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Bioreactors for cell multiplication in cultured meat product development

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1 Introduction
2 The bioreactor: principles and structure
3 Bioreactor operation modes
4 Creating a stem cell niche in a bioreactor: important bioprocess parameters
5 Cultivating anchorage-dependent cells in stirred-tank bioreactors
6 Scale-up of a bioprocess in bioreactors
7 Considerations for cost reduction and process optimization in bioreactors
8 Conclusion and future trends
9 Where to look for further information
10 References

1 Introduction

Cultured meat holds great promise for the future with its potential to satisfy the growing demand for meat while reducing harm to animals and the negative environmental impact of livestock husbandry at the same time (Post, 2014). However, the innovative idea of isolating livestock muscle and fat stem cells and growing them into muscle tissue poses challenges that need to be overcome.

High cell numbers are required for sufficient meat production; estimates suggest 10^{12}–10^{13} cells are needed to produce 10–100 kg of cultured meat (Bodiou et al., 2020). Consequently, high cell-density cultures are essential in order to satisfy the anticipated market. Thus, while the first cultured meat burger was literally created on a lab bench scale (Post, 2014), an increase in scale is inevitable to bring this novel food alternative to the general public.

Furthermore, the burger created by Post and colleagues utilized static two-dimensional (2D) cell culturing methods in which thousands of muscle fibers were grown in culture dishes, a strategy sufficient for proof-of-concept but one that does not offer optimal growth conditions and efficiency (Post, 2014; Bellani

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et al., 2020). In fact, the potential cell numbers that could be obtained in this system are limited by a low growth surface-to-volume ratio, as well as a lack of control over important growth parameters, such as gas and nutrient supply or pH (Bellani et al., 2020).

To that end, a suitable environment needs to be created and maintained to facilitate efficient cell proliferation and sufficient cell numbers, ideally through precise parameter control and growth surface increase by switching from 2D to three-dimensional (3D) cultures. Furthermore, all this needs to be achieved at a price acceptable to consumers.

A promising way to confront these challenges is by switching from conventional 2D cultures in flasks placed in CO_2 incubators to 3D cultures in bioreactors.

In this chapter, we explore bioreactors and their working principles, as well as their role as a vital tool to tackle the challenges of creating suitable growth conditions for anchorage-dependent stem cells and process scale-up. Furthermore, we evaluate measures with the potential to reduce the production costs of cultured meat.

2 The bioreactor: principles and structure

Even though bioreactors come in many shapes and forms, their basic principle is straightforward: a vessel providing a 3D growth environment mimicking the natural conditions for prokaryotic and eukaryotic cells due to culture parameter control (Fig. 1). Yet, in order to achieve this simple goal, a whole industry has developed providing innovative solutions for all bioprocessing needs.

As a result, the bioreactor field is quite diverse. Bioreactors can be made from stainless steel, glass, or plastic. Steel and glass reactors are re-usable, with steel favored for large vessels. Contemporary plastic reactors, on the other hand, are smaller than their steel counterparts but offer the advantages of a

Figure 1 Profile of a bioreactor. Components of a stirred tank-bioreactor (left) and a connected control station (right).

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single-use product, such as less maintenance effort and reduced downtime between batches.

Different bioreactor working principles provide a variety of options to choose from. Out of these options, we will have a look at four examples which are commonly employed for cell culture, namely hollow fiber, rocking bed, bubble column, and stirred-tank bioreactors.

The hollow fiber reactor uses the principle of mimicking blood vessel structures to provide a cellular growth environment. Here, a cylindrical vessel is stacked with semipermeable hollow fibers with varying pore sizes able, for example, to let pass medium components or cellular products while retaining the cells at the same time (Bellani et al., 2020). The fibers are commonly made of cellulose and polyethersulfone (Pajčin et al., 2022). Both adherent and suspension cells can be cultivated inside or outside of the fibers while being perfused by medium and medium components using the principle of diffusion (Frank et al., 2019; Bellani et al., 2020).

The rocking-bed or wave bioreactor utilizes a large, disposable polymeric bag as a culture vessel that is placed on a rocking platform (Pajčin et al., 2022). The bag is inflated through an air circulation system and the rocking movement creates a wave motion that enables mixing of medium components and cells, as well as oxygenation of the culture. Thus, this bioreactor type does not utilize internal stirring parts to mix the culture.

This is also true for the bubble column bioreactor type, such as air-lift bioreactors (Guan et al., 2021). Here, culture mixing within the bioreactor tank is achieved by an air bubble stream inducing a circular medium flow resulting in the lifting and sinking of the cells.

Lastly, the stirred-tank bioreactor utilizes a motorized impeller to swirl the cell suspension, thereby supporting gas exchange, nutrient distribution, and culture homogeneity (Fig. 1).

Each bioreactor type has its pros and cons. For example, hollow fiber, wave, and air-lift bioreactors are known to cause low shear stress (Bellani et al., 2020; Al-Mashhadani et al., 2015; Pajčin et al., 2022), a property especially important for shear-sensitive cells like those used for cultured meat production. However, due to the gentle nature of their working principles, mixing and aeration efficiency is lower compared to stirred tank bioreactors (Bartczak et al., 2022; Al-Mashhadani et al., 2015). Furthermore, the structure of hollow fiber bioreactors with cells growing around tubular structures can lead to gradients where cells growing closer to the fiber surface receive optimal nutrient, oxygen, and pH conditions, whereas cells growing in the periphery are not supplied adequately (Pajčin et al., 2022). This limits its use for operation at a larger scale (Rodrigues et al., 2011), for example, when compared to airlift or stirred-tank bioreactors. In fact, the stirred-tank bioreactor is one of the most widely utilized systems for mammalian cell culture on a larger scale (Bellani et al., 2020).

However, its more efficient and homogenous culture conditions come with the need to balance shear forces by choosing the right impeller type and adjusting the agitation speed properly.

Within this chapter, the stirred-tank bioreactor will be used as an example to convey the principles of bioprocessing and its role in culturing anchorage-dependent stem cells.

