# The Potential of Hydrocyclone Application for Mammalian Cell Separation in Perfusion Cultivation Bioreactors

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**Abstract:** Hydrocyclones have been traditionally applied for long times in many industrial fields, such as in mineral processing and mining, chemical and petrochemical, and food industries. They have many characteristics that favor them as separation system in solid/liquid, gas/liquid and liquid/liquid processes. During the last two decades, they have been evaluated for their possible application in the separation of microbial and mammalian cells. Nowadays, mammalian cells are widely used for the production of a large number of valuable therapeutic proteins, antibodies, hormones and vaccines. This review highlights the potential of the application of hydrocyclones for mammalian cell separation in continuous perfusion biorecators. The discussion will cover the structure of hydrocyclone, mechanism of separation inside hydrocyclones, different theories describing the separation process, as well as the effect of changing different geometrical variables on the efficiency and performance of the separation process. Furthermore, we will focus on the latest developments achieved in the field of separation of living cells in both laboratory and pilot plant cultivation scales.

**Keywords:** Hydrocyclone, cell separation, mammalian cells, perfusion bioreactors, separation efficiency.

## 1. INTRODUCTION

Mammalian cell cultures are currently the major source for the production of large numbers of very high-value recombinant therapeutic proteins, monoclonal antibodies (MAbs), viral vaccines and hormones and thus became as one of the main biofactories in wellness industries [1]. In 2005, the sales market for protein therapeutics reached about $ 55 billion, which represented about 20 percent of the $ 280 billion pharmaceutical market [2]. In 2010 the global market for therapeutic proteins was estimated to reach about $ 94 billion with an annual increasing rate of about 12 percent [3]. Accordingly, highly efficient and reliable production technologies are required to cope with the increased demand for these products. Generally, large-scale commercial mammalian cell cultivations are performed in batch, fed-batch and perfusion processes (Figure **1**) [4-7]. The perfusion cultivation mode is generally characterized by the continuous flow of culture medium through the bioreactor, in addition to a cell retention system. In such systems, *in situ* medium exchange is accomplished, and hence a better control of the culture environment (dissolved oxygen, pH, and substrate concentration) is achieved. Additionally, toxic metabolites are continuously removed with the concomitant recovery of the secreted product, thus minimizing problems caused by product inhibition or by limited product stability. Cell retention enables higher cell densities (up to $5 \cdot 10^7$ mL$^{-1}$), and, when performed for extended periods, a higher volumetric productivity per bioreactor can be achieved. Furthermore, during the whole fermentation run cell viability can be stably maintained, hence achieving consistent product quality [8-11].

## 2. SEPARATION OF MAMMALIAN CELLS IN CONTINUOUS PERFUSION CULTIVATION

Generally, there are different essential properties that affect the suitability of cell retention devices for their application in long-term perfusion cultures [9,12]. Primarily, animal cells are 10-100 times greater than microorganisms and they lack cell wall. Thus, they are more susceptible and highly sensitive to shear stress and outer stimuli. Secondly, they have the tendency to adhere to device surfaces which will result in clogging and fouling problems. An ideal cell retention device should meet most, if not all, of the following features: (1) The device should be able to operate satisfactorily with high retention efficiency for the required duration without replacement or maintenance in order to minimize the risk of contamination; (2) It should not adversely affect cell viability or productivity; (3) Stable, long-term operation should be possible; (4) The device should be easy to clean and sterilize; (5) It should selectively, if possible, retain viable cells while allowing nonviable cells to pass through; and finally (6) It should be suitable for large-scale operation [11,13].

