Chapter

Microbioreactors and Perfusion Bioreactors for Microbial and Mammalian Cell Culture

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Abstract

Screening for novel producer strains and enhanced therapeutic production at reduced cost has been the focus of most of the biopharmaceutical industries. The obligation to carry out prolonged intensive pilot scale experiments gave birth to micro-scale bioreactor systems. Screening large number of microorganisms using shake flasks and benchtop bioreactors is tedious and consumes resources. Microbioreactors that mimic benchtop bioreactors are capable not only of high throughput screening of producer strains, but also aid in optimizing the growth kinetics and expression of proteins. Modern technology has enabled the collection of precise online data for variables such as optical density (OD), pH, temperature, dissolved oxygen (DO), and adjusting in mixing inside microreactors. Microbioreactors have become an irreplaceable tool for biochemical engineers and biotechnologists to perform a large number of experiments simultaneously. Another aspect that is vital to any industry is the product yield and subsequent downstream processing. Perfusion bioreactors are one of the upcoming advances in bioreactor systems that have the potential to revolutionize biologics production. This chapter intends to take a review of different aspects of microbioreactors and perfusion bioreactors including their potential in high throughput pilot studies and microbial and mammalian cell cultivation technologies.

Keywords: microbioreactors, biopharmaceuticals, bioprocess, perfusion microbioreactors, microorganisms

1. Introduction

1.1 Need for microbioreactors in bioprocess development

Microbioreactor systems are an integral part of bioprocess engineering. In the past few years, microbioreactors have extensively been used for high throughput screening [1]. In addition to this, extensive bioprocess experiments were monitored and controlled. Industries involved in bulk production of pharmaceuticals, chemicals, enzymes for feed and food from microbial cell factories are in need of microbioreactors [2–4]. Advanced shaker microliter cultivation devices or down-scaled stirred tank reactors are two basic microbioreactors. Primary and secondary screening experiments based on microbial library are conducted in shake flasks and micro liter plates. This screening process aids in selecting the microbial strain candidates that are promising. Then, selected microbial candidates are subjected to lab-scale experimental conditions for better bioprocess control. Bioprocess method developed by microbioreactors is followed by successful lab-scale testing, and it is transferred to pilot scale. Pilot scale experiments are essential to understand the bioreactor inhomogeneities which can ultimately be addressed using simulators that can scale down the process of bioreactors [5, 6].

Traditional microbiorector experiments start from primary screening; followed by secondary screening using shake flasks; then, process development; and, finally, optimization leading to process validation and pilot scale. It is worth mentioning that microbioreactors reduce the number of steps involved in traditional bioreactors. Microbioreactors start with primary screening, followed by accelerated bioprocess development for secondary screening, resulting in process validation and pilot scale at a faster rate. Introduction of bioreactors has reduced the number of steps involved in traditional bioreactors to scale up the bioprocesses. Hence, microbioreactors have proved to me more economical as they reduce the usage of secondary screening by shake flasks, thus making the process development and optimization more efficient. Ecology and environmental concerns arising during traditional bioprocess development have also been addressed by microbioreactors. Therefore, the focus of this chapter is on new developments in microbioreactors and their impact on bioprocesses.

1.2 Advantages and expected outcome of microbioreactors

Microbioreactors have numerous advantages over traditional bioreactors during bioprocess development. Microbioreactors are necessary for high throughput quantitative microbial phenotyping under controlled experimental conditions. Microbioreactors reduce the time involved in the traditional bioprocess development by replacing the shake flasks and large lab-scale bioreactors (**Figure 1**). Microbioreactors are more than necessary with specifications for efficient and economical bioprocess development. Expected outcomes of microbioreactors are as follows: (a) reduction in volume for each cultivation experiment; (b) time conservation during experiments; (c) simple, friendly, and operational without