Culture volumes in bioreactors can range from a few milliliters to tens of thousands of liters; all these proportions have a place in process development. The initial growth conditions for a given cell type are typically determined in small-scale approaches before scale-up, the process of stepwise increasing the dimensions of the bioprocess, is initiated (Fig. 2).

The aim of this strategy is to ensure that yields and critical quality features obtained at small working volumes during process development can be reproduced at larger cell culture volumes. A common method to achieve this goal is to identify the most relevant cultivation parameters and try to replicate them at the different culture sizes. Stirred-tank bioreactors are well suited in this context, as the vessel dimensions are widely scalable and reproducibility of relevant vessel properties (for example, oxygen transfer capabilities or mixing time) is predictable at different scales.

Constant cultivation parameters are usually achieved by a control station connected to the bioreactor. By utilizing a sensor-feedback loop, it constantly monitors, adjusts, and maintains previously selected growth parameters, including temperature, gas concentrations such as dissolved oxygen (DO), pH, and culture mixing (Fig. 3). Sensor readings are transferred to the bioprocess software and compared to a setpoint. Setpoint deviations are then equalized by actuators in automated feedback control loops. Actuators can be heating

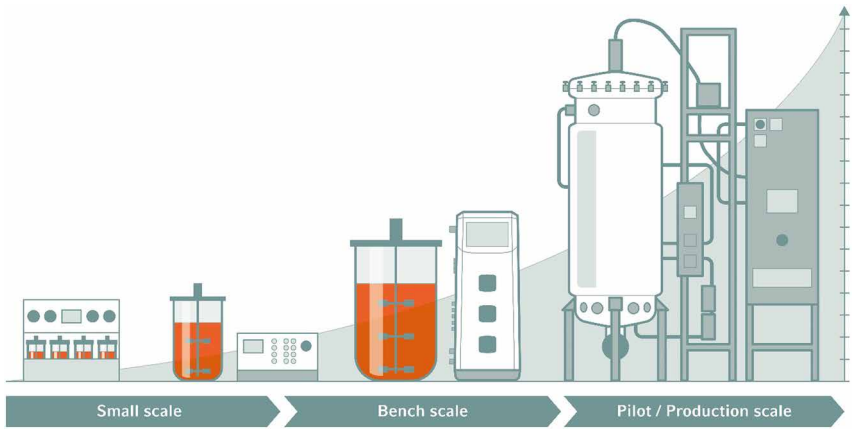

Figure 2 Bioreactor scales during process development.

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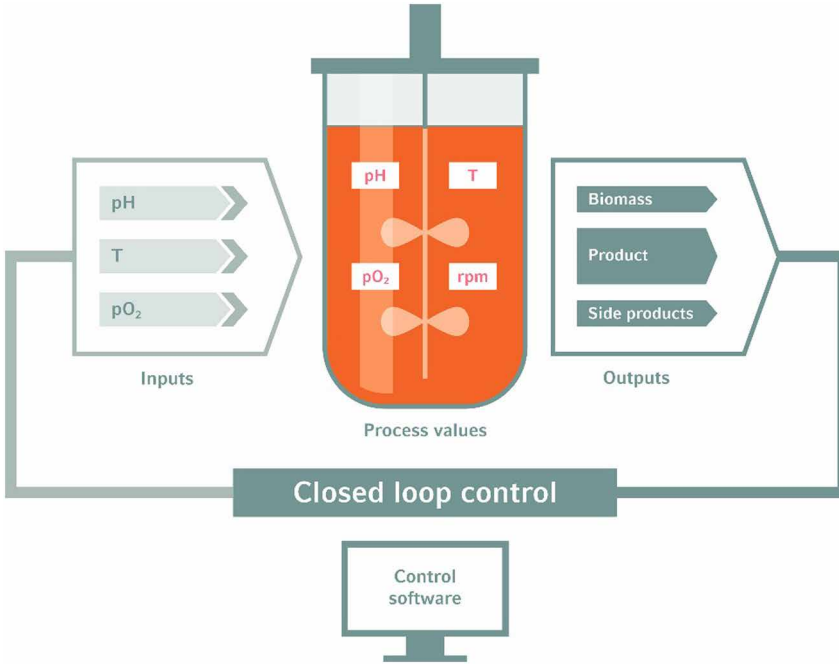

Figure 3 Working principle of a bioreactor. Regulation of parameters, such as pH, temperature (T), or oxygen (O_2) with a control station and specific software creates a suitable growth environment for cells.

devices to adjust the temperature, pumps to deliver liquids for pH control, or gassing devices to provide air, oxygen, nitrogen, or carbon dioxide to the culture.

Being able to adjust the parameters instantaneously according to the culture's needs is one of the biggest advantages of a bioreactor over static cell culture in incubators. It creates and maintains a stable and optimized environment for the cells to thrive in which ensures reproducibility between batches at different scales, thus enabling higher cell densities.

In cell culture, the availability of nutrients and the concentration of toxic by-products are crucial for the achievable cell density. Both can be influenced by the operation mode of the bioreactor. If high cell numbers need to be generated, as in the production of cultured meat, the bioreactor operation mode is, therefore, a critical parameter.

3 Bioreactor operation modes

In general, all cells, no matter if prokaryotic or eukaryotic, undergo the same growth kinetic during cultivation: an initial lag phase after inoculation of the bioreactor in which the cells adapt to the newly established environment,

followed by an exponential phase with increasing cell division and cell density, then a stationary phase in which nutrient limitations halt growth and, in case of sustained nutrient limitation, a death phase. Even though these growth phases are inevitable, their properties can be modulated, depending on the bioreactor operation mode.

Three operation modes are commonly used for bioprocessing, namely batch, fed-batch, and continuous cultivation (Fig. 4).

3.1 Batch cultivation

Batch cultivation is carried out in a fixed volume. No medium or feeding solutions are added, removed, or exchanged during the process (Yang and Sha, 2019; Reiss et al., 2021). The cells continuously consume the nutrients and undergo the typical growth phases. Once the nutrients are depleted, cellular growth comes to a halt and the process is terminated. As there are almost no external interventions, batch cultivation bears a low risk of contamination. However, the cells are presented with a constantly changing environment and accumulation of potentially toxic by-products within the medium (Yang and Sha, 2019). This may affect the growth efficiency of the culture and by-products might cause problems in downstream processes.

