

Bioprocessing Basics

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Bioreactors and Fermenters: Powerful Tools for Resolving Cultivation Bottlenecks

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APPLICATION NOTE

Bioreactors and Fermenters: Powerful Tools for Resolving Cultivation Bottlenecks

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

Introduction

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

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



What are bioreactors and fermenters?

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

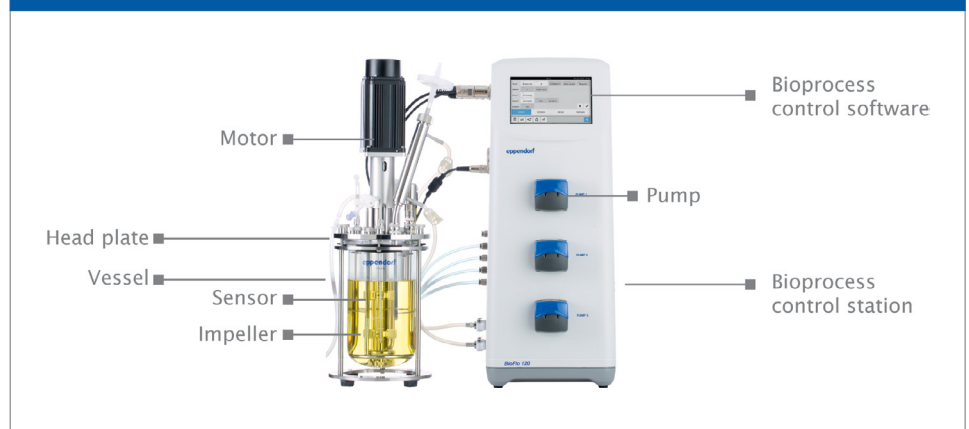
Bioreactor and *fermenter* are two words for basically the same thing. Scientists who cultivate bacteria, yeast, or fungi often use the term fermenter. The term bioreactor often relates to the cultivation of mammalian cells, but is also generically used. When we talk about bioreactors in this white paper,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

Stirred-tank bioreactors

Though many types of bioreactors exist, we will focus on stirred-tank bioreactors. The name is accurately descriptive. Cultivation takes place in the bioreactor tank—often called a vessel—and the culture is mixed by stirring (instead of shaking, for example). Stirred-tank bioreactors come in different sizes (for cultures of a few milliliters to thousands of liters) and are made of various materials (usually glass, plastic or stainless steel). The basic components and functioning of stirred-tank bioreactors are always the same. A stirred-tank bioreactor system consists of several parts (**Figure 1**):

- A vessel that is filled with the medium in which cells are cultivated
- A head plate to close the vessel
- Components within or attached to the vessel or the head plate to

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





measure and adjust the culturing conditions, such as feed lines and sensors

- A control system comprising external components used to adjust the culturing conditions (e.g., pumps) and control software

Creating optimal cultivation conditions

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

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

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

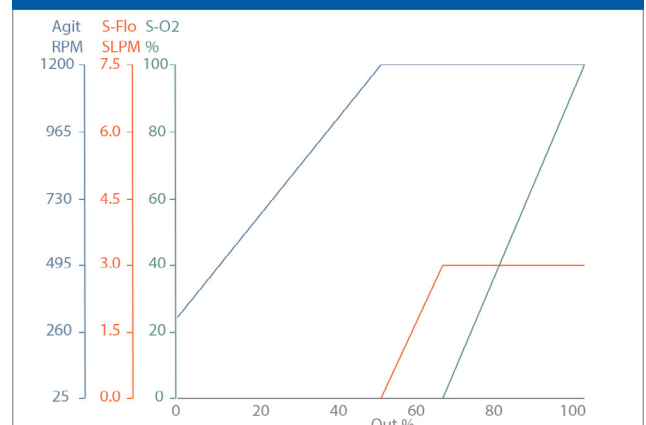
Establishing aerobic or anaerobic conditions. In a shaker or incubator,

oxygen is transferred from the surrounding air to the culture medium. This process is more efficient in shake flasks than in static cultures because shaking increases exposure of the liquid surface. In bioreactors, air or pure oxygen (coming for example from a compressed air cylinder) is usually introduced to the culture.

With the use of spargers, the gas/liquid interface can be increased and the oxygen supply maximized. Oxygen is important for culture growth, and the amount of oxygen dissolved in the medium (dissolved oxygen concentration, DO) is continuously measured with a DO sensor.

To keep DO at setpoint, a DO cascade is often set up in and executed by the bioprocess control software. **Figure 2** shows an example for a typical DO cascade. If DO drops below setpoint, first the agitation speed is gradually increased up to 1,200 rpm to increase oxygen transfer from the surrounding air. If this

Figure 2: Dissolved oxygen cascade





is not sufficient to keep DO at setpoint, the gas flow rate is increased up to three standard liters per minute (SLPM). As a final measure, the oxygen concentration in the gas mix is increased, shifting from gassing with air (containing 21% oxygen) toward gassing with pure oxygen. This is just an example. The minimum and maximum values of agitation, gas flow rate, and oxygen concentration can be optimized depending on the organism and process needs.

Anaerobic conditions can be established by gassing with N₂ or other anaerobic gases.

pH control. To regulate the pH of carbonate-buffered cell culture media, cell cultures in flasks or dishes are usually placed in CO₂ incubators. In bioreactors, the principle is the same; CO₂ is introduced to the culture from a compressed gas cylinder. In bioreactors, the medium pH is continuously measured using a pH sensor and CO₂ is added as needed. This is different from the situation in a CO₂ incubator, in which the CO₂ concentration in the internal

incubator atmosphere is measured and controlled, rather than the medium pH. Bioreactors also differ in that a basic solution is often added to compensate for acidification during culture growth.

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

Control of parameters at setpoint. In bioreactors, different components and the control software play together to control pH, temperature, and dissolved oxygen at the desired setpoint. The parameters are constantly measured using pH, temperature, and DO sensors. The sensors transmit the information to the bioprocess control software, which regulates the addition of CO₂ and liquid pH agents, the activity of tempering devices, agitation, and the gassing with air and/or O₂.

A typical bioprocess run

To maintain cultures, scientists usually keep them in cultivation systems within incubators. Bioreactors are usually used

for a specific experiment or production run, which may last hours, days, or weeks, depending on the organism and application. A bioprocess run typically comprises the following steps (**Figure 3**):

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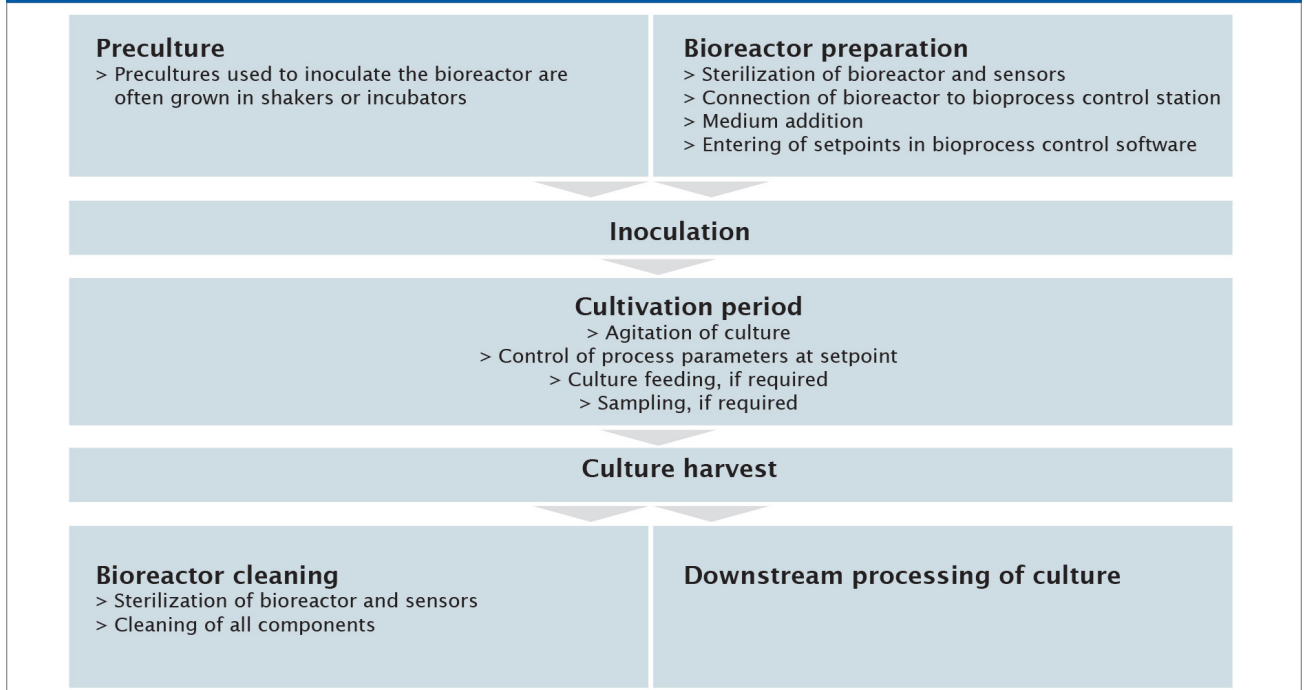