All cell retention devices depend on the same five physical and chemical properties of particles; i.e. size,

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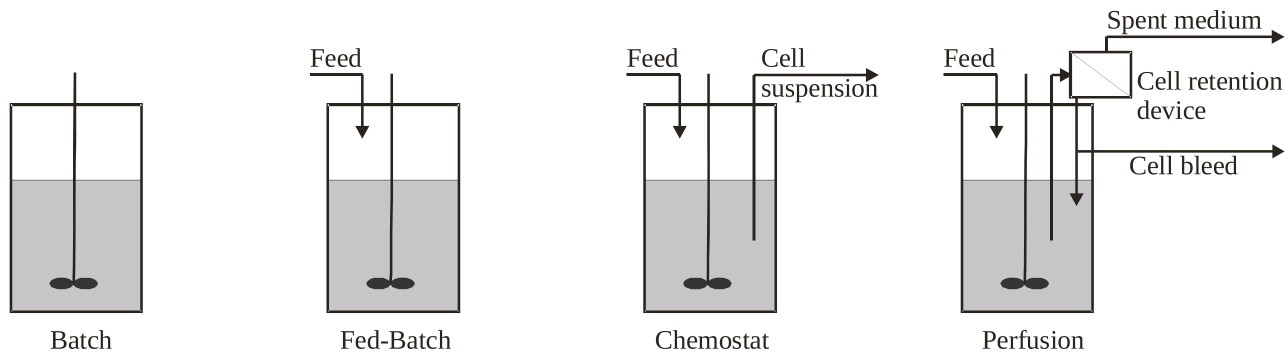



**Figure 1:** Schematic diagram of different modes of suspended-cell cultivation processes.

density, electrical charges, dielectric constant and surface properties. They are usually based on filtration, e.g. spin filters [14], tangential flow filters [15], dynamic filters [16]; gravitational sedimentation [17,18]; centrifugal action, e.g. centrifuges andhydrocyclones [19,20]; ultrasonic [21,22] and dielectrophoretic separation [23,24].

## 3. HYDROCYCLONES

Hydrocyclones are very simple devices, which are used in solid-gas and solid-liquid separation in different industries [25,26]. Although hydrocyclones use the same separation principle as centrifuges, sedimentation in a centrifugal field, they are characterized by having no movable parts. Accordingly, they operate as solid bowl centrifuges with no rotation of the main system. The vortex motion of the suspension is performed by the fluid through its tangential feeding into the cyclone [9,10,27]. Hydrocyclones consist of a conical section connected to a cylindrical part, which is fitted with a tangential inlet. The cylindrical part is closed by a plate with an axially installed overflow orifice or vortex finder. The conical portion ends with a circular apex opening or the underflow orifice (Figure **2**).

Although the first patent on hydrocyclone separation appeared more than 120 years ago [28], its first industrial application appeared only by the end of the Second World War. The hydrocyclone separation can be divided into three distinct development stages: solid-liquid separation (1890-1950), liquid-liquid separation (1950-2000), and the separation of ions and molecules (2000-present) [29]. Generally, hydrocyclones found many applications in mineral processing and mining [30,31], food [32,33], textile and pulp [34], petrochemical [35], electro-chemical [36], as well as biological industries [37]. Hydrocyclones have been originally designed to operate in solid/liquid separation processes; however, they are used nowadays in conventional solid/solid [38], liquid/liquid [39] and gas/liquid separations [40]. Table **1** lists some of the recently developed industrial applications for hydrocyclones.

### 3.1. Principle of Separation Inside Hydrocyclones

The tangential feeding of the suspension to be separated through the inlet opening generates a flow vortex in the conical section of the hydrocyclone. This vortex creates a centrifugal force and results in the dispersion of the particles, or cells, across the whole

