The Cookbook.

BASIC KNOWLEDGE, RECIPES AND STRATEGIES FOR MICROBIAL BIOPROCESSES.

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Contents

Introduction

So here we are.

Finally, you are standing in the laboratory in front of a bioreactor and are highly motivated to start all sorts of experiments with the device.

Speaking of starting: How do you even turn such a bioreactor on? And then? These questions can be settled with a glance in the manual, but how does it basically function, and are there any additional tips for preparation and implementation? Maybe one or another experiment has already been done. Couldn't you just simply "follow the recipes" to begin with, instead of losing yourself in new creations immediately?

We have also thought about this. No one is born a bioprocessing master.

With this guide, we would like to provide you with an orientation and introduce you to the bioprocess step-by-step. We will explain how a bioreactor works and what happens when microorganisms are brought together with nutrients. We will look at the basics of the bioprocess and the strategies with which a bioprocess can be guided to the desired success. Also, we take a look at the usual participants, the microorganisms. At this point, it should be noted that this document refers to bioreactors with 0.5 L to 10 L of working volume, used for aerobic, microbial bioprocesses, although some explanations may also apply to smaller or larger experiments.

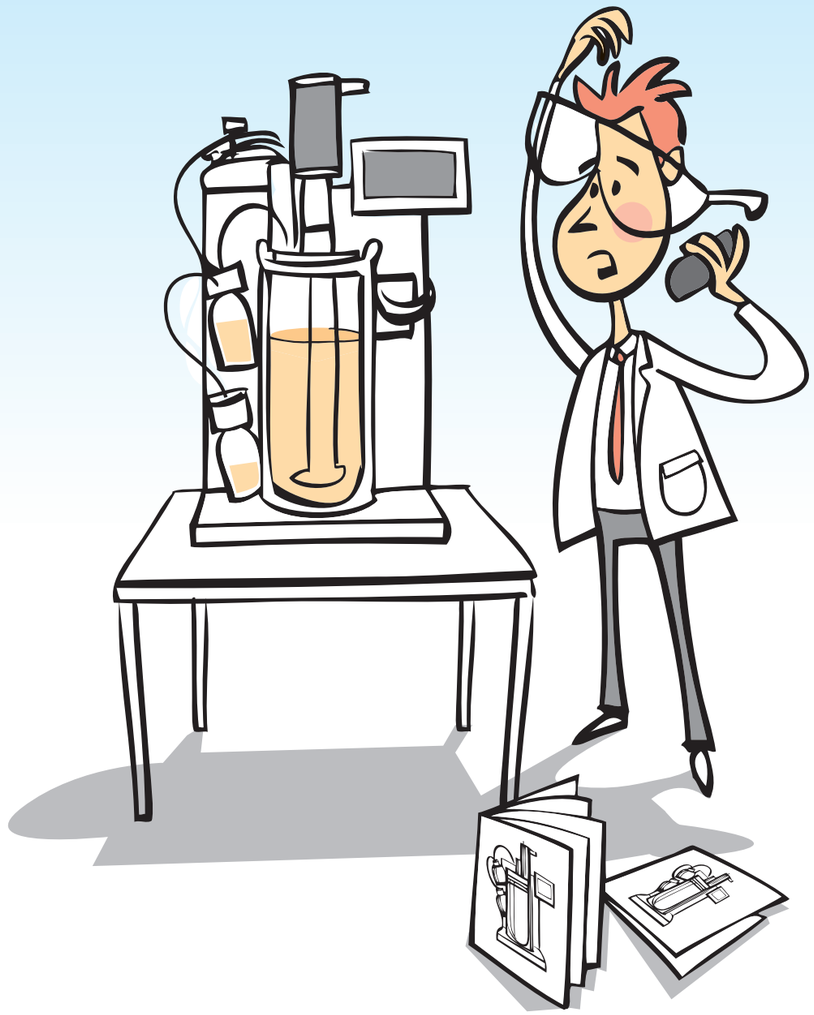
As in all good cookbooks, recipes are found in this document.

These are recipes which are exclusively designed for the best possible cultivation of microorganisms, in order to avoid any misunderstanding with regard to confusion with a conventional cookbook. Our recipes provide clear instructions, an overview of the expected outcome and some tips as well to help achieve this precise goal, as desired.

Of course, everything cannot be achieved with a small guide. The bioprocess itself is only one step preceded by the processes involved in the preparation of the microorganisms, culture media, and adjustment agents. There are many subsequent procedures where harvesting and further processing of the bioprocess results are the focus. We hope, however, that we can facilitate your move into bioprocess technology with this "cookbook", arouse your curiosity, and take away the fear of supposedly "stupid" questions – which we have already asked ourselves in the writing.

We are looking forward to feedback which will improve and expand the cookbook. We are pleased to receive comments and constructive criticism at marketing@infors-ht.com.

Have fun reading!



1. Bioreactor basics

1.1 Why a bioreactor?

At the beginning of each bioprocess is an idea. This may be to propagate cells in order to use them to manufacture proteins or certain metabolites generated by the microbes or microorganisms during the bioprocess are of interest.

To bring this idea to reality, you need a recipe, the right ingredients and well-specified kitchen equipment. It should not only mix well but also monitor the temperature and stirring speed, add one or another ingredient, and keep the environment sterile within while all this happens. In our case, this kitchen equipment is a bioreactor, or more precisely, a stirred tank reactor (STR).

A bioreactor is a multi-tasking device which helps the researcher achieve the desired bioprocess results by means of automatic monitoring and regulation of the selected process conditions. The bioreactor provides an ideal environment in which the micro-organisms can focus on what they are supposed to do: proliferate.

Like employees in the laboratory, microorganisms only provide consistently good work if the conditions are right: it should be neither too hot nor too cold and provide enough good food and fresh air. Applied to the bioreactor, this means maintaining pH and temperature, ensuring enough oxygen supply or other gases, and the addition of nutrients now and then by the user according to an automated configuration.

1.2 Setup and components

In view of the abundance of functions that a bioreactor must perform, the question arises as to how it can accomplish this? Which components does it need for this purpose? How do you know what the conditions in the bioreactor currently are and how can they be corrected? Last of all, do not lose sight of the goal: how can the data captured during a batch (bioprocess, see glossary) be displayed, structured, saved and evaluated in a meaningful way?

The most important process parameters and the mechanisms for regulating them are described in chapter 1.3. In chapter 4 we show in detail how a bioreactor is assembled. But here, we are providing a general overview of the technology, which consists of two important systems, the device itself and the SCADA (*Supervisory Control and Data Acquisition*).

Functions of a bioreactor, e.g., Minifors 2
- Low level control of sensors and actuators
- PID regulator (closed-loop controller)
- Cascades, i.e., variation of several parameters
- Operating unit for local inputs
- Depending on the model: simple imaging on the operating unit

Functions of a SCADA software, e.g., eve®
- Central collection site for all bioprocess information
- Monitoring and control of several bioreactors
- Interface to superordinate analyzers, e.g., mass spectrometers
- Planning of complex batch strategies
- Depending on the version: Integration of Design of Experiment (DoE), Process Analytical Technology (PAT)

A powerful controller manages the first stage of the process control in the bioreactor. It communicates 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. On the other hand, with actuators such as heating elements, pumps or valves, the bioreactor can interact to make corrections, if necessary. The digitalization also does not stop at bioprocess technology, a multitude of components are already addressed via digital bus systems such as Modbus. However, analogue interfaces are also absolutely necessary and are indispensable for the proven Pt100 temperature sensors, for example. The controller is also responsible for precisely maintaining all target values communicated to it. To do this, it calculates an output using a PID regulator (*proportional-integral-derivative controller*), based on the actual values versus set points. It controls also the actuators in order to set the target value as effectively and smoothly as possible.

The bioreactor also has a local operating unit available (also known as an HMI, *Human Machine Interface*). The operating unit allows the user - as its name already suggests - to interact directly with the bioreactor. Today, most of the operating units in modern bioreactors are touchscreens. With the operating unit, the user can adjust process parameters for a batch (bioprocess, see glossary) and switch control loops on and off or otherwise configure them. The interface can also be configured for connecting a SCADA software.

Nowadays, the results generated in the bioreactor should be collected and evaluated as centrally as possible. Only in this way can modern algorithms aimed at Big Data analysis be effectively implemented in order to generate more information and to improve the understanding of the process. This task is carried out by a SCADA software. With INFORS HT, this is eve®, the platform software for bioprocesses. It establishes contact with the bioreactors via a local or global network on the basis of standardized protocols like OPC UA. In the first step, all data from the bioreactor can be read, without major interventions of the user, and stored centrally in order to evaluate and compare it either individually or together with other batch data.

Here, ideas for new experiments come quickly, even with complex batch strategies, if applicable. These can be planned conveniently in eve® – certainly more comfortably with coffee in the office rather than in a lab coat in front of the bioreactor – and later used to control the bioreactor, which should occur automatically in the ideal case. Also, all information accompanying the batch in eve® can be centralized, including information on the microorganisms used, their favorite culture medium or the measurement data for the samples which were taken from the bioreactor (offline analysis).

Besides, the powerful bioprocess platform software eve® also integrates many components of the so-called "bioreactor enviroment". These include tools for process optimization using the Design of Experiment (DoE) or powerful soft(ware) sensors, which can be used, at the same time, to directly compute additional information from the batch process parameters and even to regulate them. 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.

Especially when the bioreactor and SCADA software are perfectly matched, there are so many possibilities that their description would go beyond the scope of this cookbook. If you are one of the lucky owners of both systems, have the courage to try it out – you will be able to see how ridiculously easy it is for you to benefit from the many features. Otherwise, you are welcome to browse through our website to take a look at the eve® tutorials or to request a demo version.

1.3 Functionality

With a recipe, the skilled amateur cook knows which containers and equipment he or she must use in order to achieve the desired result with the specified ingredients. Bioreactors behave in a similar manner. The practiced user puts the reactor together with the required components in such a way that the conditions specified in the recipe for the microorganisms are achieved as accurately as possible. But what are the conditions here? And how do you achieve a precise compliance? It is precisely these questions that will be clarified in the next section.

Stirred or shaken?
Continuous mixing is important for all bioprocesses in an STR. If the nutrients in the bioreactor are insufficiently dispersed, there will be significant local deviations from the ideal conditions in the bioreactor. So the pH could be too acidic, or the microorganisms might not be adequately supplied with nutrients. Such deviations not only reduce the efficiency of the planned bioprocess, but can also promote genetic modification through the selection stress acting on the microorganisms, which leads to a permanently altered and, as a rule, undesirable behavior of the microorganisms. An additional aspect is the temperature distribution. Without uniform stirring, the microorganisms at the vessel's edge will literally be boiled, while those in the middle get cold feet. Anyone who has ever heated soup in the microwave and then eaten it with great anticipation without stirring it will have had a similar experience.

The typical stirring speed varies depending on the cultivated organism. For bacteria, yeasts and fungi, this is usually 500 min^{-1} to 1500 min^{-1}. For animal, plant and insect cells – which will not be further explained here in detail – it is 30 min^{-1} to 300 min^{-1}. The adjustment of the stirring rate should be selected depending on the cells to be cultured as these react differently to shear stress, i.e., the mechanical stress caused by the stirring. By changing the stirring speed, the availability of oxygen can also be varied during the bioprocess, thus ensuring optimal growth of the cells.

Temperature measurement and control
All microorganisms have enzymes which feel best at a certain temperature – as well as a pH range. If this is exceeded or undershot, the cells do not grow as well since metabolic activities and growth largely depend on enzymes and thus on catalytically active proteins. In the worst case, these are even destroyed by the environmental conditions.

To determine the temperature, a platinum sensing resistor, a so-called Pt100 sensor, is used in the bioreactor. It has a resistance of 100 Ω at 0 °C, and may well cover the expected biologically relevant measurement range with appropriate calibration.

The control range is usually from +5 °C to +50 °C above room temperature, while typical temperatures at which organic processes operate are from 20 °C to 50 °C. If processing is to be done at temperatures near or below room temperature, then active cooling, by means of a recirculating cooler, is required. In the majority of bioprocesses, the temperature should remain constant during the entire cultivation. However, for some products, such as penicillin or recombinant proteins (i.e., bioengineered proteins using genetically modified organisms), a temperature change at the end of the growth phase activates important genes for the formation of the product and is therefore beneficial.