BioProcessing Introduction



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

Figure 3: Series of events in a typical bioprocess run





“Large amounts of a recombinantly expressed protein may be needed, for example, for biochemical or structural characterization, for ongoing use as research tools, or for ongoing use as research tools, or for evaluating medical applications.”

Cultures typically pass through four growth phases.

- In the lag phase, at the beginning of the culture, the organisms do not multiply or multiply only slowly, probably because they need to adapt to the new culture conditions.
- In the exponential growth phase, as the name says, the culture grows exponentially.
- In the stationary phase, growth stops because of the nutrient concentration, the oxygen concentration, the accumulation of byproducts, or other factors become growth-limiting.
- The stationary growth phase is followed by the death phase, in which the viable cell density decreases.

5. **Culture harvest:** Scientists typically end the bioprocess run and harvest the culture when it enters the stationary growth phase.
6. **Downstream processing:** The culture broth is further processed.
7. **Bioreactor cleaning:** The bioreactor is sterilized to inactivate culture residues and cleaned.

How bioreactors and fermenters help in resolving cultivation bottlenecks

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

Culturing large amounts of cells and microbes

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



of cells are needed for stem cell-based cell therapy and drug research applications.

Handle-less vessels

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

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

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

The availability of larger vessels is not the only advantage. In bioreactors,

“It’s critical to build evidence during the clinical trial that a differentiated outcome is possible, whether it’s versus existing treatments, a tablet form, or a less optimal form of the same treatment.”

higher cell densities can be achieved, sometimes making an increase of the working volume unnecessary.

Achieve higher cell densities

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

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

How did they manage? Using bioreactors allowed our engineers to lessen some of



“Bioreactors are valuable tools to optimize cultivation conditions.”

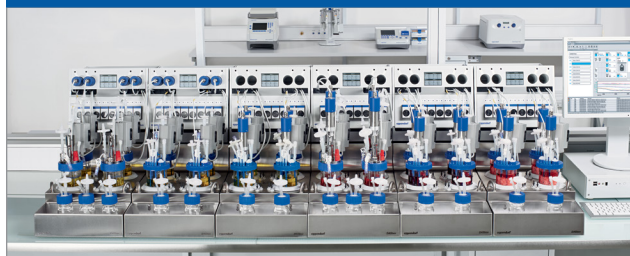
the growth-limiting factors in microbial cell cultures: They improved the supply with nutrients and oxygen, as well as the temperature control (2).

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

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

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

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



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

Comparing growth conditions

Parallel bioprocess systems are available to control more than one vessel (**Figure 4**). They often have small working volumes, which helps saving resources. Parallel systems have the advantage that cultivation parameters can be controlled independently in each bioreactor. This saves time and ensures maximum reproducibility between runs. If, for example, you would like to compare protein expression at eight different temperatures, in a parallel bioreactor system you can perform the experiments in parallel. This is more convenient than using a conventional shaker or a single bioreactor, where



you would either need to perform one experiment after the other or would need several shakers or bioreactors.

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

Increasing reproducibility

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

Let's take a simple example. You recombinantly express a protein. Let's assume the protein has the tendency to aggregate and aggregation

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

And the situation may be much more complex. Besides the temperature, process parameters including the medium pH, DO, metabolite concentrations, mechanical forces, and medium composition may influence

“Unwanted and unnoticed temperature fluctuations may be caused by repeated opening of the shaker by other users who add or remove flasks.”



culture growth, product yield, protein glycosylation patterns, byproduct formation, and more. Process parameters influence cell viability, cell behavior, and differentiation. This is especially important if the cell itself is the product of interest, for example if cells are used for cell therapy applications.

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

Home sweet home: A bioreactor for every organism

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

However, the optimal growth conditions

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

Bioreactor accessories

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

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



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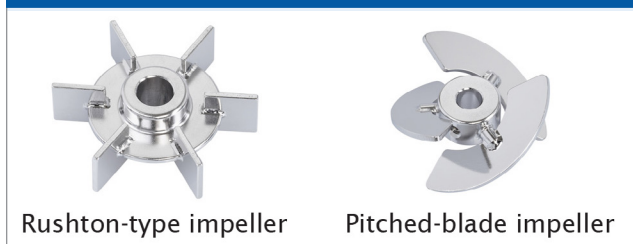
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Oxygen demand

The oxygen demand of fast-growing, aerobic microbial cultures is high. The bioprocess system needs to be capable of high gas flow rates to supply enough air and/



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



more efficiently than pitched-blade or marine impellers, but also cause higher shear forces (**Figure 5**). Therefore, the former are commonly used for microbial cultures, whereas the latter are commonly utilized for mammalian cell cultures.

Sterility

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

Setpoints

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

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

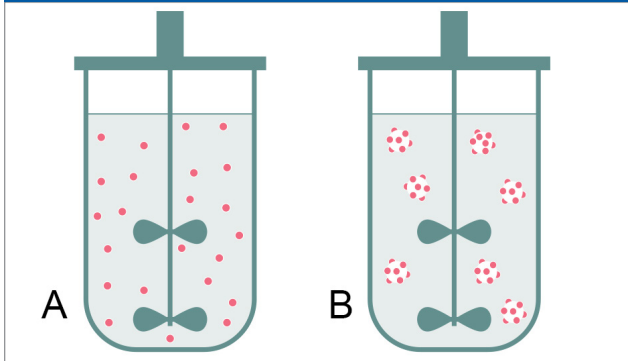
Culture mixing

Besides gassing, agitation is a critical parameter to keep DO at setpoint. Rushton-type impellers mix the culture



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Figure 6: Schematic representation of suspension cells and adherent cells grown in stirred-tank bioreactors A) Suspension cells in stirred-tank bioreactor B) Adherent cells grown on growth matrix in stirred-tank bioreactor



“They can be made of different materials, like glass, DEAE-Dextran, polystyrene, and alginate.”

glass, DEAE-Dextran, polystyrene, and alginate. There are also coated versions, whose core material is covered with peptides, proteins or protein mixtures like fibronectin, collagen or Matrigel®. The core material of non-coated microcarriers is not functionalized by the manufacturer, but may, however, bind proteins once the carrier is in contact with serum-containing culture medium. To start a cell culture process on microcarriers, they are typically added to the culture medium at a density recommended by the manufacturer and subsequently the bioreactor is inoculated with a single cell suspension. To support cell attachment to the microcarrier, the culture is only periodically agitated during the first few hours after inoculation. Once the cells have attached to the carriers the culture is continuously agitated to keep the carriers in suspension (**Figure 6**). For downstream processing, the microcarriers can be harvested and the cells detached, for example using trypsin.

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

can serve as a starting point for further optimization (5, 6).

Cultivation of adherent cells in stirred-tank bioreactors

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

Various matrices are in common usage.

