# The Cookbook

BASIC CONCEPTS, RECIPES AND STRATEGIES FOR BIOPROCESSES
INVOLVING CELL CULTURES AND MICROORGANISMS

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# Contents

# Introduction

**So here we are.**

You're finally standing in the lab in front of a bioreactor and are excited to use it for all sorts of experiments.

But speaking of starting: How do you even turn on a bioreactor like this? And then what? You could answer that question with a glance in the manual, but how does the device actually work, and are there any additional tips for preparing and implementing a bioprocess? Maybe someone's already done some of these experiments before. Couldn't you just start out by following their "recipe" instead of reinventing the wheel?

We thought about that too. After all, no one is born a bioprocessing master.

Our aim with this guide is to help you find your bearings and to give you a step-by-step introduction to bioprocessing. We'll explain how a bioreactor works and what happens during a bioprocess. We'll look at the basics of a bioprocess and offer strategies you can use to make your bioprocess a success. And we'll take a look at the stars of the show: the various microorganisms and cell cultures. This is a good place to point out that the focus of this document is on bioreactors with working volumes of 0.5 L to 10 L, even though some of our explanations are certainly applicable to bigger or smaller experiments.

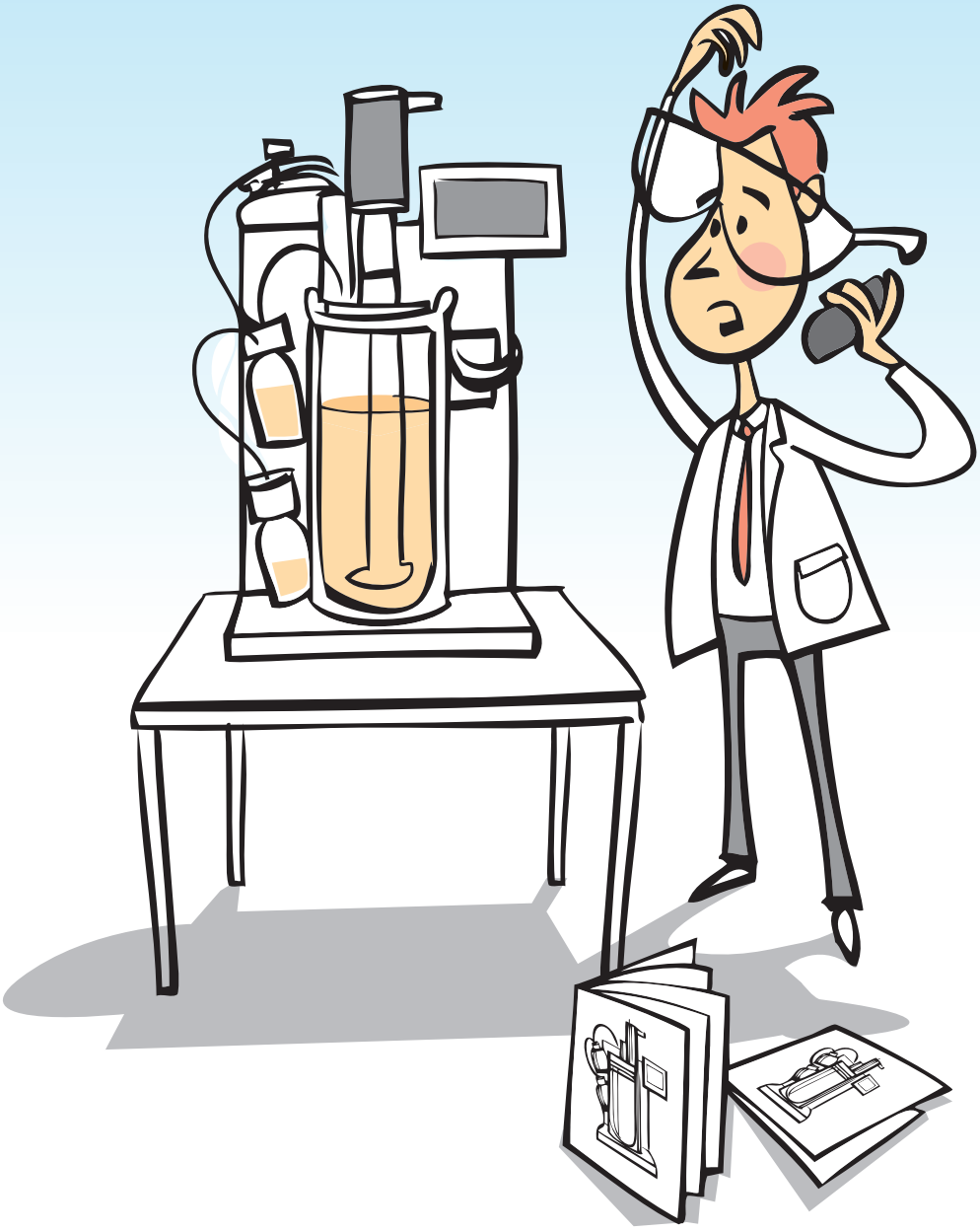
And like any good cookbook, this document comes with recipes.

Except that these recipes are designed to give you the best possible cell culture (just to make sure you don't mistake this for a conventional cookbook). Clear instructions, an overview of the expected outcome and some tips ought to help you achieve precisely this goal.

Of course, that requires more than just a little instruction book. The bioprocess itself is only one step, and it's preceded by the processes involved in preparing the cells, culture media, and adjusting agents. And there are also a lot of downstream procedures that focus on harvesting and processing your bioprocess results. We hope, however, that our "cookbook" can make the move to bioprocess technology easier for you, that it will pique your curiosity and take away the fear of asking supposedly "stupid" questions – questions, by the way, that we've already asked in the process of writing this handbook.

We look forward to feedback on how we can improve and expand the cookbook. Please feel free to send us your comments and constructive criticism at marketing@infors-ht.com.

Happy reading!



# 1. Bioreactor basics

## 1.1 Why a bioreactor?

At the beginning of each bioprocess is an idea. Like making different chemicals, vitamins or proteins, which, in turn, can be used for medicines.

To make that idea a reality, you need a recipe, the right ingredients and a pretty good kitchen appliance. One that not only stirs, but also monitors the temperature and stirring speed, adds an ingredient or two, and keeps the environment sterile where all this happens. In our case, this kitchen appliance is a bioreactor, or more precisely, a stirred tank reactor.

A bioreactor is a multi-tasking device that helps researchers achieve the bioprocess results they want by automatically monitoring and regulating select process conditions. The bioreactor provides an ideal environment where cells can focus on what they're supposed to do: proliferate.

Like lab workers, cells can only produce consistently good work if the conditions are right: it shouldn't be too hot or too cold, and they have to have enough good food and fresh air. In terms of a bioreactor, this means maintaining pH and temperature, ensuring enough oxygen and other gases, and, depending on how the instrument has been configured, manually adding nutrients now and then.

## 1.2 Setup and components

Given the abundance of functions that a bioreactor must perform, you might be wondering how it can do it all. What components does it need? How do you know what the conditions in the bioreactor currently are and how you can correct them? And finally, just for perspective: how do you take the data captured during a batch (bioprocess, see glossary), display them in a meaningful way, and then save and evaluate them while keeping them organized?

The most important process parameters and the mechanisms for regulating them are described in Chapter 1.3. We also show in detail how a bioreactor is assembled (Chapter 4). But now let's first provide a general overview of the technology, which consists of two important systems: the device itself and the SCADA (supervisory control and data acquisition) software.

The following are the features of a bioreactor (e.g., the Minifors 2):
- Low-level sensor and actuator control
- PID regulator (closed-loop controller)
- Cascades, i.e., variation of several parameters
- Operating unit for local inputs
- Depending on the model: simple imaging at the operating unit

The following are the features of SCADA software (e.g., eve®):
- Central collection site for all bioprocess information
- Monitoring and control of several bioreactors
- Interface to higher-level analyzers, e.g., mass spectrometers
- Planning of complex batch strategies
- Depending on the version: design of experiment (DoE) and process analytical technology (PAT) integration

A powerful controller takes over the first stage of process control in the bioreactor, communicating directly with the sensors and actuators of the bioreactor. The sensors, e.g., for pH and temperature, are needed in order to collect information on the current status of the system. Actuators such as heating elements, pumps or valves, on the other hand, allow the bioreactor to intervene to make corrections, if necessary. And digitalization does not stop at bioprocess technology – digital bus systems such as Modbus already communicate with a wide range of components. Still, there's no way around analog interfaces, as these are indispensable for components such as traditional Pt100 temperature sensors. The controller is also responsible for precisely maintaining all of the target values communicated to it. To do this, it uses a PID (proportional-integral-derivative) controller and actual values to determine which of its actuators it must control in order to set the target value as effectively and smoothly as possible.

The bioreactor also has a local operating unit, also known as a *human machine interface*, or HMI. As the name suggests, the HMI allows users to interact directly with the bioreactor. Today, most HMIs in modern bioreactors are touchscreens that allow the user to adjust process parameters for a batch (bioprocess, see glossary) and to switch control units on and off, or otherwise configure them. The interface can also be configured for connecting a SCADA software system.

Nowadays, the results generated in a bioreactor should be collected and evaluated as centrally as possible, as this is the only way of effectively implementing modern, big data algorithms in order to generate more information and to better understand how a process works. That's the job of the SCADA software. At INFORS HT, this is the task of eve® bioprocess platform software, which establishes contact with the bioreactors via a local or global network using standardized protocols like OPC UA. In the first step, all data from the bioreactor can be read out – with no major input on the part of the user – and stored centrally in order to evaluate it on its own or compare it with other batch data.

This quickly triggers ideas for new experiments and possibly even complex batch strategies. eve® lets you plan these easily – in the office with a cup of coffee, which is certainly more comfortable than in front of the bioreactor and in your lab coat – and then control the bioreactor, which will ideally be a fully automated process. Also, eve® allows you to centralize all information accompanying the batch, including information on the microorganisms or cell cultures used, their favorite culture medium or sample measurement data taken from the bioreactor (offline analysis).

The eve® software also integrates a number of components in the bioreactor environment. These include tools for process optimization using the design-of-experiment (DoE) technique or powerful software sensors, which can be used simultaneously to compute additional information directly from the batch process parameters and even to regulate those parameters. For example, the respiratory quotient (RQ) can be used to obtain an estimate of metabolic activity by means of the ratio of excreted carbon dioxide to absorbed oxygen.

There are so many possibilities – especially when the bioreactor and SCADA software are perfectly matched – that we couldn't possibly describe them all in this cookbook. If you are one of the lucky owners of both systems, have the courage to try it out – you'll be able to see how ridiculously easy it is to benefit from the many features. Otherwise, you're welcome to browse through our website and take a look at the eve® tutorials or request a demo version.

## 1.3 Functionality

Experienced amateur chefs look to recipes to figure out what containers and equipment they have to use to achieve the desired result with the ingredients listed. That's sort of how it works with bioreactors too. Microorganisms require certain conditions, and experienced operators assemble the reactor from the required components in a way that will achieve those conditions for as long as possible. But what are those conditions? And how do you maintain them precisely? These are precisely the questions that will be clarified in the next section.

**Stirred or shaken?**
All bioprocesses need to be mixed thoroughly at all times. If the nutrients in the bioreactor are not dispersed well enough, conditions in certain parts of the bioreactor will deviate significantly from the ideal. The pH could be too acidic, for example, or the supply of nutrients might be insufficient. Deviations like these not only reduce the efficiency of the planned bioprocess, but can also promote genetic modifications that permanently alter the behavior of the organisms in ways that are generally undesirable. Temperature distribution is an additional concern. Without uniform stirring, the microorganisms or cell cultures along the edge of the vessel will literally be boiled, while those in the middle get cold feet. If you've ever heated soup in the microwave and then eaten it with great anticipation without stirring it, you'll know what we mean.

The typical stirring speed varies depending on the cultivated organism. Speeds of 500 $min^{-1}$ to 1500 $min^{-1}$ are typical for bacteria, yeasts and fungi, whereas plant and insect cells do best at 30 $min^{-1}$ to 300 $min^{-1}$, although the development of more robust cell lines means you can now use higher stirring speeds too. Adjusting stirring speeds for cultivating either microorganisms or cell cultures is very important, as these react differently to shear stress, i.e., the mechanical strain caused by stirring. Depending on the cell line, cell cultures can have a much more intense response to overly vigorous stirring, i.e., they simply die, a tendency that scientists describe more accurately (and diplomatically) as being "sensitive to shear stress." Oxygen availability can be varied during the bioprocess by changing the stirring speed, thus ensuring optimum cell growth.

**Measuring and controlling the temperature**
Microorganisms and cell cultures alike have enzymes that feel best within certain temperature and pH ranges. If conditions fall outside of these ranges, the desired bioprocess will proceed much more slowly, because growth and metabolic performance are highly dependent on these enzymes, i.e., catalytically active proteins. In the worst case scenario, unfavorable environmental conditions may even destroy them. Cell cultures are most comfortable within a very narrow range of temperatures – one that is only present from their perspective when the temperature inside the culture vessel is 37 °C.

A platinum resistance sensor known as a Pt100 sensor is used in the bioreactor to determine the temperature. It has a resistance of 100 Ω at 0 °C, and covers the expected biologically relevant measurement range quite well when calibrated appropriately.

The control range for microorganisms usually lies between +5 °C and +50 °C above room temperature, whereas bioprocesses proceed at temperatures ranging from 20 °C to 50 °C. If working at temperatures near or below room temperature, you will need an active cooling system such as a recirculating chiller. For most bioprocesses, the temperature should remain constant during the entire cultivation. For some products, however, such as penicillin or recombinant proteins (i.e., bioengineered proteins using genetically modified organisms), changing the temperature at the end of the growth phase activates important genes for product formation and is therefore beneficial. For production methods involving cell cultures, the temperature is sometimes lowered at the end of the bioprocess as well (a technique known as "temperature shift" in biotechnology) so that the finished product will remain stabile for later use. The reasoning is the same when you put whipped cream in the refrigerator in order to decorate a cake with it later on.

There are several ways of using a heating and/or cooling circuit to regulate temperature:
- An electric heating block with built-in cooling spiral (Minifors 2 and Multifors 2)
- A silicone heating pad wrapped around the culturing vessel after sterilization (Labfors 5)
- A double jacket in which water is circulated. The temperature is adjusted via an electric heater or steam and a solenoid valve for cooling water intake (Labfors 5, Techfors-S and Techfors).

**Measuring and controlling the pH**

Measuring and controlling pH is a very important aspect of bioprocesses, as changes in the pH can significantly alter growth conditions – usually with major consequences. Some of these are desirable and provide extraordinary benefits: one example of this is lactic acid fermentation, which humans have used since Neolithic times to preserve food. This is harnessed for making fermented milk products, pickled vegetables and sourdough breads, which are acidified by the lactic acid excreted by lactic acid bacteria. This, in turn, inhibits the growth of other microorganisms below a pH of 4.5, and a further drop in pH will even inhibit the lactic acid bacteria themselves.

Part of daily lab work involves either keeping the pH stable or selectively controlling it – after all, not every bioprocess ends with fermented milk, and a sudden change in pH can ruin an experiment. That's why culture media also commonly include buffers, i.e., substances that mitigate overly sudden changes to pH caused by the addition of an acid or base. Because an acid dripping into the culture medium can damage many cell lines, scientists often carefully enrich the gas mixture used in the cell culture with $CO_2$ rather than adding a liquid acid. The gas then dissolves in the culture medium, allowing the carbon dioxide to influence the pH as well. Bacteria, yeasts and fungi used in bioprocesses usually require a pH between 4.5 and 7.0, whereas animal cells need the pH to be between 6.7 and 7.4. Insect cells are most comfortable at a pH between 6.2 and 6.9, while plant cells thrive at 5.5.

For measuring pH during the bioprocess, each bioreactor is equipped with a pH sensor known as a single-rod measuring cell for pH. The bioreactor can correct any deviations in the pH; for this purpose, an acid and/or an alkaline solution is made available and connected to the culture vessel via tubes and pumps. Depending on the need, the pumps feed phosphoric acid, sodium hydroxide, or ammonia solution, for example. The concentration of the acid and base must be skillfully selected for this to work – if it is too high, the drops of concentrated acid or base may damage the microorganisms and cell cultures before they are distributed in the bioreactor. If, on the other hand, the concentration is too low, operators will have to add more acid or base, unnecessarily diluting the culture medium.

**Adding nutrients**

During a bioprocess, microorganisms usually consume a wide array of nutrients. A batch operation is when everything is made available to the microorganisms all at once and feeding is discontinued.

Another way to keep nutrients from becoming the limiting factor is to have a nutrient solution constantly fed in during cultivation; this is called fed-batch operation. Under good growing conditions, microorganism growth doubles continually following an exponential growth curve, which is why you ideally want to increase the feeding rate exponentially as well, provided the bioreactor can sustain the other process parameters.

Continuous culturing is a special fed-batch process in which a *steady state* arises, with as much fresh culture medium added, for example, as is discharged. These types of bioprocesses are especially suitable if an oversupply of nutrients would inhibit the microorganisms. We will explore the different feed strategies more in depth in Chapter 5 of the cookbook.

**Gassing**

During the bioprocess, the bioreactor feeds a sterile gas mixture such as air into the culture medium. Constant stirring not only distributes the nutrients – it also reduces the size of the gas bubbles that arise in the culture vessel, thus efficiently releasing oxygen into the nutrient solution. This is important, because microorganisms and cell cultures can only absorb the oxygen that has been dissolved in the nutrient solution.

Oxygen demands vary: aerobic bacteria need oxygen, whereas others prefer gas mixtures such as sythesis gas ("syngas"). Anaerobic organisms, however, can do without gassing entirely, feeding only on inorganic and organic substances from the culture medium such as nitrate or fumarate.

If you've stuck with us to this point, you may already suspect that the situation with cell cultures is more complex this time too. Unlike microorganisms, cell cultures are gassed with more than just air – the oxygen content of the gas mixture can also be influenced through the use of pure nitrogen and pure oxygen. The exact composition depends on the cell culture and on the application. In order to keep the gas atmosphere constant, the bioreactor needs precise control systems.

At the beginning of the bioprocess, for example, a culture often needs less oxygen – and thus a smaller gas feed – since growth is still progressing slowly. Later, however, faster growth requires much more oxygen. In addition to ensuring a constant supply of the desired gas or gas mixture, the bioreactor also delivers the right amount of gas at the right time. To do this, the bioreactor has gas ports connected to pressurized air from the building, a compressor or a gas cylinder.