3.2 Fed-batch cultivation

Fed-batch cultivation builds upon the batch mode to address its disadvantages. Here, nutrients are replenished during the incubation period, thus establishing a more stable environment for the cells. Feeding of carbon and nitrogen sources to the cells is usually initiated after the initial lag phase. Thus, fed-batch cultivation prolongs the availability of nutrients, giving the cells more time to grow before entering the stationary phase. However, the external nutrient

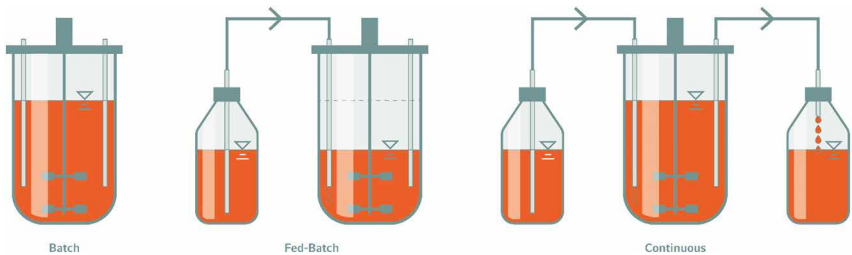

Figure 4 Bioreactor operation modes. Batch cultivation carried out in a fixed volume, fed-batch where medium and nutrients are added after the initial lag phase until the end of the process, and continuous cultivation where constant medium/nutrient replenishment is employed to ensure a stable growth environment.

supply also bears a higher contamination risk and leads to a volume increase over time (Reiss et al., 2021).

3.3 Continuous cultivation

Continuous cultivation is the most complex operation mode. However, once it is thoroughly adjusted it offers the most stable cell environment and the longest possible running times. This is obtained by the continuous removal of the used medium and cells combined with a constant flow of fresh medium and nutrients (Yang and Sha, 2019). The result is a steady state that ideally keeps the volume, nutrient concentrations, and biomass constant while removing toxic by-products at the same time (Reiss et al., 2021). The introduction of a cell retention device can be used to exchange the medium while the cells are maintained within the bioreactor. This subtype of continuous cultivation, known as perfusion (Allan et al., 2019), might be favorable for cultured meat production to obtain even higher cell densities than in a fed-batch process. However, some drawbacks of the fed-batch mode also occur here. Multiple external connections and the possibility of long-term incubation make maintaining sterile and stable growth conditions more challenging.

Nevertheless, fed-batch and continuous cultivation are the methods of choice for large-scale applications, such as cultured meat production, as they allow for easier automation and culture medium recycling (Reiss et al., 2021).

4 Creating a stem cell niche in a bioreactor: important bioprocess parameters

Even though pluripotent stem cells are an attractive option for cultured meat production, multipotent (also called adult) stem cells are the more common cell sources. Among those, muscle satellite cells, mesenchymal stem/stromal cells, and fibro/adipogenic progenitors are the cell types of choice in cultured meat production, as they can be isolated relatively easily by tissue and muscle biopsy (Reiss et al., 2021). In contrast to their pluripotent counterparts, these progenitor cells can only differentiate into specific cell types associated with the relevant organs (Reiss et al., 2021).

However, regardless of the stem cell's origin, the basic demands concerning their biological environment are similar. In this section, we will have a look at some of the important stem cell niche parameters and how they can be maintained in a bioreactor.

4.1 Temperature

A suitable temperature is the most basic parameter of any cell culture and yet one of the most important ones in order to ensure cell viability and growth. Most mammalian cells are cultivated at a temperature of 37°C (Rubio et al., 2019), even though higher temperatures were described to be adequate for myoblast development, such as using 37–39°C in a pig cell model (Metzger et al., 2021). Other possible cell sources for cultured meat production include fish or birds. Fish cells are usually cultured at 15–30°C (Rubio et al., 2019). Avian muscle cells, on the other hand, are commonly incubated at around 38°C, but studies with deviant incubation temperatures were carried out as well (Clark et al., 2016; Reed et al., 2017; Reed et al., 2017).

Also, these studies suggest an influence of temperature differences on muscle cell proliferation and differentiation (Reed et al., 2017; Clark et al., 2016; Reed et al., 2017). Such indications of temperature affecting the cell fate highlight the importance of precise temperature control during the culturing process.

Within a bioreactor, the temperature can be measured and monitored by different sensor types. The most common ones are thermocouple and resistance sensors (Djisalov et al., 2021). In both cases, the sensor readings act as feedback for the control station to maintain a previously set temperature within the bioreactor. This is achieved by electrical heating jackets (Fig. 5)

Figure 5 Two bioreactors wrapped in heating jackets and attached to a bioprocess control station. Besides electrical heating jackets, water jackets can be an option for temperature control in a bioreactor. The optimal growth temperature is regulated and maintained by the control station.

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wrapped around the bioreactor. Furthermore, certain glass bioreactors are enclosed by a water jacket, a surrounding glass cylinder in which heating or cooling water circulates.

4.2 Oxygen

Traditional *in vitro* cell cultures provide a static environment of ca. 5% CO_2 and 20% O_2 (Liu et al., 2013). However, the *in vivo* situation is much more variable with oxygen levels typically ranging from 3% to 9% in tissues and even 1.3% to 2.5% within a cell (Thomas, 2016). Such low oxygen tensions are termed 'hypoxic' and described to influence the stem cell fate.