Microcarriers

Microcarriers are spherical particles which provide a growth surface for adherent cells. Microcarriers typically have a diameter of 100–300 µm. They can be made of different materials, like



“Once the cells have attached to the carriers the culture is continuously agitated to keep the carriers in suspension.”

Fibra-Cel® disks

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

Cell-only aggregates

Instead of growing on a matrix, cells can grow in stirred-tank bioreactors as cell-only aggregates. This has been described, for example, for human

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

Summary

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

Conclusion

Stirred-tank bioreactors simplify the cultivation of large amounts of cells compared to conventional culture systems using shakers and incubators. Bioprocess control software facilitates the precise monitoring and control of



critical process parameters. Cultivating cells and microbes in bioreactors therefore can save the scientist work, time, and lab-space and improve the reproducibility and efficiency of cell growth and product formation.

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Advanced Technologies Facilitate Scale-up and Technology Transfer

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Single-use and modular technologies plus continuous manufacturing are increasingly important to biopharma scale-up and tech transfer.

It is hard to believe, but the biopharmaceutical industry is already old enough to have aging facilities that are decades old. FDA's focus on the need for updates is creating both opportunities and challenges for biologics manufacturers involved in the scale-up and transfer of production technologies. Single-use and modular technologies, along with continuous processing approaches, are helping the industry both modernize old processes and facilities and minimize the risks associated with making significant changes to existing systems.

Aging facilities attract FDA attention

Just as time passes more quickly as people age, it seems time goes by

more rapidly as industries mature. While the biopharmaceutical industry is young compared to the small-molecule pharmaceutical sector, it has been of significance for several decades. Some of the processes that are running today utilize the technologies developed when the industry was first established. "Many of these processes were licensed long ago and are quite complex, contain open steps, and are inefficient in many places," notes Parrish Galliher, CTO of BioProcess Upstream at the Life Sciences business of GE Healthcare.

FDA has recognized the need to upgrade these older processes and facilities and is imposing updates (1). Equipment suppliers are working closely with biopharmaceutical manufacturers to develop plans for implementing the needed changes. "This situation provides a great opportunity to update those systems, facilities, and practices, but also presents the



challenges associated with changing any process, including the risk of affecting product quality in some way. Fortunately, by working closely with tools and technologies suppliers and the regulators, biopharmaceutical manufacturers are better positioned to overcome such challenges,” Galliher asserts.

“These processes have high growth rates and densities, and thus the demand for oxygen is high, making it difficult to replicate a high-productivity process in a disposable reactor, according to Bird.”

Single-use solutions

One of the most efficacious ways to revamp older processes as required by FDA is to upgrade with single-use technologies, according to Helene Pora, vice-president of single-use technologies at Pall Life Sciences. “Single-use systems have proven to not only reduce capital investment, but also minimize turndown time, resulting in more effective and higher-quality manufacturing processes for scale-up and tech transfer,” she states. In fact, biopharmaceutical contract

manufacturers routinely assess single-use technologies as options when new equipment is introduced to meet a process need, according to Paul Bird, head of the manufacturing engineering group at Fujifilm Diosynth Biotechnologies’ Billingham, UK site.

Fujifilm develops and manufactures biologics using both microbial and mammalian production systems, and although single-use technology is a relatively new introduction, it has had an impact on mammalian manufacturing at the company. Mammalian cultures do not tend to be intensive; they do not have high oxygen demand and do not grow in very high cell densities, Bird explains. In addition, the culture growth takes place over a long period of time. As a result, a reactor for mammalian cell culture is not required to have high heat removal or provision for high oxygen supply; as such, single-use systems are well suited.

The situation is different for microbial cultures based on *Escherichia coli* and yeast, for example. These processes have high growth rates and densities, and thus the demand for oxygen is high, making it difficult to replicate a high-productivity process in a disposable reactor, according to Bird. “Because these cultures require very good heat removal, high oxygen transfer, and other rigorous conditions, stainless steel tends to remain the best possible option for



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microbial processes today,” he notes. Advances in single-use technologies designed specifically for microbial systems may, however, lead to their greater use in the future.

Galliher agrees that single-use technologies are not a panacea for the upgrading of older processes. “Most older facilities use stainless-steel manufacturing systems, so the conversion to disposable technologies is not automatically straightforward,” he observes. When single-use systems are chosen, however, he adds that they are fairly rapid to install and start-up, particularly relative to older legacy technologies, so the impact on facilities and utilities support systems is minimized. In addition, because the running costs are less for single-use systems, they can be considered enabling technologies.

Modular approach proves flexibility

While modular systems are a well-established concept and have been available in some form for several decades, they are attracting increasing attention in the biopharmaceutical industry today. In fact, the increasing availability of modular processing units is bridging the gap for many manufacturers with both established traditional facilities and new sites under construction, according to Pora. “For established facilities, modular processing units enable

“In addition, because the running costs are less for single-use systems, they can be considered enabling technologies.”

a quick and easy transition to the hybrid facility format, while for new facilities, they are making the fully flexible single-use facility a reality,” she says.

The popularity of modular systems is not just being driven by FDA mandates; Pora also notes that manufacturers have realized that modular solutions can help them overcome bottlenecks and become more efficient with less investment of time and money.

The global interest in standardizing facilities, harmonizing designs, and moving to a distributed facility model with a series of smaller identical facilities around the world is also driving interest in modular systems. “For this model to be successful, the facilities need to be identical in order to facilitate tech transfer, documentation, training, and essentially everything that needs to be commissioned to run these facilities in remote territories. Due to this trend, the demand for duplicate cookie-cutter-type modules has increased,” Galliher comments.

Another trend driving interest in modular technologies is a reduction in production volumes due to the switch to distributed



“Modular technologies are also increasingly available on the production equipment level and are used to build systems and processes that are identical in different locations, which again simplifies maintenance, documentation, training, and validation.”

production, increasing yields, and the trend towards personal medicine. “Smaller facilities require smaller production systems, which makes modularity increasingly possible,” says Galliher. He does note, however, that modularity will compete with stick-built facilities in places where labor is cheap. “Companies will have to consider the quality standards they want to reach and whether modules already constructed to meet cGMP and other industry requirements will better meet their needs,” he adds.

Modular technologies are also increasingly available on the production equipment level and are used to build systems and processes that are identical in different locations, which again simplifies maintenance, documentation, training, and validation.

“The modular nature of machinery makes modifying or adding options possible; their plug-and-play designs allow for easy modification with additional or different functions,” Galliher observes.

Fujifilm is a good example of a biopharmaceutical manufacturer that is taking advantage of modular production systems. In particular, the company designed a single system for the final bulk filling of cGMP products that replaces numerous existing filling systems. “By adopting a modular technology approach, we generated a unique capability for biologic drug product filling that for the first time provides flexible and adaptive manufacturing,” asserts Bird. He notes that the system is flexible because the production process supports bespoke operations meeting customer requirements in full, and adaptive because the modular nature of the system enables point-of-use increases or decreases in production capacity. “This ambitious project has extended the benefits for the most critical unit operation—final bulk filling—and has standardized the operator experience without impacting the flexibility to meet customer requirements,” he adds.

Consistent, flexible, yet rigorous business processes that introduce dependable operations on-time and in full are necessary for effective technology transfer, according to Bird.



“In addition to effective technology transfer between R&D sites and various business units, companies must have the ability to site, develop, and deliver into manufacturing correctly the first time. The collaboration of highly motivated and highly skilled people across multiple programs of activity is the key to success in both areas,” Bird states.

As one example of how Fujifilm is using technology to address current challenges, Bird cites the company’s “TAG” system for digitally managing the capture, conveyance, and retention of manufacturing knowledge to improve technology transfer, which provides controlled publication and distribution of manufacturing system standards and operational best practice.

Scale-down modeling for successful scale-up and tech transfer

One of the benefits of the use of smaller production facilities is a reduction in the scale-up factor for many biopharmaceutical processes. No longer are manufacturers required to scale-up from the lab to 20,000 L; more commonly processes are scaled up to 2000 or sometimes 5000 L. As a result, scale-up is less of a technological leap and therefore more predictable and lower-risk than in the past, according to Galliher.