The gassing rate is usually measured in liters per minute. In order to have a generic parameter applicable to various bioreactors, the rate is also frequently indicated as the specific gassing rate, which refers to multiples of the working volume (*vessel volumes per minute,* vvm) and is expressed as $L\ L^{-1}\ min^{-1}$ or just as $min^{-1}$. A typical value for microbial bioprocesses is 1 to 1.5 times the working volume per minute; the maximum is typically around $2\ L\ L^{-1}\ min^{-1}$. For a bioreactor with 4 L of working volume, the maximum gassing rate would therefore be $4\ L * 2\ L\ L^{-1}\ min^{-1} = 8\ L\ min^{-1}$. In cell cultures, by contrast, the maximum rate is often 10% to 15% of the working volume per minute as a way of keeping gas bubbles small and thus preventing foam formation and damage to cells from bursting bubbles.

Changing the gas rate, however, is not the only way of controlling the efficiency with which the bioreactor delivers oxygen and other gases to the culture medium. The greater the surface area of the total number of all gas bubbles in the bioreactor – i.e., the more finely distributed the gas bubbles themselves – the more efficiently oxygen will be transferred from the gas to the liquid phase. This means, for example, that increasing the stirring speed can improve oxygenation for microorganisms, since the stirrer makes the gas bubbles even smaller and thus increases the total surface area of all gas bubbles in the bioreactor. Another way to increase the oxygen content is to enrich the air with pure oxygen or even by gassing with pure oxygen only.

Precise regulation of $pO_2$ – and thus precise control of the gassing rate and the gas composition – is very important since, normally, the $pO_2$ should not be the growth-inhibiting factor for the culture. If insufficiently controlled, however, the $pO_2$ does become the limiting factor.

Since the gas fed to the bioreactor is usually dry, moisture from the bioreactor can dissolve in the gas during gassing. At a high gassing rate, not only would the fill level drop, but the moisture would also block the exhaust filter, preventing proper venting from taking place and allowing pressure to build up. To avoid this effect, bioreactors are equipped with an efficient exhaust cooler where the moisture in the exhaust condenses and can drip back into the bioreactor before it reaches the exhaust filter.

**Measuring and controlling the pressure**

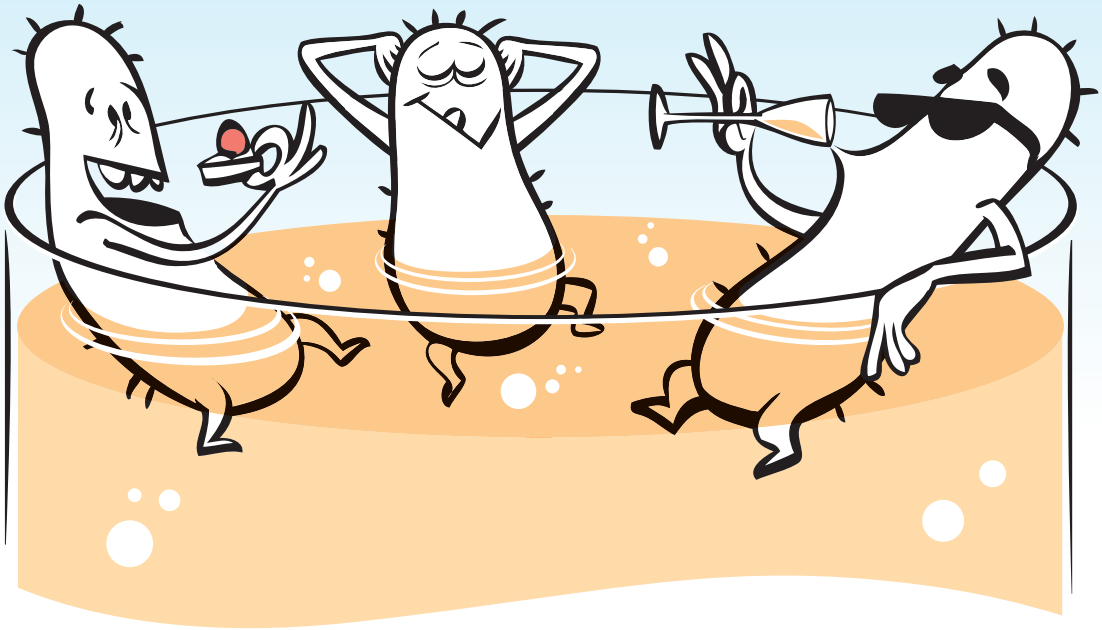
The higher the pressure in the vessel, the more oxygen is dissolved. Culture vessels made of glass are frequently only approved for a pressure of up to 0.5 bar, which is not even half the pressure of a moderately filled bicycle tire. At a higher operating pressure, slightly damaged culture vessels made of glass can burst, which not only ruins the day (and the experiment) for many a researcher, but is also a safety risk. That's why you should always ensure a free, non-pressurized exhaust line from the bioreactor by keeping the exhaust filter dry and regularly replacing it – in the process, you'll also be ensuring the integrity of the culture vessel, of course. Unlike glass culture vessels, stainless steel bioreactors are designed for higher pressures and, even in their standard configuration, are suitable for pressures up to 2 bar (a well-filled bicycle tire). Plus, systems like these are often equipped with a pressure control mechanism based on a pressure sensor in the bioreactor and a proportional valve in the exhaust line. Not only can these measure the pressure in the bioreactor – they can actively control it as well.

**Preventing foam formation**

Outside of bathtubs and beer glasses, foam is a rather unpopular side effect, especially in bioreactors. Foam forms at the interface between the liquid and gas phase in the culture vessel and can quickly find its way up under the lid. In the worst case scenario, it then blocks the exhaust filter, which in turn blocks the flow of gas. Most bioreactors are therefore equipped with a system for combatting foam formation. Mechanical foam breakers in the headspace are reserved for rather large stainless steel bioreactors, while antifoam control systems based on chemical agents (such as PPG, Struktol, or silicon-based defoamers) can also be found in smaller bioreactors.

A typical antifoam control system consists of a sensor installed at a specific height in the culture vessel. If the foam height reaches the sensor, an antifoam agent is pumped from a reservoir into the culture vessel. These antifoam agents are active at the liquid-gas interface and increase the tendency of the foam bubbles to collapse.

In particularly stubborn cases where the foam does not immediately dissolve, repeat the procedure after a preset time (a *"shot & delay"* strategy). Caution is advised when using an antifoam agent – if you dispense even slightly more than necessary, it can lie like a second skin on the surface of the liquid, which hinders the gas exchange. Antifoam agents also counteract efficient oxygen transfer, because the change in surface tension promotes the collapse of gas bubbles in the bioreactor, thus reducing the surface area available for gas exchange. The selection of the appropriate agent also depends on the bioprocess in question, because bacteria and cells react differently to certain chemicals.



# 2. Bioprocess basics

In order to understand the optimum bioreactor conditions for microorganism growth, you need to know what natural environment makes the organisms feel at home and what they need to grow and thrive. Practically all cultivable organisms of prokaryotic and eukaryotic origin (i.e., with or without a nucleus) can be cultured in a bioreactor. To do this, a preculture known as the inoculum must be produced, which, in turn, makes up 5 to 10% of the total volume of the medium to be inoculated. The inoculum is mostly produced as a shaken culture, usually in two steps, while the actual bioprocess typically runs in a bioreactor.

Since there is more than enough technical literature on microorganisms and cell cultures – literature we can neither add to nor improve on – we can refer you to the publications in Chapter 7 with a clean conscience. Instead, we're going to focus on those aspects that are important for preparing a bioprocess.

## 2.1 Microorganisms

Microorganisms can be divided up into a large number of classes depending on their source of carbon and energy, and where their electrons are coming from. In the glossary we describe the various ways they obtain energy under the entries for "phototrophic," "chemotrophic," "autotrophic," and "heterotrophic."

The following is a brief summary of the most well-known organisms used for conducting microbial bioprocesses:

### 2.1.1 Bacteria

Bacteria cover a wide range of possible growth conditions and are, so to speak, the Swiss army knife of microorganisms. In addition to "normal" bacteria, there are also those that love extreme environmental conditions, the so-called extremophiles (Lat in-*phil* = loving/having an affinity for). Examples of these include thermophilic (which like high temperatures) and halophilic (which like high salt concentrations) microorganisms.

The doubling time, i.e., the time needed until a microorganism population has doubled, can vary from minutes to days, which in turn depends on whether it is anaerobic or even genetically modified bacteria.

**Typical cultivation parameters for bacteria:**

| Parameters | Temperature | Mixing rate | Cultivation time | pH | $pO_2$ |
|---|---|---|---|---|---|
| Shaker | 20 – 60 °C | 100 – 400 $min^{-1}$ | 8 – 60 h | | |
| Bioreactor | 20 – 60 °C | 100 – 1500 $min^{-1}$ | 8 – 60 h | 7.0 | 0 – 80% |

### 2.1.2 Yeasts

Yeasts have the same essential mixing, temperature, etc., requirements as bacteria. That's why these do not normally require any special growing conditions – they typically prefer a process temperature of 30 °C and an acidic pH. Good mixing and an adequate supply of oxygen are essential for a good yield of yeast biomass. Yeast metabolism generates a great deal of heat, and you may need to cool the process in order to counteract this and maintain an acceptable operating temperature.

**Typical cultivation parameters for yeasts:**

| Parameters | Temperature | Mixing rate | Cultivation time | pH | $pO_2$ |
|---|---|---|---|---|---|
| Shaker | 25 – 30 °C | 200 – 250 $min^{-1}$ | 16 h | | |
| Bioreactor | 25 °C | 1000 $min^{-1}$ | 16 – 48 h | 6.5 | 40 – 50% |

### 2.1.3 Fungi

Fungi often grow as fibrous, mycelial cell agglomerates that are relatively sensitive to shear, which is why they tend to adhere to the vessel wall or even in the headspace – in order to escape the shear stress. On the other hand, the fibrous cell agglomerates result in a very viscous culture medium requiring considerable power input for mixing and gassing. This often makes it tricky to identify ideal operating parameters for cultivating fungi.

These days, a typical bioprocess for producing citric acid on an industrial scale uses *Aspergillus niger*, a filamentous fungus. Citric acid is excreted in large quantities when the glucose and oxygen levels of the medium are high, combined with a very low pH and low iron concentration.

**Typical cultivation parameters for fungi:**

| Parameters | Temperature | Mixing rate | Cultivation time | pH | $pO_2$ |
|---|---|---|---|---|---|
| Shaker | 23 °C | 250 $min^{-1}$ | 72 – 90 h | | |
| Bioreactor | 23 °C | 1500 $min^{-1}$ | 8 – 72 h | 5.0 – 6.0 | 25 – 50% |

## 2.2 Cell cultures

Cultivating plant or animal cells involves obtaining a primary culture of cells, tissues or organs that initially possesses its full metabolic capacity. Further subcultivation in an incubator or shaker ultimately produces cell lines capable of dividing, which can then be further cultured in the bioreactor. Within cell cultures, a distinction is drawn between adherent cells (growing on the surface) and suspension cells (floating in the culture medium); the glossary explains this in more detail.

### 2.2.1 Mammalian cells

Proteins obtained from mammalian cells play an important role in the biotech industry when it comes to manufacturing medications. The most well-known mammalian cells are HEK (human embryonic kidney) cells, which are relatively easy to handle. CHO cell lines from the ovaries of the Chinese hamster is one of the most frequently used cell lines for manufacturing therapeutic agents.

**Typical cultivation parameters for mammalian cells:**

| Parameters | Temperature | Mixing rate | Cultivation time | pH | $pO_2$ |
|---|---|---|---|---|---|
| Shaker | 37 °C | 40 – 120 $min^{-1}$ | 96 h+ | | |
| Bioreactor | 37 °C | 40 – 150 $min^{-1}$ | 20 – 70 days | 7.2 | 30 – 50% |

### 2.2.2 Insect cells

Insect cell cultures are often used for research into recombinant proteins, i.e., those produced using biotech methods, such as insulin. Relative to mammalian cells, insect cells are less susceptible to contamination and are somewhat more robust. The most well-known cell lines are Sf9 and Sf21, which are obtained from moth tissues. In order to obtain products from the bioprocess, researchers have to inoculate insect cells with the light-sensitive baculovirus.

**Typical cultivation parameters of insect cells:**

| Parameters | Temperature | Mixing rate | Cultivation time | pH | $pO_2$ |
|---|---|---|---|---|---|
| Shaker | 27 – 28 °C | 100 – 140 $min^{-1}$ | 120 h | | |
| Bioreactor | 27 – 28 °C | 100 – 140 $min^{-1}$ | 4 – 8 days | 6.15 – 6.25 | 25 – 50% |

### 2.2.3 Plant cells

Plant cells have omnipotent properties, which is to say that they can be used for regenerating intact plants. For this reason they are often used for studying differentiation processes in biology. Pharmaceutically active proteins and antibodies are derived from plant cell lines as well. Tobacco plants, for example, are especially popular as a fast, economical alternative to mammalian cells in the production of vaccines.

**Typical cultivation parameters of plant cells:**

| Parameters | Temperature | Mixing rate | Cultivation time | pH | $pO_2$ |
|---|---|---|---|---|---|
| Shaker | 20 – 28 °C | 100 – 200 $min^{-1}$ | 96 h+ | | |
| Bioreactor | 20 – 28 °C | 100 – 200 $min^{-1}$ | 4 – 6 days | 5.8 – 6 | 21 – 50% |

## 2.3 The bioprocess

The objective of a bioprocess is to use cells or their components to generate more of a "product" with added benefits. This can be the biomass itself or merely components of cells that are then further processed, sometimes with a great deal of effort.

Enzymes, i.e., substances whose structures make them suitable for use as catalysts in chemical reactions, were formerly known as ferments, which is why the term fermentation is used incorrectly as a synonym for the bioprocess. When Louis Pasteur coined the phrase *"Fermentation is life without air,"* he correctly understood that fermentation is a biotic reaction in the absence of air. In more modern times, fermentation is defined as the breakdown of organic substances, in which molecular oxygen is not the terminal electron acceptor for the production of energy. For this reason, acetic acid fermentation is not strictly fermentation in the sense of the newer definition, whereas production of ethanol and lactic acid, as well as some other forms of fermentation, definitely are included.

Everyday bioprocesses can be found in technology, agriculture, biomedical research and pharmaceutical production, with examples including cheese production, the generation of biogas, the operation of bioreactors of any type, and the production of recombinant proteins and biosimilars. Of course, there are still many, many other bioprocesses falling under the larger heading of biotechnology.

Selecting the right process strategy allows you to optimize the design of a bioprocess, while maximizing efficiency in terms of product and time. Selective genetic manipulation is another way of achieving the best possible media composition and tweaking microorganism function. When combined with the actual bioprocess, these preparatory steps are known as upstream processing.

Following the inoculation of a culture in the bioreactor, cells first have to adjust to their new environmental conditions. This period is called the latency phase or lag phase.

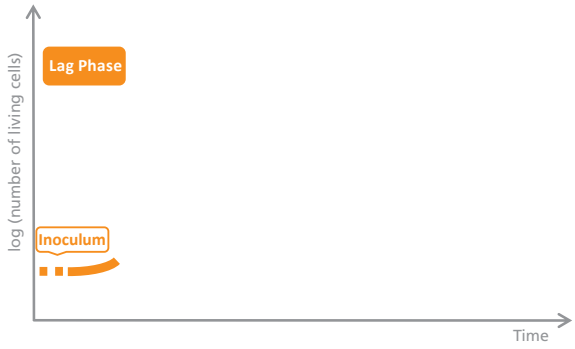



**Figure 1: Schematic representation of the lag phase.** The inoculum added to the culture medium defines the starting quantity of cells. After the bioreactor is inoculated, the living cell count initially increases slowly, because the organisms must still accustom themselves to the prevailing environmental conditions. This phase is therefore called the latency phase or lag phase.

Although there are sufficient nutrients and, most importantly, no metabolic waste products in the fresh medium, the microorganisms/cells still can't grow at full speed because they haven't perfectly adapted to the environment. They adapt by sensing environmental parameters such as temperature and nutrient supply in order to up-regulate the appropriate genes, which can take some time depending on the organism. One way to shorten the lag phase is by starting a preculture in the shake flask under the same conditions (same medium, same temperature) and rapidly transferring the cells to the bioreactor during the exponential growth phase.

In the subsequent exponential phase, called the log phase, the microorganism is ideally adapted to the environment, and the growth rate of the dividable cells is at its maximum. The term "exponential growth" stems from the fact that the number of cells does not increase linearly—it doubles constantly. In other words, it is not just 2, 3, 4, or 5 cells that appear, but rather 4, 8, 16, 32 cells, etc.

Generally, the cells divide at the greatest possible speed, and the biomass grows. Now the nutrients are absorbed and metabolized at the maximum rate, which, in an aerobic bioprocess, increases oxygen requirements and carbon dioxide production. In short, the nutrients are used up. During this process, the bacteria also produce by-products such as organic acids or excess heat. When this happens, researchers need to draw upon the bioreactor's broad range of tools in order to prevent cell growth from being impaired. In the log phase, the increasing amount of biomass and any already lysed (decomposed) cells increases the content of free proteins in the medium and therefore the risk of foam formation.

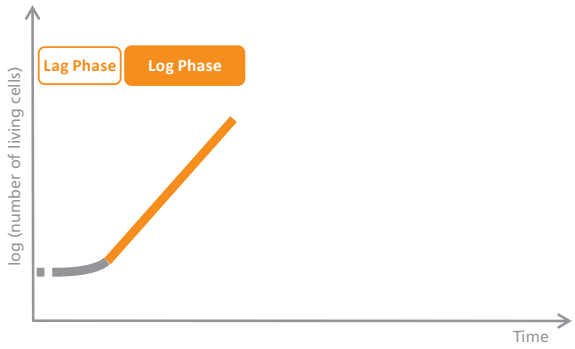



**Figure 2: Schematic representation of the log phase.**
The cells are now well-adapted and divide at the greatest possible speed since they have all the nutrients they need. For this reason, the number of living cells increases rapidly during this phase, which is called the exponential or log phase.

Unfortunately, exponential growth cannot go on forever. Nutrients are depleted, components of dead cells accumulate, and, to put it succinctly, the inside the bioreactor looks more and more like a mess of dust bunnies under the sofa.