There are several ways to regulate the temperature by means of a heating and/or cooling circuit:
- Electric heating block with built-in cooling coil (Minifors 2 and Multifors 2)
- Heating pad made of silicone, which is wrapped around the culture vessel after sterilization (Labfors 5)
- Double jacket in which water is circulated. The temperature is adjusted by an electric heater or steam and a solenoid valve for the intake of cooling water (Labfors 5, Techfors-S and Techfors).

pH measurement and control
The measurement and control of pH is very important in most bioprocesses as the microorganisms themselves often contribute to a change. Culture media for microorganisms commonly include buffer substances, i.e., substances that dampen any drastic alterations of pH when an acid or a base is added. If this is not the case, the pH often changes abruptly, causing the growth conditions for the microorganisms to change significantly – usually with fatal consequences. If the pH is outside of the preferred range, the microorganisms will no longer multiply or even die off. A strong change of pH can also cause other unwanted metabolic processes and contribute to an inhibition of the corresponding microbes. An example of this is lactic acid fermentation, which has been used since Neolithic times by humans to preserve food. This is used to produce fermented milk products, pickled vegetables and sourdough breads which are acidified by the lactic acid excretion of the appropriate bacteria. This leads in turn to a growth inhibition of other microorganisms beginning at pH 4.5, and with a further drop in pH to an inhibition of the lactic acid bacteria themselves. Bacteria, yeasts and fungi usually require a pH between 4.5 and 7.0, animal cells around 7.0, in the bioprocess.

Each bioreactor is equipped with a pH sensor, a single-electrode measuring cell for pH, for the measurement of pH during the bioprocess. The bioreactor can correct any deviations in the pH; for this purpose, an acid and/or an alkaline solution are 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 the alkali must be skillfully selected – if it is too high, the concentrated acid or alkali drops may damage the microorganisms before they are distributed in the bioreactor. If, on the other hand, the concentration is too low, a greater volume of the acid or alkali must be added, and the culture medium is unnecessarily diluted.

Adding nutrients
During the bioprocess, the microorganisms usually consume a multitude of nutrients. If everything is made available to the microorganisms all at once and in the following bioprocess feeding is not continued, this process is called a batch operation.

Another way to not allow nutrients to become the limiting factor is to have a nutrient solution constantly fed in during cultivation; this is called a fed-batch operation. In good growing conditions, the microorganisms double constantly, therefore following an exponential growth curve, which is why the feeding rate is ideally also exponentially increased as long as the bioreactor can support the other process parameters.

A special fed-batch process is continuous culturing in which a *steady state* arises. For example, just as much fresh culture medium is added as is discharged. Such bioprocesses are especially suitable if an oversupply of nutrients would entail an inhibition of the microorganisms. In Chapter 5 of the cookbook, the different feed strategies will be explored more in depth.

Gassing
The microorganisms are supplied with oxygen through sterile air introduced into the bioreactor. This is accompanied by thorough mixing with high stirrer speeds (up to 2000 min^{-1}). This not only contributes to the uniform distribution of substances and microorganisms in the bioreactor, but also breaks up gas bubbles which arise in the bioreactor, which allows the oxygen to be delivered more efficiently to the nutrient solution. This is important since only the oxygen dissolved in the nutrient solution is available to the microorganisms and can be absorbed through the cell surface. In return, the carbon dioxide formed by the microorganisms also re-enters the gas phase and is ultimately discharged from the tank through a sterile filter. To regulate the pH and to provide additional nutrients, pumps are available on the bioreactor which can inject appropriate adjustment agents or enriched nutrient solution into the culture vessel.

Gasses, and especially oxygen, are one of the most important components for aerobic microorganisms, without which no cell growth can occur. Various microorganisms have differing needs in this respect: aerobic bacteria need oxygen, while others prefer gas mixtures such as synthetic gas (“syngas”). Anaerobic organisms, however, can do entirely without gassing and use only inorganic and organic substances from the culture medium such as nitrate or fumarate.

At the beginning of the bioprocess, for example, a culture needs less oxygen since the growth is still progressing slowly. Later, with an increasing growth rate, much more oxygen is needed. The bioreactor ensures not only the permanent supply of the desired gas or gas mixture, but also supplies it in the right quantity at the right time. For this, the bioreactor has gas connectors that are connected to the a house supply line of pressurized air, a compressor or a gas cylinder.

The gassing rate is usually measured in liters per minute. In order to be able to provide a generic parameter transferrable to various bioreactors, the specific gassing rate is given which refers to multiples of the working volume (vessel volumes per minute, vvm) and is given in L L^{-1} min^{-1} or only min^{-1}. A typical value 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}.

However, the efficiency with which the bioreactor delivers oxygen to the culture medium can be controlled not only by changing the gassing rate. The greater the surface area of all gas bubbles in the bio-reactor, that is, the more finely distributed the gas bubbles themselves, the more efficient the transfer of oxygen from the gaseous to the liquid phase. Thus, for example, the increase in the stirring speed can lead to an improvement in the oxygen input since the stirrer further breaks up the gas bubbles and thus the surface area of all gas bubbles in the bioreactor becomes larger in total. The oxygen content can be further increased by enriching the air with pure oxygen or even gassing with pure oxygen only.

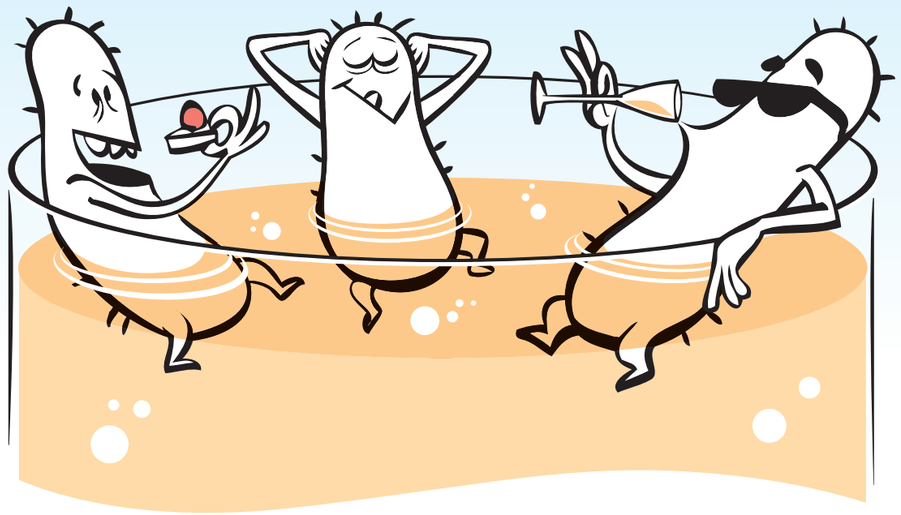
The precise regulation of pO_2 and thus the precise control of the gassing rate and the gas composition are very important since, normally, the pO_2 should not be the growth inhibiting factor for the culture. If there is insufficient control, however, the pO_2 becomes the limiting factor.

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 exhaust filter would also be blocked by the moisture so that the exit gas can no longer escape and pressure may 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.

Pressure measurement and control
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 inflated bicycle tyre. 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. Therefore, it is necessary to always ensure an unobstructed, non-pressurized exhaust line from the bioreactor, by keeping the exhaust filter dry and regularly exchanged while ensuring the integrity of the culture vessel. Unlike glass culture vessels, stainless steel bioreactors are designed for higher pressures and are suitable for pressures up to 2 bar (a well-inflated bicycle tyre) in the standard configuration. Such systems are also often equipped with pressure control which, based on a pressure sensor in the bioreactor and a proportional valve in the exhaust line, can not only measure but also actively regulate the pressure in the bioreactor.

Avoiding foam
Outside of bathtubs and the head on beer, foam is a rather unpopular side effect, especially in bioreactors. It is formed on the interface between the liquid and gas phase in the culture vessel and can quickly find its way up under the bioreactor top plate. In the worst case, it then blocks the exhaust filter, which in turn blocks the flow of gas. Therefore, most bioreactors are equipped with a system for combatting foam formation. Mechanical foam breakers in the head space are reserved for rather large stainless steel bioreactors, while anti-foam control systems based on chemical agents (e.g. PPG, struktol, silicon-based anti-foam agents) can also be found in smaller bioreactors.

A typical anti-foam control system consists of a sensor that is attached in the culture vessel at a certain height. If the foam height reaches the sensor, an antifoam agent is pumped from a bottle into the culture vessel. These anti-foam agents are active at the interface of liquid and gas and increase the tendency of the foam bubbles to collapse. In particularly stubborn cases, the foam does not immediately dissolve. In this case, the procedure is repeated after a preset time (“shot & delay” action). Caution is advised with the usage of anti-foam agent because already a minimum overdose can form a skin on the surface of the liquid, which hinders the gas exchange. Anti-foam agents also counteract an efficient oxygen input because they promote a collapse of gas bubbles in the bioreactor caused by the change in surface tension and thus reduce the surface area available for gas exchange. The selection of the appropriate agent also depends on the cultivated microogranisms because bacteria and cells react differently to certain chemicals.



2. Bioprocess basics

In order to understand what the conditions must be in a bioreactor for optimal growth of the microorganisms, it is important to know in which natural environment the organisms feel well and what they need to grow and thrive.

Depending on which carbon and energy sources are used, and where the electrons for energy transfer come from, organisms can be classified in a variety of ways. If you wish to learn more about the metabolism of microorganisms, you can find detailed information in the classics of biochemistry literature (see chapter 7). Definitions of some of the different ways of obtaining energy can be found in the glossary (see chapter 8) under the headings “phototroph”, “chemotroph”, “autotroph”, and “heterotroph”.

2.1 Microorganisms

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 pre-culture, referred to 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, in two steps maximum. The acutal biopricess typically runs in a bioreactor.

Here is a brief summary of the most well-known organisms with which microbial biopro-cesses are undertaken:

Bacteria
These 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 lovers of extreme environmental conditions, the so-called extremophiles (Latin *-phil* = loving/ori-ented). Examples are the thermophilic (which like high temperatures) or 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 of bacteria in the shaker and bioreactor:

Parameters	Temperature	Mixing	Duration of cultivation	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 %

Yeast
Yeast has the same essential requirements with regard to mixing, temperature, etc., as bacteria. Therefore, these normally do not require exceptional growth conditions because, typically, a process temperature of 30 °C and an acid pH is preferred. Good mixing, as well as an adequate supply of oxygen is essential for a good yield of yeast biomass. To counteract the strong heat generation by the yeast metabolism and to maintain an acceptable operating temperature, cooling of the process is necessary under certain circumstances.

Typical cultivation parameters for yeasts in the shaker and bioreactor:

Parameters	Temperature	Mixing	Duration of cultivation	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 %

Fungi
They often grow as fibrous, mycelial cell agglomerates which are relatively sensitive to shear. Therefore, they tend to adhere to the vessel wall or even in the head space in order to escape the shear stress. On the other hand, the very viscous culture medium, due to the fibrous cell agglomerates, requires a high power input for mixing and gassing. Therefore, it is often tricky to identify ideal operating parameters for the cultivation of fungi.

A typical bioprocess for the production of citric acid on an industrial scale is performed nowadays with *Aspergillus niger*, a filamentous fungus. Citric acid is excreted in large quantities under conditions of high glucose and oxygen content of the medium with, at the same time, very low pH and low iron concentrations.

Typical cultivation parameters for fungi in the shaker and bioreactor.

Parameters	Temperature	Mixing	Duration of cultivation	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 The bioprocess

The objective of a bioprocess is to generate more of a "product" with an additional use with the aid of cells or their components. This can be the biomass itself or merely components of cells which are then further processed, sometimes with much effort.

Enzymes, i.e., substances which can be used because of their structure as catalysts for chemical reactions, were formerly known as ferments. Thus the term fermentation is used incorrectly as a synonym for the bioprocess. Louis Pasteur, with his expression "*Fermentation is life without air*", correctly understood that fermentation is a biotic reaction with the exclusion of air. In the more recent definition, it is a breakdown of organic substances which involves a terminal electron acceptor for the production of energy which is not molecular oxygen. Strictly speaking, for this reason, acetic acid fermentation is not fermentation in the sense of the newer definition, while the production of ethanol and lactic acid, as well as some other forms of fermentation are definitely included.

Everyday examples of bioprocesses are cheese production, the generation of biogas, the operation of bioreactors of any type as well as the production of recombinant proteins and bio-similars. These examples cover the areas of food technology, agriculture, biomedical research and pharmaceutical production. Of course, there are still a plethora of other bioprocesses which are allocated to the large field of biotechnology.

By selecting the right process strategy, the bioprocess can be optimally designed and a maximum of product and time efficiency achieved. Additionally, the best possible media composition and functioning of the microorganisms can be achieved, for example, through targeted genetic manipulation. These prior steps including the actual bioprocess are called upstream processing.