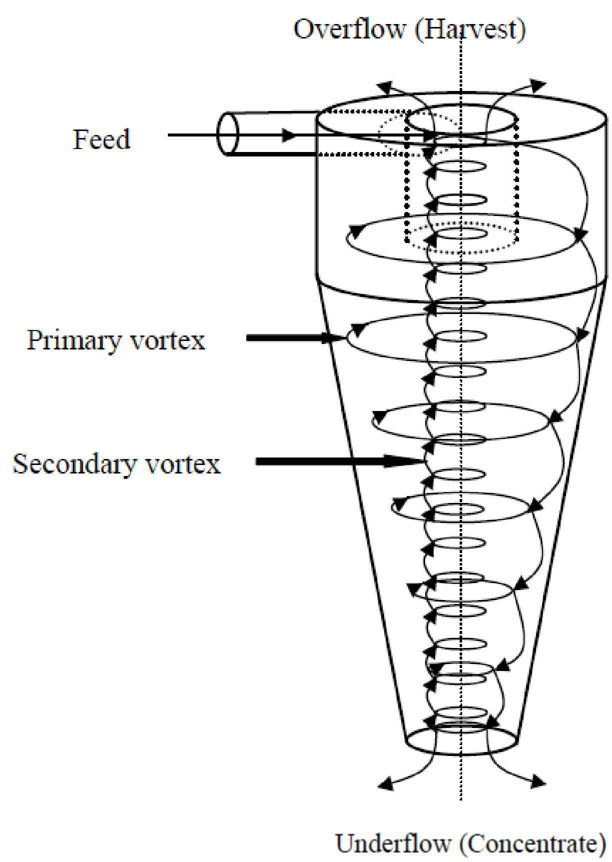



**Figure 2:** Schematic diagram of a typical hydrocyclone [11].

**Table 1: Recent Industrial Applications of Hydrocyclones**

| Application | References |
|---|---|
| Material disposal, collection of heavy metals (As, Cd, Cu, Hg, Pb, Zn) from pyrite ashes | [41] |
| Adsorption of organics with nitrogen and sulfur from wastewater | [42] |
| Treatment of laboratory wastewater mainly containing nitrate | [43] |
| Remediation of oil-contaminated soil | [44] |
| Washing of soil contaminated by a variety of heavy metals and radioactive contaminants | [45-47] |
| Improvement of the irrigation system | [48] |
| Oil–water separation | [49] |
| Purifying coke-cooling wastewater | [50,51] |
| Starch-protein separation from chickpea flour | [52] |
| Separation of invasive mudsnails from aquaculture waters | [53] |
| Integrityof animal and microbial cells | [54] |
| Separation of mammalian cells | [11,55] |

cross-sectional area [26,56]. At that point, the next separation process is characterized by the development of two equally important stages: (1) separation of solids from the main flow and their migration to the boundary layer on the wall, and (2) the removal of the separated solids from the wall into the apex and out of the cyclone. A small fraction of the flow will form a cross-circuit flow below the top cover and bypass the vortex; consequently, any particles in this flow will then directly pass through into the overflow. Contrary, most of particles, or cells, will go through the outer vortex under the influence of the centrifugal forces. Hence, the centrifugal forces must be greater than the drag forces to drive particles radially towards the wall; otherwise they will tend to move radially inward. Since centrifugal and drag forces are proportional to particle volume and size, respectively, therefore the separation performance of hydrocyclone is supposed to be strongly particle-size dependent [57]. This means that large particles will be readily separated than fine ones, which may not reach the boundary layer by the time the outer vortex reaches the bottom of the cone. Upon reaching the bottom of the cone, the outer vortex flow and its fine particles feed across into the inner vortex. Moreover, some of the fine particles may be able to leave the inner vortex resulting in a certain amount of solid circulation, while others will leave with the overflow. The boundary layer and its particle content move downwards into the apex of the cone, where they leave from the underflow orifice. The separation in hydrocyclones is not affected by gravity; particles move into the boundary layer under the influence of the flow itself [58-60].

Recently, the development of computer power and the use of computational fluid dynamics (CFD) allowed a better understanding of fluid flow inside hydrocyclones, and hence their separation mechanism and performance [61-64]. CFD modeling of hydrocyclones involves prediction of velocities of the liquid-phases, profiles of the suspension concentrations, turbulent viscosities and particle velocities, as well as swirling flow patterns inside hydrocyclones [65,66].

### 3.2. Separation Theories of Hydrocyclones

Many theories have been developed and proposed to explain the separation process inside the hydrocyclones. These theories are based either on actual physical models derived from first principles, or models derived as empirical equations from linear regression analysis. With this regard, three major theories can be described.

