achieve the highest separation possible (PCV_{Target} = 80 vol %) or the most rapid separation possible ($\dot{V}_{Feed}$ = 120 L h^{-1}), a decrease of cell viability was determined, but the concentrations of HCP, DNA, and LDH measured show that the centrate is also well suited for the following DSP at these settings.

The cell separation method chosen not only determines which impurities need to be removed in further clarification steps, but also affects the IgG recovery rate. Separation procedures based on filtration may result in a loss of IgG [6, 22]. In contrast, with centrifugation-based separation, theoretically no loss of IgG is possible due to adsorption effects. At the beginning of the separation with the kytero, the IgG concentration in the supernatant was 692 mg L^{-1}. The IgG concentration increased until the end of the separation experiments to 716 mg L^{-1} at the last separation performed with a separator speed of 4750 rpm, $\dot{V}_{Feed}$ = 90 L h^{-1} and a PCV_{Target} of 80 vol %. This 3.5 % increase (Fig. 5) is not due to any concentration effect during separation, but to IgG production by the CHO cells during the 2.2 h separation process. All the separation experiments listed were carried out directly one after the other with the cell suspension from the 200-L bioreactor.

While the adsorption-based loss of IgG is not possible with disk stack separators, there is still IgG loss due to the liquid phase fraction contained in the concentrate. For example, if a PCV of 50 vol % is aimed for in the concentrate, this results in a concentration factor of 18.5 with a PCV of 2.7 vol % in the bioreactor (Tab. 2). In the separation of 200 L cell suspension, 10.8 L concentrate is produced, of which 5.4 L are liquid phase containing mAb. Assuming the IgG production process established in Müller et al. [23] on a 200-L scale and a final IgG concentration of 3600 mg L^{-1} after 12 cultivation days, this results in an IgG loss of 19.4 g. If the PCV in the concentrate is increased to 80 vol %, the IgG loss is only 0.7 %, under the best possible assumption that the PCV no longer contains any liquid. Under the assumption that the cells in the PCV are present as a densest sphere pack, not 0.7 but 1.4 % IgG is lost. However, this does not correspond to reality, as the cells are not perfectly round and are compressed during the separation process. The real IgG recovery rate is therefore between 98.6 and 99.3 %. In contrast to the fluidized bed centrifuge kSep, there was no washing of the cells envisaged to recover the IgG contained in the concentrate in this work. By displacement washing, the IgG recovery rate could be further increased. Through direct washing, a 100 % mAb recovery would theoretically be possible with the kSep. However, Saballus et al. [20] showed that only 93.2–97.0 % of the mAb was obtained when using the kSep including an integrated washing process. The mAb concentration decreased due to washing and, thus, a lot of buffer would be necessary for complete mAb recovery. Depth filters represent an alternative as a primary clarification step. Tomic et al. tested different depth filters and antibody combinations and showed that the IgG recovery rate was between 82 and 97 % [21]. However, to keep the loss as low as possible, a suitable depth filter material must be found for each product.

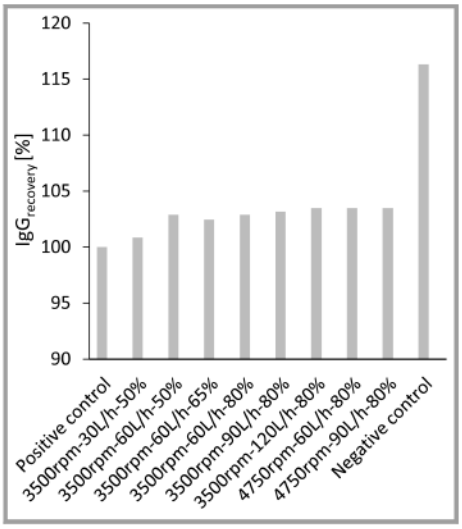

Figure 5. IgG recovery rate overall separation conditions. Nomenclature: separator speed-V_{Feed}-PCV_{Target}; positive control: bioreactor cell suspension; negative control: disintegrated cell suspension.

Table 2. Theoretical IgG loss depending on the PCV in the concentrate.

$PCV_{Concentrate}$ [vol %]	Concentration factor [–][a)]	$V_{Concentrate}$ [L]	$V_{Liquid,concentrate}$ [L]	Absolute IgG loss [g][b)]	Relative IgG loss [%]
50	18.5	10.8	5.4 (6.8)[c)]	19.4 (24.5)[c)]	2.7 (3.4)[c)]
65	24.1	8.3	2.9 (4.3)[c)]	10.5 (15.5)[c)]	1.5 (2.2)[c)]
80	29.6	6.8	1.4 (2.8)[c)]	4.9 (9.9)[c)]	0.7 (1.4)[c)]
90	33.3	6.0	0.6 (2.0)[c)]	2.2 (7.2)[c)]	0.3 (1.0)[c)]

a) Based on the PCV of 2.7 vol %; in the separated bioreactor suspension (positive control); b) assumption of an IgG concentration of 3600 mg L^{-1}; c) considering the densest sphere packing in the PCV.

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4 Conclusion and Outlook

In this paper, separation results with the single-use disk stack centrifuge kytero 500 for the separation of CHO cells are published for the first time. To determine the opportunities and limitations that can be achieved with this system for the separation of CHO suspension cells, various settings were tested. The separator speed, $\dot{V}_{Feed}$, and PCV in the concentrate were varied. With ≥ 96.6 %, a high turbidity-based *CE* was achieved for all separator settings. During the separation process, a decrease in the viability of the cells in the concentrate was observed depending on feed flow and PCV value. However, apart from one exception, no increase in quality-relevant parameters such as HCP, DNA, and LDH was detectable in the centrate. Accordingly, it can be assumed that the shear forces acting in the separator bowl are sufficiently low not to influence the quality of the centrate containing API. Further evaluation should be made to determine also the suitability for perfusion processes. A separator speed of 3500 rpm is already sufficient to separate the cells completely and $\dot{V}_{Feed}$ and the PCV can be varied over a wide range. At high flow rates of 120 L h^{-1}, the 200 L pilot bioreactor suspension can thus be completely separated in less than 2 h. Based on the very high PCV_{Real} values of 80 % achieved in the concentrate, very high IgG recovery rates can be achieved in the centrate, which in theory lie between 98.6 and 99.3 %.

We would like to thank Thermo Fisher Scientific's Gibco team for providing the cells and the media.

Symbols used

CE	[%]	clarification efficiency
T	[NTU]	turbidity
V	[L]	volume
$\dot{V}$	[L h^{-1}]	volumetric flow rate

Sub- and superscripts

Centrate	centrate phase
Concentrate	concentrate phase
Feed	bioreactor suspension
Liquid	liquid phase
Real	real measured value
Recovery	recovery
Target	target value

Abbreviations

API	active pharmaceutical ingredient
CHO	Chinese hamster ovary
DSP	downstream processing
HCP	host cell protein
IgG	immunoglobulin G
LDH	lactate dehydrogenase
mAb	monoclonal antibody
NTU	nephelometric turbidity unit
PBS	phosphate-buffered saline
PCV	packed cell volume
TFF	tangential flow filtration
USP	upstream processing

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