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

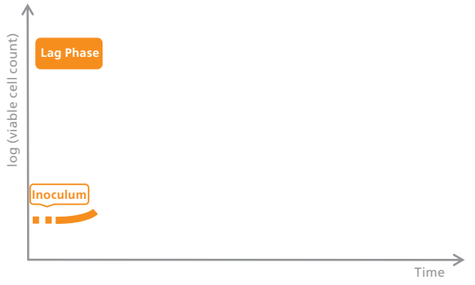


Figure 1 Schematic representation of the lag phase.
The inoculum added to the culture medium defines the starting quantity of cells. After the inoculation of the bioreactor, the living cell count increases only slowly since the organisms must still accustom themselves to the prevailing environmental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

Although there are sufficient nutrients and no metabolic waste products in the fresh medium, the microorganisms still cannot grow at full speed because they are not optimally adapted to the environment. This is achieved by sensing environmental parameters such as temperature and nutrient supply for the up-regulation of the appropriate genes, which can take some time depending on the organism. The lag phase can be shortened by starting a pre-culture in a shake flask under the same conditions (same medium, same temperature) and transferring the cells rapidly into the bioreactor during the exponential growth phase.

In the subsequent exponential phase, called the log phase, the microorganism is best 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 simply in a linear manner, but rather doubles. In other words, it is not just 2, 3, 4, 5 cells that appear, but rather 4, 8, 16, 32 cells, etc. Generally, the cells divide at the greatest possible speed, and thus the biomass grows. Now the nutrients are absorbed and metabolized at a maximum rate, which leads to increased oxygen requirements and an increased emission of carbon dioxide in the case of an aerobic bioprocess. In brief, the nutrients are used up. During this process, the bacteria also produce by-products such as organic acids or excess heat. For this, the bioreactor's broad range of tools must be used to prevent the cell growth from being impaired.

In the log phase, the increasing amount of biomass increases the content of free proteins in the medium and therefore the risk of foam formation.

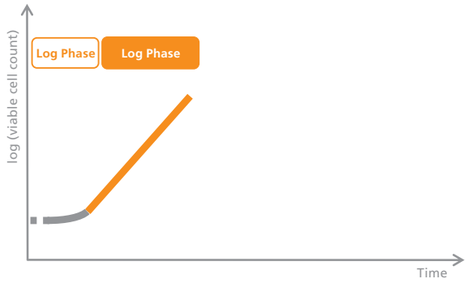


Figure 2 Schematic representation of the log phase.
The well-adapted cells divide at the greatest possible speed since they have all the necessary nutrients available. For this reason, the number of living cells increases rapidly, and this phase is also called the exponential or log phase.

Unfortunately, the exponential growth cannot go on forever. Nutrients are depleted, components of already dead cells accumulate; in short, inside the bioreactor, it looks more and more like a pigsty.

It is becoming more and more uncomfortable in the bioreactor, and so the growth rate decreases in the subsequent, stationary phase. However, in this phase, the proliferation and death of the microorganisms are still in balance.

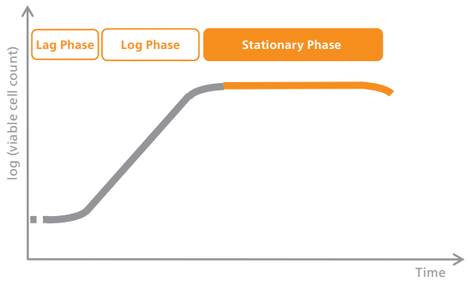


Figure 3 Schematic representation of the stationary phase.
Due to exhausted nutrients and the accumulation of harmful degradation products, the rate of division of the microorganisms decreases to such an extent that the number of dying organisms is approximately equal to the number of organisms coming in by division. Therefore, this phase is also called the stationary phase.

At the end of the bioprocess, in the so-called death phase, the growth rate drops so far that more microorganisms die than are added by the division process so there is a net loss of microorganisms. Thus, depending on the process management, the bioprocess comes to a “natural end” or the targeted termination of the bioprocess by the user.

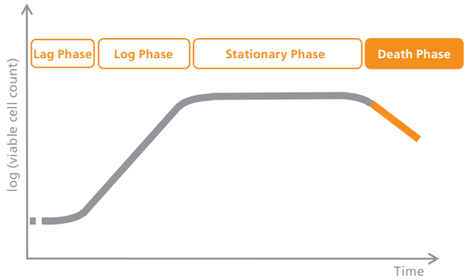


Figure 4 Schematic representation of the die-off phase.
If the bioprocess is allowed to advance to the die-off phase, this phase is characterized by a decreasing number of living microorganisms due to insufficient nutrients and the accumulation of harmful by-products. Since more microorganisms die off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

This also depends on how long such a process is economically viable to operate. If the bioprocess is not actively ended, there is a gradual die off of the cells because the nutrients simply used up and the toxic effect of accumulating metabolites does the rest.

2.3 What happens after the bioprocess?

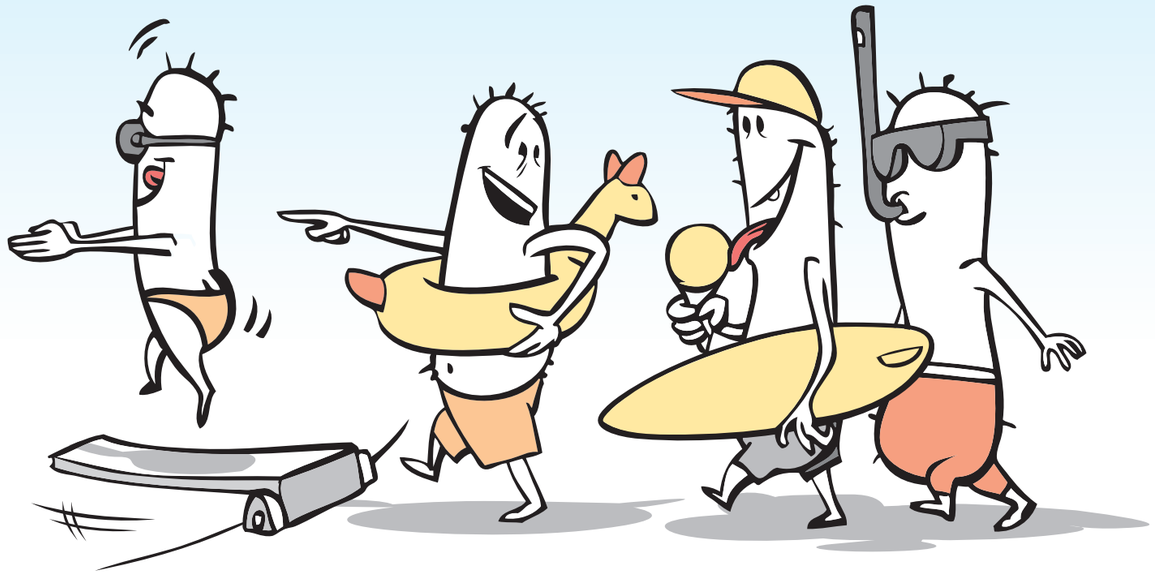
After the completion of the bioprocess, the harvesting and processing of desired products, also known as downstream processing, normally follows. Depending on the process and the desired end product, which fraction is kept and processed after a bioprocess may therefore greatly differ.

Figure 5 Embedding of the bioprocess in bioprocess engineering.
Although the bioprocess in itself already represents a complex matter, much can still be optimized in the pre- and post-processing. For example, bioprocess development also involves, as possibilities, the improvement of the producer stock, the medium and the additives as well as the feeds (upstream process), and how the product is processed in the after-treatment as gently as possible, with high purity and good yield (downstream process).

For example, only the biomass or the medium may be harvested and suitably processed to obtain the desired product. Depending on the characteristics of the product, the purification is simpler or more elaborate and involves correspondingly more or less costly procedures.

E.g. recombinant protein released from the microorganism into the medium can be recovered by simple processing of the medium, whereas a similar protein, which cannot be secreted, first has to be purified from the cell (and thus from the complex mixture of lipids, proteins, nucleic acids and sugars) with great effort. Generally, the downstream processing follows the work stages of cell separation, cell disruption with an intracellularly existing product, product extraction and concentration, purification and packaging. At the same time, of course, the cleaning and re-preparation of the bioreactor for the next process takes place by means of manual cleaning and sterilisation, CIP and/or SIP.

Notes



3. Setup, prepare and perform a bioprocess

3.1 Preparing the bioreactor

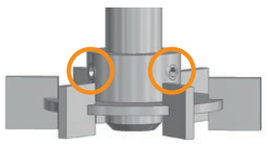
For those who are still motivated, or motivated more than ever, to start a bioprocess after the explanations above, the steps necessary for the successful operation of the bioreactor are listed below. These are provided to serve as a summary of the required steps. For a more detailed description of the procedure, the bioreactor manuals provide advice and practical help, for example, Chapter 8 “Before Cultivation” in the Minifors 2 manual.

1. Prepare culture vessel
Before we can start making our confection, the most important thing to check is the culture vessel itself. After all, it is supposed to serve as a safe home for our microorganisms in the following hours and days. Therefore, the culture vessel itself must be checked to ensure that it is undamaged and that all O-rings are seated and intact – otherwise, contamination may penetrate and disturb the process after autoclaving. For this reason it is indispensable that all sensors, inputs for gases and adjustment agents as well as ports for sampling or cell harvesting, which are required for the process, are installed, connected and secured. Once the reactor has been autoclaved, changes can only be made carefully at a sterile workbench; in this case, there is always a residual risk of contamination.

It is not uncommon, when checking the culture vessel, to break it down into its individual parts or, after cleaning, as a completion of the preceding process, for it to still be separated into its individual components. The correct assembly of the culture vessel thus quickly becomes a routine – and there is more and more envy of users of bioreactors with cleaning and sterilization-in-place (for example Labfors 5 with LabCIP).

But here are the most important points to pay attention to:

- Check the installation height and secure fastening of the agitator elements on the shaft. If necessary, loosen, move and retighten the screws



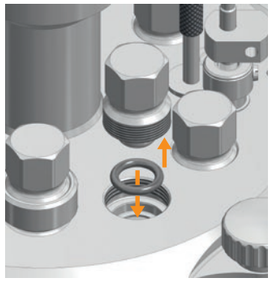
- Depending on the planned process: Insert baffle plate cage

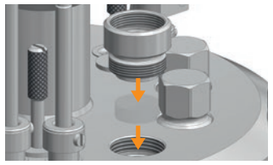
Note: In some bioreactors such as the Labfors 5, the baffle plates are not present as a baffle plate cage, but are instead fastened to the cover.

- Check O-ring for sealing the cover for damage and correct position

- Check O-rings on all cover installations, regardless of whether these are intakes, sensors or plugs

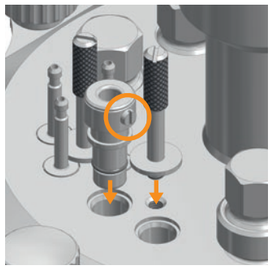
At the inoculation port, a silicone membrane is used instead of the O-ring, which can later be used for inoculation via a cannula or an optional piercing needle. Since this membrane is pierced during each process, it should be replaced regularly.





- Check the installation height of all adjustable-height fittings such as spargers or immersion pipes and ensure their correct position.

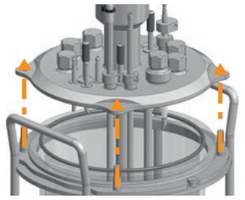
Note: Some of the installed parts have a little play. The O-rings used, however, seal correctly, so that there is no danger for the process.



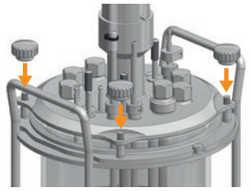
- If applicable, fill with culture medium.

Note: This is only useful and possible if the culture medium is heat-stable and will withstand autoclaving undamaged. Otherwise, the culture medium must be sterilized separately and then be transferred to the culture vessel in a sterile manner after the culture vessel has been sterilized.

- Put the cover on carefully and correctly aligned. In doing so, take care to ensure that no installed parts and baffle plates are touching each other.



- Tighten knurled screws by hand (no tool) crosswise



2. Calibrate (Part 1) and install sensors
After all mechanical installations 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 must also be calibrated prior to the process. There are often specific work instructions for this in the laboratories which differ in details. Generally, however, the calibration of the pH sensors prior to installation and before autoclaving is done in a manner similar to the calibration of ordinary pH-meters with two reference buffers. The pO_2 and the optional turbidity sensors are not calibrated, however, until after autoclaving and after filling with the culture medium since both parameters are dependent on the actual operating conditions.

