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Bioreactors and Fermentors—Powerful Tools for Resolving Cultivation Bottlenecks

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Executive Summary

Shake flasks, cell culture dishes, and T-flasks are the first vessels that come to mind, when we think about cultivation systems for growing eukaryotic and prokaryotic cells in the lab. Bioreactors and fermentors are another alternative to consider if we need larger quantities of cells, increased efficiency of cultivation, or enhanced reproducibility. In this white paper we explain the key characteristics of stirred-tank bioreactors and which organisms are typically grown in them. Using specific examples, we demonstrate how bioreactors and fermentors can help to resolve cultivation bottlenecks.

Introduction

Many applications are well served by the cultivation of bacteria or yeast in shake flasks and cells in dishes or T-flasks. Bioreactors and fermentors, however, improve productivity and save work, time, and lab space for scientists,who

> need large quantities of cells, microbes, or of the products they express
> would like to improve the reproducibility of growth, the product formation or the product quality
> would like to systematically compare different growth conditions
> would like to increase the cultivation efficiency.

What are bioreactors and fermentors?

Broadly speaking, bioreactors and fermentors are culture systems to produce cells or organisms. They are used in various applications, including basic research and development, and the manufacturing of biopharmaceuticals, food and food additives, chemicals, and other products. A broad range of cell types and organisms can be cultivated in bioreactors and fermentors, including cells (like mammalian cell lines, insect cells, and stem cells), microorganisms (like bacteria, yeasts, and fungi), as well as plant cells and algae.

Bioreactor and *fermentor* are two words for basically the same thing. Scientists who cultivate bacteria, yeast, or fungi often use the term fermentor. The term bioreactor often relates to the cultivation of mammalian cells but is also generically used. If we talk about bioreactors in this

white paper we usually mean systems for the cultivation of microbes or mammalian cells.

Stirred-tank bioreactors

Though many types of bioreactors exist, we will focus on stirred-tank bioreactors. The name is accurately descriptive. Cultivation takes place in the bioreactor tank—often called a vessel—and the culture is mixed by stirring (instead of shaking, for example).

Stirred-tank bioreactors come in different sizes, for cultures of a few milliliters to thousands of liters, and are made of various materials—usually glass, plastic or stainless steel. The basic components and functioning of stirred-tank bioreactors are always the same.

A stirred-tank bioreactor system consists of several parts (Figure 1):

> A vessel, which is filled with medium in which cells are cultivated
> A head plate, to close the vessel
> Components, within or attached to the vessel or the head plate, to measure and adjust the culturing conditions, such as feed lines and sensors
> A control system comprising external components used to adjust the culturing conditions (for example pumps) and control software

Creating optimal cultivation conditions

Like incubators and shakers, bioreactors allow for the creation of optimal environmental conditions for the growth of cells or microbes. They differ, however, in how these are established.

Culture mixing

Instead of mixing by shaking, in a bioreactor the culture is stirred with an impeller. The impeller is mounted to the impeller shaft, which in turn is connected to a motor. In a bioreactor, not only are bacterial, yeast, and suspension cell cultures constantly mixed, but also cultures of adherent cells attached to a growth matrix.

Tempering

To obtain the right cultivation temperature, a bioreactor does not need to be placed inside a shaker or incubator but can remain on the lab bench. The temperature of the culture medium is continuously monitored with a temperature sensor. To regulate it, the vessel is placed in a thermowell, wrapped with a heating blanket or has a water jacket. Cooling is possible as well.

Establishing aerobic or anaerobic conditions

In a shaker or incubator, oxygen is transferred from the surrounding air to the culture medium. This process is more efficient in shake flasks than in static cultures, because shaking increases exposure of the liquid surface. In bioreactors, usually air or pure oxygen (coming for example from a compressed air cylinder) is introduced to the culture. With the use of spargers the gas/liquid interface can be increased and the oxygen supply maximized. Oxygen is important for culture growth, and the amount of oxygen dissolved in the medium (dissolved oxygen concentration, DO) is continuously measured with a DO sensor.