The cells are becoming more and more uncomfortable in the bioreactor, and so the growth rate decreases in the subsequent, stationary phase. In this phase, however, the proliferation and death of the microorganisms or cell cultures are still in balance.

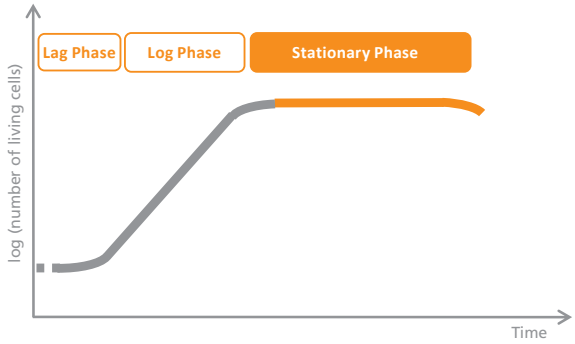



**Figure 3: Schematic representation of the stationary phase**
The nutrients are now exhausted and harmful degradation products begin to accumulate. For this reason, the cells begin to divide at a slower rate, to the point that the number of dying cells is approximately equal to the number of cells produced through division. This phase is therefore called the stationary phase.

At the end of the bioprocess, in what is known as the die-off phase, the growth rate drops so far that more cells die than are added by the division process, resulting in a net loss. As a result, depending on the process management, either the bioprocess comes to a natural end or the user deliberately stops it.

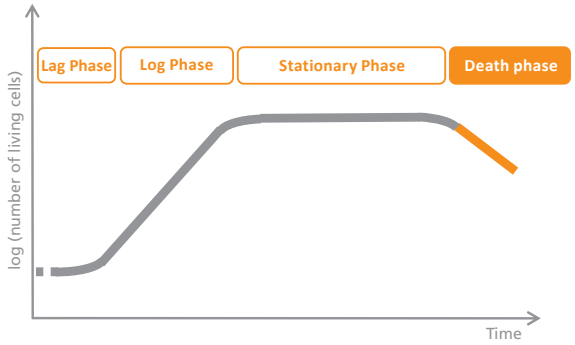



**Figure 4: Schematic representation of the die-off phase**
If the bioprocess is allowed to advance to the die-off phase, this phase is characterized by a decreasing number of living cells due to insufficient nutrients and the accumulation of harmful by-products. Since more cells die off than are added by division, the number of living organisms decreases.

This also depends on how long it makes sense for a process like this to keep going. If the bioprocess is not actively ended, the cells gradually die off because they've simply used up their food and the toxic effect of accumulating metabolites does the rest.

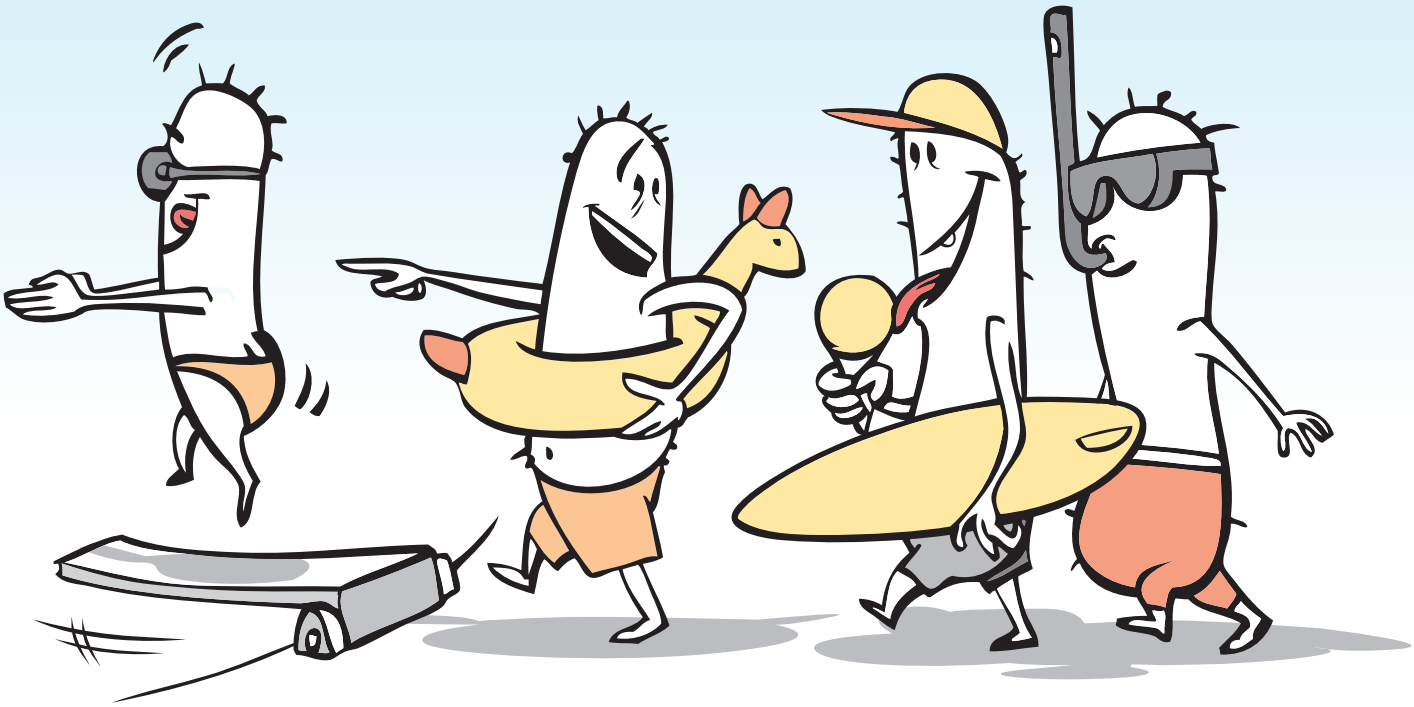
## 2.4 What happens after the bioprocess?

Once complete, the bioprocess is normally followed by the downstream processing, i.e., harvesting and processing desired products. This means that the fraction that a researcher keeps and processes can vary quite a bit depending on the process and the desired end product.


**Figure 5: Where the bioprocess fits into bioprocess engineering**
Although the bioprocess in and of itself is already complex, scientists can still optimize quite a lot during the pre- and post-processing stages. Bioprocess development also involves improving the producer stock, the medium, the additives, and the feeds, as well as the (upstream process) options for subsequently processing the product as gently as possible, while obtaining high levels of purity and good yields (downstream process).

Only the biomass or the medium can be harvested and suitably processed to obtain the desired product, for example. The product characteristics determine whether purification is simpler or more elaborate and whether it involves correspondingly more or less costly procedures.

Take for example a recombinant protein, which, when released by the microorganism or cell line into the medium, can be recovered simply by processing the medium. For a similar protein, however – one that cannot be secreted – the scientist first has to painstakingly purify the protein from the cell (and thus from the complex mixture of lipids, proteins, nucleic acids and sugars). Generally, downstream processing stages are cell separation, cell disruption with an existing intracellular product, and product extraction, concentration, purification and packaging. At the same time, of course, the bioreactor has to be cleaned and prepared for the next process, either manually of following CIP and/or SIP procedures.



# 3. Setting up, preparing, and carrying out a bioprocess

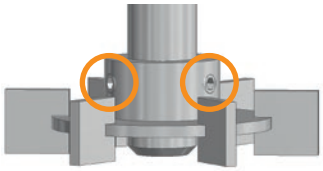
## 3.1 Preparing the bioreactor

For those who are still motivated, or motivated more than ever, to start a bioprocess after the explanations above, the steps necessary for successfully operating the bioreactor are listed below in a brief summary. For a more detailed description of the procedure, the bioreactor manuals provide advice and practical help (ex.: Chapter 8 "Before Cultivation" in the Minifors 2 manual).

**1. Preparing the culture vessel**
Before we get down to the nitty-gritty, the most important thing to do is check the culture vessel itself. After all, it is supposed to serve as a safe home for our microorganisms in the following hours and days. That's why you have to check the culture vessel to ensure that it is undamaged and that all of its O-rings are positioned properly and intact – otherwise the vessel could become contaminated after autoclaving, which would disrupt the process. For this reason you have to make sure you have installed, connected and secured all of the sensors, gas inputs, adjustment agents and ports for sampling or cell harvesting that the process needs. Any changes made once the reactor has been autoclaved have to be performed carefully at a sterile workbench.

Inspecting the culture vessel commonly involves disassembling it, although it may still all be in one piece after the post-inspection cleaning.

- Check the installation height of the agitator elements and make sure they are securely fastened to the shaft. If necessary, loosen, move and re-tighten the screws



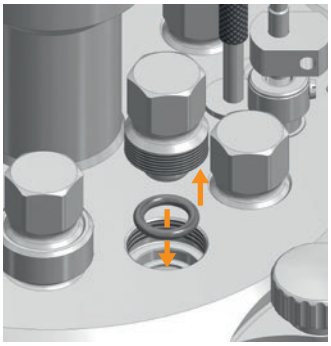
- Insert the baffle plate cage if the planned process requires it.

Note: Baffle plates are used in microbial bioprocesses to improve gas introduction by generating a turbulent flow. The baffle plate cage is not used, by contrast, for cell cultures that are sensitive to shear stress.

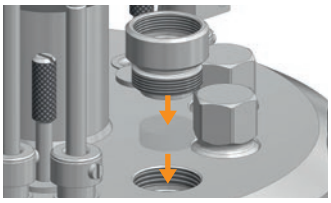
- Check the O-ring sealing the lid to make sure it is undamaged and positioned correctly.

- Check the O-rings on all components installed on the lid, regardless of whether these are intakes, sensors or plugs.

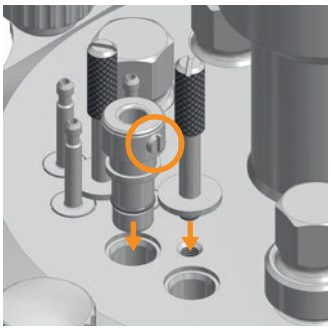
In cell cultures, on the other had, cells are often fed in through a sterile tubing connection created using a tube welding machine. At the inoculation port, a silicone membrane is used instead of the O-ring, which can later be used for inoculation via a cannula or an optional piercing needle. Since this membrane is pierced during each process, it should be replaced regularly.



Note: Some of the installed parts have a little play. The O-rings used, however, seal correctly, so that the process is not at risk.



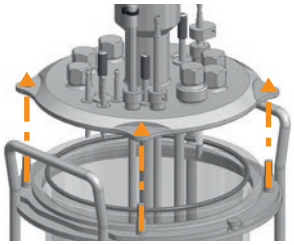
- Check the installation height of all adjustable-height fittings such as spargers or immersion pipes and ensure they are positioned correctly.



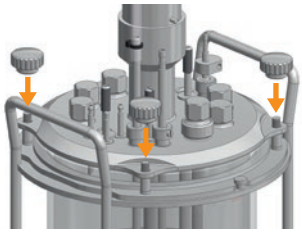
• If applicable, fill with culture medium.

Note: This is only useful and/or possible if the culture medium is heat-stable and will withstand autoclaving undamaged. Otherwise, the culture medium must be sterilized separately and then transferred (pumped, etc.) to the culture vessel via a sterile process after the culture vessel has been sterilized.

• Put the lid on carefully and align it correctly. Be very careful here to make sure the installed parts and baffle plates are not touching each other.



• Tighten knurled screws by hand (no tool) crosswise



**2. Calibrating (part 1) and installing sensors**

After all of the mechanical components are secured and the lid is closed, the open ports for the sensors are still ajar. Because these can be sensitive, they are typically installed last. The sensors also have to be calibrated prior to the process. Laboratories often have specific work instructions for this and they differ in the details. Generally, however, calibrate the pH sensors prior to installation and before autoclaving much the same way you would calibrate ordinary pH-meters with two reference buffers. Do not calibrate the $pO_2$ and optional turbidity sensors, however, until after autoclaving and dispensing the culture medium, since both parameters are dependent on the actual operating conditions.

Note:
- If you're using digital sensors, we should also mention that these can store and transport their calibration data in the sensor head. As a result, you do not necessarily have to calibrate these sensors at the bioreactor control unit; the calibration can instead be performed in a separate calibration laboratory. The last calibration is automatically available after connecting to the bioreactor.
- If, instead of optical $pO_2$ sensors, you are using amperometric $pO_2$ sensors, you will have to maintain these according to the sensor manufacturer's instructions, which is not addressed in this cookbook.
- For special applications, you may also use additional sensors. This cookbook does not address these either.

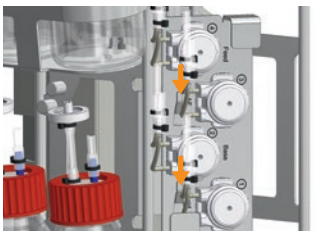
Work steps:
- Install the $pO_2$ sensor in the bioreactor
- Optional: Install the turbidity sensor in the bioreactor
- Connect the pH sensor to the base unit, call up the calibration function at the operating unit, and calibrate the pH sensor at the control unit according to the instructions (see also bioreactor manual).
- Separate the pH sensor from the base unit and install it in the bioreactor
- Note: Never cover the terminals of digital sensors with aluminum foil! Follow the sensor manufacturer's instructions!

**3. Connecting tubing and preparing for autoclaving**

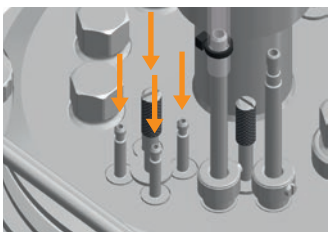
• Prepare the adjustment agent bottles

Note: Not all adjustment agents can be sterilized in the autoclave. Instead of adding these kinds of adjustment agents (ammonia solution, etc.), fill with water and, after autoclaving (at a sterile work bench, etc.), replace the water with a second adjustment agent (if applicable, one that has undergone sterile filtration).

• Take the mounting plate for the hose pump head, loosen it from the base unit and attach it to the reactor holder.



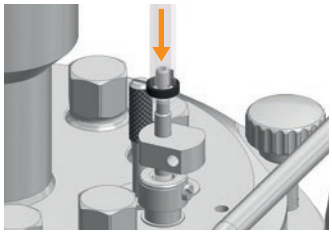
• Connect the tubing from the adjustment agent bottles to the pump heads and then to the input ports on the lid; secure the connections.



- Connect the Super Safe Sampler to the sampling pipe, clamp it off and cover it with aluminum foil.

- Connect the air filter.

- If applicable, connect any additional tubes (for cell harvesting, etc.) and clamp them off.



- Cover the filter lightly. Leave the exhaust filter open and do not clamp it off under any circumstances! This is essential for balancing the pressure during autoclaving!

**4. Autoclaving**

Take the prepared culture vessel in the culture vessel holder, including all of the connected peripherals such as adjustment agent bottles, and autoclave it according to local work instructions (ex.: 121 °C for 20 to 30 minutes).

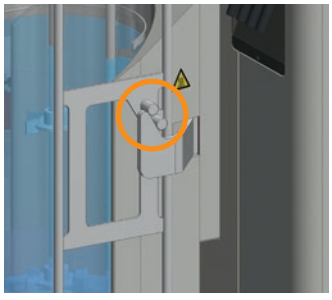
**5. Calibrating the sensors (part 2) and connecting the culture vessel holder to the base unit**

The culture vessel is now almost ready for the bioprocess. All you have to do now is connect it to the base unit, possibly fill it with culture medium, and calibrate the final sensors.

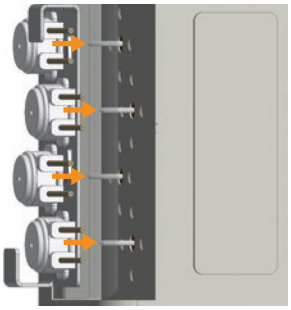
Work steps:

- Position the culture vessel holder on the base unit.

Note: Depending on the design of the bioreactor, you may need to connect temperature control tubing via the double jacket or install the heating mat. Please follow the instructions in the appropriate manual!



- Place the pump head mounting plate on the drive shafts



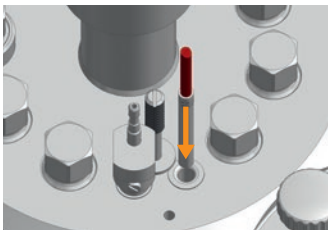
- Fill the adjustment agent tubes

Note: If the tubes are not filled with adjustment agent, the regulator may malfunction, because all it will initially feed into the culture vessel will be air instead of adjustment agent.

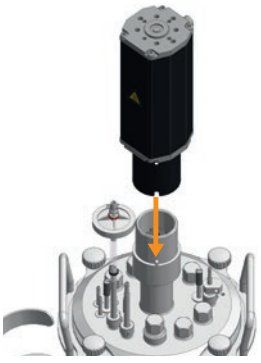
- Connect the foam, pH, $pO_2$ and turbidity sensors


Note: If, instead of optical $pO_2$ sensors, you are using amperometric $pO_2$ sensors, you will have to polarize these according to the sensor manufacturer's instructions; we do not address this here.

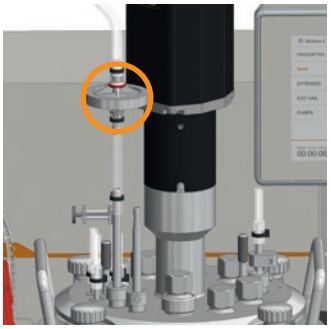
- Insert the temperature sensor into the immersion pipe.

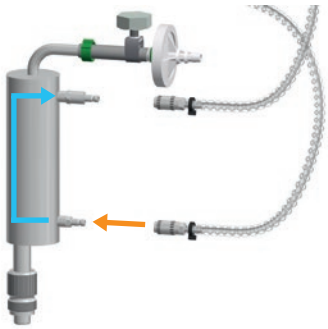


- Mount the motor.



- Connect the gas feed and the exhaust gas cooler. For cell cultures, a gas feed is often used in the headspace, and this is included in the current step.





- If not autoclaved inside the culture vessel: dispense the culture medium using a sterile filling process.
- Start the bioreactor and turn on the stirrer and temperature control, each set to the desired target values.
- As soon as the target values are reached, you may need to readjust the pH sensor or adjust the pH of the culture medium, calibrate the $pO_2$ sensor, and perform a zero-point calibration on the turbidity sensor.