Note:
- If digital sensors are used, it should also be mentioned that these can store and transport their calibration data in the sensor head. Therefore, these sensors do not necessarily have to be calibrated on the control unit of the bioreactor, and the calibration can be conducted, alternatively, in a separate calibration laboratory. The last calibration is automatically available after connecting to the bioreactor.
- If, instead of optical pO_2 sensors, amperometric pO_2 sensors are used, then these must be maintained according to the sensor manufacturer's instructions, which is not addressed in this cookbook.
- For special applications, additional sensors may also be used. This cookbook does not address these either.

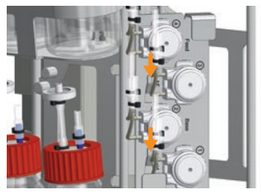
Work steps:
- Install pO_2 sensor in the bioreactor
- Optional: Install turbidity sensor in the bioreactor
- Connect the pH sensor to the basic unit, call up the calibration function on the operating unit, and calibrate the pH sensor according to the instructions on the control unit (see also bioreactor manual)
- Separate pH sensor from base unit and install in bioreactor
- Note: Do not, in any case, cover the terminals of digital sensors with aluminum foil! Follow the sensor manufacturer's instructions!

3. Attach tubes and prepare for autoclaving

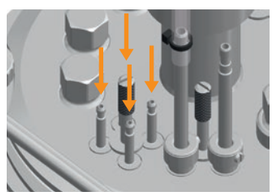
- Prepare adjustment agent bottles

Note: Not all adjustment agents can be sterilized in the autoclave. Instead of such adjustment agents (e.g., ammonia solution), fill with water and, after autoclaving, for example at a sterile work bench, exchange for a second adjustment agent that has been sterile filtered, if applicable.

- Loosen the hose pump head mounting plate from the base unit and attach to vessel holder



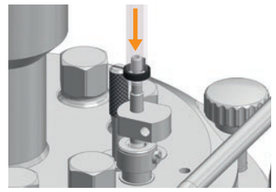
- Connect tube couplings of adjustment agent bottles to the pump heads and then to the inputs on the cover and secure



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

- Connect the air filter

- If applicable, connect and clamp additional tubes, e.g., for cell harvesting



- Cover the filter lightly. The exhaust filter remains open and must not be clamped off at any time! This is essential for balancing the pressure during autoclaving!

4. Autoclaving
Autoclave prepared culture vessel in culture vessel holder along with all connected peripherals such as adjustment agent bottles according to local work instructions, e.g., at 121 °C for 20 to 30 minutes.

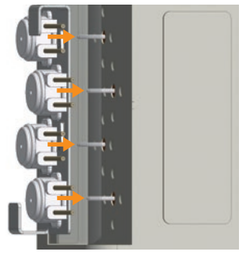
5. Calibrate sensors (part 2) and connect culture vessel holder with base unit
The culture vessel is now almost ready for the bioprocess. It still just has to be connected with the base unit, filled with culture medium (if necessary), and the last sensors must be calibrated.

Work steps:

- Position the culture vessel holder on the base unit

Note: Depending on the design of the bioreactor, tubes for temperature control via the double jacket must also be connected, or the heating mat must be installed. Please follow the instructions in the respective manual!

- Place the pump head mounting plate on the drive shafts



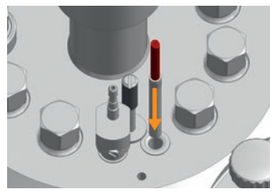
- Fill the adjustment agent tubes

Note: If the tubes are not filled with adjustment agent, the regulator may malfunction since it will feed only air from the tubes into the culture vessel at first instead of adjustment agent.

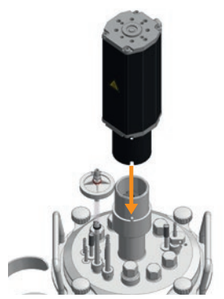
- Connect foam, pH, pO_2 and turbidity sensors

Note: If, instead of optical pO_2 sensors, amperometric pO_2 sensors are used, then these must be polarized according to the instructions of the sensor manufacturer; we do not address this here.

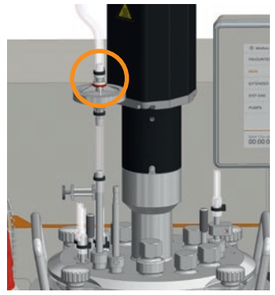
- Insert temperature sensor into the immersion pipe

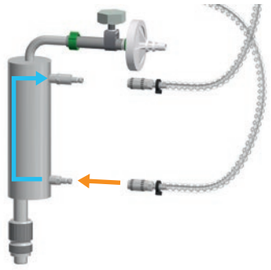


- Mount the motor



- Connect gas and exhaust gas cooler





- If not autoclaved in the culture vessel: Fill with sterile culture medium
- Start the bioreactor and turn on stirrer and temperature control with the desired target values
- As soon as the target values are reached: If necessary, readjust the pH sensor or adjust the pH of the culture medium, calibrate the pO_2 sensor and conduct a zero balance for the turbidity sensor

6. Inoculate and (finally) get properly started
Now, everything is ready for inoculation, the addition of the microorganisms. As is so often true in biotechnology, the precise procedure depends, again, on the individual case. Anyone who just wants to approach the world of bioreactors or wants to answer very simple questions, actually just adds the cells, tells this to the bioreactor or the SCADA software, and then looks at how the process develops. In most cases, however, something very definite is planned, the user already knows about the growth behavior of the microorganisms and would like, for instance, to determine an ideal strategy for the fed-batch phase. This has been prepared for in the best possible way and ideally automated, e.g., with eve®. It does not matter whether it is simple strategies like a ramp for the pH or complex strategies depending on calculated factors like substrate acquisition rates – for who wants to wait the whole time at the reactor just to adjust the target values at the right time. We won't go into the planning and preparation of such strategies in more detail here, but the examples in Chapter 6 can certainly provide inspiration.

Work steps:
- If applicable, take a "blank sample" of culture medium that is still free of cells
- Prepare cells for inoculation, e.g., from a preculture in the incubation shaker, and place them in a suitable vessel for inoculation. A relatively simple method for an inoculum of up to 25 mL or 50 mL can be a syringe with a needle, for example, with which the membrane in the inoculation port is pierced in order to insert the cells in the culture medium. It can help 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 100% certainty.

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!

- Signal that the inoculation has taken place using the button on the bioreactor or in the SCADA software, thus starting, for example, the process of the stored strategy.

3.2 Adding nutrient medium

So far, a lot of time has been spent getting the bioreactor to run in the first place. It has been connected, screwed together, assembled and steam-sterilized, and the typical, ideal running of a bioprocess has been reviewed. But do the cultures now grow as expected? As is the case with many things in life, here, also, it depends.

For example, on the nutrients we make available to the microorganisms in the medium. As a rule, a great variety of nutrients are required since the microorganisms must find everything in the medium to build themselves up and thus proliferate. Sometimes the media recipes look amazingly simple because very few components are listed. However, these are usually complex media in which "complex" components such as yeast extract or peptone are used, which in turn contain a considerable spectrum of individual nutrients to provide all the elements that make a cell. In contrast, there are defined media in which the individual nutrients are precisely weighed and mixed. In a subcategory of these, the minimal media, only that which the microorganisms really need in the bioprocess is contained. This is particularly important with regard to cost optimization and avoiding unwanted side reactions.

In contrast to the minimal medium, a selective medium is used to preferentially cultivate a certain kind of microorganism by the skillful selection of environmental parameters. This can be achieved both by a clever selection of the nutrients as well as by the addition of substances such as antibiotics which inhibit the growth of unwanted microorganisms. Nutrient media can be differentiated according to many other aspects, which would, however, be beyond the existing framework here.

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

As a supplier of nitrogen and a large number of accompanying substances, extracts from yeast, soy or casein as well as their equivalents, treated with digestive enzymes (pepsin and trypsin), so-called peptones and tryptones, are often used. Ammonium salts can be used in defined media instead, for example.

The following is a generic recipe for nutrient media, which is explained in more detail using the components of TB medium for bacterial cultivation and YPD medium for yeast cultivation:

Components	Typical concentration range	Example of bacterial cultivation TB medium	Example of 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 each list the composition of a bacterial and a yeast culture medium as an example. Since both are complex media, often the exact amount of the individual substances 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 an ingredient. This ingredient that limits growth is called a limiting factor. Usually the carbon source is 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 sterilely. 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.

In general, it is important to keep in mind that the media presented as examples are only two of a variety of nutrient media for very different requirements. Changes to these basic recipes always allow adaptation to individual needs, so please refer to the defining characteristics of the organisms and process parameters used, including the nutrient medium.

3.3 When the fun is over…

…you have to clean up again. This means that, after the bioprocess has taken place, the bioreactor must be put right again in such a way that the microorganisms can be rendered harmless and the bioreactor is restored to a clean and usable state. The regulations on how the organisms are to be killed vary depending on the institution. This happens either by autoclaving in the culture vessel and subsequent disposal of the stock, which usually involves a more complex cleaning of the vessel and the periphery afterwards. Or, the culture broth, including microorganisms, can also be killed separately, and the bioreactor and the surfaces of the periphery that came into contact with the bioreactor can be decontaminated and then cleaned.

4. Bioprocess strategies and their control

A lot of preparation has now been put into our bioprocess, but it still requires further consideration. Good planning is already half the battle. To this end, a few pieces of basic knowledge about microbial growth are necessary so that the bioprocess is ultimately carried out in the same way as was originally planned. Once again, design follows the requirements, which is why you should know the goal of the bioprocess.

In general, the growth of microorganisms is limited as soon as an essential factor becomes limiting. For example, if there is not enough oxygen and the growth rate is limited, 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

If the microorganisms are fed all the food at once, and they are not fed any more in the subsequent bioprocess, it is called a batch process or batch. During the entire bioprocess, no additional nutrients are added; it is a closed system. Only corrective reagents such as gasses, acids and bases are added. The bioprocess lasts until all nutrients are used up. 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 the oxygen input 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 are supposed to bring about an improvement in concentration, sophisticated management and control processes are required in this case. These so called cascades can be freely configured and are set according to the application. To do so, one or several parameters influencing the concentration of dissolved oxygen are set for the controller. For this reason, the first parameter in a defined sequence is varied in order to achieve the target value. If this is not possible, parameters further “downstream” are changed 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 produced only in stages. In addition to the low yield of biomass, the occurrence of a substrate or product inhibition also needs to be treated as a high risk in the batch process. The latter describes the impairment of the enzyme activity by the presence of high concentrations of substrate or product which effects metabolic feedback and therefore can drastically reduce the yield.

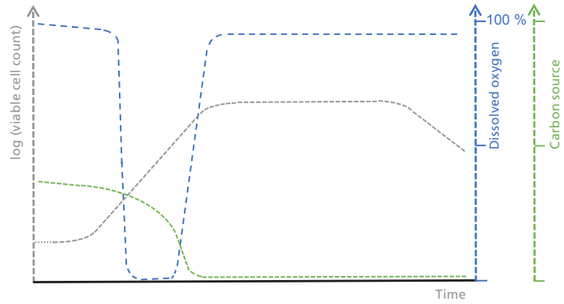


Figure 6 Schematic illustration of the connections between viable cell concentration, dissolved oxygen and the limiting carbon source during batch operation.
Initially, the viable cell count only increases slowly in the lag phase, which leads to a moderate but steady uptake of the carbon source. The oxygen consumption can increase over the period of the exponential growth phase until it exceeds the possible oxygen input. Once the carbon source is exhausted, 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 not allowing nutrients to become a limiting factor is to constantly supply them during cultivation. This is called a fed-batch process, which is a partly open system. Feeding during cultivation has the advantage of being able to achieve overall higher product quantities.

Under good growth conditions, the microorganisms constantly double and therefore follow an exponential growth curve, which is why they should also be fed in an exponentially increasing manner. Generally, the nutrient solution passes through a silicone tube from the supply bottle into the culture vessel with the aid of a pump. The quantity can be determined manually at any time by the user (constant, exponential, pulse-wise), but dosing according to defined conditions is also possible, for example 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, there is a longer processing time and potential inhibition by the accumulation of toxic by-products. It is also necessary for the implementation of these strategies to have an in-depth knowledge of bioprocesses, which should not be construed as a disadvantage here, however.

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 the batch process, it was found that excessively high substrate concentrations (in this case glucose) caused growth inhibition, mainly by the formation of ethanol. For this reason, we resort to the fed-batch process here. For this purpose, relatively small amounts of glucose are added continuously, which are immediately consumed by the microorganisms. 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.