To keep DO at setpoint, a DO cascade is often set up in and executed by the bioprocess control software. Figure 2

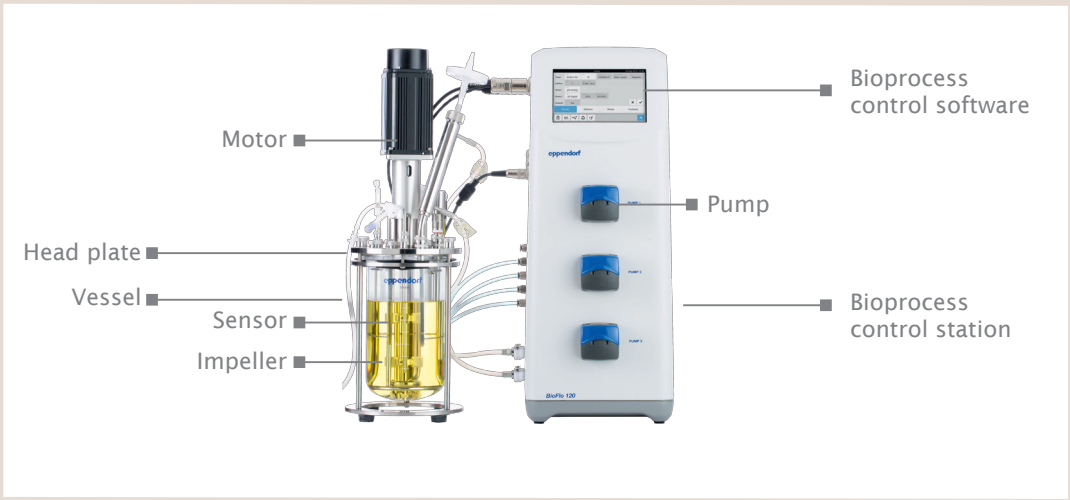

Figure 1: Stirred-tank bioreactor system consisting of bioprocess control station, vessel, and bioprocess control software. The BioFlo® 120 bioprocess control station is shown.

shows an example for a typical DO cascade. If DO drops below setpoint, first the agitation speed is gradually increased up to 1,200 rpm to increase oxygen transfer from the surrounding air. If this is not sufficient to keep DO at setpoint, the gas flow rate is increased up to three standard liters per minute (SLPM). As a final measure, the oxygen concentration in the gas mix is increased, shifting from gassing with air (containing 21% oxygen) toward gassing with pure oxygen. This is just an example. The minimum and maximum values of agitation, gas flow rate, and oxygen concentration can be optimized depending on the organism and process needs.
Anaerobic conditions can be established by gassing with N_2 or other anaerobic gases.

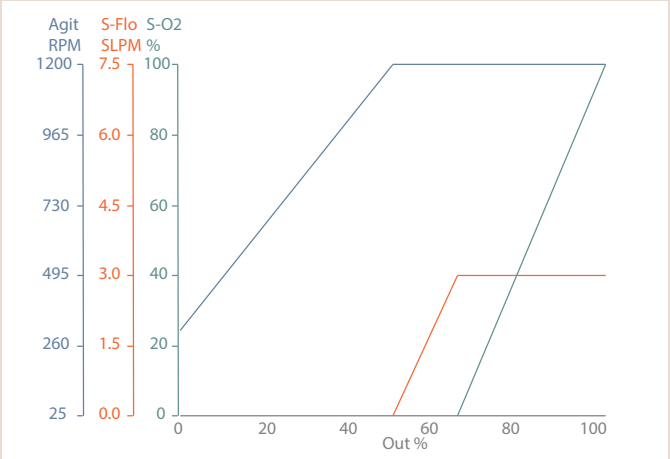

Figure 2: DO cascade. See text for details.

pH control
To regulate the pH of carbonate-buffered cell culture media, cell cultures in flasks or dishes are usually placed in CO_2 incubators. In bioreactors, the principle is the same; CO_2 is introduced to the culture from a compressed gas cylinder. In bioreactors, the medium pH is continuously measured using a pH sensor and CO_2 is added as needed. This is different from the situation in a CO_2 incubator, in which the CO_2 concentration in the internal incubator atmosphere is measured and controlled, rather than the medium pH. Bioreactors also differ in that a basic solution is often added to compensate for acidification during culture growth.