For instance, oxygen levels of 1% maintained human stem cells at a primitive progenitor state and promoted self-renewal (Ivanovic et al., 2000; Ivanovic et al., 2002). Similar observations were made for human mesenchymal stem cells cultured at 2% oxygen which expressed more stem cell-related genes compared to cells incubated with 20% oxygen (Grayson et al., 2006). Additional effects of low oxygen tension on mesenchymal stem cells include reduced deoxyribonucleic acid damage (Estrada et al., 2012), better engraftment (Hung et al., 2007), and inhibition of senescence (Tsai et al., 2011). Also, the cultivation of old muscle satellite cells in a 3% oxygen atmosphere greatly increased their proliferation potential *in vitro* (Chakravarthy et al., 2001). Moreover, different oxygen tensions can affect the differentiation behavior of stem cells. Incubation of human mesenchymal stem cells at 5% O_2 resulted in chondrocyte development whereas incubation at 20% O_2 induced adipogenic differentiation (Lovett et al., 2010).

Those examples illustrate the impact of oxygen, as certain concentrations induce cell proliferation while others shift the cells toward differentiation. As the process of cultured meat production incorporates both proliferation and differentiation, being able to precisely control DO levels is of high importance. Using a bioreactor not only provides this control but also enables mid-process adjustments, for example, if a switch from pure proliferation toward differentiation is required.

The DO concentrations in a bioreactor are usually measured by one of three sensor types: electrochemical, optical, or paramagnetic (Djisalov et al., 2021). Regardless of the measuring method, the DO levels are processed by the control station which constantly compares the actual values to a setpoint. Deviations between the setpoint and measured value can be resolved through various countermeasures. The control station can use a cascade to adjust the DO levels. Depending on the requirements of the specific culture, DO levels can be upregulated, for example, by increasing the airflow rate, adding pure oxygen, or increasing the agitation of the impeller, or downregulated by mixing the four gases used in most bioreactors (air, oxygen, nitrogen, and carbon

dioxide) accordingly, for example, by blending nitrogen into the air to lower the oxygen concentration.

4.3 pH

Another critical part of successful cell culturing is the correct pH value. The pH optimum for animal cells lies around 7.4 (Djisalov et al., 2021). However, uncontrolled pH in a bioprocess usually leads to medium pH changes due to the increase of by-products such as lactic acid (Lawson et al., 2017; Djisalov et al., 2021) and small deviations can affect cell survival and growth (Djisalov et al., 2021).

Indeed, there are indications of pH value fluctuations altering stem cell fate determination. For instance, short-term acidic pre-treatment of pH 6.8 was shown to enhance the viability and proliferation of murine bone marrow-derived mesenchymal stem cells, as well as an increased expression of stem cell markers (Hazehara-Kunitomo et al., 2019). Another study found that acidic conditions (pH 6.8) maintain the pluripotency of murine embryonic stem cells (Teo et al., 2014). Even though few studies on the direct impact of pH value exist, it is hypothesized that modulations affect stem cells fate-related phenomena, such as alternative mRNA splicing or mitochondrial activity (Kim, 2021). Thus, providing a stable pH is important to maintain optimal growth conditions.

In a bioreactor, this is typically monitored either by electrochemical or optical sensors (Djisalov et al., 2021). The feedback provided by the sensors enables the control station to adjust the pH value accordingly by either adding base or acid.

4.4 Lactic acid

Bioreactors enable dynamic feeding with nutrients such as glucose-containing solutions. However, metabolizing glucose results in increasing lactic acid levels during the culturing process with the potential to alter stem cell properties. Even though no direct mechanism has been proposed yet (Nalbandian et al., 2020), several studies suggest an impact on muscle-related stem cells and their progeny *in vitro*.

For example, treating human mesenchymal stem cells with low sodium lactate concentrations of 1 mM promoted stemness properties (Sun et al., 2020). Experiments with a murine myoblast cell line, the downstream differentiation step of muscle satellite cells, demonstrated that a daily 2 h exposure to lactic acid (10 mM and 20 mM) induces early but delays late differentiation marker expression in a time- and dose-dependent manner (Willkomm et al., 2014). Moreover, myotubes differentiated from this cell line showed reduced

myogenesis after long-term treatment with 8 mM of lactic acid for 24 h (Oh et al., 2019). Furthermore, increased lactic acid levels can alter the pH of the medium (Djisalov et al., 2021), leading to the consequences discussed earlier.

Thus, monitoring lactic acid and other compounds such as glucose in a bioreactor and controlling them accordingly by removing used and adding fresh medium is an important tool to maintain optimal culture conditions (Fig. 6).

For instance, knowledge of the medium's nutrient and by-product composition enables the determination of medium feeding time points or the evaluation of medium quality in the process of medium recycling (Djisalov et al., 2021). For that, process analytic technology can be employed to analyze critical compound levels in the medium, either by an online or offline approach. Online means the analysis device is integrated into the feedback loop of the bioreactor control station software which then adjusts medium addition and removal automatically. Offline means removing the medium manually through specific sampling ports without disturbing the sterile conditions of the culture and analyzing it in an external device. The obtained values can then be used to adjust feeding and removal rates manually. Both online and offline metabolite monitoring was shown to be viable tools for process optimization of mammalian cell culture in stirred-tank bioreactors (Craven, 2015; Escobar Ivirico and Sha, 2020).

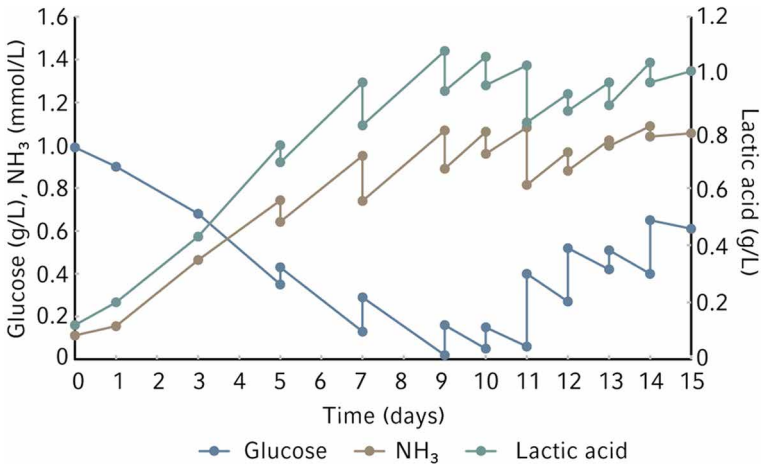

Figure 6 Example of controlling glucose, lactic acid, and ammonia levels during mesenchymal stem cell culture in a bioreactor (Escobar Ivirico and Sha, 2020). Lactic acid, a by-product of glycolysis, increases when glucose is consumed by the cells. Adjusting the glucose feeding rates and regular medium exchanges can help to keep the lactic acid levels from exceeding a critical value that can be harmful to the cells. In addition, medium exchanges can also be a measure to regulate the levels of other potentially toxic by-products, such as ammonia (NH_3).