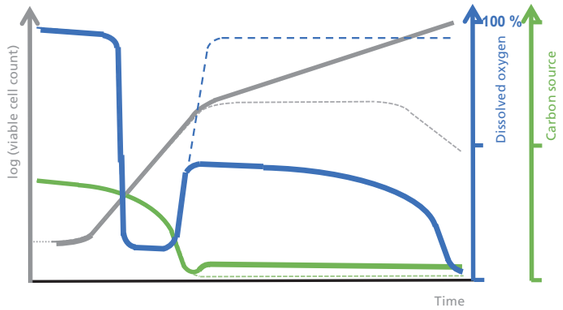


Figure 7 Schematic illustration of the relationship between the living cell concentration, dissolved oxygen and the limiting carbon source in the fed-batch process.
In fed-batch process implementation, supply of the feed starts directly after exponential phase so that the carbon source is not or only shortly exhausted (see green line vs. green broken line in the batch). An exponential feeding process is illustrated, so that the exponentially growing organisms remain in a prolonged exponential phase (thick gray line vs. gray broken line in the batch). This also means that the quantity of consumed oxygen increases, which is why there is a lower content of dissolved oxygen in the medium (thick blue line vs. blue broken line in the batch).

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 culturing operation

A special case in which the system is operated as open 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*). In this case, for example, just as much fresh culture medium is introduced as is removed (chemostat). Such bioprocesses are also referred to as a continuous culture and are particularly suitable when an excess of nutrients would result in the microorganisms overeating, and thus inhibition of the microorganisms. A minimized product inhibition and an improved space-time-yield are further advantages of this method. 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, using a spin filter), which is referred to as perfusion operation.

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

4.4 Other special forms of process management

Finally, there are also mixed forms which can be used during the operation of a bioprocess. For example, a completed (fed-) batch is harvested only up to a small residue in order to use the remaining amount of cells as an inoculum for the next filling. This type of process is then called a repeated (fed-) batch process. The distinction between the batch and fed-batch process again is only made according to whether a batch or fed-batch process is undertaken with the remnants of the bioprocess. This eliminates cleaning times of the reactor as well as the need to cultivate a fresh inoculum while simultaneously increasing productivity. However, as with other fed-batch processes, there is also a higher risk of contamination and the possibility of strain alteration.

4.5 Scale transfer in process and media development

Process development and characterization typically start with a bioreactor on the laboratory scale, in order to detect as many parameters as possible and to learn about the limits of the process. At the same time, the bioprocess can be optimized, the cells and the medium can be improved, and critical process parameters can be determined.

In the dimensioning of the bioprocess for larger culture vessels, in particular to make it more economical, a pilot scale operation is first carried out, followed by production scale operations, if necessary. This scale transfer is also called scale-up. For the generation of value-intensive recombinant proteins, a bioreactor on the laboratory or pilot scale is often also sufficient.

The inverse process of the scale-up is called a scale-down and is suitable for the simulation of model tests, in particular to explain malfunctions of a large-scale system.

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 for simple cooking. This includes not only the recipes for the media, but also the exact conditions and quantities as well as what strategy is followed. Finally, at the end of the respective bioprocess, possibilities for refining the recipe by, for example, introducing more feeding phases or other basic recipes for media are used. All the described recipes can also be found in the bioprocess platform software eve®

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

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

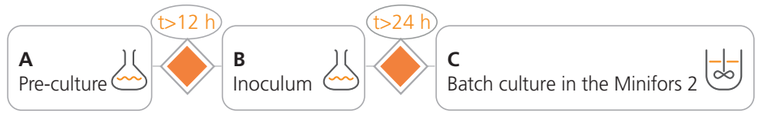
Last but not least, the methanol-utilizing yeast *Pichia pastoris* is presented for use in a high cell density fed-batch process on two substrates, which can be used as a eukaryotic expression system for recombinant proteins of a more complex structure.

5.1 *Saccharomyces cerevisiae* cultivation

5.1.1 Basic setup

Name	Cultivation of baker's yeast *(Saccharomyces cerevisiae)*
Description	As an introduction to the bioprocess technique, a bioprocess for generating yeast biomass is performed. This is used to learn about the individual components of a bioreactor, its handling and the basic process control in a batch bioprocess.
Recipe in eve®	*S. cerevisiae* cultivation
Device selection	**A** and **B** Incubation shaker **C** Bioreactor
Parameters	see separate description for **A**, **B** and **C**

5.1.2 Workflow



A Pre-culture in the incubation shaker

Organism

Name	**A** pre-culture
Description	Generation of pure *S. cerevisiae* inoculum for further expansion in the shake culture
Organism	*Saccharomyces cerevisiae* (baker's yeast)
Origin	From a supermarket (available as cubes from the refrigeration unit or as a dry yeast, crumble some pieces into the medium) or, for example, 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 250 mL Erlenmeyer flask without baffles
Biomass yield	dependent 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) 20 g L^{-1} peptone (P) 20 g L^{-1} dextrose (D)

Cultivation parameters

Shaking throw	25 mm
Shaking speed	300 min^{-1}
Temperature	30 °C
Time	min. 12 h cultivation time

B Generation of the inoculum in the incubation shaker

Organism

Name	**B** Cultivation of the inoculum
Description	Generation of sufficient *S. cerevisiae* inoculum for the subsequent cultivation in the bioreactor
Organism	*Saccharomyces cerevisiae* (baker's yeast)
Origin	10 mL of pre-culture (Step **A**)
Inoculum volume	5 mL of pre-culture in 100 mL fresh YPD medium, divided into 2 1000 mL Erlenmeyer flasks with baffles
Biomass yield	6–8 g L^{-1}

Culture medium

Type	Complex medium
Name	YPD (Yeast extract-peptone-dextrose)
Composition	10 g L^{-1} yeast extract (Y) 20 g L^{-1} peptone (P) 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	Biomass generation with a *S. cerevisiae* culture using the Minifors 2
Organism	*Saccharomyces cerevisiae* (baker's yeast)
Origin	Inoculum (Step **B**)
Inoculum volume	100 mL of inoculum in 1000 mL of fresh YPD medium in the 2.5 L stirred reactor with 2 impellers, Minifors 2
Biomass yield	6–8 g L^{-1}, depending on the strain and aeration 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) 20 g L^{-1} peptone (P) 20 g L^{-1} dextrose (D)

Cultivation parameters

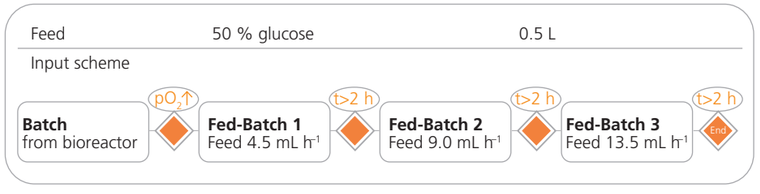
Temperature	30 °C
Stirring speed	1200 min^{-1}
pH	5.5
Gassing rate	2 min^{-1} (vvm)
Overpressure	0 bar
pO_2	≥ 20 %

Additional parameters to regulate on the bioreactor or in eve®

Pump	Additive	Goal	Specification	Trigger
a	*Base*	pH regulation	25 % NH_4OH	action-based
b	*Acid*	pH regulation	20 % H_3PO_4	action-based
c	*Anti-foam*	Anti-foam agent	Biospumex 153	action-based

Suggestions for the modification of this recipe
For reasons of simple handling, in this first attempt, there is no programming of a pO_2 cascade. All the parameters applied were set to the corresponding maximum values in order to ensure the highest possible pO_2, so that the maximum yield of biomass is possible.

To increase the yield of biomass, the batch phase can be extended by three fed-batch phases. This is done by the supply of needed nutrients in a so-called feed solution. In the three successive phases, the feed rate increases 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 jumps again accordingly. From this point, the addition of feed is manually dosed according to the following scheme or can be programmed in eve® under Batch Strategy as a function.



For a further increase in the biomass yield and the running time of the bioprocess, the medium can be successively supplied with additional components that are rapidly exhausted during the cultivation process (such as magnesium). Alternatively, a more abundant base medium such as YUM or BMGY can also be used to start the batch.

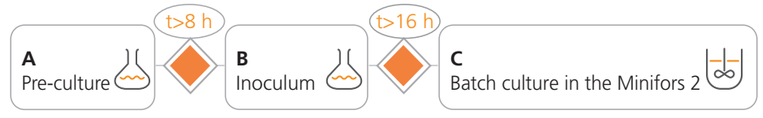
5.2 *Escherichia coli* cultivation

Escherichia coli has always been considered the workhorse of molecular biology due to the short generation time and easily managed cultivation requirements and tools. Therefore, it is often used for the production of recombinant proteins. So that its yield is as high as possible, as large an amount as possible of *E. coli* biomass must be created. The production of recombinant proteins as a function of the used promoter construct behaves proportionally.

5.2.1 Basic setup

Name	Propagation of *E. coli*
Description	With the proposed three-step process, *E. coli* can be selectively propagated and used for protein production after appropriate genetic modification. Alternatively, this bioprocess can also be executed in two stages, by producing 20 mL of inoculum in the first step which is then used for the inoculation of the bioreactor.
Recipe in eve®	*Escherichia coli* cultivation
Device selection	**A** and **B** Incubation shaker **C** Bioreactor
Parameters	see separate description for **A**, **B** and **C**

5.2.2 Workflow



A Pre-culture in the incubation shaker

Organism

Name	**A** Pre-culture
Description	Generation of a pure *E. coli* inoculum for further expansion as a shake culture
Organism	*Escherichia coli* such as the strains K-12, BL21, DH5α, etc.
Origin	10–100 µl of a liquid culture
Inoculum volume	10 mL YPD medium in 250 mL Erlenmeyer flask with baffles
Biomass yield	dependent on the added quantity of *E. coli*

Culture medium

Type	Complex medium	
Name	TB medium (*Terrific broth* medium)	
Composition	24.00 g L^{-1}	Yeast extract
	12.00 g L^{-1}	Soy peptone
	12.54 g L^{-1}	K_2HPO_4
	2.31 g L^{-1}	K_2HPO_4
	20.00 g L^{-1}	Glycerin (anhydrous)

Cultivation parameters

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

B Inoculum in the incubation shaker

Organism

Name	**B** Inoculum
Description	Generation of sufficient *E.coli* inoculum for subsequent cultivation in the bioreactor
Organism	*Escherichia coli*
Origin	from 10 mL of pre-culture (Step **A**)
Inoculum volume	10 mL of pre-culture in 100 mL of fresh PAN medium in 1000 mL Erlenmeyer flask with baffles
Biomass yield	2.5 to 10 g L^{-1} dry cell weight

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}	$NaH_2PO_4 \cdot H_2O$	autoclave in advance
	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}	$CaCl_2$ x H_2O	
	5 g L^{-1}	Glycerin	
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

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	*E. coli* biomass production during batch phase using Minifors 2 with simultaneous and subsequent generation of recombinant protein in the glucose feeding phase
Organism	*Escherichia coli*
Origin	from inoculum culture (Step **B**)
Inoculum volume	100 mL of inoculum in 1000 mL of fresh PAN medium with trace elements in the 2.5 L stirred reactor with 2 impellers, Minifors 2
Biomass yield	10 to 100 g L^{-1} dry cell weight

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}	Glycerin	
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

Parameter	Description	set value	Min. value to control	Max. value to control
1	*Stirring speed*	500 min^{-1}	500 min^{-1}	1200 min^{-1}
2	*Gassing rate*	1 vvm	1 vvm	2.5 vvm
3	*Overpressure*	0 bar	0 bar	0 bar
4	*pO_2*	≥ 20 %		

Feed schema

Phase	Description	Start	End	Action	Feed rate in percentage	Feed rate in 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 regulate on the bioreactor (a,b,c) or in eve® (d, also manually)

Pump	Additive	Goal	Specification	Trigger
a	*Base*	pH regulation	25 % NH_4OH	action-based
b	*Acid*	pH regulation	20 % H_3PO_4	action-based
c	*Anti-foam*	Anti-foam agent	Biospumex 153	action-based
d	*Glucose feed*	Carbon source (C) 500 g L^{-1} glucose	50 % glucose	after batch phase

5.3 *Pichia pastoris* cultivation

As a yeast with the potential to metabolize methanol, a low priced raw material, bioprocesses with *Pichia pastoris* for the generation of recombinant proteins is indispensable these days. The utilization of methanol makes it possible to decouple the production of biomass from product formation, and thus to specifically initiate the phase of product formation by feeding methanol.