For microbial cultures in bioreactors, basic and acid solutions are commonly used for pH adjustment. This is different from cultures in shake flasks, where the culture pH is usually not controlled.

Control of parameters at setpoint
In bioreactors, different components and the control software play together to control pH, temperature, and dissolved oxygen at the desired setpoint. The parameters are constantly measured using pH, temperature, and DO sensors. The sensors transmit the information to the bioprocess control software which regulates the addition of CO_2 and liquid pH agents, the activity of tempering devices, agitation, and the gassing with air and/or O_2.

A typical bioprocess run

To maintain cultures, scientists usually keep them in cultivation systems within incubators. Bioreactors are usually used for a specific experiment or production run, which may last hours, days, or weeks, depending on the organism and application. A bioprocess run typically comprises the following steps (Figure 3).

1. **Preculture:** The medium in the bioreactor is inoculated with a preculture. Often, the preculture is grown in a shaker or incubator. Sometimes smaller bioreactors are used to grow precultures for the inoculation of larger bioreactors.
2. **Bioreactor preparation:** The bioreactor is prepared in parallel to inoculum preparation. Preparations include the sterilization of bioreactor, feed lines, and sensors; medium addition to the bioreactor; the connection of the bioreactor with the bioprocess control station; and the definition of process parameter setpoints in the bioprocess control software.
3. **Inoculation:** Once the bioreactor is prepared, the medium is inoculated.
4. **Cultivation period:** During the cultivation period, agitation, pH, temperature, and DO are typically monitored and controlled in real time via the bioprocess control software. In addition, scientists often take culture samples to analyze, for example, the biomass and the concentration of metabolites. Eventually researchers feed the culture by adding nutrient solutions.

   Cultures typically pass through four growth phases.
   - > In the lag phase, at the beginning of the culture, the organisms do not multiply or multiply only slowly, probably because they need to adapt to the new culture conditions.
   - > In the exponential growth phase, as the name says, the culture grows exponentially.
   - > In the stationary phase growth stops, because the nutrient concentration, the oxygen concentration, the accumulation of byproducts, or other factors become growth-limiting.
   - > The stationary growth phase is followed by the death phase, in which the viable cell density decreases.
5. **Culture harvest:** Scientists typically end the bioprocess run and harvest the culture when it enters the stationary growth phase.
6. **Downstream processing:** The culture broth is further processed.
7. **Bioreactor cleaning:** The bioreactor is sterilized to inactivate culture residues and cleaned.