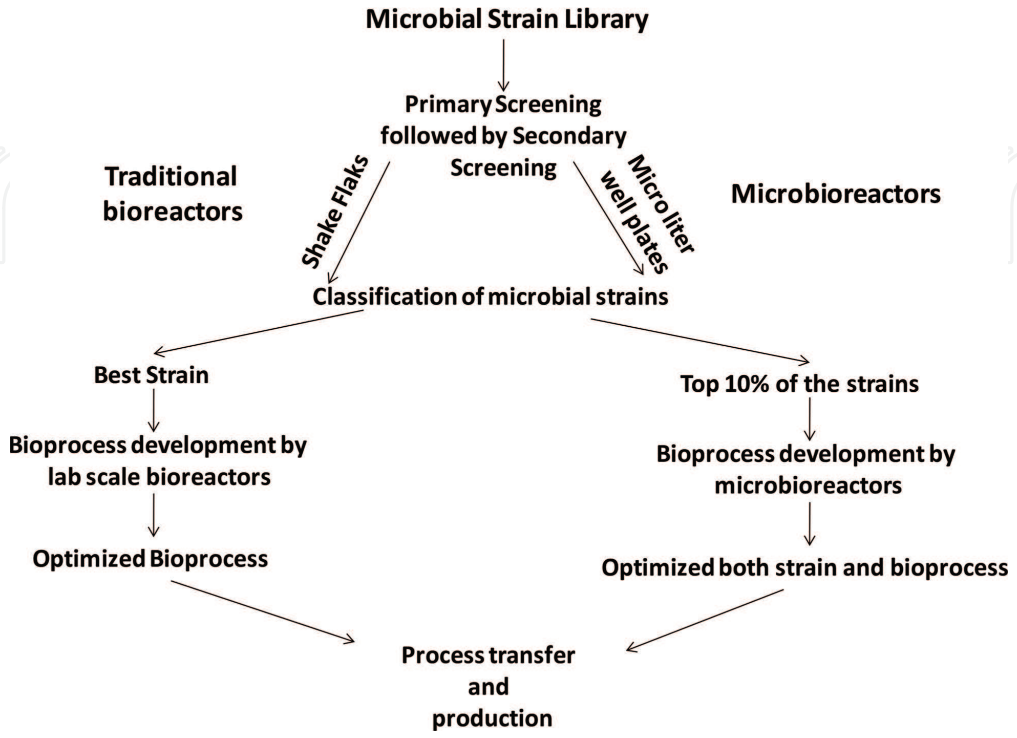

Figure 1.
Comparison of workflow for traditional bioreactors and microbioreactors.

fail; (d) automated operation with minimal or no supervision; (e) examination of bioprocess variables with high resolution; (f) temperature, pH, and feeding profiles for controlled cultivation process; (h) culture accessibility for sampling and dosing; (i) cultivation by fed-batch, batch, and continuous modes; (j) robotic systems with advanced hardware and software; and (k) scalability is compared to laboratory bioreactors [7].

For realizing all the above-mentioned desired properties of microbioreactors, researchers are emphasizing on applied and basic research. Extensive research has resulted in various microbioreactors with different technologies and applications.

2. Types of microbioreactors

Commercially available microbioreactors are based on their applications, specifications, and capabilities. Different types of microbioreactors are born out of extensive basic and applied research.

Biolector (m2p-labs.com), a commercial microbioreactor manufacturer, had developed microbioreactors for organism phenotyping, screening the strains, toxicity screening, and optimization of feed and growth parameters. These bioreactors support single-use 48-well plates that can hold culture volume of 0.8–2.4 ml. Biomass formation can be monitored via fluorescence and by optodes pH and dissolved oxygen (DO). This system is integrated with liquid handlers for pH adjustment; feeding; sampling; and control over temperature, gas, and humidity [8].

Microbioreactors (32 plate and 48 plate) [9] from Biolector were used for optimization of feeding rate, media screening, and fermentation parameters for anaerobic and microaerophyllic organisms. Few other applications that are possible with biolector microbioreactors are growth characterization, high throughput protein characterization, enzyme and cell activity tests, functional genomics and proteomic studies, inhibition and toxicity studies, and quality control.

RoboLector [10] is another microbioreactor where microbioreactor system from biolector is interfaced with liquid handling robot. These microbioreactors with the aid of 48 or 96 parallel cultivations through microplates provide fermentation data repeatedly for every 5–15 minutes. In addition to this, a robotic system also controls nutrient feeding, adjusts pH by adding acid or base, and does sampling based on user definition.

Another commercial manufacturer Micro-24 (pall.com) [11] developed a microbioreactor for screening strains and cell lines besides optimization of growth and feed parameters. These microbioreactors support single-use cassettes with 24 columns with culture volume ranging from 3 to 7 ml and regulate pH using ammonia, carbon dioxide, and control over temperature.

Microbioreactors to study biotransformation, phenotyping, strain and toxicity screening were developed by commercial manufacturer Bioscreen C (bioscreen.fi). These microbioreactors consist of two parallel 100-well plates with a capacity to hold culture volume of 0.4 ml. Optical density was used for quasi-continuous monitoring of biomass.