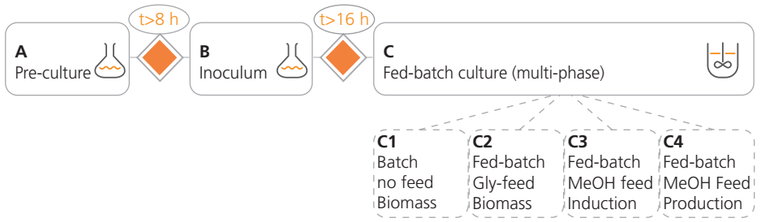
Pichia, as a eukaryotic organism, is also able to produce and secrete into the medium correctly folded proteins with pronounced posttranslational modifications, which represents an additional advantage over production in the *E. coli* system. In addition, *Pichia* has *GRAS (generally recognized as safe)*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

For reasons of consistency and multiple uses in the field, the (old) name *Pichia pastoris* will be maintained in the remainder of this document. A 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 the production of recombinant proteins. First of all, a fed-batch bioprocess for a high cell density bioprocess will be presented. Then, the understanding of the specificity of the cultivation of this methylotrophic yeast on glycerol and methanol and how these should be implemented as phases in the bioprocess will be shown.
Recipe in eve®	*P. pastoris* cultivation
Device selection	**A** and **B** Incubation shaker **C** Bioreactor
Parameters	see separate description for **A**, **B** and **C**

5.3.2 Workflow



A Pre-culture in the incubation shaker

Organism

Name	Pre-culture
Description	Generation of a pure, reproducible *P. pastoris* inoculum 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 of fresh YPD in a 250 mL Erlenmeyer flask without baffles
Biomass yield	dependent 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)
	20.0 g L^{-1}	Peptone	(P)
	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 Generation of the inoculum in the incubation shaker

Organism

Name	**B** Cultivation of the inoculum
Description	Generation of sufficient *P. pastoris* inoculum for subsequent cultivation in the bioreactor
Organism	*Pichia pastoris*, neuer Name *Komagataella phaffii*
Origin	10 mL of pre-culture from Step **A**
Inoculum volume	10 mL *Pichia* pre-culture in 100 mL of fresh BMGY medium in a 1000 mL Erlenmeyer flask with baffles
Biomass yield	10 to 30 g L^{-1} dry cell weight

Culture medium

Typ	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}	Glycerin	(G)
	10.0 g L^{-1}	Yeast Nitrogen Base (without 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** Main culture in the bioreactor
Description	Upstream biomass generation of *P. pastoris* culture using the Minifors 2 and subsequent generation of recombinant protein in the methanol feeding phase
Organism	*Pichia pastoris*, new name *Komagataella phaffii*
Origin	from inoculum culture (Step **B**)
Inoculum volume	100 mL of inoculum in 1000 mL of fresh BSM/PTM1 medium in the 2.5 L stirred reactor with 2 impellers, Minifors 2
Biomass yield	More than 120 g L^{-1} dry cell weight possible

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%	sterilize in the bioreactor
	0.93 g L^{-1}	$CaCl_2 \cdot 2\ H_2O$	
	18.2 g L^{-1}	K_2SO_4	
	14.9 g L^{-1}	$MgSO_4 \cdot 7\ H_2O$	
	4.13 g L^{-1}	KOH	
	40.0 g L^{-1}	Glycerin	
Composition PTM1	6.0 g L^{-1}	$CuSO_4 \cdot 5\ H_2O$	add 4.35 mL sterile PTM1 solution each per L BSM
	0.08 g L^{-1}	NaI	
	3.0 g L^{-1}	$MnSO_4 \cdot H_2O$	
	0.2 g L^{-1}	$Na_2MoO_4 \cdot 2\ H_2O$	
	0.02 g L^{-1}	H_3BO_3	
	0.5 g L^{-1}	$CoCl_2$	
	20.0 g L^{-1}	$ZnCl_2$	
	65.0 g L^{-1}	$FeSO_4 \cdot 7\ H_2O$	
	0.2 g L^{-1}	Biotin	
	5.0 ml L^{-1}	H_2SO_4	

Cultivation parameters

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

pO_2 cascade on the bioreactor touchscreen

Parameter	Description	Set value	Min. value to control	Max. value to control
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	Action	Feed rate in percentage	Feed rate in 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	Glycerin	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 regulate on the bioreactor (a, b, c) or in eve® (d, also manually)

Pump	Additive	Goal	Specification	Trigger
a	*Base*	pH regulation	25 % NH_4OH	action-based
b	*Acid*	pH regulation	20 % H_3PO_4	action-based
c	*Anti-foam*	Anti-foam agent	Biospumex 153	action-based
d	*Glycerin feed*	Carbon source (C2+3) 550 g L^{-1} glycerin	50 % glycerin 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 the modification of this recipe

To increase the biomass yield, modified media and compositions for the batch phase and the feeds can be used. In terms of product yield, the optimization of the methanol feed is also worthwhile in order to limit toxic effects. The latter should never rise above 32 °C since protein production stops at that point. Therefore efficient cooling is necessary during the propagation and product formation phase. Testing product stability in the chosen pH range is just as important in order to adjust the pH value if necessary, or adding protease inhibitors such as casamino acids to the medium, which increase the stability of the product.

In the transition phase between glycerol and methanol metabolism, a short "famine phase" can be helpful. In other cases, it might be helpful to reduce the glycerol feed while slowly increasing the methanol feed. It is important here to wait for the PO_2 peak, which indicates that all the glycerol has been consumed.

A second methanol feeding phase with reduced methanol feed can also be helpful. This should follow phase C4 and bypass the cell toxicity effect of methanol.

The evaluation of the strain for its ability to efficiently use methanol (Mut^+ versus Mut^S) and the targeted use of USP methods can likewise represent a significant difference in yield with regard to biomass and product.

Notes

6. Tips for a successful bioprocess

6.1 Avoiding contamination

- Contaminations can have very different causes. The most frequent is a contamination of the starter culture. This can happen in many ways: insufficiently cleaned or autoclaved culture vessels or reactor components, contaminated starter cultures or media components as well as improper handling.
- In addition, an organism which grows only slowly as a contaminant in a static culture suddenly finds the perfect growth conditions in the bioreactor and overgrows the organisms to actually be cultivated within a very short time.
- Ensure that the target temperature in the bioreactor is kept for a sufficient time span while autoclaving. The temperature sensor should be placed into the immersion pipe of the vessel.
- For a double-walled culture vessel, sufficient water must be present in the jacket so that a good heat transfer during sterilization can be ensured.
- All flexible seals should be in perfect condition, i.e., there should be no signs of kinks, flattening or any burr formation; in case of doubt, it is better to exchange one time too many, especially if a seal is not seated properly.

6.2 Growth maximization through control of the dissolved oxygen concentration

- In aerobic bioprocesses, the amount of dissolved oxygen is an important parameter which decides the success of the bioprocess. Depending on the requirements of the organisms, this must be individually configured and controlled by means of various parameters, e.g., Total_Flow, stirrer speed and gas mix. A pO_2 control with a precision in the range of ±5 % to 10 % of the target value is accepted by most users.
- Was pO_2 switched on as a parameter and configured in the cascade on the bioreactor?
- Are the limits of the individual parameters selected broadly enough to meet the high oxygen demand during the exponential growth phase?
- Is the gassing rate selected sufficient?
- Was a lot of anti-foam agent added? This can hinder an 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.
- The supplementation of pure oxygen may be required for fast-growing, high-density cultures. Most bioreactors are configured to allow for oxygen supplementation ex works. If yours isn't, request a quote for retrofitting.
- After the nutrients are depleted, the dissolved oxygen concentration increases again. Such behavior can be used in a fed-batch process for determining the feed timing. However, the remaining concentration of the carbon source present in the reactor should be investigated so that there is no confusion with a short-term jump in pO_2.

6.3 Maintaining the culture volume

- When autoclaving the medium in the reactor, a volume loss of up to 10% may occur. So that the medium does not become too concentrated, it should be balanced by adding sterile water before inoculating the reactor.
- If a decrease in the culture medium becomes evident during cultivation (in particular) without feed, the status of the exhaust cooler, its coolant inflow and whether the temperature regulation is switched on should be checked.
- Any sampling should be limited to 10 mL in smaller culturing vessels. The use of the SuperSafe sampler can help with this.

6.4 Avoiding foam formation

- Foam formation tends to occur in protein-rich media with higher gassing rates and/or stirrer speeds. This can disturb not only the bioprocess and the damage the microorganisms, but can also be detrimental to the product.
- At the beginning of a batch culture, active gassing might not yet necessary. The gassing can continually be increased later. This guarantees that sufficient oxygen is available without forming unnecessary foam.
- If possible, a small portion of anti-foam agent should be added to the medium in order to effectively prevent foam formation from the beginning. If the dosing of anti-foam agents is too high, the efficient transfer of oxygen is limited and thus also the microbial growth of aerobic organism. As a general rule, avoiding foam formation is simpler than getting rid of it later, which is why, in summary, the early addition of anti-foam agents can keep the additional use low.

6.5 Functional exhaust filter

- It is very important to keep the exhaust filter dry. If this is not the case, the hydrophobic membrane becomes clogged, which, in turn, prevents the gas flow, thus leading to an unwanted pressure build-up in the culturing vessel or even to a stop of the gassing.
- If moisture gets into the exhaust filter during the bioprocess, there are several options for eliminating this:
  – Reduction of the gas flow, if possible,
  – Increase of the coolant supply in the exhaust cooler or use of a better refrigerant, e.g., with a lower temperature,
  – Use of a larger filter,
  – Use of a depth filter, which cannot become clogged in this manner.
- If the problem is caused by too much foam formation, a foam trap between the exhaust cooler and the exhaust filter can help. This can be provided simply by means of a bottle through which exhaust gas runs before reaching the filter.

6.6 Constant pump speed

- If Marprene tubes are used, the pump should be connected about 30 minutes in advance, before the flowthrough rate is determined. Marprene tubes have the property of stretching over time; repeated autoclaving can also have such an effect.
- The flow rate may vary with the viscosity of the liquid. A feed medium with a high sugar concentration can therefore have a different flowthrough rate than a solution with a low sugar content.
- It is also important that the feed tubes for feed or other solutions are neither kinked nor squashed on their way to the cover plate of the bioreactor.
- The clamp applied for autoclaving must also not block the tubing line where it is attached.
- If a high precision for the supply of the feed is necessary, this should be monitored as a gravimetric feed using a weighing scale. The bioreactor can therefore use the feedback of the decreasing weight on the scale to adjust automatically the pump rate in order to reach the desired flow-through-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 the inoculation of the bioreactor in order to achieve a maximum growth rate.
- If any nutrient has a limiting effect (and this does not necessarily have to be the carbon source), the growth of the microorganisms will be limited. Therefore, a sufficient supply of all nutrients and a good supply of oxygen should always be ensured.
- Genetically modified strains may react significantly more sensitively to shear stress, for example, or grow more slowly due to the genetic modifications.
- By ensuring that the starter culture is sufficiently vital and cultivated with high cell counts and best adapted to the conditions in the bioreactor, the lag phase can be kept as short as possible.

6.8 Successful protein yield

- If the nutrient supply is so excessive that the metabolism of the microbes is only geared towards growth, no metabolites are excreted. Managing the bioprocess as a fed-batch process can provide a remedy here.
- Often the growth conditions of the microorganism are not optimal for the production of the targeted protein, for example, so that, if necessary, the temperature or the pH must be adjusted to it in order to improve the protein yield and stability.
- In addition, it should be considered very carefully whether the correspondingly cultured strain can produce the desired yields, which can be evaluated in advance using the professional literature.
- It is just as important to know whether the cultivated strain excretes toxins or growth-inhibiting substances, which can be produced by the growth, the disintegration of cells or their metabolism. Such influencing factors can impair protein production over a period of a few days.

6.9 Tips for fed-batch processes

- A fed-batch process should not be started below the minimum volume in order to guarantee that all sensors and the impeller are sufficiently covered with culturing fluid.
- An overfilled culturing vessel is also of little use. There should always be a minimum of 20 to 30 % head room in the culturing vessel.
- A calculation of the quantity of the required culture liquid prevents cumbersome post-production and/or ordering and ensures a smooth running of the bioprocess. It is equally important that the pump used achieve the desired flow rates.
- If a very precise flow rate is to be maintained, it is advisable not to determine and set this in 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. Thus, for example, a higher biomass yield can be achieved with *E. coli* if an ammonium solution is used as a base in the process.
- The resulting (waste) heat, especially in high cell density processes, should not be underestimated. Sufficient cooling can be achieved by a heat exchanger with water intake.
- The metabolic state of the culture can be more accurately determined by the calculation of the respiratory quotient if an exhaust analyzer is connected to the bioreactor.
- If the gas flow rate is increased too much (not over 2 min^{-1}), too much fluid is carried off from the culture liquid. On the one hand, this allows the culture volume to shrink unnecessarily and leads, on the other hand, to a clogged exhaust filter.