#### *3.2.1. Equilibrium Orbit Theory*

This theory is based on the concept of the equilibrium radius of the particle, and the fact that the motion inside the hydrocyclone is developed by the liquid flow itself and not forced as in the case of centrifuges. Inside hydrocyclones, the particle will find a position in an equilibrium orbit depending on the equilibrium between the radial velocity of the fluid toward the center and the tangential velocity of the liquid. Accordingly, larger particles will be found toward the outside and enter the outer vortex moving downwards toward the underflow orifice. Smaller particles, which will be found toward the center, will enter the inner fluid vortex moving upwards toward the vortex finder and will be then removed through the overflow orifice [37,57]. Due to very short residence times (usually 0.03 s) and high solid concentrations inside the hydrocyclone, the equilibrium orbits may not be achieved. Moreover, this theory takes no account of the turbulences, which may affect the separation process [59].

### *3.2.2. Residence Time Theory*

This theory describes the dependence of particles entering the cyclone at the inlet centre on its residence time, and hence whether they will settle to cyclone wall reporting to the underflow. This theory takes into account non-equilibrium conditions [67]. However, the residence time theory does not consider the effect of particle concentration, the so-called hindered settling. Moreover, it neglects the inertial effects and radial fluid flow.

### *3.2.3. Crowding Theory*

This theory describes hydrocyclone separation of particles at higher feed concentrations. It assumes that the size distribution of the feed and the underflow determine the separation efficiency. Accordingly, any cut size within the feed size distribution can be obtained by controlling the dimensions of the outlet [68,69].

### 3.3. Effect of Hydrocyclone Geometry on its Performance and Efficiency

During the last three decades, many investigations have been carried out to study the effect of changing the geometrical dimensions (Figure **3**) on the hydrocyclone performance. Nowadays, it is well established that the structure of the hydrocyclone is the determining factor [33]. Table **2** summarizes the effects of changing different hydrocyclone dimensions on the separation process.

Many attempts have been carried out to improve the separation efficiency by changing the structure of the hydrocyclone. Jirun *et al.* [70] inserted a solid cone into the hydrocyclone resulting in an increase in the performance as well as reduction of energy loss through the stabilization of the tangential flow and the omission of air core. Furthermore, a spiral inner surface was developed to improve the separation sharpness, where the boundary layer flow was interrupted [71]. Bai *et al*. [72] applied a small-diameter hydrocyclone for the separation of small particles.

### 3.4. Biological Applications of Hydrocyclones

Hydrocyclones have been traditionally and widely used for a long time in mineral processing industry [73]. They have been generally used for suspension concentration, liquid clarification, thickening, classification, sorting of solids by size and density, liquid-liquid separation and liquid-gas separation [29]. This can be attributed to their simple design, low cost, easy operation and low maintenance [59,74,75]. Although, many investigations have been carried out to study the separation and the working principles of hydrocyclones, however, most of this work was related to their application in mineral processing industry [58,76-78].