The use of scale-down modeling, in which a large-scale system is reverse-engineered down to the lab scale so that it can be operated to model the large-scale process, has also significantly reduced the difficulties associated with process scale-up. With this approach, data that are representative of large process behavior can be collected early in the development process and at lab-scale costs, according to Galliher.

“The use of scale-down modeling, in which a large-scale system is reverse-engineered down to the lab scale so that it can be operated to model the large-scale process, has also significantly reduced the difficulties associated with process scale-up.”

He adds that better-designed model bioreactor, chromatography, and filtration systems are allowing for even smoother scale-up and tech-transfer operations.

Raw material choices are also being adjusted to improve scale-up processes. “Instead of buying small amounts of lab quality reagents and then moving to large-scale suppliers once a process



has been developed, today some manufacturers are beginning the development process with materials purchased from their eventual large-scale suppliers. This approach eliminates the need to change their raw materials at the point of tech transfer and scale-up and thus avoids any potential impact on performance and product quality," Galliher explains.

Continuous processing has potential to overcome scale-up and tech-transfer issues

The adoption of continuous processes and intensified manufacturing may have a significant impact on technology transfer and process scale-up. Pora believes that single-use technologies can also be aligned with the concept of continuous processing to address scale-up issues. "Many of the recent investments at Pall have been driven by this expectation, and our new single-pass tangential flow filtration modules and systems and inline concentrator are examples of technologies that have

resulted from our recent efforts," she says.

Galliher sees both advantages and disadvantages associated with continuous processing, and he remains uncertain whether these techniques and technologies will graduate to the commercial manufacturing stage. He does, however, believe that with their knowledge and skills, service providers can support biopharmaceutical manufacturers with the assessment of these new technologies and help them with scale-up and tech transfer into their own facilities. "When you drill down," concludes Pora, "the particular challenge is reducing complexity.

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A Beginner's Guide to Bioprocess Modes: Batch, Fed-Batch and Continuous Fermentation

Ying Yang and Ma Sha

*In this application note, we explain the differences between batch, fed-batch, and continuous fermentation and how these influence culture growth. As an example, we look at *E. coli* fermentation processes at bench scale. We track the biomass and nutrient concentrations during batch, fed-batch, and continuous fermentation runs. We explain different methods to analyze the process, including determination of biomass, growth rate, productivity, yield, and analysis of process costs. The comparisons can help bioprocess engineers to select the most appropriate method to meet their needs.*

*In our examples we studied *E. coli* fermentation at bench scale. The principles may also apply to bioprocesses using other microbes or mammalian cells, at both smaller and larger scales.*

Introduction

Fermentative microorganisms consume carbon sources, mainly sugar, to produce various acids, alcohols, and gases. In industry, fermentation is used to produce biopharmaceuticals, food and feed supplements, biofuels, and chemical building blocks. To establish a cost-effective process, bioprocess engineers have to consider various factors, including the costs for media and supplements, the process runtime, bacterial growth and viability, product titer and yield, and product quality. The concentrations of nutrients and by-products in the culture medium are important influencing factors. This is why, during process development, bioprocess engineers decide whether to apply a batch, fed-batch or continuous bioprocess.



“Disadvantages are the comparatively low cell densities which can be achieved and the relatively long downtime between batches, due to cleaning, vessel setup, and sterilization.”

In batch fermentation, microorganisms are inoculated to a fixed volume of medium in a fermenter. With microbial growth, the nutrients are gradually consumed and by-products accumulate. Therefore, the culture environment is continuously changing. The broth is removed at the end of the run. The growth curve is usually divided into distinct phases. During the initial lag phase, growth is slow, as the organism needs to adapt to the new environment. During the exponential growth phase, the microbes divide at a constant rate. When nutrients are getting depleted and by-products accumulate, growth slows down, and the culture enters the stationary growth phase. At this point, bioprocess engineers usually harvest the culture. If the culture continues, it would finally enter the death phase, which is characterized by a decrease in the viable cell density.

The advantages of batch processing are ease of operation and low risk of

contamination. Disadvantages are the comparatively low cell densities which can be achieved and the relatively long downtime between batches, due to cleaning, vessel setup, and sterilization. Batch fermentation is a convenient starting point for beginners in this field, and is often used to optimize conditions in the early stages of experimental design.

Fed-batch fermentation is a modified version of batch fermentation. It is the most common mode of operation in the bioprocess industry. Microorganisms are inoculated and grown under batch regime for a certain amount of time, then nutrients are added to the fermenter in increments throughout the remaining duration of fermentation to feed them. The entire culture suspension is removed at the end of each run. The start of feeding is normally determined by substrate limitation in the broth, and the time profile of feeding should be designed in a way that the substrate remains non-excessive while microbial growth is fully supported. Because of the addition of fresh nutrients, extensive biomass accumulation normally occurs in the exponential growth phase. Therefore, fed-batch fermentation is very useful for bioprocesses aiming for high biomass density or high product yield when the desired product is positively correlated with microbial growth. Also, because the substrate is not overfed during the process, by-product accumulation is limited.



“Cultures in steady-state can last for days, weeks or even months, thus greatly reducing the downtime and making the process more economically competitive.”

In continuous fermentation, fresh medium is continuously added to the fermenter, while used medium and cells are harvested at the same time. Consumed nutrients are replaced and toxic metabolites are removed from the culture. When addition and removal are at the same rate, the culture volume stays constant. Therefore, in contrast to fed-batch fermentation, the maximum working volume of the vessel does not limit the amount of fresh medium or feed solution which can be added to the culture in the course of the process. Keeping the working volume constant furthermore simplifies culture scale-up based on constant-power-to-volume strategy (1).

The rate of medium exchange can be optimized to reach a steady state. In steady state, the cellular growth rate and environmental conditions, like the concentrations of metabolites, stay constant. Cultures in steady-state can last for days, weeks or even months, thus greatly reducing the downtime and making the process more economically competitive. Due to the long cultivation, sterility maintenance can be challenging, and downstream processing is complicated (2).

For this application note, we cultured *E. coli* in Eppendorf BioBLU 3f Single-Use Vessels and directly compared these three modes of fermentation operation.

Material and methods

Bacterial strain

The bacterial strain used in this study was *E. coli* ATCC® 25922GFP™. A mini cell bank was prepared from the cryovial obtained from ATCC, as described previously (2). This strain has an ampicillin resistance gene (*bla*) encoded on a plasmid. A

concentration of 0.1 g/L ampicillin must be maintained in all cultures, since absence or low concentrations of ampicillin will result in plasmid loss (3). Therefore, from

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Determination of k_L Values of Single-Use Bioreactors

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“We carried out two different types of batch fermentation, one in complex medium and one in chemically defined medium. The medium recipe was the only difference between these two batch fermentations.”

inoculum preparation in shake flasks to fermentation runs in 3 L vessels, we always added 0.1 g/L ampicillin to the medium.

Media preparation

We prepared different types of media for various purposes in this study. All chemicals were purchased from Sigma Aldrich® USA, unless mentioned otherwise.

We carried out two different types of batch fermentation, one in complex medium and one in chemically defined medium. The medium recipe was the only difference between these two batch fermentations. The complex medium was a water solution containing 47.6 g/L Terrific Broth Modified (Thermo Fisher Scientific®, USA), 0.4% (v/v) glycerol, and 0.03% (v/v) Antifoam 204 which is a mixture of organic polyether dispersions. The detailed composition of each medium is described in the appendix in **Table A1**.

The chemically defined medium was prepared as follows. We prepared 10 x citrate-phosphate buffer, 100 x trace element solution, thiamin (vitamin B1) stock solution, and magnesium sulfate stock solution in advance, as described in the appendix in **Table A2**. For each run, we first filled the vessel with a DI water solution containing citrate-phosphate buffer and antifoam. After autoclave sterilization, we aseptically added $MgSO_4$ stock solution, 100 x trace element solution, and thiamine stock solution to the vessel. Glucose and ampicillin were also aseptically added to reach a final concentration of 90 g/L and 0.1 g/L, respectively (**Table A1**).