Growth profiler (enzyscreen.com), Applikon (applikonbio.com), SensorDish Reader (presens.de), ambr 15 (tapbiosystems.com), bioReactor (emag.de), and Sartorius (Sartorius.com) are other manufacturers of microbioreactors for various applications as per the requirements of clients.

2.1 Microbioreactors for fed-batch cultivation of *Escherichia coli*

During recombinant protein expression, *E. coli* synthesizes proteins at a faster rate with low multiplication rate. In traditional microbioreactors, fed-batch cultivations of *E. coli* were performed by stirred tank reactors [1]. Carbon source, magnesium, and ammonium were used as feeding solutions to match the nutritional requirements. It is worth mentioning that two fed-batch cultivations can be performed simultaneously with different nutrient compositions in the RoboLector systems. For example, feeding solution of one set of reaction is constituted with 1 M sodium hydroxide, 400 g/l glycerol, 100 g/l ammonium phosphate, and 1 g/l of magnesium sulfate $7H_2O$ whereas that of another set is composed of 200 g/l glycerol, 100 g/l ammonium phosphate, 2 g/l of magnesium sulfate $7H_2O$, and 1 M NaOH. Cultivations were performed at a shaking frequency of 1100 rpm with diameter 3 mm and temperature was set at 30°. Modified medium with 10 g/l glycerol and 100 mM MOPS with minimum salt was used. Online-monitored DO measurements were used to control the repeated additions of feeding solutions and both the feeding methods resulted in high cell densities of approximately 80 OD besides pH stabilization in between 6.5 and 7 for favorable growth of *E. coli*. Both the cultivations, one with high volume of glycerol and another with low volume of the same, exhibit high biomass concentration. Traditional bioreactors need huge amounts of energy for mixing, heating, and cooling during scale-up process but RoboLector microbioreactors are efficient and precise to hasten the development of bioprocesses using microbial cells [1].

2.2 Cultivation of *Pichia pastoris* using microbioreactors

Secreted proteins during bioprocesses undergo proteolysis and fragmentation; therefore, samples are generally removed from fermentation broth to obtain a kinetic growth curve. The purpose is to identify the optimum cultivation setup to reach maximum activity for the recombinant enzyme. RoboLectors are used to understand the kinetics of *Pichia pastoris* fed-batch cultivation [1]. Kinetics was monitored online by drawing 20 μl automatically and the concentrations of secreted enzymes were plotted against time to identify the space-time yield. During the automatic pipetting process, robotic tips immersed in the solution without any shaking, thus preventing artifacts of sedimentation.

To increase the productivity, softwares are programmed to provide outline of the experiments as per the input parameters. Dosing volume and variations in the concentrations of glycerol, ammonium hydroxide, and methanol can be given in the inputs. In 48 parallel fed-batch cultivations, a factorial design play gives a value, $2^4 + 3 = 19$; these different possibilities can be performed in one single analysis. Once the experiment is completed, the system automatically generates a contour plot and summarizes the activity profile of the recombinant enzymes with respect to the dosing volume and feeding solutions. For a particular recombinant product from *Pichia pastoris,* following factors were summarized at the completion of the experiment: 35% v/v methanol, 25% w/w ammonium hydroxide, and 150 g/l as feed composition for a dosing volume of 5 μl. The above-mentioned experiment is repeated for a particular design to identify and confirm the variable factors and increase the productivity [1].

2.3 Microbioreactors to produce monoclonal antibodies

Monoclonal antibodies are one of the major products among biotherapeutics. Organisms such as Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells are utilized to produce monoclonal antibodies, and several bioprocess parameters need to be optimized [12, 13] for their production. Composition of culture media, cell growth rate, and antibody growth rate need favorable physicochemical parameters such as pH, temperature, and dissolved oxygen. Physicochemical parameters are controlled to initiate and sustain recombinant expression in the CHO cell culture and to provide nutrients and relevant growth factors to bioreactors. During the production of monoclonal antibodies, possibilities of forming various variants of immunoglobulin (IgG) molecule and glycosylated IgG forms can be seen. Similar to monoclonal antibodies, other protein therapeutics also pass through issues such as oxygenation, amination, and degraded product molecules. Each of these above-mentioned processes is lengthy and consumes enormous time during research and development (R&D).