Figure 6 illustrates how metabolite monitoring and feeding rate adjustment can maintain a balance of nutrients such as glucose and potentially harmful by-products, including lactic acid and also ammonia, a by-product of glutamine metabolism known for its toxic effects on mammalian cells (Escobar Ivirico and Sha, 2020; Mirabet et al., 1997; Moritz et al., 2015).

4.5 Culture mixing

Efficient nutrient distribution, gas-liquid mass transfer, and culture homogeneity are the key factors for successful bioprocessing. A stirred-tank bioreactor achieves this through the use of rotating impellers. However, mammalian cells are known to be shear-sensitive (Allan et al., 2019) and factors such as wrong impeller design, increasing agitation speeds, and bursting bubbles from an oxygen sparger can induce cell damage (Martens et al., 1996; Fan et al., 2015).

Furthermore, it was observed that certain shear forces can affect cell differentiation. For instance, fluid flow-induced shear stress was shown to drive human and goat mesenchymal stem cells toward osteogenic differentiation (Yourek et al., 2010; Knippenberg et al., 2005). While this is a noteworthy observation, it bears the risk of directing the cells to an unwanted cell fate determination.

Thus, shear stress regulation is crucial to steer the cells toward the optimal growth and differentiation path. In a stirred-tank bioreactor, shear forces can be regulated by choosing the right impeller type and adjusting the agitation speed. In our studies, pitched blade impellers (Fig. 7) combined with low agitation speed have emerged as a proven tool to efficiently mix shear-sensitive mammalian cells in a stirred-tank bioreactor, including mesenchymal and pluripotent stem cells (Olmer et al., 2015; Dufey et al., 2016; Siddiquee and Sha, 2014; Roldão et al., 2018; Escobar Ivirico and Sha, 2020).

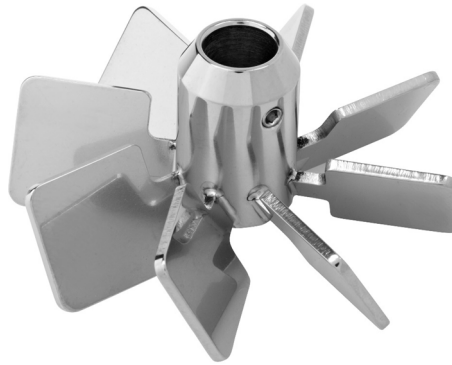

Figure 7 A pitched eight-blade impeller. This impeller type with blade angles of 60°C is suitable for stem cell cultivation within a stirred-tank bioreactor.

5 Cultivating anchorage-dependent cells in stirred-tank bioreactors

Like most other tissue-derived cells, stem cells employed for cultured meat production are anchorage-dependent, meaning attachment to a surface is needed for normal proliferation (Merten, 2015). Therefore, scaffolds enabling cell adherence are required for efficient culturing. This aspect can be tackled by employing microcarriers. Microcarriers are beads with diameters of 60–300 µm (Bellani et al., 2020) that can be added to the culture medium, thereby providing a growth surface to the cells (Fig. 8) and increasing the surface-to-volume ratio within the bioreactor.

Microcarriers are available with varying porosity from a range of materials, including polystyrene, gelatin, dextran, and collagen (McKee and Chaudhry, 2017). Besides those materials, research is performed on biodegradable and edible microcarriers (Bodiou et al., 2020). These would be a valuable addition to cultured meat production as they have the potential to facilitate or even eliminate downstream microcarrier removal and cell-detachment steps which are laborious and can affect cell integrity (Reiss et al., 2021).

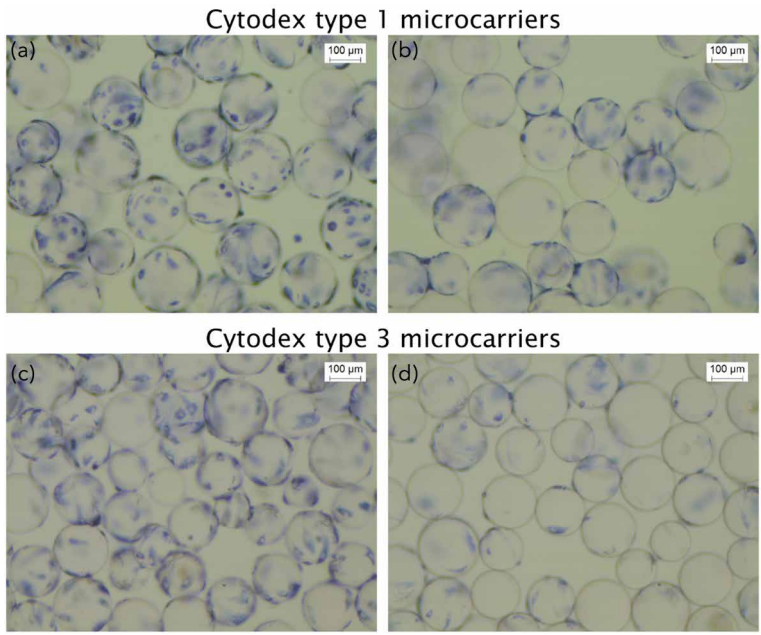

Figure 8 Viable mesenchymal stem cells (blue) on different microcarrier types (transparent spheres) (Dufey et al., 2016). Cells were stained with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Light microscopy pictures of cells (a) (c) before and (b) (d) 1 day after microcarrier addition to the culture demonstrate the quick transfer of cells to the new beads.