For fed-batch fermentation, we used chemically defined medium as well, as described in **Table A1**. We prepared 1.5 L of feeding medium containing $MgSO_4$, thiamine, trace elements, and 614 g/L glucose.

For continuous fermentation, the preparation of chemically defined medium was almost the same as for fed-batch fermentation except the volume for citrate-phosphate buffer and the original total volume to start with (**Table A1**). The 1.5 L feeding medium was prepared the same way as for the fed-batch fermentation (**Table A1**). **Table 1** summarizes the media prepared for each operational mode.



Table 1: Medium composition

Operation mode	Culture medium	Feeding medium
Batch fermentation, complex medium	Complex medium containing Terrific Broth Modified and 0.4 % (v/v) glycerol	n/a
Batch fermentation, chemically defined medium	Chemically defined medium containing 90 g/L glucose, citrate-phosphate buffer, MgSO ₄ , thiamine, and trace elements	n/a
Fed-batch fermentation	Chemically defined medium containing 15 g/L glucose, citrate-phosphate buffer, MgSO ₄ , thiamine, and trace elements	Concentrated feeding medium containing 614 g/L glucose, MgSO ₄ , thiamine, and trace elements
Continuous fermentation	Chemically defined medium containing 8 g/L glucose, citrate-phosphate buffer, MgSO ₄ , thiamine, and trace elements	Concentrated feeding medium containing 614 g/L glucose, MgSO ₄ , thiamine, and trace elements

Table 2: Process parameters

Parameter	Configuration/setpoint
Vessel	BioBLU 3f Single-Use Vessel
Inoculation density	5 % (v/v)
Dissolved oxygen (DO)	30 %
Agitation	Magnetic drive; controlled by DO cascade; maximum 1,200 rpm
Gassing	Automatic gas flow and mix controlled by DO cascade
Temperature	37 °C; cooling controlled by stainless steel cooling finger
pH	7.0 ± 0.1; controlled by 25 % (v/v) ammonium hydroxide solution
Impeller	Three Rushton-type impellers
Sparger	Macrosparger

Inoculum preparation

For inoculum preparation, 14.28 g Terrific Broth Modified and 1.2 mL glycerol were dissolved in DI water to make a total volume of 300 mL, and sterilized through autoclaving. After cooling to room temperature, we transferred 150 mL of the medium and added 3 mL of the 0.2 µm membrane filtration-sterilized 50 x ampicillin stock solution (5 g/L, **Table A2**) to each of the two 500 mL sterile Erlenmeyer flasks. We inoculated each flask with 500 µL *E. coli* suspension from a single pre-thawed cryovial from the cell bank. We put the two Erlenmeyer flasks into an Eppendorf Innova® S44i incubator shaker, set the temperature at 37 °C and agitation at 200 rpm, and left it

overnight for inoculum growth. We transferred the entire 150 mL of growing culture from one Erlenmeyer flask to a pre-sterilized bottle, and pumped the *E. coli* suspension into the vessel to reach an inoculation ratio of 5% (v/v) for each run. The starting optical density at 600 nm (OD₆₀₀) was between 0.1 and 1.0. We had done a preliminary study to demonstrate that varying the starting biomass density between 0.1 and 1.0 OD₆₀₀ had no significant effect upon harvest in a normal 8+ hour 3 L fermentation run of an overnight *E. coli* inoculum (data not shown).

BioBLU® 3f Single-Use Vessel

Eppendorf BioBLU 3f Single-Use Vessels have rigid walls that maintain the performance and scalability of traditional stirred-tanks. BioBLU 3f vessels are specifically designed for microbial applications using bacteria,



Figure 1: Dissolved oxygen cascade

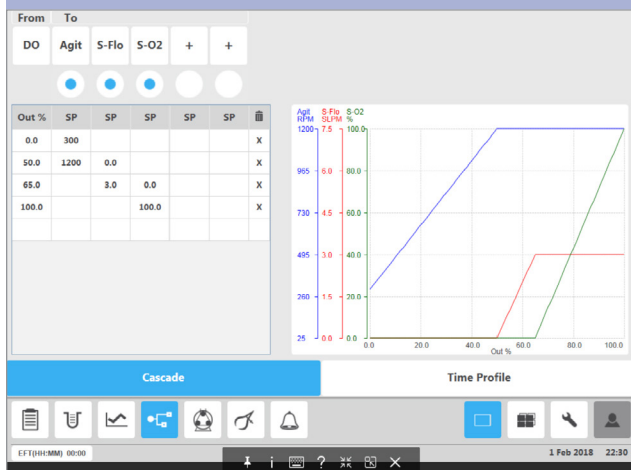
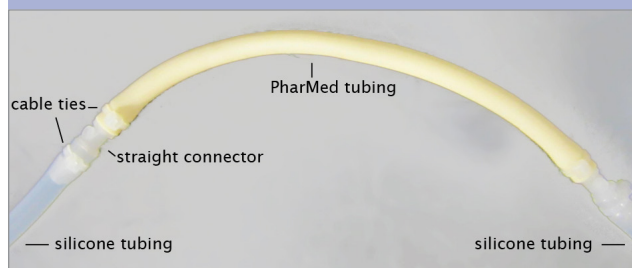


Figure 2: Silicone tubing connected with PharMed tubing



yeasts, and fungi. The vessels are made of an autoclavable material which allows medium sterilization within the vessel, similar to traditional glass vessels.

In this study, we ran all fermentations using the BioBLU 3f Single-Use Vessel. The vessel has a working volume range of 1.25–3.75 L and is equipped with a macrosparger and three Rushton-type impellers.

Process parameters

We used a BioFlo® 320 as the control station in this study. The process parameters applied in all fermentations are listed in **Table 2**.

Normally in aerobic microbial applications, DO control uses a cascade of agitation, air flow, and oxygen flow. By setting a DO cascade, the control station automatically adjusts the assigned process loops to maintain the DO level

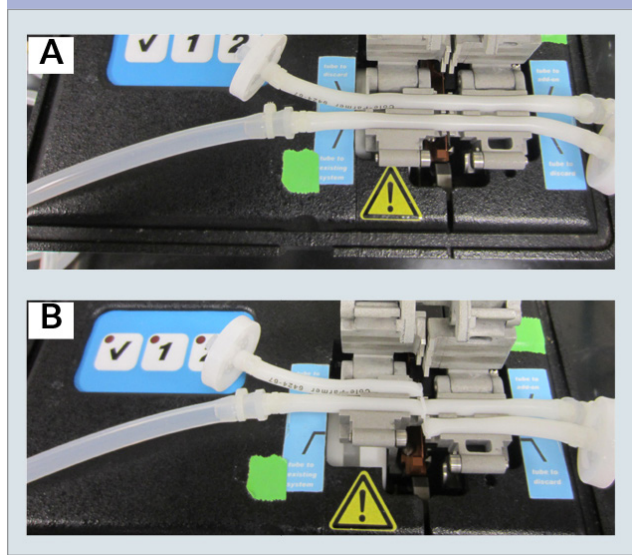
at a specific setpoint (4). In this study, we set a cascade to maintain the DO at 30%, as shown below for a DO output range between 0 and 100 (**Figure 1**).

Since acids are produced during *E. coli* fermentation, we only prepared 25% (v/v) ammonium hydroxide solution as the base for pH control (**Table A2**). For each run, 1 L base solution was prepared and sterilized through 0.2 µm membrane filtration. We used feed lines made of silicone for liquid addition to the bioreactor via the system's pumps. For base addition, we added a section of PharMed® tubing (Saint-Gobain®, France) between silicone tubing connections and fitted it to the peristaltic pumps on the BioFlo 320. PharMed and silicon tubing were connected by straight connectors and fixed with cable ties (**Figure 2**).

PharMed tubing better resists the base than silicone tubing and by inserting it into the pump head we avoid the risk of damage of the mechanically stressed part of the feed line. The pump used for base addition was specifically assigned as a base pump through the control station.