Microbioreactors, due to their miniature size and parallel testing, are needed to accelerate research and development (R&D) at a faster pace. Microbioreactors encompassed with sensors [14] that can hold volume in the range of 1–20 μl are ideal to monitor the bioprocess parameters. Microbioreactors need to be designed as per the needs of users. For example, for users to develop bioprocess procedures for mammalian cell cultures, the list of biological functions of mammalian cells and technical functions of bioreactors becomes essential.

Biological functions and requirements include: (a) nature of the cells (CHO or HEK cells) with concentration ranging from 10,000 to 10,000,000, (b) expression of proteins such as IgGs, (c) serum-free medium, and (d) culture time of 7–14 days [15].

Technical information parameters must include: (a) shaking for better mixing, (b) oxygen transfer value >100 h^{-1} to aerate the culture so that it can be extended to a large-scale process, (c) oxygen permeability <1%, (d) surface hydrophobicity (10°) along with a confocal microscope installed in situ without disturbing the reaction, (e) cell culture volume, (f) transportation of culture media to contained culture, and (g) measurement of chemical and physical conditions of culture and media [15].

Information function, namely online or offline information, is also essential: (a) online information about sensors to control and detect pH, temperature, and pO_2 is significant and (b) offline information such as monomers in culture media, forms of IgGs, excreted metabolites, products formed, and residual nutrients is needed for analysis of analytes. It is important that microbioreactors should be designed as per the specifications based upon the above requirements.

These specifications also cater to the requirements of microbioreactors in laboratories with 2–5-liter capacity. Therefore, such processes could be scaled up to laboratory level with effortless ease at reduced cost. CHO cell culture microbioreactors used to optimize the production of monoclonal antibodies are schematically shown in **Figure 2**. This is a part of Hubka-Eder map [16] focused on biological and technical functions. Hubka-Eder map shows the importance of integration among the subsystems, that is, the expression systems, cell line, medium with various technical and information functions for the design of the microbioreactors for the maximum production of monoclonal antibodies [15].

2.4 Microbioreactors in drug discovery

Organ-on-a-chip [17] is an ongoing research to hasten the process of drug discovery and development. In vitro drug screening and safety testing [18–20] are essential to minimize extensive studies on laboratory animals. Microbioreactors designed as per the need of the study will be highly beneficial to understand the potency and toxicity of the developed drug molecules. Drug molecules upon absorption get distributed to various organs such as lung, liver, gut, intestine, etc. These organs majorly consist of enzymes that can metabolize the drug molecules to metabolites [21] which can either be potent or toxic. After metabolism, the drug and its metabolites need to reach the target site for a particular time and get eliminated from the circulating system [22]. Kidneys play an important role in excreting drugs and its metabolites. Hence, developing various organs on a chip, programmed through a microbioreactor will be very helpful to understand the safety of drugs and metabolites.

Heart-on-a-chip is one such device which is developed to understand the efficacy of drug molecules with cardiac cells. Studies have proved that these devices have the capability to lend a helping hand in drug discovery and development process.