**6. Inoculating and (finally) getting properly started**

Now you have everything ready for inoculation – for adding the microorganisms or cells. As is so often true in biotechnology, the precise procedure depends, again, on the individual case. If you just want to get a feel for the world of bioreactors or want to answer very simple questions, then all you actually have to do is add the cells, send a signal to the bioreactor or SCADA software, and then see how the process develops. In most cases, however, scientists have very definite plans, already understand the growth behavior of their microorganisms, and would maybe like to determine the perfect strategy for the fed-batch phase. They will also undertake the best possible preparations and, ideally, automate the process using software like eve®. It doesn't matter whether you're just implementing simple strategies like a pH ramp, or complex strategies that depend on calculated factors like substrate acquisition rates – after all, who wants to wait around next to their reactor all that time just so they can adjust their target values at just the right time? We won't go into the planning and preparation involved in strategies like that here, but the examples in Chapter 6 can certainly provide inspiration.

Work steps:

- Run a blank culture medium sample containing no cells.
- Prepare the inoculum (from a preculture in the incubation shaker, etc.) and place it in a suitable vessel for inoculation. For microbial bioprocesses, a relatively simple method of introducing the cells into the culture medium is to use a syringe with a needle that can pierce the membrane in the inoculation port. It can help here to disinfect the membrane with an alcohol solution, for example, or to work in the vicinity of a flame, but there is no such thing as a 100% guarantee against contamination. In a cell culture, by contrast, the risk of contamination is larger and the inoculum is introduced via a sterile tubing connection that can either extend under a sterile workbench or be created at the bioreactor locally (using a tube welding machine, etc.)

Note: Disinfectant should never be used on the membrane! Even a small drop of it may be sufficient to ruin the entire experiment if, for example, it gets into the culture medium when the membrane is pierced!

- Press the button on the bioreactor or in the SCADA software to signal that you have inoculated the sample; this will launch the work sequence (from the strategy you have saved, for example).

## 3.2 Adding culture medium

So far, you've spent a lot of time getting the bioreactor to run in the first place. You've hooked it up, screwed its parts together, assembled it, steam-sterilized it, and reviewed the typical, ideal process for running a bioprocess. But does that mean your cultures will now grow the way you expect? As is the case with many things in life, the answer here is: it depends.

It depends on the culture medium, for instance. As a rule, a great variety of nutrients are required since the microorganisms and cell cultures must find everything in the medium to build themselves up and thus multiply. Sometimes the media recipes look amazingly simple because very few components are listed. However, these are usually complex media involving "complex" components like yeast extract or peptone, which, in turn, contain a considerable spectrum of individual nutrients, providing all of the elements that make up the macromolecules of a cell. By contrast, there are also defined media in which individual nutrients are precisely weighed and mixed. In a subcategory of these, there are also "minimal media," which only contain precisely what the bioprocess needs. This is particularly important when it comes to optimizing costs and avoiding unwanted side reactions.

Unlike the minimal medium, what is known as a selective medium is used for selectively cultivating a certain kind of microorganism or cell line by skillfully selecting the environmental parameters. This can be achieved both by cleverly selecting the nutrients or by adding substances like antibiotics, which inhibit the growth of unwanted microorganisms. Nutrient media can be differentiated according to many other aspects, but that would go well beyond the scope of this cookbook.

As far as the nutrients go, compounds containing the elements carbon, oxygen and nitrogen are particularly important as macronutrients (C, O, H, N, S, P, K, Ca, Mg, Fe). In addition, what are known as micronutrients and trace elements such as copper, molybdenum, vitamins or amino acids are also needed in smaller quantities. Carbon sources include sugars or sugar alcohols (e.g., glucose, glycerol), corn starch or potato starch, syrups from sugar cane or sugar beets, and cellulose waste.

Other common sources of nitrogen and a large number of accompanying substances include extracts from yeast, soy or casein, as well as their counterparts – peptones and tryptones – when treated with digestive enzymes (pepsin and trypsin). Ammonium salts are an example of an alternative that can be used in defined media.

The following is a generic recipe for nutrient media, along with the additional components needed for a TB medium (bacterial cultivation) and a YPD medium (yeast cultivation):

| Components | Typical concentration range | Sample bacteria cultivation: TB medium | Sample yeast cultivation: YPD medium |
|---|---|---|---|
| Hydrogen acceptor | 50 – 100% | Oxygen | Oxygen |
| Carbon source | 1 – 20 g $L^{-1}$ | Peptides* | Glucose |
| Nitrogen source | 0.2 – 2 g $L^{-1}$ | Peptides* | Peptides* |
| Inorg. nutrients (S, P, etc.) | 50 mg $L^{-1}$ | Sulphur*, magnesium* | Sulphur*, magnesium* |
| Trace elements | 0.1 – 1 µg $L^{-1}$ | Salts* | Salts* |
| Growth factors such as amino acids, purines, vitamins | 0.1 – 1 mg $L^{-1}$ | e.g., vitamin B* | e.g., vitamin B* |
| Solvent | | Water | Water |
| Buffer components | | $KH_2PO_4$ – $K_2HPO_4$ | n.a. |

* Components come from complex media ingredients such as peptone or yeast extract

The two right columns list examples of components for a bacterial and a yeast culture medium. Since both are complex media, the exact amount of the individual substances often cannot be precisely determined, and their origin can only be attributed to the peptone and yeast extract. Media compositions are typically chosen so that there is a slight surplus of everything except for one ingredient. This ingredient limits growth, and is called a limiting factor. The carbon source is usually selected for this.

In practice, it is important to mention that the carbon source (and under certain circumstances other components such as potassium phosphate buffer) must be separately autoclaved and later added via a sterile process. This prevents reducing sugars from interacting with the nucleophilic group of amino acids under the heat effect of autoclaving (see glossary: Maillard reaction). It smells delicious, as if you had baked or roasted something, but it unnecessarily reduces the amount of available sugar and amino acids for the microorganisms.

Cell cultures are frequently just given "pre-packaged foods." By this we mean purchased, premixed culture media. There are two reasons for this: Cell cultivation is often performed for producing medications in pharmaceutical applications, where you have to provide precise details on what the medium contains, and you can't do that on your own, given the constantly changing composition of complex media components like yeast extract. Secondly, using premade culture media is also a way of attempting to reduce the risk of contamination.

## 3.3 When the fun is over…

…you have to clean up again. This means that, after the bioprocess has taken place, you have to put the bioreactor right again in such a way that renders the microorganisms harmless and restores the bioreactor to a clean and usable state. Different institutions have different policies on how to kill the organisms. This is either done by autoclaving in the culture vessel and then disposing of the stock, which usually involves a more complex cleaning process for the vessel and the periphery afterwards. Alternatively, you can also kill the culture broth separately, and first decontaminate and then clean the bioreactor and the surfaces of the peripherals that came into contact with the bioreactor.

# 4. Bioprocess strategies and their control

We've done a lot of prep work for our bioprocess, but there are still things we need to think through. Good planning is already half the battle. To this end, you need a few pieces of basic knowledge about growth so that you can ultimately carry out the bioprocess as you had originally planned. Once again, design follows the requirements, which is why you should know what the goal of the bioprocess is.

In general, the growth of microorganisms is limited as soon as an essential factor becomes limiting. For example, if the growth rate is limited by insufficient oxygen, then it will increase again if more oxygen is provided due to changed process management, until the same (or another) factor has a limiting effect.

## 4.1 Batch

A batch process is when you provide all of the nutrients at once without adding any more in the subsequent bioprocess. During the entire bioprocess, no additional nutrients are added – just adjuvants like gases, acids and bases; it is a closed system. The bioprocess then lasts until the nutrients are consumed. This strategy is suitable for rapid experiments such as strain characterization or the optimization of the nutrient medium. The disadvantage of this convenient method is that the biomass and product yields are limited. Since the carbon source and/or oxygen transfer are usually the limiting factor, the microorganisms are not in the exponential growth phase for long.

To improve the availability of dissolved oxygen, the oxygen transfer rate must be increased. This is achieved by increasing the stirring speed, the gas flow, the proportion of oxygen in the gas mix, or the pressure, as long as the bioprocess takes place in a steel bioreactor. Because the combination of the various parameters is intended to improve the concentration, you will need sophisticated management and control processes. You can configure these processes, known as cascades, however you like and adjust them to your specific application. One or more parameters that can be used for adjusting the concentration of dissolved oxygen will be predefined for the controller in this case. The first step toward reaching the target value is to vary the first parameter within the defined range. If that doesn't work, the next step is to change downstream parameters until the target value can be maintained.

After the end of a bioprocess run in batch mode, only the biomass or medium is then harvested and appropriately processed to obtain the desired product. From the reactor point of view, the process is repeatedly interrupted by cleaning and sterilization steps, and the biomass is only produced in stages. In addition to the low yield of biomass, batch processes can also give rise to substrate or product inhibition, which also needs to be treated as a major risk. The latter describes the impairment of enzyme activity by the presence of high concentrations of substrate or product, which induces metabolic feedback and can therefore drastically reduce the yield.

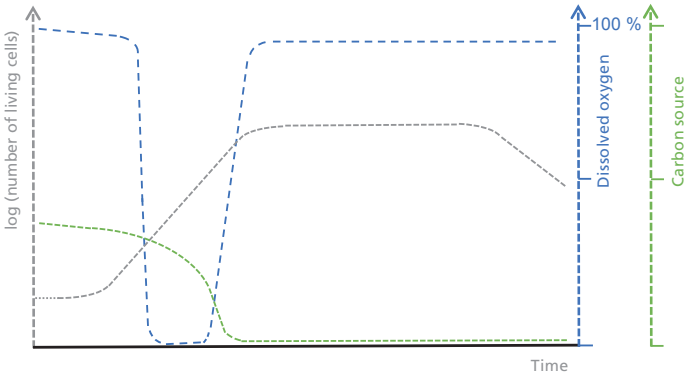



**Figure 6: Schematic illustration of the correlations between living cell concentration, dissolved oxygen and the limiting carbon source in batch operation.**
In the initial lag phase, the living cell count only increases slowly, which leads to a moderate but steady uptake of the carbon source. Oxygen consumption increases during the exponential growth phase until it exceeds possible oxygen input. Once the carbon source is depleted, the stationary phase starts and is followed by a die-off phase, during which the living cell count drastically decreases.

## 4.2 Fed-batch

Another way of keeping nutrients from becoming a limiting factor is to constantly supply them during cultivation. This is called a fed-batch process, which is a partly open system. The advantage of feeding during cultivation is that it allows you to achieve higher product quantities overall.

Under good growth conditions, the microorganisms and/or cells constantly double and therefore follow an exponential growth curve, which is why the feed rate should increase exponentially as well. Generally, the nutrient solution is pumped into the culture vessel through a silicone tube leading from the supply bottle. The user can manually determine the quantity at any time (constant, exponential, pulse-wise); users can also introduce nutrients when specific conditions are met, however, such as when a certain biomass concentration is reached or when a nutrient is used up. The process offers a wide range of control strategies and is also suitable for highly specialized applications. On the other hand, however, this may increase the processing time and potentially lead to inhibition through the accumulation of toxic by-products. The user also needs to have a more in-depth understanding of bioprocesses to do this, but we certainly don't mean to portray that as a disadvantage.

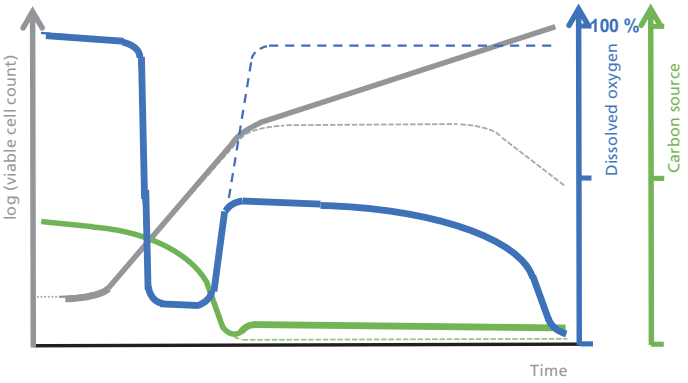



**Figure 7: Schematic illustration of the relationship between the living cell concentration, dissolved oxygen and the limiting carbon source in the fed-batch process.**
When implementing a fed-batch process, you will need to begin introducing the feed immediately after the exponential phase, to prevent the carbon source from being exhausted either at all or for only a short time (thick green line vs. dashed green line). Shown here is an exponential feeding process in which exponentially growing organisms remain in a prolonged exponential phase (thick gray line vs. dashed gray line). This also means that the quantity of consumed oxygen increases, which is why the content of dissolved oxygen in the medium is lower (thick blue line vs. dashed blue line). In this example, a growth rate μ less than $\mu_{max}$ has been selected for the feed phase.

While the batch process is classified as a discontinuous process, a fed-batch process is a semi-continuous process.

In attempts at the beginning of the last century to produce as much biomass as possible from baker's yeast in a batch process, excessively high substrate concentrations (in this case glucose) were found to inhibit growth, mainly by the formation of ethanol. On the other hand, this property of baker's yeast can be used to produce ethanol. At high glucose concentrations and sufficient dissolved oxygen in the medium, alcoholic fermentation still occurs, which is called the Crabtree effect. This effect is used in some food production processes with yeast.

Due to their advantages, fed-batch processes per se are now used in all areas of biotechnological production, in particular for the production of recombinant proteins and antibiotics.

## 4.3 Continuous culture

In one special reaction design, one in which system operation is open, the design is modified according to the bioprocess requirements in such a way that a flow equilibrium is established with respect to a particular component (also referred to as *steady state*). Under these conditions, as much fresh culture medium is added, for example, as is discharged. These kinds of bioprocesses are also referred to as continuous cultures, and are particularly suitable when an excess of nutrients would result in the microorganisms overeating, thus inhibiting them. Other advantages of this method include reduced product inhibition and an improved space-time yield. When the medium is removed, cells are also discharged, which is why the inflow and outflow rates must be less than the doubling time of the microorganisms. Alternatively, the cells can be retained in a wide variety of ways (for example, in a spin filter), which is referred to as perfusion operation.

In a continuous process, the space-time yield of the reactor can be improved still further over that of a fed-batch process. However, the long cultivation period also increases the risk of contamination and long-term changes in the cultures. Likewise, continuous processes are ideal tools for gaining a better understanding of the process, since all of the process parameters are constant when the system is operating correctly.

## 4.4 Other special forms of process management

Finally, there are also hybrid methods that can be used when running a bioprocess. One example is to harvest all but a small residue of a completed (fed) batch in order to use the remaining cells as an inoculum for the next filling. This type of process management is called a repeated (fed-)batch process. The distinction between a batch process and a fed-batch process again lies only in whether the remnants of the bioprocess are used for a batch or fed-batch process. This eliminates the cleaning time for the reactor and the need to cultivate a fresh inoculum, while simultaneously increasing productivity. However – as with other fed-batch processes – this is also associated with a higher risk of contamination and the possibility of strain alteration.

## 4.5 Dimensioning during process and media development

Process development and characterization typically start with a laboratory-scale bioreactor in order to detect as many parameters as possible and to learn the limits of the process. This also allows you to optimize the bioprocess, improve the cells and the medium, and determine critical process parameters at the same time.

When dimensioning the bioprocess for larger culture vessels – particularly to make it more economical – you must first carry out a pilot study, possibly followed by a production-scale study. This dimensioning process is also called scale-up. In order to generate value-intensive recombinant proteins, a bioreactor on the laboratory or pilot scale may also be sufficient.

The inverse of the scale-up process is called a scale-down and is suitable for simulating model tests, in particular for explaining malfunctions of a large-scale system or for optimizing an existing process even further.

# 5. Application examples

The following chapter is intended to present practical application examples for aerobic microbial bioprocesses. As befits a cookbook, these are beginner-friendly instructions that you can simply follow. Not only does this include media recipes – it also provides the precise conditions, quantities, and notes on which strategy to use. At the end of each bioprocess, you will also find more notes on how to refine a recipe by, for example, introducing more feed phases or drawing upon other basic media recipes. All of the recipes given here can also be found in eve® bioprocess platform software.

We begin with baker's yeast – *Saccharomyces cerevisiae* – whose biomass we want to grow in a batch process.

This is followed by a proposal for fed-batch cultivation of *Escherichia coli*, which is often used as a prokaryotic expression system for the production of proteins.

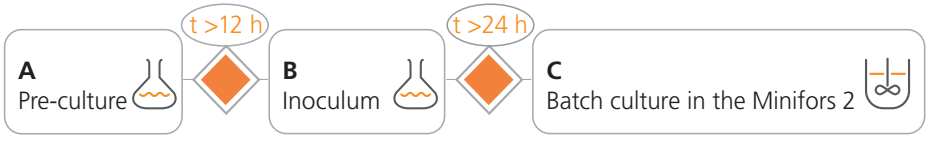
Last but not least, we will present *Pichia pastoris*, a methylotrophic yeast for use in a high-cell-density, fed-batch process on two substrates. This can be used a eukaryotic expression system for recombinant proteins of a more complex structure.