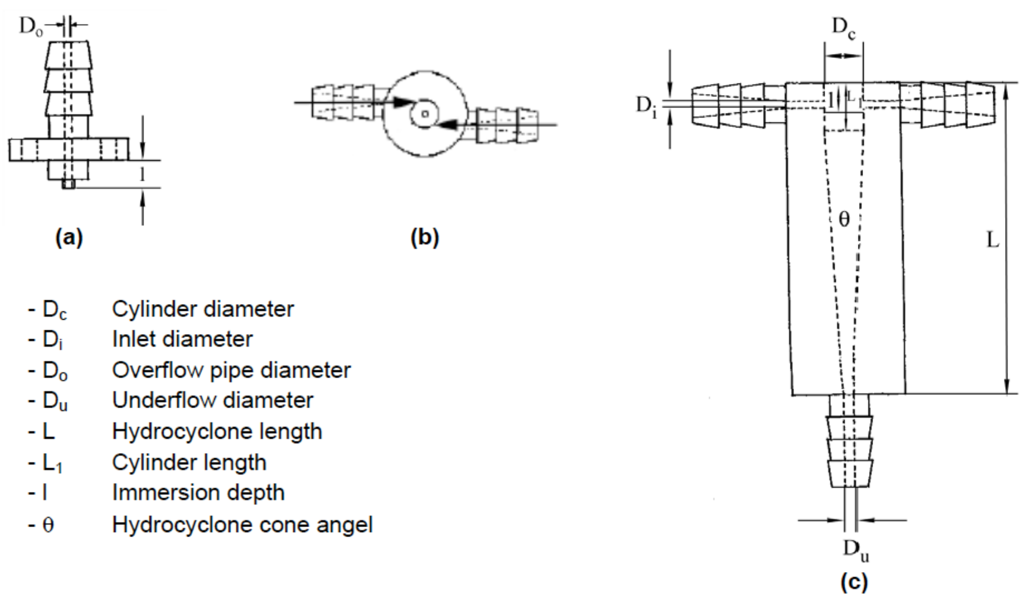



**Figure 3:** Schematic diagram of a typical double-inlet hydrocyclone with its corresponding geometrical dimensions, (**a**) cover Side-view, (**b**) body Top-view, (**c**) body Side-view [11].

**Table 2: Effect of Changing the Geometry of the Hydrocyclone on the Separation Performance [11]**

| Geometrical dimension | Performance of hydrocyclone | | |
|---|---|---|---|
| | Increased capacity | Increased separation efficiency | Increased flow ratio |
| Cyclone diameter | Increase | Decrease | - |
| Inlet diameter | Increase | Decrease | - |
| Overflow diameter | Increase | Decrease | Decrease |
| Body length | Increase | Increase | Increase |
| Vortex finder length | Decrease | Decrease | Decrease |
| Cone angle | - | Decrease | - |

In the last two decades, hydrocyclones have been tested for their application in solid-liquid separation processes involving biological materials. They have been used in the food industry for starch refining, recycling of silica gel for filters in the brewing industry, separation of gossypol from cottonseed protein in cottonseed oil processing, and as multi-stage separator systems for soluble coffee production [26,60]. Additionally, hydrocyclones have been applied in the separation of starch-protein from chickpea flour with a separation efficiency of 99.8% [52], as well as the separation of invasive species from aquacultures in fish hatcheries [53,79,80]. Recently, Show *et al*. [81] used hydrocyclones among four different centrifugation systems in the process of algal biomass separation and dryness.

On the other hand, hydrocyclones have been used to separate living microbial cells in continuous yeast cell cultivation and in beer industry [82,83]. Cilliers and Harrison [37] investigated the application of mini-hydrocyclones in the concentration of yeast suspensions. They studied the effect of several process parameters, e.g. pressure, temperature as well as hydrocyclone geometry on the recovery and concentration of yeast cells. Moreover, Weuster-Botz *et al.* [84] developed a fermentation process with high oxygen transfer for the continuous methanol controlled-production of formate dehydrogenase with *Candida boidinii*. They applied three geometrically similar cyclones with different liquid volumes (0.5, 2.5 and 15L) and obtained more than 100% improvement in dry cell mass concentration and about 100% improvement in the enzyme space-time yield. Only two years later, a new method for yeast recovery in batch ethanol fermentation has been developed. This method uses a filtration method followed by separation of yeast from filter aid using hydrocyclones [85].

Medronho *et al*. [86] investigated the separation of both microorganisms and mammalian cells depending of CFD-based numerical simulation of hydrocyclones. Their studies revealed the presence of a cylindrical air core running the whole length of the cyclone, which resulted from the low-pressure region developed along the central axis. Additionally, they found that *Escherichia coli* and *Saccharomyces cerevisiae* cells could not be efficiently separated with the tested hydrocyclone. However, they were able to separate the BHK-21 mammalian cell line using a 10 mm-Bradley hydrocyclone with separation efficiencies as high as 90%.