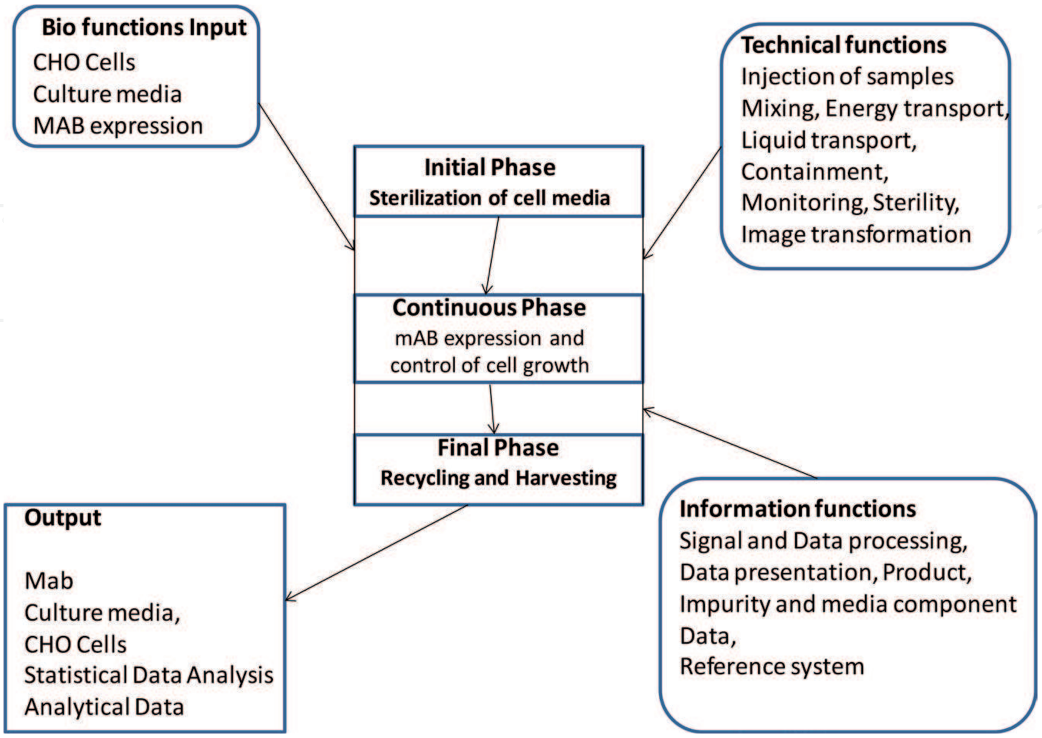

Figure 2.
Bioprocess optimization of a CHO cell culture to produce monoclonal antibodies.

3. Perfusion Bioreactors

3.1 Perfusion technology for biopharmaceutical production

The leading focus of R&D of any biopharma-based industry is to develop production process at reduced cost of production or rather work toward a minimal Cost of Goods (CoG) for cell culture-based products. Fed-batch mode of production has been the most tried and tested one, documented and dominantly followed methodology for production of biologics or biopharmaceuticals from mammalian cell culture. Due to the presence of expertise on this process within the industry, it has been proved to be the efficient process with judicious use of media coupled with a hassle-free downstream process. However, fed-batch mode has a severe limitation of inhibition of product formation and cell growth due to accumulation of inhibitory products during culture, especially ammonium ions, lactates, and proteases [23]. This leads to loss of key nutrients and incurs massive financial and resource loss to the production setup. This is especially significant if the product is a sensitive one. An alternative to this technology that came up in the early 1990s is perfusion technology. It involves addition of media or key media constituents at regular intervals along with retention of cells in the reactor and harvesting of formed products. However, in the initial phase, due to less advancement in media formulation and process development as well as dearth of efficient expression systems, not much advantage was offered by perfusion-based systems as compared to the well-established fed-batch systems and, hence, perfusion technology faced many failures and could not progress significantly. Many companies involved in manufacturing of perfusion-based systems especially for hybridoma-based monoclonal antibodies, like Endotronics, Cellex, and Biosyn, also folded up due to low market for perfusion bioreactors [24]. However, the pharma sector was on a constant lookout for newer or more effective production processes and hence advancements were continuously being made in perfusion technology. Now, with major advancements in media formulation, multiple technological options are available for cell retention and a rising trend has been witnessed in pharmaceutical industry for investment in adoption of new technologies and the cost effectiveness of perfusion-based processes, and their product yield increased significantly in comparison to conventional fed-batch processes [25]. Hence, this technology is on a comeback for the last few years and numerous life science companies are advocating the use of perfusion for biologicals production.

Factor VIII (ReFacto®) and IgG (Remicade® by Jansen Biotech and Simulect® by Novartis) are two of the leading products being produced commercially using perfusion technology [26, 27]. Apart from these, other products include interferon β-1a (Rebif from merck Sereno) and Ab Golimulab (Simponi® by Jansen Biotech), Factor VIII (Kogenate from Bayer Schering); anti-platelet MAb (ReoPro) and tumor necrosis factor MAb (Remicade from Centocor/J&J); CD52 a MAb (Campath from Genzyme/Sanofi); and a modified Factor VIII (Xyntha/ReFacto from Wyeth/Pfizer) among others. This substantiates the wide application of perfusion-based technologies for a variety of biologicals. The growing need of the pharma sector for reduced cost and enhanced productivity led to the resurgence of perfusion-based production technologies which have the key to revolutionize biopharmaceutical production process from mammalian cells.