Figure 3: Tube welding. Silicone tubing is extended with weldable connectors made of C-Flex via straight connectors. **A:** Before welding **B:** After welding



airflow cabinet. If you have bottles mounted at the base of the vessel, you can also autoclave them with the vessel, without detaching their tubing from the head plate. Alternatively, tubing can be connected outside a laminar air flow cabinet using a tube welder. We did all tubing connections in this study by using a SCD®-II Sterile Tubing Welder from Terumo BCT, USA as shown in **Figure 3**. The welding was carried out at temperatures up to 300 °C for sterility maintenance. To be welded, tubing needs to be made of weldable material like C-Flex®, or needs to be extended with a weldable connector (**Figure 3**).

Vessel set-up and sterilization

We used a pH/Redox ISM® sensor (12 mm diameter with 225 mm insertion depth) for pH monitoring and an analog polarographic DO sensor (12 mm diameter with 220 mm insertion depth)

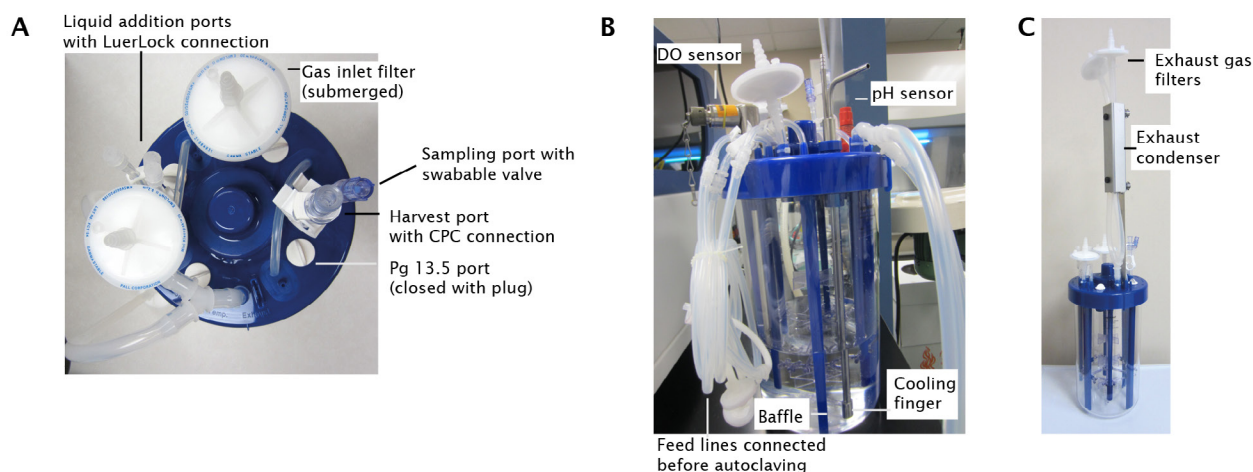
“We used a pH/Redox ISM® sensor (12 mm diameter with 225 mm insertion depth) for pH monitoring and an analog polarographic DO sensor (12 mm diameter with 220 mm insertion depth) for dissolved monitoring (Mettler Toledo®, Switzerland).”

We also prepared and sterilized 100 mL 10% (v/v) Antifoam 204 solution to add to the ongoing fermentation when needed, as excessive antifoam may reduce the oxygen transfer rate and affect microbial growth. In all the runs presented in this study, the 0.03% (v/v) antifoam added at the beginning of medium preparation were sufficient to control foaming throughout the entire culture, so no additional antifoam was added during the actual run.

Connection of addition and harvest bottles

The fermenter and addition and harvest bottles can be connected under aseptic conditions, for example in a laminar

Figure 4: BioBLU 3F Single-Use Vessel preparation. **A:** Head plate of a BioBLU 3f Single-Use Vessel. Please refer to the text for details. **B:** BioBLU 3f Single-Use Vessel filled with a colorless medium. A pH and a DO sensor and a stainless-steel cooling finger are installed before autoclave sterilization. Feed lines are also connected before autoclaving. **C:** Exhaust condenser setup. The exhaust condenser should be installed post-sterilization to avoid heat damage to the head plate by the metal bracket



“We calibrated the pH sensor outside of the vessel, before sterilization, following the 2-point calibration method.”

for dissolved oxygen monitoring (Mettler Toledo®, Switzerland). The two sensors were installed on the head plate of the BioBLU 3f vessel, through Pg 13.5 ports, before sterilization of the vessel. **Figure 4** shows the setup of a BioBLU 3f Single-Use Vessel. Its head plate has four Pg 13.5 ports which can be used for installing DO and pH sensors, stainless-steel cooling fingers, and more. In addition, there are one harvest tube, one

sample port, one thermowell, two ports for overlay liquid addition, one port for submerged liquid addition, one gas inlet with filter, one exhaust with two filters, one additional exhaust for pressure release during autoclaving, and four baffles (**Figure 4**). **Figure 4B** shows a medium-filled BioBLU 3f vessel with a pH sensor and a DO sensor and a stainless steel cooling finger, installed before autoclave sterilization. **Figure 4C** shows how the exhaust condenser is set up to keep the two exhaust filters standing up straight. The exhaust condenser is installed after autoclave sterilization to avoid heat damage to the head plate by the metal bracket.



Sensor calibration

We calibrated the pH sensor outside of the vessel, before sterilization, following the 2-point calibration method. We used a buffer at pH 7.00 to set ZERO, and a buffer at pH 4.00 to set SPAN.

We calibrated the DO sensor inside the vessel after sterilization, just before the inoculation. Before vessel assembly, we filled the sensor, through its cap, with fresh electrolyte solution that was separated from the medium by a permeable membrane at the tip. Six-hour sensor polarization was accomplished by connecting the sensor with the control station, which provided a voltage to establish an anode and a cathode within the sensor (5). The pH and temperature of the fresh medium were adjusted to 7.0 and 37 °C, respectively, to simulate the actual *E. coli* culture condition. As with the pH calibration, DO calibration was performed by applying the 2-point calibration method. We sparged pure nitrogen at three standard liters per minute (sL/m), with the

maximum 1,200 rpm agitation until the DO value stabilized, to set ZERO at 0%; then we switched the gas supply from nitrogen to air at three sL/m, with 1,200 rpm agitation until the DO value stabilized, to set SPAN at 100%.

Pump calibration

Pump calibration was performed before the run. The same tubing applied to the peristaltic pump head for liquid addition or harvest during fermentation should be used in pump calibration. Pump calibration was performed by pumping DI water into a fully filled section of tubing for a set period of time and tracking the water volume collected in a graduated cylinder at the end of tubing. Then the maximum pump speed specific to the tubing used can be recorded in the system. Pump calibration is critical, especially for fed-batch and continuous fermentations in which a detailed time profile of feeding is applied. Since the BioFlo 320 has multiple bi-directional pumps, it is easy to simultaneously perform both feeding and harvest using one control station, without the need for

external pumps.

Feeding strategy

For fed-batch and continuous fermentations, nutrient feeding and broth harvest were carried out following a pre-set time profile of pump speed. This

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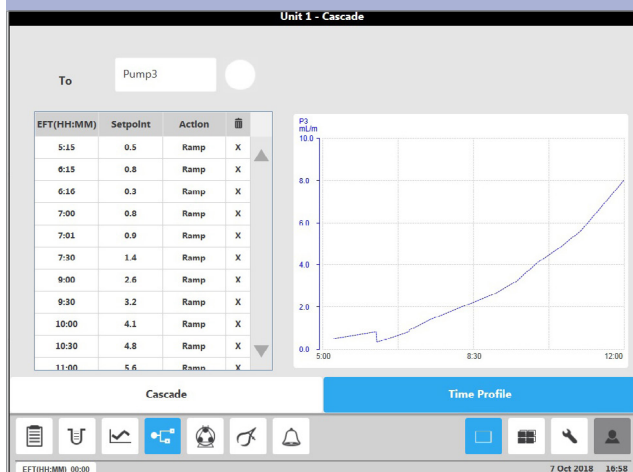
Scale-Up of *Escherichia coli* Fermentation

The parameters described include proportional vessel transfer geometry, oxygen transfer rate (OTR), impeller power number (Np) and impeller power consumption per volume (P/V).