## 5.1 Cultivating *Saccharomyces cerevisiae*

### 5.1.1 Basic setup

| | |
|---|---|
| Name | Cultivating baker's yeast *(Saccharomyces cerevisiae)* |
| Description | Perform a bioprocess for generating yeast biomass to familiarize yourself with bioprocess technology. This will help you learn about the individual components of a bioreactor, how to handle it and basic process control in a batch bioprocess. |
| Recipe in eve® | *S. cerevisiae* cultivation |
| Device selection | Incubation shakers **A** and **B**, bioreactor **C** |
| Parameters | See separate description for **A, B** und **C** |

### 5.1.2 Workflow



## A. Preculture in the incubation shaker

*Organism*

| | |
|---|---|
| Name | **A** Preculture |
| Description | Generate a pure *S. cerevisiae* inoculum for further expansion in the shake culture |
| Organism | *Saccharomyces cerevisiae* (baker's yeast) |
| Origin | From the supermarket (available as cubes from the refrigeration unit or as a dry yeast; crumble a few pieces into the medium); you may also use laboratory wild type strains S288c (or FY1679), W303 and CEN.PK2 (use 10 to 100 µL of the maintenance culture) |
| Inoculum volume | 10 mL YPD medium in a 250 mL Erlenmeyer flask without baffles |
| Target yield of biomass | Depends on the quantity of yeast used |

*Culture medium*

| | |
|---|---|
| Type | Complex medium |
| Name | YPD (Yeast extract-peptone-dextrose) |
| Composition | 10 g $L^{-1}$ yeast extract (Y)<br>20 g $L^{-1}$ peptone (P)<br>20 g $L^{-1}$ dextrose (D) |

*Cultivation parameters*

| | |
|---|---|
| Shaking throw | 25 mm |
| Shaking speed | 300 $min^{-1}$ |
| Temperature | 30 °C |
| Time | At least 12 h of cultivation |

## B. Cultivating the inoculum in the incubation shaker

*Organism*

| | |
|---|---|
| Name | **B** Inoculum |
| Description | Generate enough *S cerevisiae* incolulum for subsequent cultivation in the bioreactor |
| Organism | *Saccharomyces cerevisiae* (baker's yeast) |
| Origin | 10 mL preculture (step **A**) |
| Inoculum volume | 5 mL of preculture in 100 mL fresh YPD medium, divided into 2 * 1000 mL Erlenmeyer flasks with baffles |
| Target yield of biomass | 6 – 8 g $L^{-1}$ |

*Culture medium*

| | |
|---|---|
| Type | Complex medium |
| Name | YPD (Yeast extract-peptone-dextrose) |
| Composition | 10 g $L^{-1}$ yeast extract (Y)<br>20 g $L^{-1}$ peptone (P)<br>20 g $L^{-1}$ dextrose (D) |

*Cultivation parameters*

| | |
|---|---|
| Shaking throw | 25 mm |
| Shaking speed | 300 $min^{-1}$ |
| Temperature | 30 °C |
| Time | 24 h |

## C. Main culture in the bioreactor

*Organism*

| | |
|---|---|
| Name | **C** Main culture in the bioreactor |
| Description | Generate biomass with a *S. cerevisiae* culture using the Minifors 2 |
| Organism | *Saccharomyces cerevisiae* (baker's yeast) |
| Origin | From the inoculum (step **B**) |
| Inoculum volume | 10 mL YPD medium in a 250 mL Erlenmeyer flask without baffles |
| Target yield of biomass | 6 – 8 g $L^{-1}$, depending on the strain and gassing of the culture from step **B** |

*Culture medium*

| | |
|---|---|
| Type | Complex medium |
| Name | YPD (Yeast extract-peptone-dextrose) |
| Composition | 10 g $L^{-1}$ yeast extract (Y)<br>20 g $L^{-1}$ peptone (P)<br>20 g $L^{-1}$ dextrose (D) |

*Cultivation parameters*

| | |
|---|---|
| Temperature | 30 °C |
| Stirring speed | 1200 $min^{-1}$ |
| pH | 5.5 |
| Gassing rate | 2 $min^{-1}$ (vvm) |
| Overpressure | 0 bar |
| $pO_2$ | ≥ 20 % |

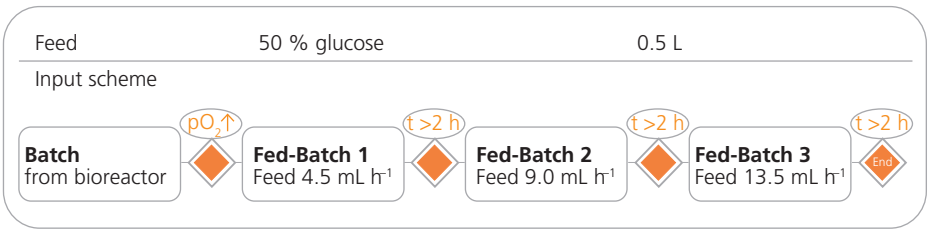
*Additional parameters to regulate (via either the bioreactor or eve®)*

| Pump | Addition of | Goal | Compound added | Trigger |
|---|---|---|---|---|
| a | *Base* | pH regulation | 25% $NH_4OH$ | Specific conditions met |
| b | *Acid* | pH regulation | 20% $H_3PO_4$ | Specific conditions met |
| c | *Antifoam agent* | Foam control | Biospumex 153 | Specific conditions met |

*Suggestions for modifying this recipe*

For the sake of simplicity, this first experiment does not involve programming a $pO_2$ cascade. All the parameters applied were set to the corresponding maximum values in order to ensure the highest possible $pO_2$ and thus potentially maximize the biomass yield.

To increase the yield of biomass, the batch phase can be extended by three simple fed-batch phases. This is done by supplying the needed nutrients in what is known as a feed solution. In the three successive phases, increase the feed rate by 1% each time. The end of the batch phase is marked by the depletion of the nutrients, which is why less oxygen will now be needed and the dissolved oxygen content in the media will suddenly rise again. From this point onward, either manually introduce the feed according to the following scheme or program it as a function in eve® (under Batch Strategy).



To further increase biomass yield and bioprocess running time, use additional components that will be rapidly exhausted during the cultivation process (such as magnesium) and add these to the medium one at a time. Alternatively, a more abundant base medium such as YUM or BMGY can also be used to start the batch.

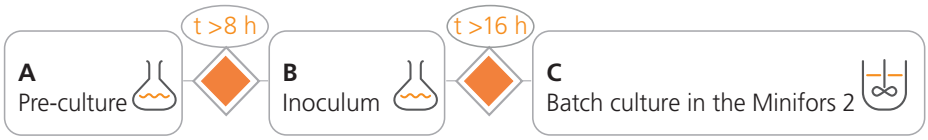
## 5.2 Cultivating *Escherichia coli*

*Escherichia coli* has always been considered the workhorse of molecular biology due to its short generation time and easily managed cultivation requirements and tools. As a consequence, it is often used for producing recombinant proteins. You must generate as much E. coli biomass as possible to produce the highest possible yield. Production of recombinant proteins will be proportional to this and will depend the promoter construct used.

### 5.2.1 Basic setup

| | |
|---|---|
| Name | Proliferation of *E. coli* |
| Description | Follow the proposed three-step process to selectively propagate *E. coli* after appropriate genetic modification; use for protein production. Alternatively, this bioprocess can also be executed in two stages by producing 20 mL of inoculum in the first step, which is then used immediately for inoculating the bioreactor. |
| Recipe in eve® | *Escherichia coli* cultivation |
| Device selection | Incubation shakers **A** and **B**, bioreactor **C** |
| Parameters | See separate description for **A**, **B** und **C** |

### 5.2.2 Workflow



## A. Preculture in the incubation shaker

### *Organism*

| | |
|---|---|
| Name | **A** Preculture |
| Description | Generate a pure *E. coli* inoculum for further expansion as a shake culture |
| Organism | *Escherichia coli*, such as strains K-12, BL21, DH5α etc. |
| Origin | 10 – 100 µL of a liquid culture |
| Inoculum volume | 10 mL YPD medium in a 250 mL Erlenmeyer flask with baffles |
| Target yield of biomass | Depends on the amount of *E. coli* added |

### *Culture medium*

| | |
|---|---|
| Type | Complex medium |
| Name | TB Medium (*Terrific broth* medium) |
| Composition | 24.00 g $L^{-1}$ Yeast extract<br>Soy peptone<br>12.00 g $L^{-1}$ $K_2HPO_4$<br>$K_2HPO_4$<br>12.54 g $L^{-1}$ Glycerol (anhydrous)<br>2.31 g $L^{-1}$<br>20.00 g $L^{-1}$ |

### *Cultivation parameters*

| | |
|---|---|
| Shaking throw | 25 mm |
| Shaking speed | 300 $min^{-1}$ |
| Temperature | 37 °C |
| Time | 8 h |

## B. Cultivating the inoculum in the incubation shaker

*Organism*

| | |
|---|---|
| Name | **B** Prepare the inoculum |
| Description | Generate enough *E. coli* inoculum for subsequent cultivation in the bioreactor |
| Organism | *Escherichia coli* |
| Origin | From the 10 mL preculture (step **A**) |
| Inoculum volume | 10 mL of preculture in 100 mL of fresh PAN medium in a 1000 mL Erlenmeyer flask with baffles |
| Target yield of biomass | 2.5 to 10 g $L^{-1}$ dry cell mass |

*Culture medium*

| | | |
|---|---|---|
| Type | Complex medium | |
| Name | PAN medium (or, alternatively, TB as in the previous step) | |
| Composition PAN medium, pH 7.0 | 1.6 g $L^{-1}$ $NaH_2PO_4 \cdot H_2O$<br>3.2 g $L^{-1}$ $KH_2PO_4$<br>2.6 g $L^{-1}$ $K_2HPO_4$<br>0.2 g $L^{-1}$ $NH_4Cl$<br>2.0 g $L^{-1}$ $(NH_4)_2SO_4$<br>0.6 g $L^{-1}$ $MgSO_4$<br>0.2 g $L^{-1}$ $CaCl_2 \times H_2O$<br>5 g $L^{-1}$ Glycerol | Autoclave in advance |
| Composition of trace element solution | 5 mL $L^{-1}$ $H_2SO_4$ (conc.)<br>6 g $L^{-1}$ $CuSO_4 \cdot 5H_2O$<br>0.08 g $L^{-1}$ KI<br>3 g $L^{-1}$ $MnSO_4 \cdot H_2O$<br>0.3 g $L^{-1}$ $Na_2MoO_4$<br>0.02 g $L^{-1}$ $H_3BO_3$<br>0.5 g $L^{-1}$ $CoCl_2$<br>20 g $L^{-1}$ $ZnCl_2$<br>65 g $L^{-1}$ $FeSO_4 \cdot 7H_2O$ | Add 1.0 mL of sterile trace element solution per L of PAN medium |

*Cultivation parameters*

| | |
|---|---|
| Shaking throw | 25 mm |
| Shaking speed | 300 $min^{-1}$ |
| Temperature | 37 °C |
| Time | 16 h |

## C. Main culture in the bioreactor

### *Organism*

| | |
|---|---|
| Name | **C** Main culture in the bioreactor |
| Description | Use the Minifors 2 to produce *E. coli* biomass during the batch phase and next generate recombinant protein in the glucose feeding phase |
| Organism | *Escherichia coli* |
| Origin | From the inoculum culture (step **B**) |
| Inoculum volume | 100 mL inoculum in 1000 mL fresh PAN medium with trace elements in the 2.5 L stirred tank with 2 impellers, Minifors 2 |
| Target yield of biomass | 10 to 100 g $L^{-1}$ dry cell mass |

### *Culture medium*

| | | | |
|---|---|---|---|
| Type | Complex medium | | |
| Name | PAN medium (or, alternatively, TB as in the previous step) | | |
| Composition PAN medium, pH 7.0 | 1.6 g $L^{-1}$ | $NaH_2PO_4 \cdot H_2O$ | Sterilize in the bioreactor |
| | 3.2 g $L^{-1}$ | $KH_2PO_4$ | |
| | 2.6 g $L^{-1}$ | $K_2HPO_4$ | |
| | 0.2 g $L^{-1}$ | $NH_4Cl$ | |
| | 2.0 g $L^{-1}$ | $(NH_4)_2SO_4$ | |
| | 0.6 g $L^{-1}$ | $MgSO_4$ | |
| | 0.2 g $L^{-1}$ | $CaCl_2 \cdot H_2O$ | |
| | 5 g $L^{-1}$ | Glycerol | |
| Composition of trace element solution | 5 mL $L^{-1}$ | $H_2SO_4$ (conc.) | Add 1.0 mL of sterile trace element solution per L of PAN medium |
| | 6 g $L^{-1}$ | $CuSO_4 \cdot 5H_2O$ | |
| | 0.08 g $L^{-1}$ | KI | |
| | 3 g $L^{-1}$ | $MnSO_4 \cdot H_2O$ | |
| | 0.3 g $L^{-1}$ | $Na_2MoO_4$ | |
| | 0.02 g $L^{-1}$ | $H_3BO_3$ | |
| | 0.5 g $L^{-1}$ | $CoCl_2$ | |
| | 20 g $L^{-1}$ | $ZnCl_2$ | |
| | 65 g $L^{-1}$ | $FeSO_4 \cdot 7H_2O$ | |

### *Cultivation parameters*

| | |
|---|---|
| Temperature | 37 °C |
| Stirring speed | 500 $min^{-1}$ |
| pH | 5.0 |
| $pO_2$ | > 20% |

*$pO_2$ cascade on the bioreactor touchscreen*

| Parameters | Description | Target value | Controlled value, min. | Controlled value, max. |
|---|---|---|---|---|
| 1 | *Stirring speed* | 500 $min^{-1}$ | 500 $min^{-1}$ | 1200 $min^{-1}$ |
| 2 | *Gassing rate* | 1.0 vvm | 1.0 vvm | vvm |
| 3 | *Overpressure* | 0 bar | 0 bar | 0 bar |
| 4 | *$pO_2$* | ≥ 20% | | |

*Feed schema*

| Phase | Description Goal | Start | End | Work sequence | Percent feed rate | Feed rate, mL/h |
|---|---|---|---|---|---|---|
| C1 | *Batch* | 0 h | 10 h | No feed | | |
| C2 | *Fed-batch* | 10 h | 12 h | Glucose | 1% | 4.5 mL $h^{-1}$ |
| C3 | *Fed-batch* | 12 h | 14 h | Glucose | 2% | 9.0 mL $h^{-1}$ |
| C4 | *Fed-batch* | 14 h | 16 h | Glucose | 3% | 13.5 mL $h^{-1}$ |
| C5 | *Fed-batch* | | 18 h | Glucose | 4% | 18.0 mL $h^{-1}$ |
| C6 | *Fed-batch* | 18 h | 20 h | Glucose | 5% | 22.5 mL $h^{-1}$ |
| C7 | *Fed-batch* | 20 h | 22 h | Glucose | 6% | 27.0 mL $h^{-1}$ |

*Additional parameters to be regulated via either the bioreactor (a,b,c) or eve ® (step d can be also be performed manually)*

| Pump | Addition of | Goal | Compound added | Trigger |
|---|---|---|---|---|
| a | *Base* | pH regulation | 25% $NH_4OH$ | Specific conditions met |
| b | *Acid* | pH regulation | 20% $H_3PO_4$ | Specific conditions met |
| c | *Antifoam agent* | Foam control | Biospumex 153 | Specific conditions met |
| d | *Glucose feed* | Carbon source (C) 500 g $L^{-1}$ glucose | 50% glucose | After batch phase |

## 5.3 Cultivating *Pichia pastoris*

This yeast's potential for metabolizing methanol, an economical raw material, has made bioprocesses with *Pichia pastoris* indispensable tools for generating recombinant proteins. Utilizing methanol allows scientists to decouple the production of biomass from product formation, and thus to specifically initiate the phase of product formation by feeding methanol.

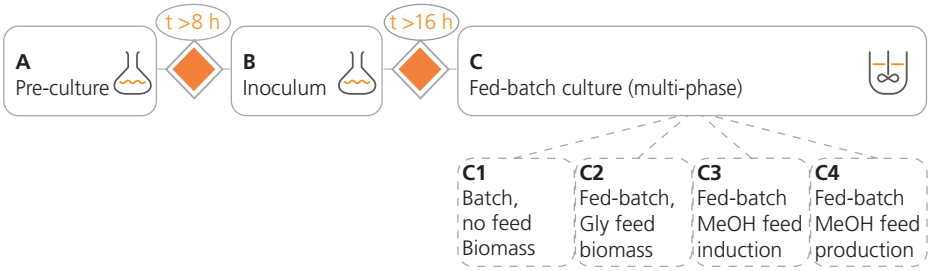
As a eukaryotic organism, *Pichia* is also capable of producing correctly folded proteins with pronounced posttranslational modifications and releasing these into the medium, which represents an additional advantage over production in the *E. coli* system. In addition, *Pichia* has *GRAS* (*generally recognized as safe*) status and neither endotoxin contamination of the product nor bacterial or viral contamination problems are to be expected.

For reasons of consistency and because of its multiple uses in the field, we will retain the (old) name – *Pichia pastoris* – in the remainder of this document. Reclassification made it necessary to assign this type of yeast to the genus *Komagataella*, which is why the correct name is *Komagataella pastoris*. Further molecular biological investigation of mitochondrial RNA has revealed, however, that the strains originally marketed as *Pichia pastoris* are actually *Komagataella phaffii*.