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8. Glossary

A

Actuator
A physical component of a control system or loop which actually provides the desired change in a process value, e.g., a heater element or cooling valve.

Aerobic
In the presence of air/oxygen. Most bioprocesses involved with protein production are aerobic and some cultures.

Anaerobic
Without air/oxygen. Growth of microorganisms can occur in the absence pf oxygen. Even aerobic cultures can have periods when growth is anaerobic if the level of dissolved oxygen reaches zero due to excessive demand.

Antifoam
A chemical reagent used to reduce the amount of foam generated by a bioreactor culture. It can be a silicone oil, an alcohol, a mineral oil or a special blend of chemicals. Excess antifoam can interfere with gas transfer.

Aseptic Technique
Involves the manipulation of cultures in such a way as to minimise the chances of contamination during procedures such as seed preparation, inoculation or sampling.

Autotrophic
If the carbon required for the cellular building blocks is provided by the fixation of carbon dioxide, we speak of autotrophy.

B

Batch Culture
A mode of operation in a closed system. All the nutrients and supplements are added before starting the bioprocess and are then depleted over time. There are no additions are made into the vessel beyond reagents for e.g. pH control and foam.

Baffles
EFlat blades (usually 3 or 4) inside the vessel near the walls, which help break up the bubbles generated by flat-bladed Rushton impellors and sparged gas in microbial bioreactors. Baffles help to generate a turbulent flow, to mix the liquid phase in an optimum way, and to crush the gas bubbles of the microbial bioprocess caused by the flat blade Rushton impeller and the sparger.

Base Unit
This is the part of the bioreactor which contains the actuators and sensors as well as the controller needed to provide control of process variables. A base unit can be used for one or several vessels (see parallel-bioreactor).

Bench-top bioreactor
Typically bioreactors of 0.1–10 litres working volume.

Biomass
A term for the quantity of living organisms. In the context of a bioprocess biomass refers to the quantity of microbes in a culture. It which may be expressed directly by measurements such as dry weight, wet weight, total cell counts, viable cell counts or indirectly by measurements of e.g. optical density and an according correlation.

Bioreactor
A system consisting of a base unit and one or several vessels on whose peripheries further sensors and actuators can be connected. A bioreactor ensures the control of a bioprocess and provides optimum growth conditions for microorganisms. As a bioprocess is not necessarily a fermentation, the expression “fermenter” is used less and less.

C

Calibration
A method of providing known reference points for adjustment of the readings from a sensor to give accurate measurements around or between the reference values. Most sensor measurements drift over time and calibration minimises this source of inaccuracy. The type of sensor and the nature of the reference standards can determine how frequently sensors must be calibrated, as can effects of the sterilisation procedure used. Also some sensors age more quickly than others and can reach a point where calibration is no longer possible. Depending on the sensor a calibration can be made directly at the control unit of the bioreactor, on a separate device; or a qualified technician of the manufacturer is required.

Carbon
Typically a sugar, such as glucose, which the microorganism utilizes for metabolic process during growth and production of metabolites. The choice of carbon source is important, as the (non)formation of side products can be influenced. This is the case of e.g. glycerol in E. coli bioprocesses as acetate is not produced – unlike glucose. Also, a change of carbon source may be used as trigger for induction of production of a specific protein, e.g. switching to methanol from glucose in *P. pastoris* bioprocesses.

Cascade
Describes a control method in the bioreactor which controls the variation of several parameters within a limited range. This can be used e.g. for pO_2 control for which parameters like stirrer speed, gasmix, pressure and gassing rate are taken into account.

Catalyst
Substance, which reduces the activation energy without being used within 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.

Chemotrophic
If the energy comes from organic compounds, then the organism is chemotrophic.

Cleaning-in-Place (CIP)
Used for larger, *in-situ* sterilisable bioreactors where disassembly and manual cleaning would be impractical. A succession of cleaning solutions are pumped in sequence into the vessel and/or around the pipe work which has been in contact with the culture. CIP units like the one for Labfors 5 can be mobile or integrated into the skid of the bioreactor. Spray balls are used to clean the vessel interior.

Continuous Culture
After a fixed period of batch growth, medium is pumped into the vessel and harvest removed at the same time. The flow rate influences the growth of the microbe by supplying one key nutrient in a set concentration which determines the growth rate. This is chemostat operation during which all parameters including the biomass concentration remain constant.

Crabtree-Effect
This metabolic phenomenon was first discovered in Baker’s Yeast *(Saccharomyces cerevisae)* and is an overflow reaction producing ethanol, when there is too much glucose under aerobic conditions available. Thus, this effect is also called glucose-effect. Repressing respiratory genes, pyruvate cannot be oxidized via the citric acid cycle and the electron transport chain, but becomes reduced to ethanol which lowers energy yields. Because of this outcome the Crabtree-effect is also economically relevant. Yeast is an important organism and has to be propagated while avoiding the Crabtree-effect to enhance the cost effectiveness of the process.

Culture
The biomass being grown in the bioreactor vessel, usually in a liquid medium.

D

DCW (Dry Cell Weight)
As the fluid content of microorganisms can vary depending on their state, the dry cell weight is used for a precise estimation of the total biomass. A known volume is taken, washed in a defined buffer if needed, and dried in an oven before weighing. The generated dry cell weight concentration can be used for similar bioprocesses to correlate optical density, turbidity as well as total cell numbers and viable cell numbers.

Death/Decline Phase
After a stationary phase the death rate exceeds the growth rate. Thus the number of viable microorganisms decreases during the so called death phase.

Dissolved oxygen concentration (DO)
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. The dissolved oxygen concentration ranges between 0 % und 100 % and is monitored for instance by a polarographic sensor. Sometimes it is also indicated in mbar.

Double Jacket
A jacketed vessel allows for water to be circulated through an outer cylinder via inlet and outlet pipes. This can be in either closed or open circulation systems.

Downstream Processing
Processes following the bioprocess phase which involve extraction and purification of products.

Drift
The quality of measured values can change when sensors are used over a longer period. E.g. the measured value of a pH sensor can change during a longer bioprocess. A readjustment during the bioprocess, the so called product calibration can prevent the drift in order to ensure a continued precise measurement and therefore accurate control.

E

Electrode
see Sensor.

Error
In control terms, the difference between the set-point value and the actual value of a process variable or parameter. Its size and rate of change over time influence the output of the controller.

Exit Gas Analyser
Determines the amount of carbon dioxide and oxygen generated by a culture by measuring these values in the gas stream leaving the bioreactor. This can be used to make indirect assessments of the metabolism of the growing microbes. The measurement of the oxygen and carbon concentration is typical for anaerobic bioprocesses. For some bioprocesses, e.g. of *Pichia pastoris,* methanol concentration can be measured as an indirect indicator of concentration of the metabolite in the liquid culture.

Exponential Phase
See. Log Phase

F

Fed Batch Culture
The supply of nutrients or supplements to a batch culture to either extend production of biomass or increase the production of secondary metabolites/recombinant proteins.

Fermentation
Literally, the metabolic breakdown of acids, gasses or alcohols by microbes. Fermentation derives from the latin expression *fermentum*. Often it is wrongly used as a synonym for technical bioprocesses with microorganisms, cells or enzymes.

Fermenter (Fermentor)
See Bioreactor.

Foam
The froth formed on the surface of many cultures during a bioprocess. It is caused by proteins in the medium and the sparging of gas through the culture. It can be removed with liquid antifoams and/or a mechanical foam breaker.

Foam Sensor
A conductivity sensor which is used to detect foam above the liquid phase of an culture. The sensor is equipped with an insulating coat, from which only the uncovered top protrudes. If foam is detected, mostly a antifoam is added via a peristaltic pump with a programmed time lag. The time lag is used in order to prevent overdosing.

G

Gas Mixing
The control of gas combination is typically used for animal cell culture, and is also applied during microbial bioprocesses. Depending on the configuration bioreactors are equipped with a combination of mass flow controllers and/or magnetic valves allowing the introduction of blends of air, oxygen, nitrogen and carbon dioxide gases in any combination. Gas mixing allows to control of dissolved oxygen concentration. Especially bioreactors for cell cultivation often dispose of an additional gas line for carbon dioxide, which replaces a liquid acid solution for pH control.

H

Head Space
A proportion of the total volume of a bioreactor vessel usually left empty to allow for gas to leave the surface of the culture and provide some room for foam formation. This is typically 25–30 % of the total volume.

Heater Block
Normally made of aluminium, a heater block fits positively on a vessel so that a good heat transition is ensured. The block contains a heating element which increases the bioreactor's temperature during the liquid phase. For cooling purposes the block is equipped with cooling loops through which a coolant, e.g. water from a connected chiller, is circulated. Heating blocks are used with single-walled vessels and provide a better handling than the combination of cooling mat and cold finger.

Heterotrophic
If the carbon comes from organic carbon compounds, we speak of heterotrophy.

I

Impellors
Blades attached to the drive shaft of a bioreactor which provide stirring and mixing action. They can be different shapes and diameters, according to application. The most common are flat-bladed "Rushton" turbines for bacterial culture and the "Marine" impellors used for cell culture work. "Rushton" turbines provide maximum disruption of bubbles, "Marine" impellors tend to be used singly for low-shear, low-speed mixing.

Inoculation Port
A dedicated inlet allowing seed culture to be introduced aseptically into a bioreactor. It typically incorporates a silicone membrane seal which is pierced by a needle or syringe after disinfection with EtOH.

Inoculum
The quantity of biomass added to a bioreactor at the start of a bioprocess. The seed culture should ideally have a high viable cell count and be growing vigorously (in logarithmic phase). A typical inoculum volume is 5–10% of the working volume.

In-Situ Sterilizable (ISS) Bioreactors
see Sterilisation-in-Place/Sanitisation-in-Place (SIP)

Instrumentation
The measurement and control elements of a bioreactor. A typical control loop would have a sensor with operational amplifier, a local display and control actuator(s), e.g. a valve/pump.

K

k_La (Volumetric Mass Transfer Coefficient)
A measurement of the efficiency of transfer of oxygen into a culture. k_La is a physical property of the vessel geometry, sparger design, power input from the stirrer plus the number and design of the impellors and baffles. Many different methods of measurement can be used and the values obtained can vary greatly. The usual definition of k_La is expressed as h^{-1}, and values of 100–200 h^{-1} would be typical for stirred tank reactors (see Oxygen transfer rate also).

L

Lag Phase
A period at the beginning of a fermentation when the seeded microorganisms adapt to the prevailing operating conditions and preparing for exponential cell growth by producing new proteins. This can last from 1–2 hours for microbial cultures to 24 hours or more for cell cultures.

Log Phase
The period during which microorganisms grow with a maximal possible growth rate. This corresponds to an exponential increase in cell numbers. In batch-processes where all nutrients are provided in excess there is no limit for the cell division and the growth rate is at its maximum. If a nutrient solution is added exponentially but with lower rate – as e.g. during a fed-batch-process – the growth rate is constant, however lower. In both cases this phase may be accompanied by production of heat, acid, rapid depletion of nutrients and oxygen limitation for some bacterial/yeast cultures. Log phases can last from a few hours to many days, depending on the organism.

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 especially important for the cooking/ baking process and in the food industry, since this is where the colour and flavour of roasted, baked or fried food stems from. When preparing a bioprocess this reaction is also happening when media containing proteins and sugar is autoclaved, which is why these components should be autoclaved separately.

Mass Flow Controller (MFC)
A device that measures and controls the volumetric gas flow precisely, hands the actual value electronically to the bioreactor and retrieves set points from the machine. If there are several gasses connected, they can be controlled either via clocked magnetic valves and one MFC or a MFC for each gas. MFCs are thus ideal to control the dissolved oxygen concentration.

O

Oxygen Transfer Rate (OTR)
Defines the rate of transfer of oxygen into a bioreactor culture medium from the bubbles of gas sparged into the vessel. The size of the bubbles and its retention time are critical factors, as is the initial level of saturation of the medium with oxygen at a given temperature. A mathematical definition is the k_La oxygen solubility, and the value is usually expressed as mmol L^{-1} h^{-1}. Typical values for a standard stirred tank reactor are between 50–100 L^{-1} h^{-1}

Oxygen Supplementation
A procedure used to increase cell densities by increasing the percentage oxygen in the gas (usually air) going into the bioreactor by blending it with pure oxygen in proportion to the error in dissolved oxygen value compared with its set point. This can be realised with a second gas line equipped with a mass flow controller.