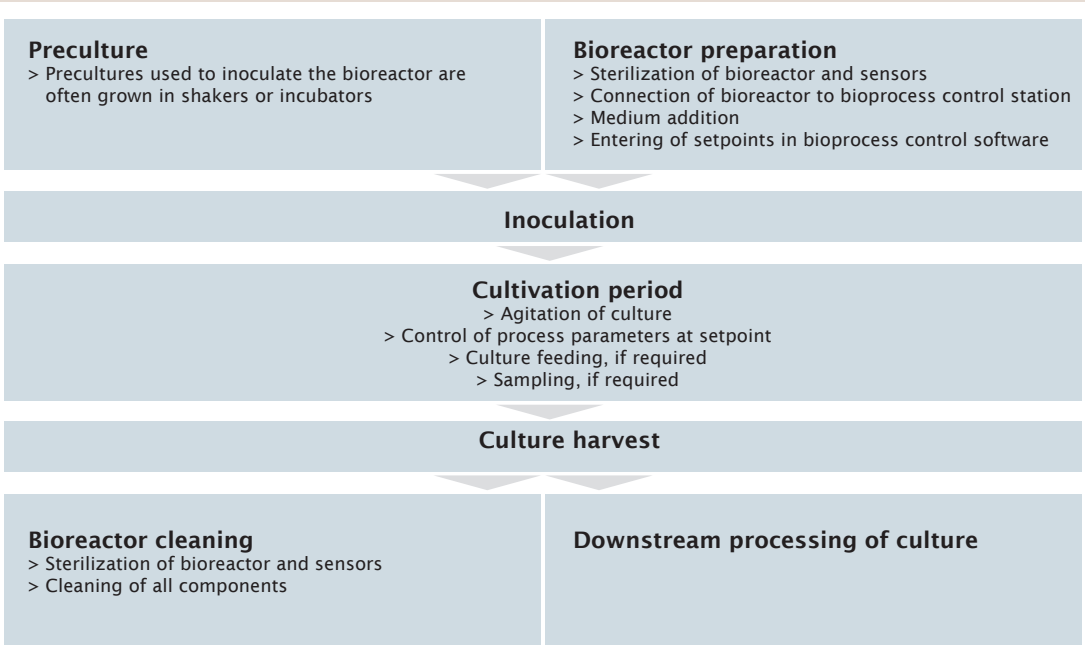

Figure 3: Series of events in a typical bioprocess run.

How bioreactors and fermentors help in resolving cultivation bottlenecks

Bioreactors may save work, time, and lab-space of scientists who need large quantities of cells, microbes, or of the products they express. Furthermore it can improve reproducibility of growth, product formation, and product quality and increase cultivation efficiency.

Culturing large amounts of cells and microbes

Sometimes researchers require large amounts of cells or of the product they produce. Large amounts of a recombinantly expressed protein may be needed for example for biochemical or structural characterization, for ongoing use as research tools, or for evaluating medical applications. Small molecules produced by microbes and intended for use as chemical building blocks, fuels or food and feed additives may be required in small quantities in the R&D phase, but usually large quantities are then needed for their commercial use. Large quantities of cells are needed for stem cell-based cell therapy and drug research applications.

Handle less vessels

Producing cells in a couple of T-flasks or preparing a few liters of a bacteria or yeast culture in shake flasks are feasible. But if dozens or even hundreds of flasks are needed to produce the required amount of biomass, lab space becomes limiting and the amount of manual work explodes. Stirred-tank bioreactors are scalable, meaning they allow increasing the size of the cultivation vessel instead of the number of vessels, so that the work and space requirements stay manageable.

As an example: In conventional cell culture consumables, CHO cells typically reach a density of 2-4 x 10^5 cells per cm^2 [1]. This corresponds to 3.5 – 7 x 10^7 cells per T-175 flask. In bioreactors we can easily reach cell densities of up to 1 x 10^7 cells per mL [2, 3]. This corresponds to 3 x 10^9 cells per bioreactor with a working volume of 250 mL. In this example, one comparably small bioreactor replaces 250 T-175 flasks.

For microbial cultures in shake flasks the situation is similar. Typically, Erlenmeyer flasks with capacities up to 5 L are used. In contrast, bioreactors with working volumes of hundreds and thousands of liters are available.

The availability of larger vessels is not the only advantage. In bioreactors, higher cell densities can be achieved, sometimes making an increase of the working volume unnecessary.

Achieve higher cell densities

At some point, the growth of cells and microbes in flasks and dishes reaches a stationary phase. The cell concentration does not increase further. In bioreactors, cultures reach a stationary phase as well (unless you perform a continuous bioprocess), but much higher culture densities can be achieved.

One example: When our application engineers cultivated *E. coli* in a complex medium in a shake flask, the culture entered the stationary growth phase overnight and typically had an optical density at 600 nm (OD_{600}) of around 12. When they cultivated *E. coli* in a bioreactor, after 12 hours the culture had also entered the stationary phase but reached an OD_{600} of 240 [2].

How did they manage? Using bioreactors allowed our engineers to lessen some of the growth-limiting factors in microbial cell cultures: They improved the supply with nutrients and oxygen, as well as the temperature control [2].