### 5.3.1 Basic setup

| Name | Cultivating a methylotrophic yeast *(Pichia pastoris)* |
|---|---|
| Description | This model organism is often used for producing recombinant proteins. First we will present a fed-batch bioprocess for a high-cell-density bioprocess. Next we intend to help you understand the special considerations involved in cultivating this methylotrophic yeast on glycerol and methanol, and show how these should be implemented as phases in the bioprocess. |
| Recipe in eve® | *P. pastoris* cultivation (fed batch) |
| Device selection | Incubation shakers **A** and **B**, bioreactor **C** |
| Parameters | See separate description for **A**, **B** und **C** |

### 5.3.2 Workflow



## A. Preculture in the incubation shaker

*Organism*

| | |
|---|---|
| Name | **A** Preculture |
| Description | Generate a pure *P. pastoris* inoculum capable of proliferation for further expansion as a shake culture |
| Organism | *Pichia pastoris*, new name *Komagataella phaffii* |
| Origin | $Mut^+$ strain, 10 – 100 µL of a liquid culture |
| Inoculum volume | 1 mL *Pichia* maintenance culture in 10 mL fresh YPD in a 250 mL Erlenmeyer flask without baffles |
| Target yield of biomass | Depends on the density of the maintenance culture |

*Culture medium*

| | |
|---|---|
| Type | Complex medium |
| Name | YPD (yeast extract peptone dextrose) |
| Composition | 10.0 g $L^{-1}$ Yeast extract (Y)<br>20.0 g $L^{-1}$ Peptone (P)<br>20.0 g $L^{-1}$ Dextrose (D) |

*Cultivation parameters*

| | |
|---|---|
| Shaking throw | 25 mm |
| Shaking speed | 300 $min^{-1}$ |
| Temperature | 30°C |
| Time | 8 – 16 h |

## B. Cultivating the inoculum in the incubation shaker

*Organism*

| | |
|---|---|
| Name | **B** Cultivating the inoculum |
| Description | Generate enough *P pastoris* incolulum for subsequent cultivation in the bioreactor |
| Organism | *Pichia pastoris*, new name *Komagataella phaffii* |
| Origin | 10 mL preculture from step **A** |
| Inoculum volume | 10 mL *Pichia* preculture in 100 mL fresh BMGY medium in a 1000 mL Erlenmeyer flask with baffles |
| Target yield of biomass | 10 to 30 g $L^{-1}$ dry cell mass |

*Culture medium*

| | | |
|---|---|---|
| Type | Complex medium | |
| Name | BMGY (buffered glycerol complex medium) | |
| Composition | 11.5 g $L^{-1}$ $KH_2PO_4$, pH 6.0 | (B) |
| | 20.0 g $L^{-1}$ Peptone | (M) |
| | 10.0 g $L^{-1}$ Glycerol | (G) |
| | 10.0 g $L^{-1}$ Yeast nitrogen base (with no amino acids) | (Y) |
| | 40 µg $L^{-1}$ Biotin | |

*Cultivation parameters*

| | |
|---|---|
| Shaking throw | 25 mm |
| Shaking speed | 300 $min^{-1}$ |
| Temperature | 30°C |
| Time | 16 h |

## C. Main culture in the bioreactor

*Organism*

| | |
|---|---|
| Name | **C** Cultivating the main culture in the bioreactor |
| Description | Use the Minifors 2 for upstream generation of *P. pastoris* culture biomass, and then generate recombinant protein in the methanol feeding phase |
| Organism | *Pichia pastoris*, new name *Komagataella phaffii* |
| Origin | From the inoculum culture (step **B**) |
| Inoculum volume | 100 mL inoculum in 1000 mL fresh BSM/PTM1 medium in a 2.5 L stirred tank with 2 impellers, Minifors 2 |
| Target yield of biomass | Potential for over 120 g $L^{-1}$ dry cell mass |

*Culture medium*

| Type | Synthetic full medium | |
|---|---|---|
| Name | BSM (basal salt medium) and PTM1 mineral supplement | |
| Composition BSM pH 5.0 | 26.7 mL $L^{-1}$ $H_3PO_4$, 85 %<br>0.93 g $L^{-1}$ $CaCl_2 \cdot 2\ H_2O$<br>18.2 g $L^{-1}$ $K_2SO_4$<br>14.9 g $L^{-1}$ $MgSO_4 \cdot 7\ H_2O$<br>4.13 g $L^{-1}$ KOH<br>40.0 g $L^{-1}$ Glycerol | Sterilize in the bioreactor |
| Composition PTM1 | 6.0 g $L^{-1}$ $CuSO_4 \cdot 5\ H_2O$<br>0.08 g $L^{-1}$ NaI<br>3.0 g $L^{-1}$ $MnSO_4 \cdot H_2O$<br>0.2 g $L^{-1}$ $Na_2MoO_4 \cdot 2\ H_2O$<br>0.02 g $L^{-1}$ $H_3BO_3$<br>0.5 g $L^{-1}$ $CoCl_2$<br>20.0 g $L^{-1}$ $ZnCl_2$<br>65.0 g $L^{-1}$ $FeSO_4 \cdot 7\ H_2O$<br>0.2 g $L^{-1}$ Biotin<br>5.0 mL $L^{-1}$ $H_2SO_4$ | Add 4.35 mL sterile PTM1 solution per L of BSM hinzufügen |

*Cultivation parameters*

| | |
|---|---|
| Temperature | 30 °C |
| Stirring speed | 500 $min^{-1}$ |
| pH | 5.0 |
| $pO_2$ | >20% |

*$pO_2$ cascade on the bioreactor touchscreen*

| Parameters | Description | Target value | Controlled value, max. | Controlled value, min. |
|---|---|---|---|---|
| 1 | *Stirring speed* | 500 $min^{-1}$ | 500 $min^{-1}$ | 1200 $min^{-1}$ |
| 2 | *Gassing rate* | 1 vvm | 1 vvm | 2 vvm |
| 3 | *Overpressure* | 0 bar | 0 bar | 0 bar |
| 4 | *$pO_2$* | ≥ 20% | | |

*Feed schema*

| Phase | Description | Goal | Start | End | Work sequence | Percent of feed rate | Feed rate, mL/h |
|---|---|---|---|---|---|---|---|
| C1 | *Batch* | Build-up of biomass | 0 h | 20 h | No feed | | |
| C2 | *Fed-batch Gly* | Build-up of biomass | 20 h | 43 h | Glycerol | 1% | 4.5 mL $h^{-1}$ |
| C3 | *Fed-batch Gly / MeOH* | Induction phase | 43 h | 49 h | MeOH | 1% | 3.6 mL $h^{-1}$ |
| C4 | *Fed-batch MeOH* | Production phase | 49 h | 97 h | MeOH | 1% | 10.9 mL $h^{-1}$ |

*Additional parameters to be regulated via either the bioreactor (a,b,c) or eve® (step d and e can be also be performed manually)*

| Pump | Addition of | Goal | Compound added | Trigger |
|---|---|---|---|---|
| a | *Base* | pH regulation | 25% $NH_4OH$ | Specific conditions met |
| b | *Acid* | pH regulation | 20% $H_3PO_4$ | Specific conditions met |
| c | *Antifoam agent* | Foam control | Biospumex 153 | Specific conditions met |
| d | *Glycerol feed* | Carbon source (C2+3) 550 g $L^{-1}$ glycerol | 50% glycerol and 12 mL $L^{-1}$ PTM1 | During phase C2/3 |
| e | *Methanol feed* | Carbon source (C3+4) 395 g $L^{-1}$ methanol | 100% methanol and 12 mL $L^{-1}$ PTM1 | During phase C3/4 |

*Suggestions for modifying this recipe*

To increase the biomass yield, you can make use of modified media and formulations for batch phases and feeds. In terms of product yield, optimizing the methanol feed to keep toxic effects in check is worth the effort. This feed should never rise above 32 °C, since protein production stops at that point. You will therefore need efficient cooling during the propagation and product formation phase. Testing product stability in the chosen pH range is just as important if you need to adjust the pH or add protease inhibitors such as casamino acids to the medium, which increase the stability of the product.

A brief "hunger phase" could be helpful during the transition between glycerol and methanol metabolism. In other situations, shutting down the glycerol feed while simultaneously increasing the methanol feed may also be effective. It is important here to wait for the $pO_2$ peak, which indicates that all the glycerol has been consumed.

A second methanol feed phase in which the methanol feed is reduced may also be helpful. This should follow the C4 phase and circumvent the toxic effect of methanol on cells.

Evaluating the strain will indicate its capacity for making efficient use of methanol ($Mut^+$ versus $Mut^S$). Making strategic use of USP methods is another way of producing a considerable difference between biomass and product.

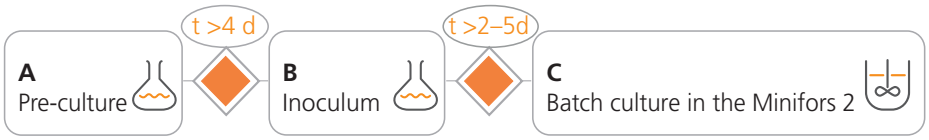
## 5.4 Cultivating CHO

The CHO cell line is by far the most important for producing biopharmaceuticals and medications. The reason why the hamster cells are so popular is because they are very similar to human cells. Plus, the risk of infecting a CHO cell line with a virus is far smaller than is the case for other cell lines.

### 5.4.1 Basic setup

| | |
|---|---|
| Name | Cultivating CHO cells (clone XM-111) |
| Description | Follow the proposed three-step process to propagate CHO cells and then cultivate them in a bioreactor. If genetically modified, these cells could be used for producing recombinant proteins. You may skip the first step if you already have a batch of passaged cells. |
| Recipe in eve® | CHO cultivation |
| Recipe | CHO batch bioprocess |
| Device selection | **A** Preculture, **B** Inoculum cultivation, **C** Batch culture in bioreactor |
| Parameters | See separate description for **A, B** und **C** |

### 5.4.2 Workflow




A. Producing the preculture in the incubator

*Organism*

| | |
|---|---|
| Name | **A** Preparing the preculture |
| Description | Generate a pure CHO inoculum for further expansion in the shake culture |
| Organism | CHO (clone XM-111) |
| Origin | Cell lines such as CHO-K1, CHO/dhFr- (approx. 4 x $10^6$ cells from a cryoculture) |
| Inoculum volume | 10 mL HP-1 medium in a T75 flask |
| Target yield of biomass | Depends on the number of cells added, 1 – 2 x $10^6$ cells/mL |

*Culture medium*

| | |
|---|---|
| Type | Serum and protein-free medium |
| Name | HP-1 medium (Cell Culture Technologies GmbH) |
| Composition | HP-1 medium<br>2 g $L^{-1}$ Pluronic F-68<br>2.5 mg $L^{-1}$ tetracycline |

*Cultivation parameters*

| | |
|---|---|
| Relative humidity (RH) | 85% |
| $CO_2$ saturation | 5% |
| Temperature | 37 °C |
| Time | Passage at least 2 x (approx. 4 days) |

## B. Cultivating the inoculum in the incubation shaker

*Organism*

| | |
|---|---|
| Name | **B** Cultivating the inoculum |
| Description | Generate enough CHO incolulum for subsequent cultivation in the bioreactor |
| Organism | CHO (XM-111) |
| Origin | From the 10 mL preculture (step **A**) |
| Inoculum volume | Use the preculture to inoculate 50 mL HP-1 medium two times for a live cell concentration of 3 x $10^5$ cells/mL, divided in to 2 * 250 mL shaker flasks (Corning) |
| Target live cell concentration | Approx. 2 x $10^6$ cells/mL |

*Culture medium*

| | |
|---|---|
| Type | Serum and protein-free medium |
| Name | HP-1 medium (Cell Culture Technologies GmbH) |
| Composition | HP-1 medium<br>2 g $L^{-1}$ Pluronic F-68<br>2.5 mg $L^{-1}$ tetracycline |

*Cultivation parameters*

| | |
|---|---|
| Shaking throw | 50 mm |
| Shaking speed | 122 $min^{-1}$ |
| Temperature | 37°C |
| Relative humidity (RH) | 85% |
| $CO_2$ saturation | 5% |
| Feed | 30 mL medium after 24 h |

## C. Cultivating the main culture in the bioreactor

*Organism*

| | |
|---|---|
| Name | **C** Cultivating the main culture in the bioreactor |
| Description | Use the Multifors Cell to produce protein with a CHO culture |
| Organism | CHO (XM-111) |
| Origin | From the inoculum culture (step **B**) |
| Inoculum volume | For each Minifors 2 reactor (1 l TV), inoculate 700 mL fresh HP-1 medium with preculture for a live cell concentration of approx. 0.5 x $10^6$ cells/mL. |
| Target live cell concentration | Approx. 3 – 4 x $10^6$ cells/mL after 2–3 days |

*Culture medium*

| | |
|---|---|
| Type | Serum and protein-free medium |
| Name | HP-1 medium (Cell Culture Technologies GmbH) |
| Composition | HP-1 medium<br>10 mL $L^{-1}$ Pluronic F-68<br>2.5 mL $L^{-1}$ tetracycline |

*Cultivation parameters*

| | |
|---|---|
| Temperature | 37°C |
| Stirring speed | 75 $min^{-1}$ |
| pH (by controlling $CO_2$) | 7.2 |
| Gassing rate, sparger<br>Gassing rate, headspace | 0.06 $min^{-1}$ (vvm)<br>up to 0.7 $min^{-1}$ (vvm) |
| Overpressure | 0 bar |
| $pO_2$ | 40% |

*Additional parameters to regulate (via either the bioreactor or eve®)*

| Pump | Addition of | Goal | Compound added | Trigger |
|---|---|---|---|---|
| a | *Base* | pH regulation | None | Specific conditions met |
| b | *Acid* | pH regulation | $CO_2$ gassing | Specific conditions met |

*Results (examples)*

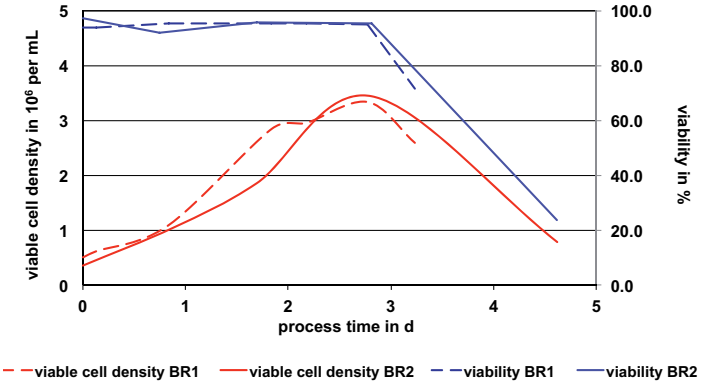



**Figure 1: Comparison between CHO XM-111 cell cultivation in bioreactor 1 and bioreactor 2**

*Example for using this recipe*

Use fed-batch cultivation for increasing cell concentrations: regularly add fresh medium to the culture, which prevents nutrient limitations. Begin with a CHO culture volume in the Minifors Cell of 600 mL, feeding 300 mL HP-1 medium both on the first day (approx. 30 h) and on the second day (approx. 46 h). Feed an additional 400 mL on the second day (approx. 54 h). Switching from the HP-1 growth medium to the HP-5 production medium (w/o tetracycline) on day 3 (65 h) initiates the switch over to the production phase of a recombinant protein (in this case secreted, alkaline phosphatase, i.e., SEAP). At this point, shift the temperature from 37 °C to 30 °C in order to prevent proliferation.

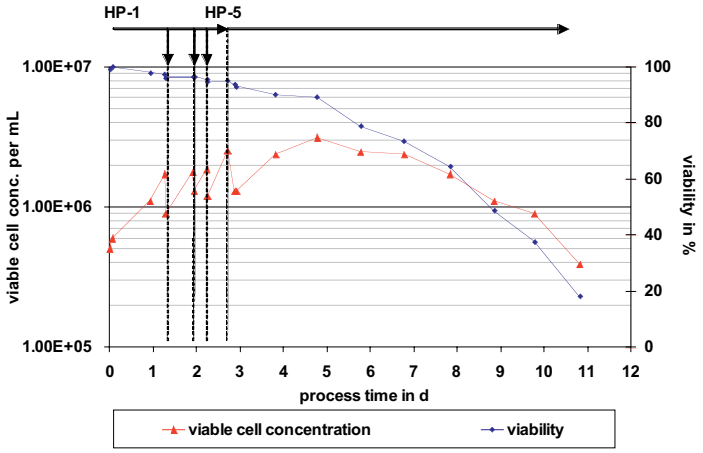



Figure 2: Comparison of live cell concentration and vitality

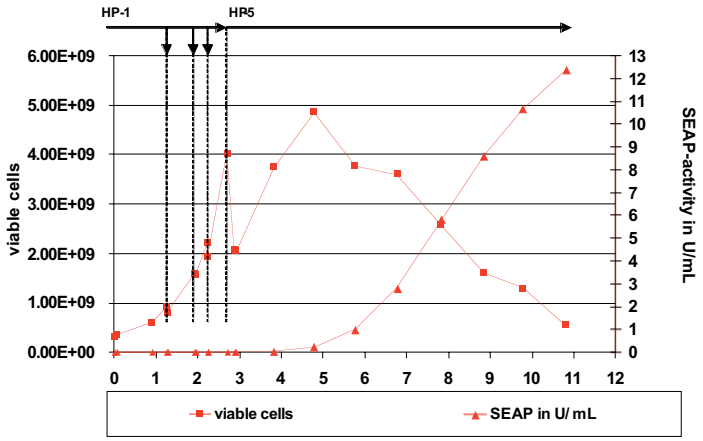



Figure 3: SEAP activity

# 6. Tips for a successful bioprocess

## 6.1 Prevent contamination

- Contamination can have very different causes. The most frequent cause is contamination of the starter culture. This can happen in many ways: improper handling, insufficiently cleaned or autoclaved culture vessels and/or reactor and media components, or contaminated starter cultures or media components.
- In addition, an organism that only grows slowly as a contaminant in a static culture will suddenly find the perfect growth conditions in the bioreactor, quickly overwhelming the organisms you are actually cultivating. This problem frequently arises in cell cultures, because cells grow very slowly and can be easily overwhelmed by rapidly growing bacteria. One possible solution is to use antibiotics to kill off bacteria.
- Here you have to be careful to maintain the bioreactor target temperature long enough during the autoclaving process. The temperature sensor should be inserted into the immersion pipe on the culture vessel.
- For a double-walled culture vessel, there must be enough water present in the jacket to ensure proper heat transfer during sterilization.
- All sealing rings should be in perfect condition, i.e., there should be no signs of kinks, flattening or any burr formation; when in doubt, it's better to replace them one time too many, especially if a seal is not seated properly.

Special recommendations for cell cultures:
- Buy a sterile medium and 70% ethanol for disinfecting.
- Wear gloves
- Work at a sterile bench
- Work with single-use equipment

## 6.2 Maximize growth by controlling the concentration of dissolved oxygen

- In aerobic bioprocesses, the amount of dissolved oxygen is an important parameter – one that determines the success or failure of your bioprocess. Depending on the requirements of the organisms, this must be individually configured and controlled by means of various parameters, e.g., TotalFlow, stirrer speed and gas mix. Most users accept $pO_2$ control precision in the range of ±5% to 10% of the target value.
- Did you switch on $pO_2$ as a parameter and configure it in the cascade on the bioreactor? Oxygen control cascades for microbial processes generally include the stirrer, since higher stirring speeds improve oxygenation. In a shear-sensitive cell culture, however, that could damage cells, which is why the gas composition and potentially the gas flow rate are most often used for controlling oxygen concentration.
- Are the limits you selected for individual parameters broad enough to meet the high oxygen demand during the exponential growth phase?
- Did you select a sufficient gassing rate?
- Did you add a lot of antifoam agent? This can hinder efficient oxygen transfer. Alternatively, anti-foaming agents can be added to the starting medium in the amount of 1:20,000 in order to prevent foam formation.
- You may need to introduce pure oxygen for fast-growing cultures in which the cell density is high. Most bioreactors are configured at the factory for an oxygen feed; if not you should request an upgrade.
- After the nutrients are depleted, the dissolved oxygen concentration increases again. You can use this kind of behavior to help you determine the feed timing in a fed-batch process. You should, however, check the residual concentration of the carbon source so that you don't confuse it with a sudden jump in $pO_2$.

## 6.3 Maintain the culture volume

- When autoclaving the medium in the reactor, you may lose up to 10% of the volume. To prevent the medium from becoming too concentrated, you should compensate by adding sterile water before inoculating the reactor.
- If you notice a decrease in the culture medium during cultivation (particularly when there is no feed), check the status of the exhaust cooler, its coolant inflow and whether the temperature regulation is switched on.
- Any sampling should be limited to a quantity of 10 mL in smaller culturing vessels. The use of the SuperSafe sampler can help with this.