In another study, a hydrocyclone with 15 mm internal diameter was applied for the separation of *Saccharomyces cerevisia* cells from the fermentation broth [87]. The authors obtained a better separation efficiency of 3.84 (g cell dry weight in: underflow/overflow) at a volumetric flow rate of about 112 $cm^3/s$. In 2012, Bicalho *et al.* [88] applied three hydrocyclone modules with different geometries for the separation of yeast cells from fermentation broth. Their work aims at developing empirical mathematical models that describe capacity, total separation efficiency and flow ratio as functions of different geometric variables and pressure drops. They obtained total separation efficiencies between 36.54% and 92.02%, with capacity range from 0.1183 to 0.4579 $m^3/h$ and flow ratios ranging from 31.74% to 84.30%. They also found that the maximum centrifugal forces generated within these devices only reduced cell viability by about 7%. Very recently, Pinto *et al.* [89] investigated the application of two commercial hydrocyclones (Doxie® Type-A and AKW® type RWK21) for the separation of *S. cerevisiae* cells from fermentation broth under different operation parameters, i.e. inlet pressure, underflow pressure, and the underflow diameter. Their results showed that the Doxie hydrocyclone gave better separation performance up to 89.13% with a processing capacity of more than 239.25 kg/h.

### 3.5. Application of Hydrocyclones for Separation of Mammalian Cells in Perfusion Cultivations

During the last decade, the application hydrocyclones for separating mammalian cells in continuous perfusion bioreactors has been evaluated. Hydrocyclones possess many advantages that make them suitable competitors for other cell separation devices. The fact that they do not contain any movable parts favors them in terms of contamination problems. They are easy to install, require very limited space, *in situ* sterilizable and easy to clean and reuse. Moreover, they are easily manufactured and modified and provide stable, long-term operation [11,90].

Hydrocyclone separation is based on the generation of centrifugal sedimentation that can reach a maximum of several thousand times the force of gravity, which is similar in magnitude to the centrifugal forces used in centrifuges. Accordingly, the generated shear forces or the rapid pressure drop inside the hydrocyclone can have damaging effect on the cells. Nevertheless, it has been found that low shear operating hydrocyclones does not appear to damage the cells, which indicates that hydrocyclones running at moderate conditions have a potential for cell separation in animal cell continuous cultivations [54].

In 2000, hydrocyclone was evaluated for the first time for mammalian cell separation [91]. The authors investigated the separation of HeLa cells by three different hydrocyclones (10 mm-Dorr-Oliver, 10 mm-Mozley and 7 mm-Bradley) working at pressure drop ranging from 1 to 4 bar. They showed that until a pressure drop of 4 bar, HeLa cells were not adversely affected. These results were explained based on the fact that cells have a very short residence time inside hydrocyclones (0.03-0.1 s), therefore, cells are only subjected to the generated shear stress for a very short time. Additionally, applying the maximum pressure of 4 bar gave a separation efficiency of 81% using the Dorr-Oliver hydrocyclone. They were also able to achieve separation efficiencies of about 94% at moderate pressure drop (2 bar) by connecting two hydrocyclones together. Despite the fact that the authors used commercially available hydrocyclones (not specially designed for animal cell separation), their results reflected the effect of hydrocyclone geometry optimization on their potential application in perfusion cultures.

In 2001, Jockwer *et al.* [92] published their work on the separation of animal cells using hydrocyclone. They studied nine different hydrocyclone geometries specially designed by computational fluid dynamics for mammalian cell separation. They reported a decrease in the viability of separated CHO cells ranging from 5-12% at 1 bar and 7-21% at 1.35 bar. However, higher separation efficiency of 94.2% was obtained with a hydrocyclone having an underflow orifice diameter of 2.5 mm and an overflow pipe diameter of 2.0 mm. Furthermore, the authors showed, for the first time, that hydrocyclone can be used as a perfusion system in continuous cultivation mammalian cells using bubble-free membrane bioreactors. They were able to cultivate CHO cells for 23 days with their optimal hydrocyclone configuration with a viability range of more than 90%. The viable cell concentration increased by 50% with more than 3-fold increase in the concentration of recombinant antibody.