Moreover, microcarriers can be used in many bioreactor types and are a vital tool to facilitate scale-up. This is because the available growth surface during bioprocessing can be increased by simply adding fresh microcarriers to the bioreactor culture, a phenomenon facilitated by bead-to-bead transfer (Bodiou et al., 2020). Bead-to-bead transfer is the exchange of cells from one bead to another, probably either by direct bead-to-bead contact or pick-up of detached floating cells by microcarriers moving in the cell suspension (Verbruggen et al., 2018). Several studies, including our own, demonstrated the feasibility of microcarriers in culturing stem cells and their progeny in bioreactors. The cell types in these studies also include myoblasts, mesenchymal stem cells, and pluripotent stem cells (Verbruggen et al., 2018; Dufey et al., 2016; Siddiquee and Sha, 2014; Escobar Ivirico and Sha, 2020; Tejerina et al., 2021). Besides that, the differentiation of microcarrier-attached satellite cells and cells of a myoblast cell line into myotubes was observed in different bioreactor models (Molnar et al., 1997; Torgan et al., 2000; Moritz et al., 2015).

Another study explored the possibility of bead-to-bead transfer as a scale-up strategy with human mesenchymal stem cells. The study successfully employed the bead-to-bead transfer to scale up from 4 L to 50 L in stirred-tank bioreactors. Moreover, the work demonstrated that the addition of fresh microcarriers to a culture of cell-populated microcarriers followed by intermediate agitation increased proliferation compared to continuous agitation (Chen et al., 2021).

An alternative to culturing anchorage-dependent cells on microcarriers is letting them grow as cell aggregates. Aggregates are free-floating 3D spheroid structures consisting of agglomerated cells which act as growth surfaces to each other and are formed at high initial cell density (Moritz et al., 2015). In theory, this organization as a united cell structure more closely resembles the natural *in vivo* environment of stem cells than microcarrier cultures (Bodiou et al., 2020). Furthermore, with the possibility of protecting individual cells from shear forces, aggregate culture bears the potential to reach high cell densities (Allan et al., 2019).

However, the structure of aggregates can lead to spatial gradients of nutrients and especially oxygen (Allan et al., 2019; Reiss et al., 2021) resulting in heterogeneity concerning viability, proliferation, and differentiation (Fan et al., 2015; Wu et al., 2014). Understandably, the effects of nutrient diffusion seem to be dependent on the aggregate size (Wu et al., 2014). Therefore, it is important to control aggregate dimensions during cultivation, a process that is difficult to handle (Allan et al., 2019; Bodiou et al., 2020). Despite that, it was shown that step-by-step optimization of process conditions in a stirred-tank bioreactor can enable high numbers of viable induced pluripotent stem cells (iPSC) while maintaining their differentiation capability and controlling aggregate size. The study demonstrated an almost 70-fold increase of human

iPSC numbers from initially 5 × 10^5 cells/mL to 33 × 10^6 cells/mL after 7 days of culture by systematically optimizing pH, culture feeding, and agitation rates in combination with *in silico*-directed process modelling (Manstein et al., 2021). The aggregate size was controlled by adjusting the agitation speed while using shear-protective substances to reduce cell stress.

At present, aggregates seem to be more relevant to pluripotent stem cell culture (Reiss et al., 2021). However, studies on other cell types are gaining momentum. Contemporary reports investigated the potential of mesenchymal stem cell aggregate culture in terms of their therapeutic use (Sart et al., 2014; Follin et al., 2016; Schäfer et al., 2016; Petrenko et al., 2017), yet an extensive evaluation in terms of cultured meat production is still pending. Also, a few reports on myogenic cell aggregates exist (Westerman et al., 2010; Wei et al., 2011; Hosoyama et al., 2013), even though cultivation was performed under static conditions. Suspension culture studies utilizing the myogenic C2C12 cell line resulted in aggregate formation. However, these aggregates expressed markers of quiescent satellite cells (Aguanno et al., 2019) which do not meet proliferation requirements (Bodiou et al., 2020). Future studies might shed light on the role of aggregate cultures in cultured meat production.

In this context, it is worth mentioning that microcarriers in cell culture can also form aggregates associated with cell proliferation inhibition (Bodiou et al., 2020). On the other hand, positive aspects of microcarrier aggregates are reported as well. A study suggests that inoculating a new culture with myoblast-loaded microcarrier aggregates might decrease the initial lag phase and promote scaling-up due to the protection of the cells from mechanical stress (Boudreault et al., 2001).

It is proposed that microcarrier aggregates might be a problem of mesenchymal stem cells rather than satellite cell culture, as the latter produces less extracellular matrix and therefore bears a reduced aggregation potential (Bodiou et al., 2020). Furthermore, other than cell aggregates the size control of microcarrier aggregates can be achieved more easily by simply adding more microcarriers to the culture (Verbruggen et al., 2018).

6 Scale-up of a bioprocess in bioreactors

As mentioned previously, scale-up is the increase of the bioprocess dimensions to meet the demand for larger working volumes and higher cell numbers. While all the described cell niche parameters are applicable for both small- and larger-scale approaches, scaling up is not just a one-step process of simply enlarging bioreactors and volumes.

Increased bioreactor geometries and working volumes can aggravate establishing homogeneous cell cultures by prolonged mixing times and induced temperature, nutrient, or oxygen gradients. To minimize the effects

specified in the previous sentence and thus ensure reproducibility for different bioreactor scales, it is critical to maintain similar properties, such as geometry, but also special physiochemical scale-up parameters that were established over time (Suttle et al., 2021).

Among the commonly employed scale-up parameters are power input/volume ratio (P/V), impeller tip speed, constant volumetric mass transfer, and constant oxygen transfer rate (OTR).