## 6.4 Prevent foam formation

- Foam formation tends to occur in protein-rich media with higher gassing rates and/or stirrer speeds. Not only can this disrupt the bioprocess – it can also damage the microorganisms/cell cultures and the final product.
- Under certain circumstances, you may need little or no gas flow at the beginning of the batch culture. You can then continually increase the gas flow later. This guarantees sufficient oxygen availability without forming unnecessary foam.
- If possible, you should add a small portion of antifoam agent to the medium in order to effectively prevent foam formation right from the start. Dispensing too much antifoam agent will limit efficient oxygen transfer and thus the growth of aerobic organisms as well. As a general rule of thumb: keeping foam from forming is simpler than getting rid of it later, which is why adding antifoam agents early on can minimize the amount you need to use later.

## 6.5 A functioning waste gas filter

- Keeping the exhaust filter dry is very important. Failure to do so will clog the hydrophobic membrane, which, in turn, will inhibit the gas flow, causing unwanted pressure to build up in the culturing vessel to the point that the flow of gas is blocked entirely.
- If moisture gets into the exhaust filter during the bioprocess, there are several options for eliminating it:
  – Reduce the flow of gas if you can
  – Increase the coolant supply in the exhaust cooler or use a better refrigerant, e.g., with a lower temperature
  – Use a bigger filter
  – Use a depth filter, which cannot become clogged in that way.
- If the problem is caused by too much foam formation, a foam trap between the exhaust cooler and the exhaust filter can help. You can do this quite easily simply by feeding the waste gas through a flask containing antifoam agent before the gas passes through the filter.

## 6.6 Constant pump speed

- If using Marprene tubes, you should allow the pump to run for approx. 30 minutes before determining the flow rate, since Marprene tubes expand over time. Repeated autoclaving can have this kind of effect as well.
- The flow rate may vary with the viscosity of the liquid. The flow rate of a feed medium high in sugar can therefore differ from that of a solution with a low sugar content, even if the pumping rate is the same.
- It is also important to make sure the supply tubes for feed or other solutions are neither kinked nor squashed on their way to the cover plate of the bioreactor.
- Likewise, the clamp applied for autoclaving must not block the feed lines at the point of attachment.
- If the feed line needs to be highly precise, you should monitor it as a gravimetric feed using a weighing scale. This will allow the bioreactor to use the decreasing weight recorded at the scale as feedback for automatically adjusting the pump rate and maintaining the desired flow rate.

## 6.7 Successful biomass yield

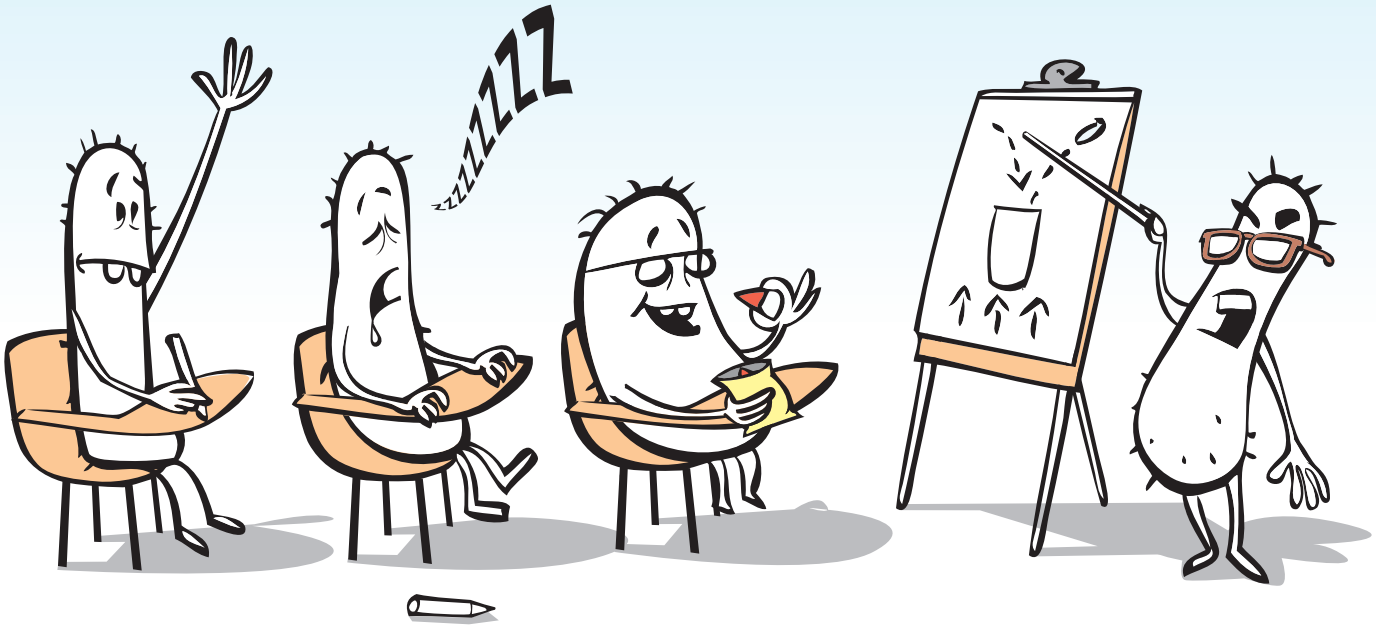
- A fed-batch cultivating system can be significantly better suited for producing sufficient biomass quantities.
- The starter culture should be in the exponential growth phase before you inoculate the bioreactor in order to maximize the growth rate.
- If any nutrient has a limiting effect (and this does not necessarily have to be the carbon source), the growth of the microbes will be limited. You should therefore always make sure you have an adequate supply of all of the nutrients and a good supply of oxygen.
- Genetically modified strains may be far more sensitive to forces like shear stress, or their genetic modifications may cause them to grow more slowly.
- You can keep the lag phase as short as possible by ensuring that the starter culture is sufficiently vital and cultivated with high cell counts, and that it is adapted as well as possible to the conditions in the bioreactor.

## 6.8 Successful protein yield

- If the nutrient supply is so excessive that microbial metabolism is only geared towards growth, the microbes will not excrete any metabolites. Managing the bioprocess as a fed-batch process can provide a remedy here.
- Growth conditions are often not ideal for producing, say, the targeted protein; as a result, you may need to adjust the temperature or the pH to the protein in question in order to improve protein yield and stability.
- In addition, you should check very carefully (by reviewing the literature ahead of time, etc.) to determine whether the strain can produce the desired yields when cultured in this way.
- It is just as important to know whether the cultivated strain excretes toxins or growth-inhibiting substances, which can be produced by growth, by the disintegration of cells or by cell metabolism. These kinds of influencing factors can impair protein production over a period of a few days.

## 6.9 Tips for fed-batch processes

- In order to guarantee that all of the sensors and the impeller are sufficiently covered with culturing fluid, you should not start a fed-batch process with the minimum volume.
- An overfilled culturing vessel is also of little use. There should always be a minimum of 20 – 30% headspace in the culturing vessel.
- Calculating the quantity of the required culturing liquid will prevent the annoying problem of having to produce and/or order more after the fact and will ensure the bioprocess runs smoothly. It is equally important that the pump you use can reach the desired flow rates.
- If you need to maintain a very precise flow rate, we do not recommend determining and setting the rate within the first hour of operation because the tubes can still stretch. Gravimetric feeding can be used to bypass these unwanted side effects.
- The carbon source is not necessarily the limiting factor. Trace elements and a sufficient supply of a nitrogen source are also essential for the success of the process. You can achieve a relatively high biomass yield of *E. coli*, for example, if you use an ammonium solution as a base in the process.
- Do not underestimate the resulting (waste) heat, especially in high-cell-density processes. Sufficient cooling can be achieved by a heat exchanger with water intake.
- A more accurate way of determining the metabolic state of the culture is to calculate the respiratory quotient, provided an exhaust analyzer is connected to the bioreactor.
- If you raise the gas flow rate too much (no more than 2 $min^{-1}$ for microorganisms and 0.1 $min^{-1}$ for cell cultures), you will introduce too much fluid from the culturing liquid. Besides allowing the culture volume to shrink unnecessarily, this can lead to a clogged exhaust filter.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# 8. Glossary

## A

**Actuator**
These are drive elements that convert electrical signals into mechanical movement or other physical phenomena. Its actuators are what allows a bioreactor to control the bioprocess.

**Adherent cells**
Most cells of vertebrates are adherent, which is to say that they cling to the surface when proliferating, as they do in the body. They are therefore cultivated on a suitable substrate – otherwise they will not be able to grow.

**Aerator**
See Sparger

**Aerobic**
In the presence of (ambient) oxygen. Most bioprocesses used for protein production are aerobic.

**Anaerobic**
In the absence of (ambient) oxygen. Even aerobic processes can go through anaerobic phases, such as when the level of dissolved oxygen drops to zero due to unfavorable process management.

**Antifoam agent**
A chemical used for preventing foam formation in the bioreactor. Antifoam agents, which can be silicone fluids, petroleum-based oils, or a special blend, have surfactant properties and increase the tendency of a foam to collapse. Excess antifoam agent can have a negative impact on the gas-transfer rate.

**Antifoam sensor**
See Foam sensor

**Aseptic technique**
This refers to handling cultures in a way that minimizes contamination with other species of microorganisms when preparing the strain, inoculation, taking samples, etc.

**Autotrophic**
Autotrophy is when the carbon required for the cellular building blocks is provided by carbon dioxide fixation.

## B

**Baffles**
Flat blades (usually 3 or 4) inside the stirred tank that dissipate energy. Baffles help generate a turbulent flow, provide ideal mixing for the liquid phase, and break down the gas bubbles in the microbial bioprocess caused by flat blade Rushton impellers and the sparger.

**Base unit**
The central component of the bioreactor consisting of the controller, sensors and actuators or that controls, i.e., regulates, process variables. A given base unit may be responsible for one or more culture vessels (see Parallel bioreactor).

**Batch culture**
A mode of operation in a closed system. All of the nutrients and additives are present in the medium before the bioprocess begins and are then depleted over the course of the bioprocess. Nothing is added to the system apart from gas, pH correcting reagents and antifoam agents.

**Benchtop bioreactor**
This term typically refers to bioreactors with working volumes between 0.1 and 10 liters.

**Biomass**
This term refers to the material mass of living organisms. In the context of a bioprocess, biomass refers to the quantity of organisms in a culture, and may be expressed directly by measurements such as dry weight, wet weight, total cell counts, or viable cell counts, or indirectly by measuring properties such as optical density and applying an appropriate correlation.

**Bioreactor**
A system consisting of a base unit and one or more culture vessels, to which additional sensors and actuators can be connected as peripherals. A bioreactor makes it possible to control and regulate a bioprocess, thus providing optimum growth conditions for microorganisms. As a bioprocess is not necessarily a fermentation, and the once common term "fermenter" is gradually falling from use.

## C

**Calibration**
A method that takes known reference points and correlates them to those of a sensor for the purpose of adjusting the sensor within a known range of tolerances. Most sensor measurements drift over time, and regular calibration is recommended as a way of minimizing this source of inaccuracy. Calibration frequency varies according to the type of sensor, the reference standards, and the effect of the sterilization process. Some sensors also age more quickly than others and can reach a point where they can no longer be calibrated. Depending on the sensor, calibration can be done directly from the control unit of the bioreactor, using a separate device, or by a qualified technician from the manufacturer.

**Carbon source**
Typically a carbohydrate such as glucose, which the organism utilizes as part of a metabolic process for biomass production and

growth. The choice of carbon source is a very important aspect of the bioprocess, as it influences whether by-products will be formed (or not). This is the case with *E. coli*, which does not produce acetate on glycerol, but does when it grows on glucose. Changing the carbon source can also trigger production of a specific protein, as happens when the carbon source is switched from glucose to methanol during *Pichia pastoris* cultivation.

**Cascade**
This describes a control method that the bioreactor uses for regulating the variation of several parameters within a limited range. An example of an application for this is $pO_2$ control, which can take parameters such as stirrer speed, gas composition, pressure and gassing rate into account.

**Catalyst**
A substance that lowers activation energy without being consumed by the reaction. In the context of a cell, catalysts are mostly enzymes.

**Cell culture**
A generic term for the culture of mammalian or insect cells.

**Cell proliferation**
Cell proliferation refers to (rapid) cell growth and division.

**Certification**
A collection of documents and test results verifying that the bioreactor has been manufactured and tested according to corresponding standards. This is often required as part of a more comprehensive validation process.

**Chemotrophic**
An organism is chemotrophic if it derives its energy from organic compounds.

**CHO cells**
An immortalized cell line derived from the Chinese hamster ovary and used in cell biology and biotechnology for producing recombinant proteins such as therapeutic antibodies.

**Cleaning-in-place (CIP)**
In classic applications, this is performed in relatively large, *in situ* sterilizable bioreactors where disassembly and manual cleaning would be impractical. With the advent of the LabCIP for the Labfors 5, this technology has now entered the world of small-scale bioreactors as well. CIP involves pumping a variety of cleaning solutions through the bioreactor and its pipe and tubing lines for the purpose of cleaning all of the surfaces that have been in contact with the product. CIP systems can be mobile or integrated directly into the reactor.

**Continuous culture**
This describes a method in which the amount of medium pumped into a system after a batch phase is the same as the amount discharged. Microbial growth in this case depends on the flow rate selected and the concentration of nutrients in the feed, a configuration known as chemostat, in which all parameters, including biomass concentration, are kept constant.

**Crabtree effect**
This metabolic phenomenon, which was first discovered in baker's yeast *(Saccharomyces cerevisae)*, is an overflow reaction producing ethanol when too much glucose is available under aerobic conditions – hence it is also called the glucose effect. Because the effect represses respiratory genes, the pyruvate intermediate cannot be oxidized via the citric acid cycle and the electron transport chain, but is instead reduced to ethanol, which significantly lowers energy yields. As a result, the Crabtree effect is also economically relevant: yeast needs to be propagated in a way that circumvents the Crabtree effect, thereby making the process more cost-effective.

**Culture**
All of the biomass grown in the bioreactor, usually in a liquid medium.

**Culture vessel**
The culture vessel is typically made of borosilicate glass or stainless steel, and is sealed by a cover plate with a sealing ring. The bottom of the culture vessel can either be flat or round. Those with a working volume greater than 10 L are double-walled; the temperature is controlled by means of a liquid or vapor circulating in the double jacket. The size and proportion of the culture vessel is a key parameter that characterizes certain bioprocesses (and thus bioreactor specifications).

## D

**Die-off phase**
After the stationary phase, the die-off rate becomes greater than the growth rate, resulting in period known as the die-off phase, which is characterized by a drop in the number of viable microorganisms.

**Dissolved oxygen concentration**
The dissolved oxygen concentration is a key parameter for aerobic bioprocesses, since only oxygen that is dissolved in the culture medium is available to the microorganisms. This is why bioreactors have a specific sensor for measuring the partial pressure of oxygen ($pO_2$, also sometimes referred to as DO). This value is generally indicated relative to the maximum achievable partial pressure of oxygen, which is why it usually ranges between 0% and 100%. It is occasionally indicated as an absolute value (in mbar, etc.).

**DO**
Abbreviation for dissolved oxygen. See Dissolved oxygen concentration

**Double jacket**
Many culture vessels are double-walled. Water is circulated within this double jacket for heating or cooling purposes. The jacket has an inlet and an outlet, and can be used as either a closed or an open circulation system.

**Downstream processing**
This refers to product processing, which follows the actual bioprocess.

**Drift**
The quality of measured values can change when sensors are used over a longer period. The measured value of a pH sensor, for example, can change during a relatively long bioprocess. Performing a readjustment during the bioprocess, a process known as product calibration, can counteract drift, ensuring continued precise measurements and accurate control.

**Dry bath**
See Heating block

**Dry cell mass**
Because the liquid content of microorganisms can vary depending on the conditions, dry biomass is used for determining quantities precisely. This is done by taking a known volume of sample, possibly washing it in a defined buffer, drying it in an oven and then weighing it. Correlations can be derived between dry biomass concentration determined in this way and the optical density or turbidity, as well as to the total or living cell count.

## E

**Electrode**
See Sensor

**Error**
In control technology, error refers to the difference between the target value and the actual value of a process variable. The error allows the controller to calculate the degree to which it needs to control its actuators in order to achieve the predefined target value.

**Exhaust analyzer**
This device determines the concentration of one or more gases in the waste gas from a culture, allowing researchers to draw indirect conclusions about the metabolic state of the culture. The concentrations of oxygen and carbon dioxide are typically measured for aerobic processes. In other bioprocesses, such as cultivation of *Pichia pastoris,* the reactor can use the concentration of methanol present as an indirect indicator of the metabolite concentration in the culture medium.

**Exponential phase**
See Log phase

## F

**Fed-batch culture**
Feeding nutrients or supplements into a batch culture can improve the biomass yield and/or the production of secondary metabolites or recombinant proteins.

**Fermentation**
This literally means the enzymatic conversion of organic materials into acids, gases or alcohols by microorganisms. Fermentation derives from the Latin word *fermentum* and is frequently used incorrectly as a synonym for bioprocesses involving microorganisms, cells or enzymes.

**Fermenter**
See Bioreactor

**Foam**
Sparger bubbles that reach the surface and proteins dissolved in the culture medium can cause foam to form during the bioprocess. Addition of a liquid antifoam agent and/or the use of a mechanical foam breaker are helpful here.

**Foam sensor**
A conductivity sensor which is used to detect foam above the liquid phase of a culture. The sensor is coated with an insulating material from which only the uncovered tip protrudes. If foam is detected, the usual response is to add an antifoam agent using a peristaltic pump with a programmed time lag. The purpose of the time lag is to keep from adding too much.

## G

**Gas mix/composition**
While controlling the gas composition is most often done when culturing animal cells, it can be applied to microbial bioprocesses as well. Depending on the configuration, bioreactors are equipped with a combination of mass flow controllers and/or magnetic valves, allowing researchers to introduce any blend of air, oxygen, nitrogen or carbon dioxide gases they wish. In this way, the gas composition also serves as an actuator for regulating the dissolved oxygen concentration. Bioreactors for cultivating animal cells are particularly likely to have an additional gas line for carbon dioxide, which is used in place of a liquid acid solution for controlling the pH.

## H

**Headspace**
A portion of the total volume of a bioreactor vessel, typically 25 – 30% of the total volume. The reason the bioreactor headspace is not filled with the culture liquid is to allow enough room for gas transfer and any foam that may form.