Fast-growing aerobic cultures consume lots of oxygen. If, at a high cell density, the culture needs more oxygen than is transferred to the medium, growth is impaired. In bioreactors, air or pure oxygen can be supplied by gassing, which is more efficient than shaking or agitation alone.

Growing cultures produce heat. To keep the temperature at setpoint, high-density bacterial cultures often do not need to be heated up any more, but cooled down. Bioreactors facilitate culture cooling, in contrast to conventional shakers.

If nutrients become limiting, growth stops. Cultures in bioreactors can quite easily and automatically fed by adding feed solutions using the system's integrated pumps. In bioprocessing we distinguish different process modes: In a batch process the culture grows in the initially supplied batch of medium. In a fed-batch process the culture is fed to keep the concentration of nutrients constant. In a continuous process the culture medium is continuously exchanged.

This is just one example. Dependent on the cell line or microbial strain other parameters may be critical for culture growth and/or product formation, for example the medium pH, metabolite concentrations, redox potential, and mechanical forces. Bioreactors are valuable tools to optimize cultivation conditions.

Comparing growth conditions

Parallel bioprocess systems are available to control more than one vessel (Figure 4). They often have small working volumes which helps saving resources. Parallel systems have the advantage that cultivation parameters can be

controlled independently in each bioreactor. This saves time and ensures maximum reproducibility between runs. If for example you would like to compare protein expression at eight different temperatures, in a parallel bioreactor system you can perform the experiments in parallel. This is more convenient than using a conventional shaker or a single bioreactor, where you would either need to perform one experiment after the other or would need several shakers or bioreactors.

The possibility of comparing multiple process conditions in parallel make bioreactors well suited to systematically analyze the influence of several parameters on the culture outcome (e.g., culture growth, product formation, byproduct formation). In this way, researchers can gain comprehensive process understanding, which in turn allows optimization of culture conditions to achieve the best possible results.

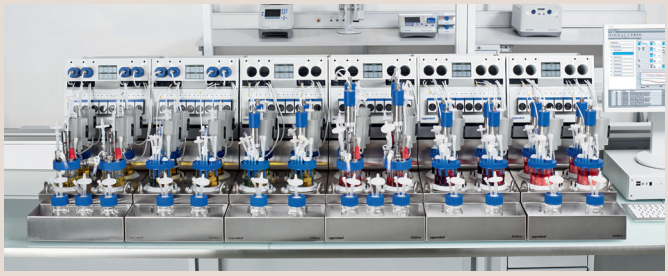

Figure 4: DASbox Mini Bioreactors System allows parallel operatition of up to 24 bioreactors.

Increasing reproducibility

In bioreactors, process parameters like pH, temperature, and dissolved oxygen can be constantly measured using sensors. The sensors transmit the information to the bioprocess control software, which regulates the action of actuators, like pumps, tempering devices, and gassing devices, to keep the parameters at setpoint. The software also continuously saves process values, making it possible to analyze them later. Monitoring, control, and recording of process values help increasing the reproducibility of culture growth, product formation, product characteristics, and more.

Let's take a simple example. You recombinantly express a protein. Let's assume the protein has the tendency to aggregate and aggregation strongly depends on the temperature. Let's further assume that you found the optimal temperature profile for growth and expression phase to balance growth, protein expression, and aggregation. You may employ that temperature profile in a shaker; however, it is prone to error. Unwanted and unnoticed temperature fluctuations may be caused by repeated opening of the shaker by other users who add or remove flasks. In a bioreactor the temperature of the culture medium is continuously monitored and adjusted as needed. As a result, the temperature profile is maintained more reliably, leading to more reproducible results. Furthermore, the temperature sensor data is recorded. Eventually, temperature deviations are detected, making it possible to identify the source of error.

And the situation may be much more complex. Besides the temperature, process parameters including the medium pH, DO, metabolite concentrations, mechanical forces, and medium composition may influence culture growth, product yield, protein glycosylation patterns, byproduct formation, and more. Process parameters influence cell viability, cell behavior, and differentiation. This is especially important if the cell itself is the product of interest, for example if cells are used for cell therapy applications.