A constant (impeller) P/V is one of the most widely used scale-up strategies; it can be calculated from the formula:

$$P/V = (Np \times \rho \times N^3 \times d^5)/V$$

where Np is the dimensionless impeller power or Newton number, ρ is the density of water (1000 kg/m³), N is the agitation speed (rps), d is the impeller's outer diameter (m), and V is the bioreactor's working volume (m³) (Suttle et al., 2021). The power number is experimentally determined for a given impeller by measuring its torque but can also be obtained from standard impeller references or vendors (Li et al., 2016; Yang, 2014).

The tip speed of an impeller influences oxygen transfer and mixing times but is also linked to shear forces, an important point to be kept in mind when working with sensitive cells. It can be calculated from the formula:

$$\text{tip speed} = \pi \times d \times N$$

where π is the ratio of a circle's circumference to its diameter (~3.14159), d is the impeller's outer diameter (m), and N is the agitation speed (rps) (Suttle et al., 2021).

The OTR describes the oxygen-to-liquid transfer in the bioreactor and is dependent on the volumetric mass transfer coefficient (k_La). k_La is a measure of the oxygen-to-bioreactor culture medium delivery efficiency for a given combination of parameter conditions (Suttle et al., 2021).

Ideally, all these parameters should be kept constant between different scale sizes. However, due to the physical properties of a bioreactor, this is not possible (Yang, 2014; Suttle et al., 2021). Therefore, the common practice is to select one or more parameters to be kept constant during the scale-up process (Suttle et al., 2021). As the choice depends on the process approach, it is important to test and identify critical culture parameters early in small-scale experiments before moving to larger dimensions.

Such small-scale bioreactors able to accommodate working volumes as small as 60 mL have been successfully used by us and others for efficient stem cell culture establishment (Olmer et al., 2015; Manstein et al., 2021; Dufey et al., 2016; Escobar Ivirico and Sha, 2020). Figure 9 showcases how the growth of a

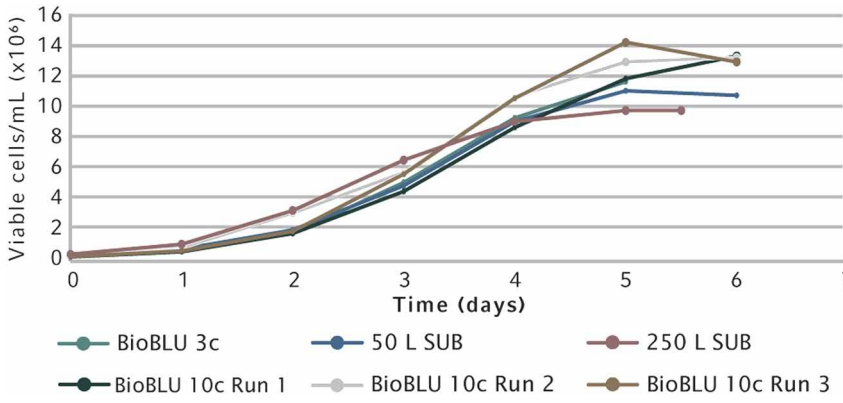

Figure 9 Examples of a scale-up experiment using a mammalian Chinese hamster ovary (CHO) cell line (Suttle et al., 2021). Scale-up was performed using four different single-use bioreactor (SUB) models with working volumes of 3 L (BioBLU 3c), 10 L (BioBLU 10c), 40 L (50 L SUB), and 150 L (250 L SUB). Applying a constant impeller power input per unit volume (P/V) resulted in comparable cell growth between all volumes over the course of the bioprocess run.

mammalian cell line can be kept consistent between different bioreactor sizes with working volumes of 3 L, 10 L, 40 L, and 150 L by maintaining a constant impeller power input per unit volume (P/V) (Suttle et al., 2021).

7 Considerations for cost reduction and process optimization in bioreactors

In order to create general acceptance in society for alternative meat options, it is important to offer the product at a competitive price. While production costs for cultured meat have tremendously decreased over the last years, they still surpass those of conventionally produced meat (Bodiou et al., 2020; Guan et al., 2021). During bioreactor cell cultivation and upscaling, resource efficiency is crucial to maintain the costs at reasonable levels. This means generating maximized yields with minimal use of resources. In this section, we will consider three possibilities with the potential to reduce production costs.

7.1 Medium recycling

In cultured meat production, the most impactful cost driver is the culture medium (Treich, 2021). One possibility to reduce its cost is changing the medium's composition and replace costly ingredients, such as the ethically questionable animal-derived fetal bovine serum, an issue that is reviewed elsewhere in this book. Another option in a bioreactor could be conserving the

medium as efficiently as possible. This can be achieved by creating a loop to re-introduce the used medium back into the bioreactor multiple times.

Of course, for this recycling process, pretreatment of the medium is needed before its re-introduction to the cell culture. Utilized nutrients, like glucose and glutamine, need to be replenished and unwanted by-products, such as lactic acid or ammonia, need to be removed. In relation to by-product removal, this could be facilitated, for instance, by chromatographic methods (Moritz et al., 2015). For this reconditioning process, close monitoring of the medium properties is necessary to enable proper adjustments accordingly (Guan et al., 2021). In a bioreactor, critical parameters can already be monitored routinely. Others may be determined by online or offline sampling.

This optimization effort could result in a more resource- and cost-efficient production process. Medium recycling could be, for example, implemented in the continuous cultivation operation mode. Furthermore, it has been suggested that the used medium might be enriched with growth factors produced by the cultured cells which, in turn, could promote subsequent cell proliferation upon medium re-introduction (Moritz et al., 2015). Thus, medium recycling bears the potential to reduce process cost and enhance cell growth.

7.2 Combining cell proliferation and differentiation in one bioreactor

Another consideration for cost reduction is to establish an approach that combines stem cell proliferation and differentiation within the same bioreactor. The usual practice of growing myotubes from muscle stem cells is to expand the cells in one bioreactor before transferring them into another reactor for differentiation (Moritz et al., 2015). This process is laborious and includes a relatively sudden change in the stem cell growth environment.