**Heating block**
Normally made of aluminum, a heating block fits snugly around its culture vessel to ensure good heat transfer. A heating element is built into the heating block for increasing the temperature of the liquid phase of the bioreactor. For cooling purposes, a coolant (such as water from a connected chiller) circulates through cooling loops in the block to lower the temperature. Heating blocks are used with single-walled culture vessels and are easier to handle than the combination of heating sleeve and cold finger.

**Heterotrophic**
An organism is heterotrophic if it obtains its carbon from organic carbon compounds.

**HEK cells**
Human embryonic kidney cells, a cell line used especially for virus production.

## I

**Impeller**
A rotor with a number of attached blades used for stirring the contents of a bioreactor. A number of different impeller designs are common: microbial bioprocesses frequently use Rushton impellers, whereas cell culture processes usually involve marine impellors with angled blades. The latter are especially good for gentle, low-shear mixing, even at low speeds, whereas Rushton impellers are used at higher speeds and do a very good job of mixing materials thoroughly.

**Inoculation ports**
A special inlet providing an aseptic route for introducing the inoculum into the bioreactor. In its most common form, an inoculation port will consist of a silicone membrane pierced by a needle or syringe once the surrounding area has been sterilized using ethanol, a flame, or another means.

**Inoculum**
The quantity of biomass added to a bioreactor at the start of a bioprocess. Ideally, the viable cell count of the inoculum should be high, and it should be in the logarithmic growth phase. A typical inoculum volume is 5 – 10% of the working volume.

***In situ* sterilizable (ISS) bioreactor**
See Sterilization-in-place (SIP).

**Instrumentation**
The measurement and control elements of a bioreactor. A typical control loop would have a sensor (possibly with a signal amplifier), a local display, and actuators such as valves or pumps.

## K

**$k_La$ (volumetric mass transfer coefficient)**
A measurement of oxygen transfer efficiency. The $k_La$ depends on vessel geometry, sparger design, power input from the stirrer plus the number and design of the baffles. There are many different ways of measuring $k_La$, but the results can vary greatly. As it is commonly defined, $k_La$ is expressed in $h^{-1}$. Stirred tank reactors typically reach values of 100 and 200 $h^{-1}$. See Oxygen transfer rate.

## L

**Laboratory scale**
This typically refers to bioreactors with working volumes between 0.1 L and 10 L.

**Lag phase**
A phase immediately following inoculation of the medium, this is when the microorganisms adapt to the prevailing operating conditions and prepare for the exponential growth phase. The lag phase can last from 1 – 2 hours for microbial cultures to 24 hours or more for cell cultures.

**Log phase**
The phase in which microorganisms grow at the maximum possible rate (i.e., exponential growth). In batch processes where all of the nutrients are available in excess, cell division is not limited by any factors whatsoever and the specific growth rate is at its maximum. In a fed-batch process in which a nutrient solution is added exponentially but at a lower rate, the growth rate will be constant, albeit lower. In both cases, this phase may be accompanied by the development of heat, acidification, rapid depletion of nutrients and oxygen limitation – this is especially true in bacterial and yeast cultures. Log phases can last from a few hours to many days, depending on the organism cultivated.

## M

**Maillard reaction**
Through this non-enzymatic browning reaction, amino acids or peptides react with reducing substances such as sugars to form new, mostly delicious smelling compounds. The Maillard reaction is essential for the cooking and baking process and in the food industry, since this is where the color and flavor of roasted, baked or fried food stems from. When preparing a bioprocess, this reaction takes place when media containing proteins and sugar are autoclaved, which is why these components should be autoclaved separately.

**Mass flow controller (MFC)**
A device that precisely measures and controls the volumetric gas flow, sends the actual value electronically to the bioreactor and retrieves target values from the bioreactor. If the bioreactor has multiple gas inputs, they can be connected via clocked magnetic valves and one MFC, or equipped with an individual MFC in order to produce different mixes of gas. That makes MFCs ideal for controlling the dissolved oxygen concentration.

## O

**Oxygen supplementation**
This procedure involves the addition of pure oxygen as a way of elevating the cell count by increasing the percentage of oxygen in the gas (usually air) going into the bioreactor. A second gas line equipped with a mass flow controller is one example of how this could be set up.

**Oxygen transfer rate**
The oxygen transfer rate (OTR) defines the rate of oxygen transfer into a bioreactor culture medium from gas bubbles. The size of the bubbles, their retention time in the reactor and the level of oxygen saturation at a given temperature are critical factors in the oxygen transfer rate. Mathematically, the OTR can be defined in terms of $k_La$ (the volumetric mass transfer coefficient), the concentration of oxygen in the introduced gas, and the equilibrium concentration. Typical OTR values for a stirred tank reactor lie between 50 and 100 mmol $L^{-1}h^{-1}$.

## P

**Parallel bioreactor**
Parallel bioreactors consist of two or more bioreactors operating in groups and equipped with common measurement and control systems. Depending on the model, these are simplified, highly miniaturized stirred tank reactors capable of performing only the most standard functions of a (generally stirred tank) bioreactor, such as pH measurement and mixing. Other models, such as the Multifors 2 utilize the same technology as large-scale bioreactors – despite their smaller working volume – and, like these, can be extended with powerful options such as additional sensors. General selling points are how easy they are to use and how quickly they can run bioprocesses. Applications of parallel bioreactors include process optimization, statistical analysis, scaling down large-scale reactors, and studies on the impact of varying individual parameters.

**Peristaltic pump**
A pump driven by a stepper motor with rollers mounted in a fixed housing. Suitable tubing is squeezed between the rollers and the housing to generate peristaltic action and move liquids (e.g., from a reservoir bottle to the bioreactor vessel).

**Phototrophic**
Organisms are phototrophic if they derive their energy from sunlight.

**PID controller**
A controller consisting of a proportional, integral and derivative component and used for achieving a target value as quickly as possible without going over. Applications include cascades.

**Pilot scale**
Typical pilot-scale working volumes range between 20 and 500 L, and are used for the testing and optimizing involved in scaling up production processes.

**$pO_2$**
See Dissolved oxygen concentration

**Port**
This is an opening in the bioreactor vessel (it can be in the top or sides or even at the bottom) that accommodates accessories such as electrodes, sampling systems, or spargers. The port is sealed by an O-ring seal or a flat membrane. When a port is not in use, it must be sealed with a plug to prevent contamination. The number and size of each port are important factors in bioreactor design, as they determine what can be added. Common sizes are 10 mm, 12 mm/13.5 PG thread, 19 mm and 25 mm.

**Preculture**
See Starter culture

**Primary culture**
A culture consisting of cells, tissues or organs taken directly from an organism. After the initial passaging, the primary culture is referred to as a cell line.

**Probe**
See Sensor

**Production scale**
This typically refers to bioreactors larger than 500 L and can range up to several cubic meters in volume. With the advent of high-quality recombinant proteins, however, even a 10 L bioreactor could be considered production scale.

**Pt100 (Sensor)**
See Temperature sensor

## R

**Recombinant proteins**
A protein expressed by an animal or bacterial cell following a process of selective genetic modification. The location where the protein is expressed determines the extraction and purification strategy. If multiple copies are present of the gene to be expressed or if a gene that disrupts cell metabolism has been deleted, this can focus the cells' metabolic processes towards production of the recombinant protein.

**Respiratory quotient (RQ)**
The RQ is calculated from the ratio of the amount of oxygen consumed by a culture to the amount of carbon dioxide produced, and serves as an indicator of the metabolic state of a culture. The RQ can be used to control the feed rate of the carbon source and to improve the balance between biomass production and metabolite formation. Modern bioprocess software comes standard with soft sensors that allow researchers to calculate the RQ in real time.

**Rotameter**
A needle valve for manually controlling the gas volume flow. Flow is indicated by a steel or plastic ball floating on the inlet gas inside a tube of increasing width with a calibration scale. The scale is used for reading the gas flow, with a typical error of up to 10%, especially at at low flow rates. Gases of different densities need different calibration scales. Because the measured values cannot be transferred to the controller and the gas flow setting can only be changed manually, gas lines with rotameters cannot be used for automatic control of dissolved oxygen concentration.

**Rushton impeller**
See Impeller

## S

**Sampling device/system**
See Super safe sampler

**SCADA**
SCADA stands for supervisory control and data acquisition. Generally speaking, a SCADA is a computer system that collects and analyzes real-time data. In the field of biotechnology, SCADA is now considered to be the standard for optimizing and controlling bioprocesses.

**Scale down**
This allows key parameters of a large-scale bioprocess to be applied to small vessels through direct extrapolation.

**Scale up**
This refers to taking a process from laboratory-scale bioreactors to large production units in several steps. At each stage, factors such as handling, energy input, gassing, mixing time, stirring speed and temperature control may have to be addressed and modified according to size.

**Sensor**
A measurement device (such as a pH sensor) inserted into the bioreactor and in direct contact with the culture medium, or an external instrument that takes indirect measurements (such as an exhaust analyzer).

**Sf21**
An immortalized line of insect cells from the ovary cells of *Spodoptera frugiperda* (a species of moth). These cells are robust and withstand shear stresses well. In cell cultures they are either adherent or grow in a suspension, and are used for producing recombinant proteins.

**Shear stress/sensitivity**
Friction arises in liquids, where it transmits force. In a bioreactor, shear stress is generated by constant stirring and gassing, and can destroy cells. Mammalian cells, which have only a thin cell membrane rather than a stable cell wall, are far more sensitive to shear forces than microorganisms.

**Single-walled culture vessel**
A single-walled culture vessel has the advantage of being lighter and therefore easier to handle. Unlike double-walled vessels, the base unit of a single-walled vessel has to have a heating block or a sleeve and cold finger in order to control the temperature.

**Sparger**
A tube extending toward the bottom of a bioreactor to just below the impeller. A sparger has tiny holes at the (top or bottom) end for the purpose of introducing a gas, typically air. Spargers are usually curved, ring-shaped or L-shaped.

**Spin filter**
An insert used especially in bioreactors for animal cell cultures. The spin filter is normally attached to the drive shaft in such a way that culture liquid can be withdrawn continuously without removing any cells from the reactor. A mesh, typically 10–30 µm in size, forms a cage through which the culture medium passes along with products and metabolites – cells, however, cannot pass. Researchers can use spin filters to continuously harvest the product and feed in fresh culture medium while allowing the slow-growing cells used for producing biopharmaceuticals to remain in the bioreactor for extended periods of time.

**Stationary phase**
A phase in a typical growth cycle for a batch culture during which nutrients are depleted over time. During this phase cells die at roughly the same rate as they are being produced by division, leading to a stagnant cell count. Given the lack of nutrients and the accumulation of toxic by-products, this equilibrium cannot last, and the bioprocess transitions over to the die-off phase.

**Starter culture**
This describes the production of a quantity of biomass under aseptic conditions to provide an inoculum for a larger bioreactor. This frequently involves several stages, often in a shaker incubator, in order to produce a sufficiently large inoculum. If, for example, the starter culture is being made in a smaller bioreactor for use in a production bioreactor, the smaller reactor is referred to as a seed bioreactor.

**Startup phase**
See Lag phase

**Sterilization/Steam/Sanitization-in-Place (SIP)**
SIP can stand for various terms, all of which describe similar concepts while differing in their nuances. In its most basic sense, the bioreactor is connected to a steam source so that it does not need to be sterilized/sanitized in an autoclave (steam-in-place). The steam is used to sanitize the equipment, i.e., to deplete the live cell count, on site (sanitization-in-place). If the SIP process has been validated for a given bioreactor and shown to result in sterility, the term used then is sterilization-in-place.

Culture vessels that can be sterilized *in situ* are usually made of (316L) stainless steel and can be sterilized using the in-house steam supply or an on-site steam generator built into the base unit (*in situ*). Peripheral components such as filters, sampling devices and the drive seal are sterilized during this process as well. ISS bioreactors frequently have working volumes over 10 L. SIP is used for more than just stainless steel bioreactors, however: solutions are now available for benchtop bioreactors as well, such as the LabCIP for the Labfors 5. These solutions do not employ steam, of course – they instead rely on chemical methods, as the use of elevated pressure would risk damaging glass culture vessels.

**Stirred tank reactor**
A stirred tank reactor is a specific type of bioreactor that mixes the liquid phase using a stirrer and impellors. This is the traditional design used for the first cultures for antibiotic production in the 1940s.

**Substrate**
In a bioprocess, this usually refers to the carbon source added as a feed.

**Super Safe Sampler**
Sampling devices such as the INFORS HT Super Safe Sampler allow scientists to sample small amounts of a culture from a bioreactor without contamination or dead volume.

**Suspension cells**
Unlike adherent cells, cells in a suspension culture do not require direct contact with the surface – they instead float freely in the nutrient medium like microorganisms.

## T

**Temperature sensor**
Usually a platinum resistance electrode providing precise temperature measurements both in the bioreactor vessel and connected peripheral equipment. A Pt100 resistor relates changes in electrical resistance to temperature, whereby 0°C has a value of 100 Ω.

**Total volume**
The total internal volume of a culture vessel, including the headspace.

**Transfer line**
A connection for moving a culture (without contaminating it) from one vessel to another by means of either a rigid or flexible tube, ideally designed as an element that can be sterilized in place.

## U

**User requirement specifications (URS)**
A detailed list of user requirements for a bioreactor; the manufacture uses the URS for designing the features of the bioreactor.

**Upstream processing (USP)**
This branch of bioprocess engineering comprises all methods for cultivating and optimizing organisms for the actual bioprocess. This includes cell isolation, cultivation, expansion, and development/optimization of the inoculum, media and processes; genetic manipulation may be used here as well.

## V

**Validation**
A procedure ensuring a process meets approved standards of productivity, reproducibility and safety. Documentation certifying equipment such as bioreactors is an essential part of this process.

**Vessel volumes per minute (VVM)**
A value for gas flow rate into a vessel based on its working volume in liters, allowing for comparisons and/or for scaling processes up and down. Bioreactors for microbial bioprocesses are typically designed for VVMs up to 2 L $L^{-1}$ $min^{-1}$ (simplified to 2 $min^{-1}$). This means that a bioreactor with a working volume of 10 L would be designed for a maximum gas volume 10 L * 2 $min^{-1}$ = 20 L $min^{-1}$. Animal cell cultures, by contrast, generally need much lower VVMs of up to 0.1 $min^{-1}$.

**Volumetric mass transfer coefficient**
See $k_La$

**vvm**
See Vessel volumes per minute

## W

**Working volume**
This typically amounts to two-thirds of the total volume of the bioreactor. For animal cell cultures, where gas transfer from the headspace requires a larger ratio of liquid surface area to culture volume, the working volume often consists of 50% or less of the total reactor volume.

# Notes


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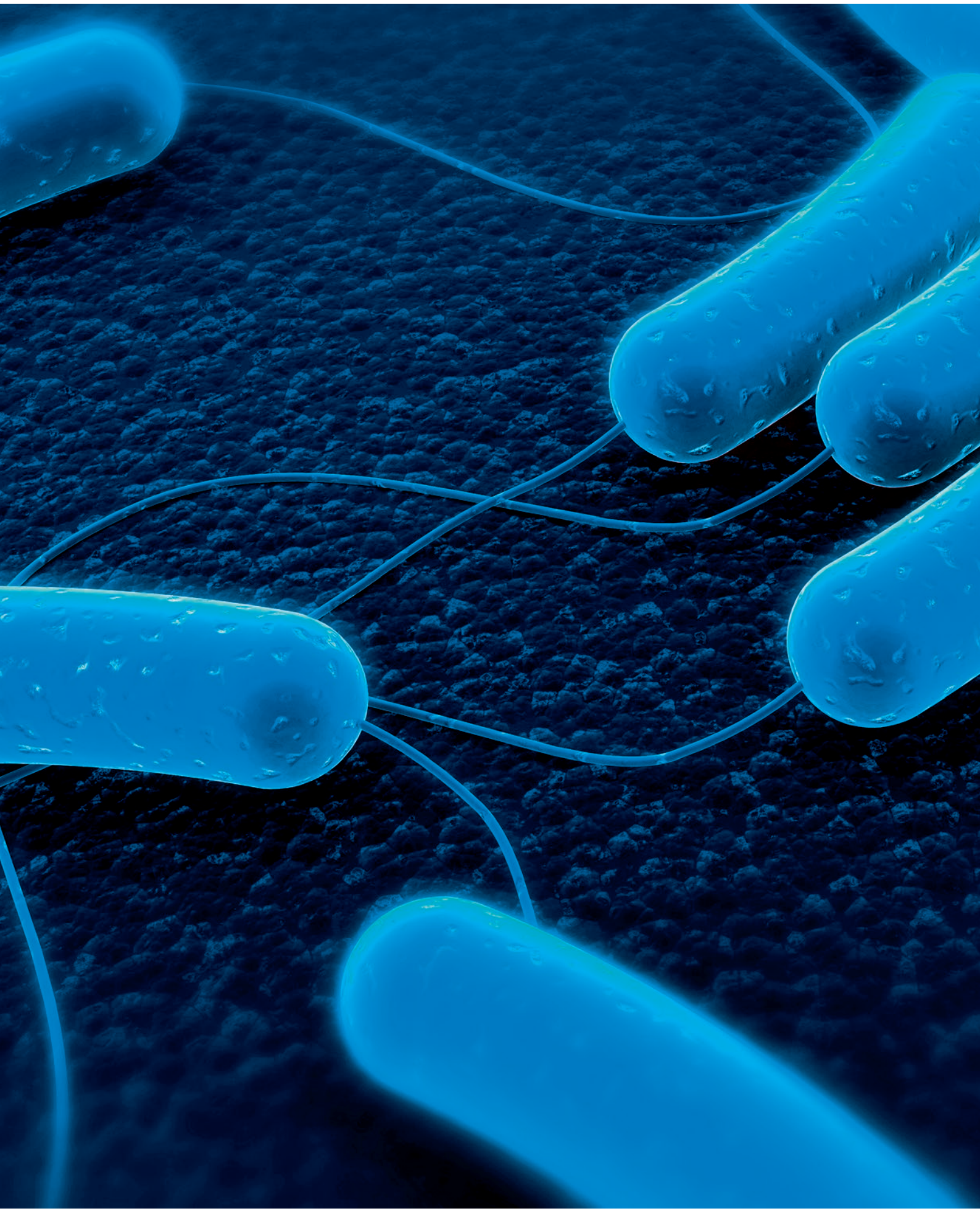

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