3.2 Why perfusion technology?

Perfusion-based bioreactors are one of the upcoming reactor technologies based on continuous bioprocessing that offers the ease of continuous culturing of cells without nuisance of filter clogging or low throughput. In addition, there are less possibilities of waste accumulation and, hence, minimized chances of any product inhibition, especially while dealing with proteins prone to instability. Since nutrients are continuously exchanged and product harvest is maintained throughout along with cell retention, the availability of key media constituents is maintained consistently by providing host cells a stable environment leading to a high cell density and higher productivity with respect to desired compound. Typically, $3–10 \times 10^7$ cells/ml is the titer achieved using perfusion as compared to $5–25 \times 10^6$ in fed-batch cultures. Besides that, cost of goods in the 10,000–20,000-l fed-batch reactor is equivalent to that achieved using 1000 -l perfusion bioreactor [28]. Since the cells are subjected to a more stable and consistent environment, the recombinant proteins and other molecules produced are more like native compounds with similar glycosylation pattern and biological activity. This further increases the stability of the product and gives a high product yield. In a recent economical comparison between fed-batch and perfusion mode for the production of a glycosylated protein, CoG was analyzed and compared for both the processes based on BioSolve software. Continuous perfusion was calculated and found to be the most productive technology giving product at the rate of 265 kg/year as compared to 130 kg/year in fed-batch mode. Perfusion was also found to be the most cost-effective mode with the lowest overall CoG of \$87/g as compared to \$118/g for the continuous fed-batch process [29]. Also, there are less possibilities of failure and economic loss. Even if a problem is encountered, only the part being processed would need to be discarded, saving the rest for further processing. In a comparative study on production by both perfusion and fed-batch modes, CMC Biologics reported yield of 425 mg/l/day from perfusion bioreactor as compared to a yield of only 55 mg/l/day from fed-batch system for the same period of time [30]. Since most of the pharma companies thrive on economic profits, perfusion technology offers a lucrative mode of production especially as it beats the conventional fed-batch system in terms of productivity, efficiency, and capital investments.

3.3 Cell retention in perfusion

The prominent aspect which makes perfusion systems different and more valuable than fed-batch systems is the ability to yield a high cell mass due to the presence of cell retention devices. There are various ways through which cell retention is achieved [31]. Cells can be retained by making them grow inside bioreactor on hollow capillary fibers, flat plates, sponge-like materials, microcarrier particles, or other membranes. It can also be done by use of various cell separation devices like gravity-based cell settlers, spin filters, centrifuges, cross-flow filters, alternating tangential-flow filters, vortex-flow filters, acoustic settlers (sonoperfusion), and hydrocyclones [32]. Spin filter was one of the earliest available devices for cell retention which used a two-dimensional screen to retain the cells. However, it had limited scale-up potential especially in the scenario where rapid feed rate is needed. Gravity-based cell settlers are cost-effective but are marked by inefficient cell separation and significant cell loss, which lowers output and increases cost. Centrifuges have been known to give good performance but increase the production cost. Alternating tangential-flow filters (TFFs) have emerged as the most effective and practical means of high-density cell retention in a perfusion bioreactor [33]. The alternating tangential-flow action in these filters and location of diaphragm in the system prevent clogging as well as ensure a faster return of cells back to the reactor, bringing complete clarification. However, what need to be worked upon are other reactor specifications for handling large cell load at reduced volume and culture time. Also, scalability complications are a deterrent for many manufacturers. Many companies are targeting advancements in ATF system to handle increased cell load at smaller reactor volume. In a recent report on biologics development and manufacturing, the advancement in perfusion and its leading incorporation in manufacturing processes by leading biologics-based companies was attributed mostly to the advancements made in ATF systems which enhance the cell titers by multiple folds over extended periods of time, leading to higher volumetric productivity [34]. Acoustic wave separation (AWS) is another technology used by many companies for cell separation. Applikon Biotechnology and Pall Life Sciences are two such manufacturers advocating the use of acoustic waves to clump and settle down cells leading to their eventual separation. Sigma Aldrich Co. LLC (Merck & Co. Inc.), FiberCell Systems Inc., Zellwerk GmbH (Glen Mills in the United States), Cell Culture Company, ATMI Incorporated, PBS Biotech, Inc., GE Healthcare Life Sciences, Applikon Biotechnology, WAVE Life Sciences Biovest, AmProtein, Xcellerex, etc. are few of the leading manufacturers of perfusion bioreactors [35]. These reactors are revolutionizing the biopharmaceutical production industry and have established their presence in this sector preferably to stay for many years to come.