P

Parallel Bioreactors
Collection of two or more vessels, operating in groups, with common measurement and control systems capable of performing most of the standard functions of a bioreactor (often a stirred tank reactor), e.g. a minimum of pH measurement and control, nutrient feeding and sampling. Ease of handling and speed are the key benefits. Applications include process optimisation, statistical analysis, excursion testing and scaling-down of larger systems. Other models such as the Multifors 2 utilize despite a smaller working volume the same technology as seen in large scale bioreactors and can be modified with further powerful options such as additional sensors. Key points are the ease of use and the speed with which bioprocesses can be run. Applications of parallel bioreactors are process optimisation, statistical analysis, scale down of large scale reactors and research on single parameters that need to be optimised.

Peristaltic Pump
A pump which uses a stepper motor to drive a pump head with rollers mounted in a fixed housing. Silicone or other soft synthetic tubing is squeezed between the rollers and the housing to provide a peristaltic action and move liquids, e.g. from a reservoir bottle to the bioreactor vessel.

PID Control
A controller consisting of a proportional, integral and derivative part. It is used to adjust a set point as fast as possible an without exceeding the set point. PID control is used e.g. for programming cascades.

Pilot Scale
Typical sizes of bioreactors are from 20–500 l. Pilot scale bioreactors are often used for testing and optimisation of production techniques.

Phototrophic
Organisms are phototroph when their source of energy generation is sunlight.

pO_2
see DO.

Port
This is an opening in the bioreactor vessel (it can be in the top or sides or even at the bottom) which allows the fitting of vessel accessories such as electrodes or provides points of entry and exit for culture gas etc. An O ring seal or flat membrane protects the port opening. When a port is not in use it must be fitted with a blanking "plug" to ensure freedom from contamination. The number and size of each port are important factors in bioreactor design, as they determine what can be added. Common sizes are 12 mm/13.5 mm Pg, 19 mm and 25 mm.

Probe
see Sensor.

Production Scale
This typically refers to bioreactors larger than e.g. 500 L and can be up to several metres cubed. However, with the arrival of high value recombinant proteins, even a 10 l bioreactor present a production volume scale.

Pt100(-Sensor)
see Temperature Sensor.

Q

Qualification
A collection of documentation and test results which provide a bioreactor user with certification that a particular unit has been manufactured and tested to agreed standards. This is often required as part of a more comprehensive validation of a process.

R

Recombinant Protein
A protein expressed by a mammalian or microbial cell which has been introduced into the cell by a process of genetic manipulation. Where the protein is expressed, i.e. intracellular, extracellular or on a membrane, determines how it is extracted and purified. There may be several copies of the genes to express the protein introduced to increase its production, and genetic deletions may also be used to focus the cells' metabolic processes towards production of the required protein.

Respiratory Quotient (RQ)
RQ is a calculated value, expressed as a ratio between the oxygen used by a culture compared to the amount of carbon dioxide evolved and is an indicator for the metabolic state of a culture. RQ can be used to control feed rates of sugars and adjust the balance between biomass production and formation of metabolites. Modern bioprocess software offer soft sensors as a standard which allow a RQ calculation in real time.

Rotameter
A needle valve for the manual control of the gas volume flow. A steel or plastic ball rises up a tube of increasing width, floating on the inlet gas, until the required flow rate value is reached on a calibration scale marked on the glass wall of the tube. Rotameters have a typical error of up to 10 %, with the greatest inaccuracies at low flow rates. Gases of different density need different scale calibrations. The measured values cannot be transferred to the controller and the adjusted gas volume flow can only be changed manually. This being the reason why gas lines with rotameters cannot be used for automatic control of dissolved oxygen concentration.

Rushton-Impellor
see Impellor

S

Sample Device
see Super Safe Sampler

Scale Down
This allows key parameters for a large-scale fermentation to be determined using small vessels by directly extrapolating the information obtained, either in real time or as a template for future work.

Scale Up
Taking a process from laboratory-scale bioreactors to large production units in several steps. At each stage, factors such as handling, energy input, aeration, mixing, stirring and temperature control may have to be addressed and modified according to size.

SCADA
Stands for Supervisory Control and Data Acquisition. Generally spoken it is a computer system to collect and analyse real time data. In the field of biotechnology SCADA are considered nowadays as standard to optimise and control bioprocesses.

Seed
Production of a quantity of biomass under aseptic conditions to provide an inoculum for a larger bioreactor. Often, several stages are involved with the last one typically being grown in relatively large shake flask cultures. When the starter culture for e.g. a pilot bioreactor is produced in a smaller bioreactor, the latter is often called seed-bioreactor.

Sensor
A measurement device which can be a probe or electrode which penetrates the vessel and is in direct contact with the culture medium or an external device taking indirect measurements of e.g. concentrations of molecules in the exit gas stream such as carbon dioxide or methanol.

Single-Walled Vessel
A single-walled has the advantage of being lighter and therefore easier to handle. Unlike double-walled vessels the base unit a heater pad/block to provide temperature control. A cold finger or coil inside the vessel or block provides cooling.

Sparger
An inlet pipe carrying a gas, usually air, to the bottom of a bioreactor vessel, where it emerges just below the bottom impellor through a series of small holes. Spargers can have different shapes (e.g. ring, straight, curved and L-shaped) and the holes may point upwards or downwards.

Spin Filter
A device normally attached to the drive shaft of an animal cell bioreactor which allows culture liquid to be withdrawn continuously while leaving the cells behind. A mesh, typically 10–30 µm in size, forms a spinning cage which prevents cells either attaching or passing through. A spin filter allows slow-growing mammalian cells to produce e.g. a protein or antibody, over prolonged periods while harvesting a product and feeding with fresh nutrients.

Stationary Phase
A phase in a typical growth cycle for a batch culture where nutrients have been depleted. During this phase cells are dying at roughly the same rate as they are being produced by division, leading to a static situation. This equilibrium does not last due to the lack of nutrients, moving thus the cells into decline/ death phase.

Steam Sterilisation/Steam in Place (SIP)
See In-situ sterilisation.

Stirred Tank Reactor (STR)
A specific type of bioreactor which uses a drive shaft with impellors to provide mixing. This is the "traditional" design used for the first deep cultures for antibiotic production in the 1940's.

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

Super Safe Sampler
Using sampling devices such as the INFORS HT Super Safe Sampler small amounts of a culture can be sampled from a bioreactor without contamination or dead volume.

T

Temperature Sensor
A platinum resistance electrode which is used to give accurate temperature measurements both in the bioreactor vessel and peripheral pipe work. A pt-100 relates changes in electrical resistance of the sensor to temperature and 0°C has a value of 100 Ω, hence its name.

Total Volume
The total internal volume of the bioreactor, including any head space.

Transfer Line
A means of moving a culture from one vessel to another without contamination, either by flexible tubing or fixed pipe work which can be steam sterilised.

U

Upstream-Processing (USP)
This branch of bioprocess engineering comprises all methods with a focus on cultivation and optimisation that are destined to produce product in the actual bioprocess. That includes cell isolation, cultivation, expansion, development and optimisation of the inoculum, media and processes, also with the help of genetic manipulation.

URS (User Requirement Specification)
A detailed list of requirements by which a bioreactor can be defined for passing to manufacturers.

V

Validation
The procedures and protocols necessary to ensure a process meets approved standard of productivity, reproducibility and safety. Use of qualification documentation and test results for bioreactors and associated equipment is a part of this process.

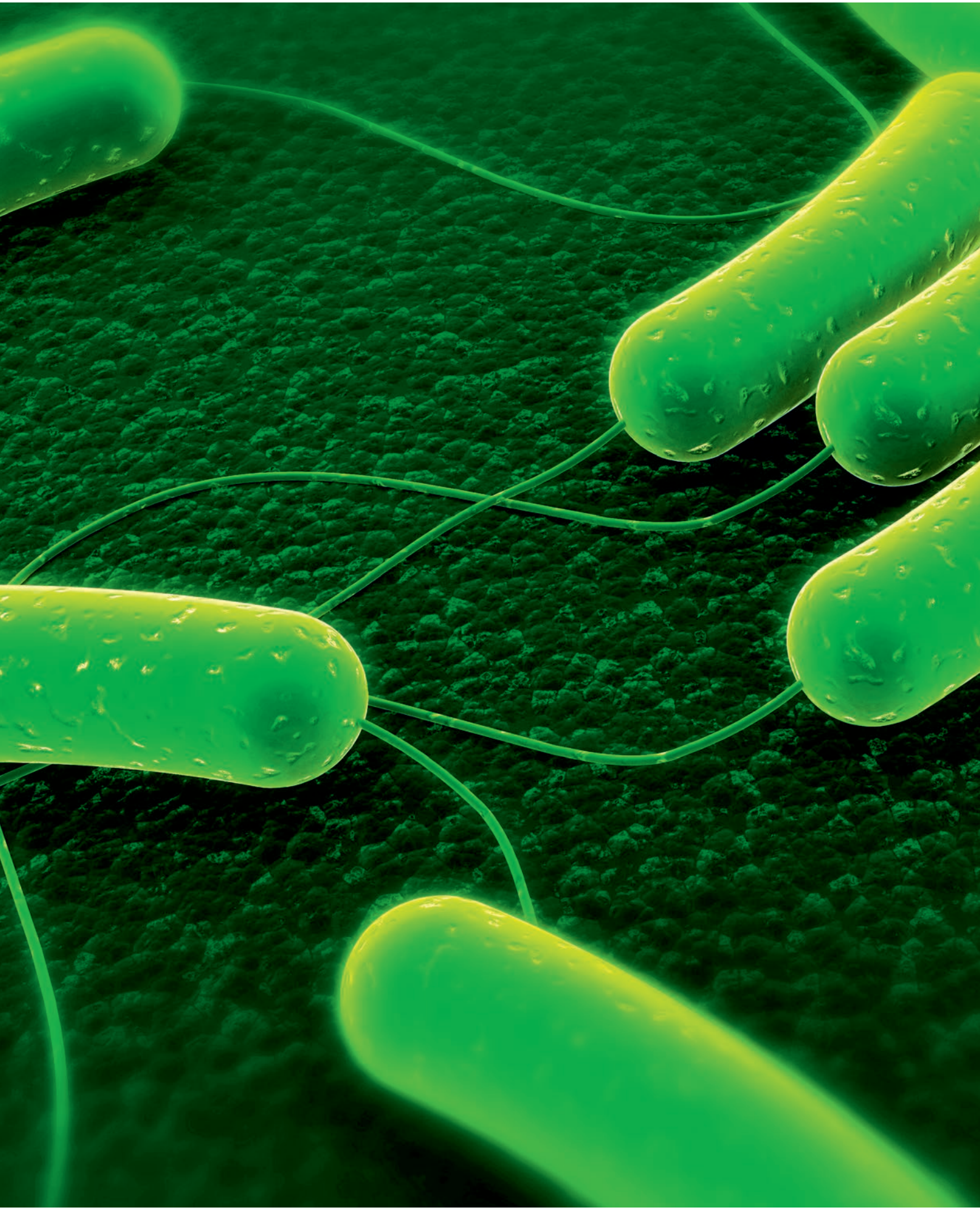
Vessel
Vessels are made from borosilicate glass or stainless steel with a flanged top onto which is fitted a top plate. An O ring separates the vessel body and top plate sections. The bottom of the vessel can be flat or rounded and most vessels above 10 l working volume have double jackets for liquid circulation to provide temperature control. The vessel size and its proportions are key elements in a bioreactor specification.

Vessel Volumes per Minute (VVM)
A value for gas flow rate into a vessel, based on its working volume in litres. Therefore it serves for scaling processes up and down. Typical air flow rates for microbial applications are 1–1.5 VVM which can be also expressed in L min^{-1}. Rates above 2 VVM are rarely used due to entrainment of water droplets in the exit gas stream. A bioreactor with 10 L working volume at 2 VVM requires a gas flow of 20 L min^{-1} (10 L * 2 min^{-1} = 20 L min^{-1}). Lower rates, e.g. 0.5 VVM, may sometimes be needed at the start of a fermentation to allow a period of CO_2 build-up and mammalian cell culture may need much lower rates, e.g. only up to 0.1 VVM, to avoid excessive foaming.

W

Working Volume
The working volume is typically 2/3 of the total volume of the bioreactor. For animal cell culture, a bigger surface area is usually required, and up to 50% of the total vessel volume may be used for gas transfer via the head space.

Notes



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