We carried out *E. coli* fermentation runs at three different scales (1, 10, and 100 L) following a constant P/V strategy, and represented the *E. coli* biomass growth trends by plotting optical density at 600 nm (OD₆₀₀) versus run time. The fermentation time at each of the three scales produced very similar biomass over time, indicating excellent scalability within the Eppendorf fermenter product family.



Figure 5: Time profile of pump speed



the anticipated DO spike caused by carbon source exhaustion. It is a common practice to rely on the DO spike to signal fed-batch feeding start, however, the carbon source exhaustion that causes a DO spike and may also shift *E. coli* metabolism and reduce peak biomass potential.

For continuous fermentation, two pumps were assigned to follow the same time profile but with opposite directions, one for feeding, and the other one for harvest.

Optical density measurement

After DO sensor calibration and right before inoculation, 20 mL of fresh medium were taken from the vessel. One milliliter of medium was used to set blank for measurement of optical density at 600 nm on an Eppendorf BioSpectrometer® kinetic, and the rest was used to dilute the dense *E. coli* suspension collected in the later phase during fermentation. Samples

can be achieved in the “Time profile” setting under “Cascade” to a designated pump (**Figure 5**). For time duration calculation, EFT (elapsed fermentation time) was initiated at inoculation.

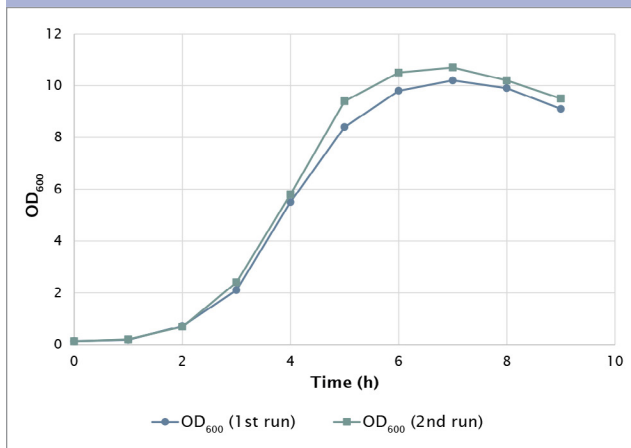
The detailed time profiles of pump speed are displayed in **Tables 3 and 4** (1, 6). For highest biomass potential, we adjusted the feeding start time before

Tables 3 & 4: Time profile of feeding pump speed at different EFT during fed-batch fermentation **3:** Time profile of feeding pump at different EFT during continuous fermentation

Elapsed fermentation time (EFT, hh:mm)	5:15	6:15	6:16	7:00	7:01	7:30	9:00	9:30	10:00	10:30	11:00	11:30	12:00
Pump speed (mL/min)	0.5	0.8	0.3	0.8	0.9	1.4	2.6	3.2	4.1	4.8	5.6	6.8	8.0
Elapsed fermentation time (EFT, hh:mm)	3:30	4:30	5:30	6:30	7:00	7:30	8:00	8:30	10:00	11:30			
Pump speed (mL/min)	0.2	0.4	0.6	1.2	1.6	2.0	2.6	3.1	5.1	8.0			



Figure 6: Batch fermentation in complex medium



were taken every 0.5 to 1 hours until a decreasing trend of OD₆₀₀ was observed.

Glucose measurement

Samples taken out for optical density measurement were also used for glucose analysis. We first pelleted the bacteria from the collected suspension by centrifugation in a Centrifuge MiniSpin® plus (Eppendorf, Germany), saving the supernatants, and then measured the glucose concentration in the supernatant using a Cedex® Bio Analyzer (Roche Diagnostics®, Germany).

Cell wet weight (CWW) and cell dry weight (CDW) measurement

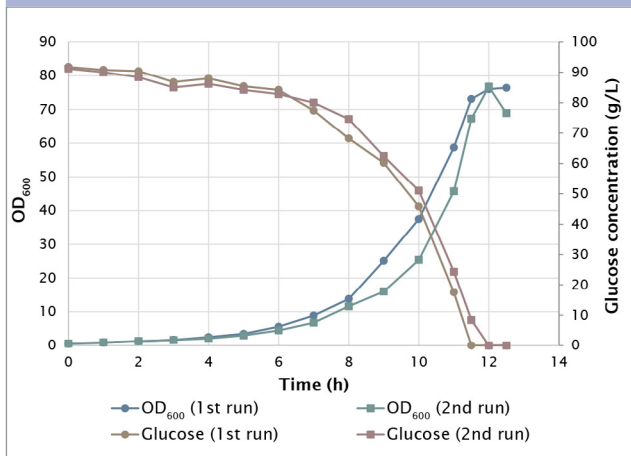
E. coli suspension samples with different OD₆₀₀ ranging from 0 to 160 were taken during continuous fermentation for cell weight measurement. For cell wet weight, we transferred 6 mL of each

suspension to a pre-weighed 15 mL Eppendorf conical tube, pelleted down *E. coli* cells using the Centrifuge 5920 R (Eppendorf, Germany), discarded the supernatant, and weighed the pellet-containing tube again. The weight of each pellet was calculated accordingly. For cell dry weight, we transferred 5 mL of each suspension to a pre-weighed 15 mL Eppendorf Conical Tube, pelleted down using the Centrifuge 5920 R, discarded the supernatant, washed the pellet twice by resuspension and centrifugation, and finally resuspended the washed pellet to make a final volume of 5 mL. We transferred 3 mL of this suspension to a pre-weighed aluminum weighing dish, dried it at 80 °C in an oven overnight for 16 hours, and weighed each dish again. Then we calculated the dry mass of each sample.

Results

For each fermentation mode, we ran the experiments in duplicate and presented the data obtained from both runs.

Batch fermentation in complex medium is shown in **Figure 6**. *E. coli* growth was limited, with the highest OD₆₀₀ being 11 at t = 7 h. To keep the DO at setpoint, only air sparging was required upon reaching the maximum agitation at 1,200 rpm, and no oxygen enrichment was needed throughout the process. We observed a standard growth curve for 9 h, with distinct lag, exponential

Figure 7: Batch fermentation in chemically-defined medium

growth, stationary, and death phase (Figure 6).

Batch fermentation in chemically defined medium

The growth curve of the batch fermentation in chemically defined medium is shown in **Figure 7**. Though we observed a relatively extended lag phase of 6 to 7 hours, the highest OD₆₀₀ was 77 at t = 12 h, which was a 7-fold increase of biomass compared to the batch fermentation in complex medium. The exponential growth of *E. coli*, with a 70-fold OD₆₀₀ increase, was accompanied by the drastic consumption of 80 g/L of glucose between 7 and 12 h. After glucose depletion at t = 11.5–12 h, bacterial growth stopped. For the DO cascade, we observed oxygen enrichment after air sparging reached 3 sL/min. Oxygen enrichment reached up to 40% toward the end of cultivation.

Figure 8: High-density *E. coli* culture in the BioBLU 3f Single-Use Vessel. Fed batch fermentation process 11 hours post inoculation

By comparing batch fermentations in complex and chemically defined medium, we found that medium composition plays a significant role in shaping the bacterial growth and biomass potential. Since batch fermentation is a relatively easy operation, medium optimization can be done in small scale batch mode to reduce time and cost on process development.

Fed-batch fermentation

In the fed-batch fermentation process (**Figure 8**) we achieve a maximum OD₆₀₀ of 240 at t = 11 h. This was the highest cell density among all fermentations in this study, which verified the capability of fed-batch fermentation to achieve very high biomass density.