Few years later, Elsayed [11] extensively evaluated the application of specially-designed hydrocyclones [93] for the separation of mammalian cells in continuous perfusion cultivation. The author investigated different hydrocyclone geometries (different overflow and underflow orifices diameter), operating parameters (pressure drop and flow rate) as well as different cell lines (HeLa, CHO, NS0, BHK21). The author proved that changing the hydrocyclone diameter has a great impact on the separation efficiency of the hydrocyclone with regard to cell viability. Moreover, it was shown that hydrocyclone separation gave similar results when the perfusion process was compared with traditional cell separation (hollow-fiber membrane).

Furthermore, Elsayed *et al.* [94] published their work on hydrocyclone separation for CHO and HeLa cells cultivated on lab scale (6 L) and pilot scale (30 L) bioreactors. The authors demonstrated that hydrocyclone can be applied in continuous perfusion cultivations for the production of monoclonal antibodies. They were able to maintain an average cell concentration of up to 10 million per mL with cell viability above 93%. The hydrocyclone was operated at pressure drop ranging from 0.85 to 1.0 bar, resulting in separation efficiency above 85%, and a maximum dilution rate of 1.83 reactor volume $d^{-1}$.

In 2006, Elsayed *et al*. [95] characterized the performance of a hydrocyclone having an internal volume of 2.56 $cm^3$ with regard to flow split and flow ratio. Depending on the pressure drop applied, the authors were able to separate BHK and HeLa cells with a separation efficiency ranging from 77 to 97%. Moreover, their results demonstrated for the first time, that only cells passing from the primary vortex downwards into the inner secondary vortex and from there upwards could be damaged. Consequently, this has no impact on the overall cell viability in the bioreactor, since the overflow cell fraction is leaving the bioreactor. Additionally, they proved that continuous hydrocyclone application (for 3 h) has no adverse effect on cell viability or apoptotic cell fractions, and that cells suffer from the shear stress inside the hydrocyclone only for the time required for each cell to pass through the hydrocyclone. In other words, the time required for the whole bioreactor content to pass through the hydrocyclone, where the cells adapt themselves to the new conditions.

Pinto *et al.* [55,96] investigated the effect of hydrocyclone separation using different geometries on the viability and integrity of CHO cells by following cell viability, released lactate dehydrogenase (released in case of cell lysis) and the concentration of apoptotic cells. Their results showed that cells maintained higher viabilities (above 97%) with a selective separation for viable cells. They also used the same hydrocyclone for the separation of NS0 cells in 300 L bioreactors for the production of a monoclonal antibody (unpublished data).

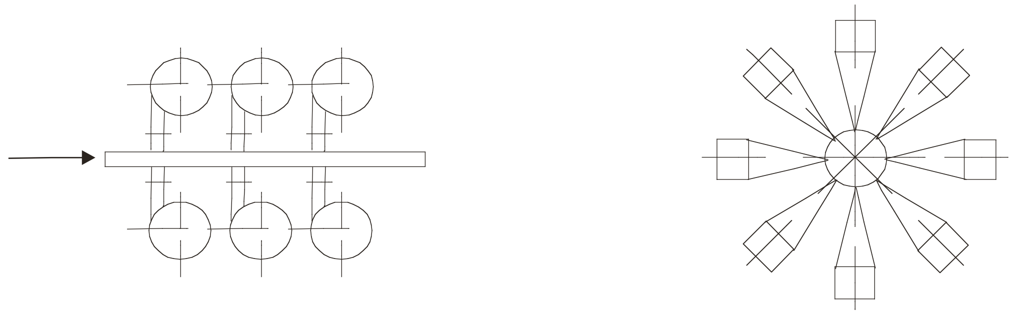
In 2010, the specially designed hydrocyclone which has been applied in the work cited from 2001 to 2008 has been commercialized by Sartorius-BBI Systems GmbH, Melsungen, Germany. This system (Figure **4**) was then evaluated for cell separation in the cultivation of a hybridoma cell line producing a monoclonal antibody in protein free medium [97]. The 10 cm hydrocyclone was directly installed on top of highly-automated, balance-controlled 20-L and 200-L stirred tank bioreactors. The hydrocyclone was operated at a flow rate ranging from 0.3 to 0.5 L $min^{-1}$, giving a maximum process-specific perfusion performance of 450-720 L $d^{-1}$.