In a bioprocess, temperature, pH, and DO are routinely monitored and controlled. Advanced bioprocess control software allows the integration of additional sensors, for example to monitor biomass and metabolites, as well as the setup of tailored process control strategies.

Home sweet home - A bioreactor for every organism

Eukaryotic cells, like mammalian cell lines, stem cells, insect cells, and plant cells, and microbial organisms like bacteria, yeasts, fungi, and algae can all be cultivated in bioreactors.

However, the optimal growth conditions differ and therefore differ the optimal bioreactor design as well as setpoints of agitation, temperature, DO, pH, and other parameters. Are you cultivating mammalian cells or microorganisms? This is the first important question when setting up a process, and will influence some basic decisions regarding certain bioreactor accessories. Dependent on the cell line or strain, parameters will then need to be finetuned.

Bioreactor accessories

Although generalized, much of the following applies for many microbial strains and cells:

Many of the microbial strains which are routinely used in industrial bioprocess applications (e.g *E. coli*, *C. glutamicum*, and *S.cerevisiae*, *P. pastoris*) grow much faster than commonly used mammalian cell lines (e.g. CHO and HEK293). For example, the growth rate of *E. coli* is in the range of 1/few hours or less; the growth rate of a CHO cell line is closer to 1/day. This has important implications for the design of the bioprocess system.

Oxygen demand

The oxygen demand of fast-growing, aerobic microbial cultures is high. The bioprocess system needs to be capable of high gas flow rates to supply enough air and/or oxygen to keep DO at setpoint. To maximize the dissolved oxygen concentration in microbial cultures air/oxygen are usually introduced to the culture medium through submerged gassing, either through a dip tube with an open end or a porous sparger. The pores of the gassing device determine the size and number of the gas bubbles and therefore the surface available for gas exchange with the medium. The oxygen demand of mammalian cell cultures is lower. Often it is not required to maximize the gas/medium interface through sparging, but air/oxygen supply to the bioreactor headspace is enough. An important advantage of headspace gassing is the avoidance of shear force-causing air bubbles which may damage sensitive mammalian cells.

Culture mixing

Besides gassing, agitation is a critical parameter to keep DO at setpoint. Rushton-type impellers mix the culture more efficiently than pitched-blade or marine impellers, but also cause higher shear forces. Therefore the former are commonly used for microbial cultures, whereas the latter are commonly utilized for mammalian cell cultures.

Figure 5: Two impeller types. Rushton-type impellers are commonly used for microbial bioprocesses. Pitched-blade impellers are commonly used for mammalian cell cultures.

Sterility

Because of their slower growth, the contamination risk in mammalian cell cultures is much higher than in microbial processes. The material and connection of feed lines are two factors to consider to ensure sterility. To minimize the contamination risks in cell culture bioprocesses, many scientists prefer feed lines made of autoclavable material (instead of feed lines which need to be chemically cleaned) and which can be safely connected by welding [5].

Setpoints

The above paragraph provides some general recommendations regarding the bioreactor accessories for microbial and cell culture applications. But this is not the whole story. Suitable setpoints for temperature, pH, DO, agitation, and strategies to control them differ between organisms, cell lines, and strains, and may even be different for a single strain used in different applications. Setpoints and control strategies need to be optimized on a case by case basis. Methods described in literature can serve as a starting point for further optimization [5, 6]

Cultivation of adherent cells in stirred-tank bioreactors

Many mammalian cell types need to attach to a growth surface to survive and multiply. Expansion of adherent cells in stirred-tank bioreactors sounds counterintuitive at first, but is feasible if an attachment matrix is provided. The cells attach to the matrix, which is kept in suspension by gentle agitation.
Various matrices are in common usage.