3.4 Perfusion and microbioreactors

Integration of perfusion technology with microbioreactors enhances the advantages associated with microbioreactors effectively. It further minimizes the losses associated with batch failure due to contamination. Even if contamination occurs earlier in the process, lesser media and other consumables would be wasted. However, the compatibility of the setup is amenable to technology development, scale-up, optimization, parameter sensitivity studies, and validation. Advancements in cell retention systems at microfluidic levels continue to be made. In a recent report, a novel microfluidic cell retention device based on inertial sorting was tested positively for retention of IgG1-producing Chinese hamster ovary (CHO) cell line. Parameters tested were cell retention efficiency, biocompatibility, and scalability. This was a spiral membrane-less system accomplishing cell retention based on hydrodynamic forces. The device was fabricated with polydimethylsiloxane (PDMS) and connected to spinner vessel-containing cells. There was also the flexibility of configuring the device to separate different-sized cells with a specific input flow rate. This gave an added advantage of flushing out non-viable cells, thus creating a healthier environment for the productive cell batch, leading to an increased productivity and product specificity [36]. In a first-of-its-kind study on feasibility of growth of human embryonic stem cells (hESCs), in continuous-flow microbioreactors, key cellular behavioral factors were assessed for robust cell growth under a range of flow rates. This would provide an assay platform for screening multiple cell lines for their capability to function in perfusion culture conditions as well as would aid in identifying optimum flow rates for their application in other microfluidic cell culture systems. Such microfluidic reactor setups would help in better understanding of stem cells maintenance and differentiation under varying stimulation conditions [37]. Perfusion microfluidic systems can also be used for growth and expression of proteins from bacterial cells. Growth of suspended cultures of *Pichia pastoris* in microbioreactor, integrated with perfusion devices, established the feasibility of use of such integrated systems for suspended bacterial growth and expression. Expression of recombinant human growth hormone (rhGH) or recombinant interferon alfa-2b (rIFNα-2b) by *Pichia pastoris* was found to be maintained after 11 days of growth which is close to the duration of many industrial processes with duration lasting from 7 to 30 days. Success of this study establishes the feasibility of use of perfusion-capable bioreactors to study impact of perfusion culture on expression and process optimization of many other microbial systems [38] at microfluidic levels, thus saving the initial process development costs [39].

3.5 Future of perfusion technology

Despite being in the sector for many years, perfusion technology did not gain much usage. However, with new methods for cell retention in the market and growing need for high throughput mammalian cell-based production systems, there is a renewed interest in the use of perfusion bioreactors. With a low capital and start-up cost, smaller setup, a smaller requirement for upstream and downstream processes, and reduced cost of failures, more and more compounds are being commercially produced using this technology. From leading biological manufacturers like Pfizer to upcoming ones like CMC Biologics, many companies are propagating the use of this technology with setup ranging from 250 to 2000 liters. Current research, however, would focus on scalability of the technology, creating a robust cell retention system, high-yield cell lines used in single-use technologies, cutting down on the initial establishment cost and creating the availability of expertise in perfusion-based devices and also the ease of bioprocessing modeling during continuous mode of operation. Also of relevance and on the rise is the use of single-use perfusion-based reactors for production. There are many manufacturers dealing with development of single-use bioreactors, sensors, and other systems to simplify setup and operation of perfusion cell culture. These include among Pall Life Sciences, Applikon Biotechnology, Sartorius, AcuSyst, GE Healthcare, and many others.

4. Conclusion

Microbioreactors are essential for high throughput screening of various strains and optimize the bioprocess development in industries. Microbioreactors assist in identifying the appropriate experimental conditions to scale up the production process. Production of biopharmaceuticals, enzymes, proteins, and medicinally important chemical compounds from organisms was attempted successfully. Microbioreactors have been demonstrated as a suitable technology to cultivate *Pichia pastoris*, *Escherichia coli*, monoclonal antibodies, etc., at large scale that are important for biopharmaceutical industries. Incorporation of microbioreactors in drug discovery and development will reduce the cost and time for developing therapeutic molecules. The growing need for a high production process and efficient production strategy has also championed the cause of perfusion-based technology interfaced with microbioreactors. Perfusion microfluidic bioreactors have been extremely beneficial in maintaining stem cells, expression of proteins, growth of cells, and microbial cultures. Thus, microbioreactors aid in developing high-quality products at affordable cost with minimal resources.

Acknowledgements

Authors thank Symbiosis International (Deemed University) for funding and research facility.

Conflict of interest

None declared.

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