Elsayed and Wagner [98] evaluated hydrocyclone for the separation of SP-2/0 cells in perfusion bioreactors under different operating pressure drops. According to the applied pressure drop, the authors achieved from 3.5- to 5-fold increase in the cell concentration from the batch cultivation. The cells were not adversely affected and the viability ranged from 92-98%. Concerning process performance, the hydrocyclone was operated with a separation efficiency ranging from 89 to 95%. Moreover, results showed that the hydrocyclone preferably separates viable cells into the underflow, and hence, back to the bioreactor, resulting in an improved system viability and product quality. Furthermore, Elsayed *et al*. [99] investigated the effect of feed flow pulsation on cell viability and separation efficiency. They changed the pulsation-free feed pumphead to a conventional pulsating pumphead. The results revealed that both pumpheads gave comparable results in terms of hydrocyclone separation efficiency and cell viability under different investigated pressure drops.

**Figure 4:** Prototype of mammalian cell culture hydrocyclone [97].

### 3.6. Scaling Up of Hydrocyclones

Concerning mammalian cell perfusion cultivations, there is always a need to scale up the fermentation process to cope with higher demands for the production of pharmaceutical proteins. Usually, scaling up is required in processes requiring higher dilution rates or applying the hydrocyclones for larger mammalian cell bioreactors. Accordingly, it can be proposed that the hydrocyclone itself should be scaled up. Normally, scaling up is usually obtained by changing the configuration of the device used for cell retention. However, in the case of hydrocyclones this concept cannot be applied [11,37]. The performance of the separation process is strictly controlled by the geometrical dimensions of the hydrocyclone itself. Many reports described the effects of changing the geometries of the hydrocyclone on the separation performance [76,77,100,101].



Linear arrangement
(plan view)

Circular arrangement
(plan view)

**Figure 5:** Different arrangements used for hydrocyclone batteries [11].

As described in Table **2**, it is shown that in order to have higher separation efficiencies, most of the hydrocyclone geometries, except for the body length, should be decreased. Hence, the smaller the nominal diameter of the hydrocyclone is, the higher the efficiency will be and the smaller the handling capacity as well [102]. In such case, another concept should be applied, which is the use of the so-called multi-cyclone arrangements or parallel hydrocyclones. Multi-cyclone arrangements have been largely used on industrial scale [59,102]. In the multi-cyclone arrangements, hydrocyclones are connected together to cope with the large flow rates required. This may be done by linearly or circularly arranging a series of two or more hydrocyclones. Figure **5** shows linear and circular arrangements of the hydrocyclone batteries.

## CONCLUSIONS AND FUTURE PROSPECTS

Hydrocyclones have unique characteristics that make them attractive devices to replace the traditional cell separation equipments that have been used in mammalian cell perfusion bioreactors. Hydrocyclones are characterized by their small size, ease of installation, suitability for *in situ* sterilization, and easy cleaning and reusing. These all together make them attractive separation devices in bioprocess industries. In addition, they do not have movable parts which reduced the risk of contamination during operation. This make them also easily approved to be used in cGMP facilities. They have been evaluated over the last two decades for cell separation in perfusion production cultivations with different mammalian cell lines under different operational conditions. However, with the exception of few reports, most of the published work is related to laboratory investigations. Therefore, further studies on their potential use in semi-industrial and industrial scale should be investigated to evaluate and understand the scalability of these devices. Once they are further studied and optimized for large scale mammalian cell separation, they will change in part the current pharmaceutical practice regarding cell separation systems applied in long-term perfusion cultivations.

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