Microcarriers

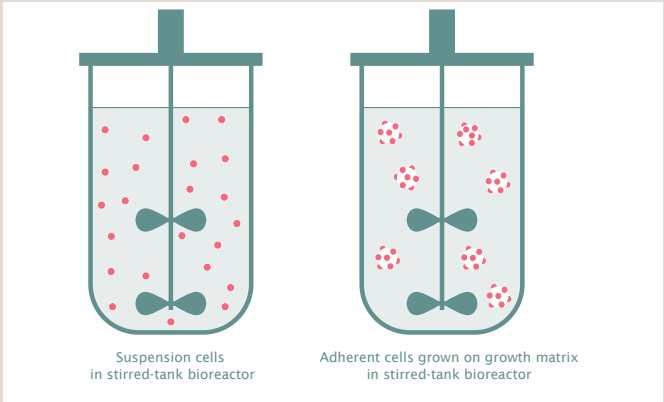

Figure 6: Schematic representation of suspension cells and adherent cells grown in stirred-tank bioreactors.

Microcarriers are spherical particles which provide a growth surface for adherent cells. Microcarriers typically have a diameter of 100 – 300 µm. They can be made of different materials, like glass, DEAE-Dextran, polystyrene, and alginate. There are also coated versions, whose core material is covered with peptides, proteins or protein mixtures like fibronectin, collagen or Matrigel®. The core material of non-coated microcarriers is not functionalized by the manufacturer, but may, however, bind proteins once the carrier is in contact with serum-containing culture medium. To start a cell culture process on microcarriers, they are typically added to the culture medium at a density recommended by the manufacturer and subsequently the bioreactor is inoculated with a single cell suspension. To support cell attachment to the microcarrier, the culture is only periodically agitated during the first few hours after inoculation. Once the cells have attached to the carriers the culture is continuously agitated to keep the carriers in suspension. For downstream processing, the microcarriers can be harvested and the cells detached, for example using trypsin.

Vero cells and mesenchymal stem cells are only two examples of adherent cells, which have been cultivated in stirred-tank bioreactors on microcarriers [7, 8].

Fibra-Cel® disks

Like microcarriers, Fibra-Cel disks provide a growth support for adherent cells. Fibra-Cel disks are made of a meshwork of polyester and polypropylene, which is electrostatically pretreated to support cell attachment. In contrast to many microcarrier types, Fibra-Cel provides a three-dimensional growth surface with a high surface-to-volume-ratio, and protects cells from damaging shear forces, thus increasing the total biomass which can be maintained in the bioreactor. Fibra-Cel disks have a diameter of 6 mm. They provide a growth matrix in packed-bed bioreactors, and in principle can also be used free-floating in shake flasks or disposable bags. Fibra-Cel is predominantly used for cell culture processes for the production of secreted products, like recombinant proteins and viruses. They have been used for example for the cultivation of Vero cells [7].

Cell-only aggregates

Instead of growing on a matrix, cells can grow in stirred-tank bioreactors as cell-only aggregates. This has been described, for example, for human induced pluripotent stem cells and cells differentiated thereof [9, 10], and certain tumor cell lines [11]. For expansion as cell-only aggregates, bioreactor cultures are usually inoculated with a single-cell suspension. Cell expansion leads to the formation and growth of aggregates. The attachment of cells to each other is influenced by the agitation speed and impeller type, among other factors. Stem cell-derived cell spheroids, neurospheres for example, can reach a remarkable degree of maturation and are promising model systems in basic research and drug screening applications [12]. The absence of a synthetic matrix may simplifies downstream processing, for example for cell therapy applications.

Summary

The advantages of cell cultivation in stirred-tank bioreactors, like simplified scalability and improved process control are not limited to suspension cells. By providing a growth matrix or cultivating as cell-only aggregates, adherent cells can be expanded in stirred-tank bioreactors, too.

Conclusion

Stirred-tank bioreactors simplify the cultivation of large amounts of cells compared to conventional culture systems using shakers and incubators. Bioprocess control software facilitates the precise monitoring and control of critical process parameters. Cultivating cells and microbes in bioreactors therefore can save the scientist work, time, and lab-space and improve the reproducibility and efficiency of cell growth and product formation.

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