Thus, combining the two steps into one bioreactor would reduce work and open the possibility to gradually change the environment from proliferation to differentiation, for example, by altering growth factor concentrations within the medium during continuous cultivation. Molnar and Torgan identified precursor differentiation into myotubes taking place on microcarriers (Molnar et al., 1997; Torgan et al., 2000). While microcarriers are mainly used for the cell expansion step, this may provide a model for combining proliferation and differentiation steps in one bioreactor by altering the culture conditions. It is an interesting observation, yet it remains to be determined if such structures are able to develop into mature muscle fibers with the same physical properties as the ones grown by the established process (Moritz et al., 2015). Also, by having differentiated myotubes attached to the surface, detachment for downstream

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processing bears the risk of damaging the structures. Thus, the availability of edible microcarriers would gain importance in this setting.

Further investigation is still needed to clarify this possibility. However, if it could be realized, combining the proliferation and differentiation process in one vessel would save time, material, and costs during cultured meat production.

7.3 Single-use technology

It might appear counterintuitive at first, but another cost-saving and environmentally friendly strategy could be found in disposable products. When it comes to choosing a bioreactor, two main options exist: reusable units, such as traditional stainless steel bioreactors, or single-use bioreactors, such as bags or vessels usually made from plastic compounds (Fig. 10). Re- or multi-usable bioreactors are an established and reliable tool for bioprocessing, but single-use technology also has its advantages.

As cultured meat technology is still in the beginning stages of establishing large-scale production processes and facilities, an evaluation of established industries might be helpful for decision-making.

A possible model is the pharmaceutical industry, where single-use technology gained momentum over the years, for instance, in the production of monoclonal antibodies. Here, reduced downtime between batches, minimal

Figure 10 Examples of a single-use stirred-tank bioreactor using a disposable plastic bag for cell culture (left) and a multi-use stainless steel stirred-tank bioreactor (right), both attached to a bioreactor control system.

cleaning effort and decreased contamination risks are strong arguments for single-use products (Allan et al., 2019). Admittedly, even though available working volumes are increasing, the current maximum working volumes of single-use bioreactors are typically smaller than their stainless steel counterparts, ranging from several thousand liters in single-use to tens of thousands of liters in stainless steel reactors. However, scale-out, the process of using multiple smaller units in parallel, might be an alternative to the classical scale-up approach and could compensate for reduced volumetric capacities. Furthermore, operating multiple bioreactors in parallel potentially reduces the risk of contamination-induced batch loss. In general, scale-out offers a more flexible process operation and performance improvement, while scale-up enables better reproducibility at the expense of handling simplicity (Bellani et al., 2020).

Single-use technology also bears the potential for cost reduction at the bioreactor facility level. Compared to a stainless steel bioreactor facility for monoclonal antibody production, the use of disposable bioreactors was estimated to reduce costs significantly, mainly due to less labor, maintenance, and waste (Jacquemart et al., 2016). Another study concluded that despite increased production of plastic waste, a single-use facility would have an overall reduced environmental impact compared to a traditional stainless steel bioreactor facility. The authors explain this by reduced water and energy consumption, less space requirements, and an overall smaller carbon footprint (Sinclair et al., 2008). However, in order to keep a single-use facility productive, consistent quality, reproducibility, and availability of single-use components need to be ensured by the supplier. Additionally, the plastic material needs to comply with threshold values for extractable and leachable compounds (Jacquemart et al., 2016), a criterion with special significance for food products like cultured meat.

Thus, future cost analyses will be essential to determine if single-use or reusable bioreactors are the right approach for cultured meat production (Allan et al., 2019).

8 Conclusion and future trends

The world's population is projected to reach 8 billion in late 2022 and further growth is expected in the years to come (United Nations Department of Economic and Social Affairs; Population Division, 2022). Such an expansion of humanity will increase the demand for meat, a trend that will be harder and harder to satisfy with conventional meat production methods. Moreover, conventional meat production is increasingly tied to negative impacts on animal welfare, environment, and climate. The concept of cultured meat offers

an exciting new avenue to address the social, ethical, and environmental challenges.

However, tacking up challenges like those mentioned in the previous sentences entails its own trails. Introducing cultured meat to the general public requires a step-up in cell numbers and productivity, as well as ensuring a stable and maintainable growth environment for the cells to provide consistent quality; all at a price that customers are willing to pay.

To this end, bioreactors are a powerful tool to help reach this goal. Established in other industries for decades and with an ever-expanding application repertoire, bioreactors are both a proven and future-proof technology that can also aid cultured meat production.

Their ability to precisely control a diversity of growth parameters facilitates the creation of a 3D environment for various cell types to thrive. Several bioreactor types, operation modes, and accessories enable process structures tailored to one's specific needs. Scale-up and scale-out options, as well as cell density-increasing measures, including the use of microcarriers, can help to augment cell yields and efficiency. Furthermore, process adaptation can be used to reduce costs and the environmental footprint.

Constant improvement in bioreactor and process control design, material options, and culturing methods will continue to push the boundaries of what is possible and what is feasible in cell culture technology.

In conclusion, bioreactors are a vital tool to overcome current and future challenges that the relatively new field of novel food is facing these days.

9 Where to look for further information

A useful introduction to stirred-tank bioreactors, bioreactor accessories, incubation parameters, and a typical bioprocess run can be found in this white paper by Eppendorf:

- Rasche, U. (2019). Bioreactors and fermentors-powerful tools for resolving cultivation bottlenecks. *Eppendorf White Paper* 21.

A detailed explanation of the different bioreactor operation modes along with a comparative cost analysis are available in the following application note by Eppendorf:

- Yang, Y. and Sha, M. (2019). A beginner's guide to bioprocess modes-batch, fed-batch, and continuous fermentation. *Eppendorf Application Note* 408.

Information about important scale-up parameters can be accessed by reading the following publications:

- Li, B., Becken, U. and Sha, M. (2016). Fermentation systems for bioprocess scale-up from small to pilot/production. *Genetic Engineering and Biotechnology News* 36(9).
- Suttle, A., Rasche, U. and Sha, M. (2021). From R&D to production: How the bioreactor choice can streamline biologics scale-up. *International Pharmaceutical Industry* 13(3), 60–62.

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