Figure 9: Fed-batch fermentation

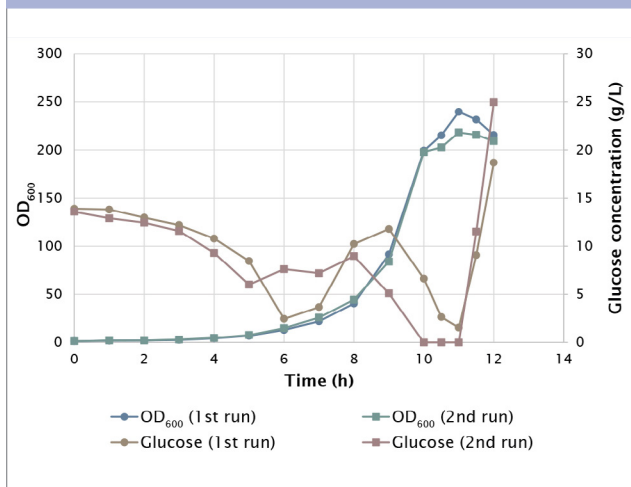


Figure 11: Continuous fermentation

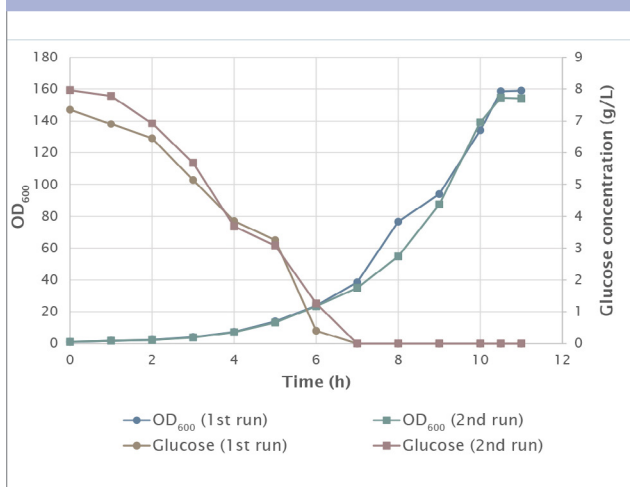
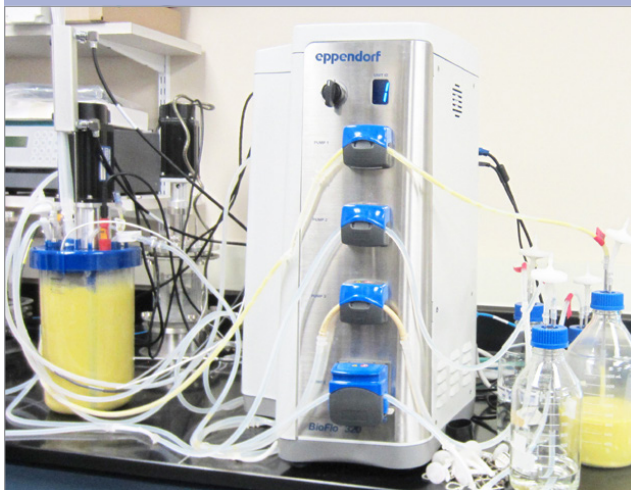


Figure 10: Setup of the continuous fermentation process



throughout most of the fermentation process and we observed a significant accumulation of biomass corresponding to a more than 150-fold OD₆₀₀ increase between 8 and 10 h. Glucose was depleted or almost depleted when the maximum OD₆₀₀ was reached, then it started to accumulate during death phase. For the DO cascade, oxygen demand increased to 100% at t = 10.5 h. The run during which we managed to avoid glucose depletion throughout the entire fermentation process achieved the highest peak OD.

Continuous fermentation

The maximum OD₆₀₀ observed in continuous fermentation (**Figure 10**) was 159 at t = 10.5 h in one run. The detailed growth curve and glucose consumption pattern are displayed in **Figure 11**. As with fed-batch

The detailed growth curve and glucose consumption pattern are shown in **Figure 9**. We initiated feeding before glucose depletion, which triggered strong exponential *E. coli* growth. With the exponential increment of feeding rate, we avoided early glucose depletion



Figure 12: The correlation between *E. coli* cell wet weight and OD₆₀₀

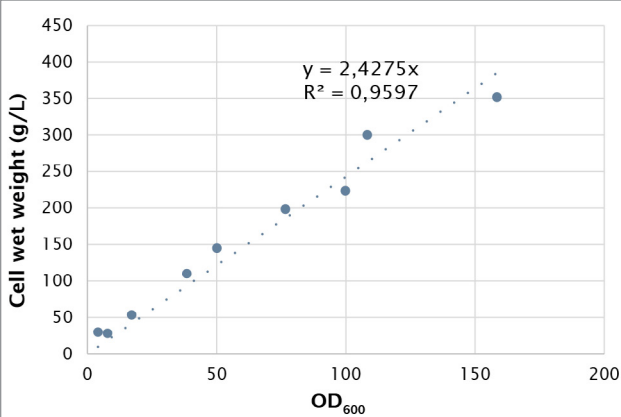
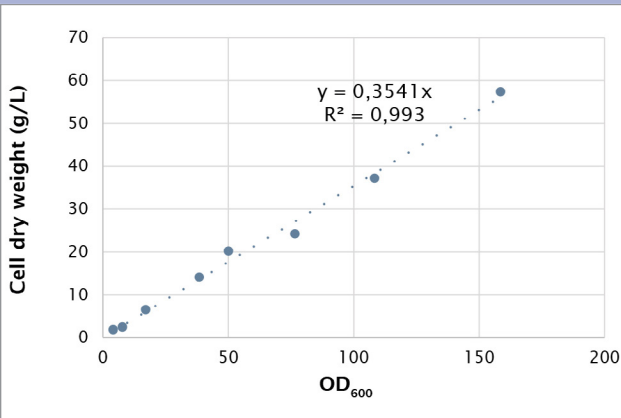


Figure 13: The correlation between *E. coli* cell dry weight and OD₆₀₀



fermentation, we initiated feeding before glucose depletion, and began broth harvest simultaneously. Glucose depletion occurred at $t = 7$ h and stayed depleted throughout the rest of the cultivation, indicating that the newly fed glucose was completely consumed by the growing *E. coli* during this period. For the DO cascade, oxygen demand increased to 100% at

Table 5: Maximum biomass concentrations gained in different operation modes

Operation mode	Maximum wet weight (g/L)	Maximum dry weight (g/L)	Maximum OD ₆₀₀
Batch fermentation, complex medium	26.7	3.9	11
Batch fermentation, chemically defined medium	186.9	27.3	77
Fed-batch fermentation	582.6	85.0	240
Continuous fermentation	386.0	56.3	159

$t = 10.5$ h. The culture volume stayed constant throughout the culture. The OD₆₀₀ of the harvest broth was 95. The concentrations of biomass and glucose changed, meaning that the culture did not enter the steady state.

Cell wet weight, cell dry weight, and OD₆₀₀

We analyzed the cell wet weight and dry weight during a continuous culture run. The corresponding cell wet weight and dry weight are measured and summarized in **Figures 12** and **13**.

The correlations are: CWW (g/L) = $2.4275 \times OD_{600}$, CDW (g/L) = $0.3541 \times OD_{600}$. Based on the correlations, we calculated the maximum wet weight and dry weight of each run (**Table 5**).

Specific growth rate and doubling time

Specific growth rate μ and cell doubling time τ_d can be calculated based on biomass concentration in



Table 6: Volumetric biomass productivity of different operation modes

Operation mode	CDW per vessel at the end of the run (g)	Total volume (L)	Process run time (h)	Volumetric biomass productivity (g/(L·h))
Batch, complex medium	9.87	3	9	0.37
Batch, chemically defined medium	77.16	3	12.5	2.2
Fed-batch	225.6	3	12	6.27
Continuous	210.0	4.3	11	4.44

In batch and fed-batch fermentations, the biomass is collected from the 3 L working volume of the BioBLU 3i vessel. In continuous fermentation, the biomass is collected from the culture in the vessel (3 L) and harvest (1.3 L).

terms of dry weight as shown in equations (1) and (2) (7)

$$\mu = \frac{1}{X} \frac{dX}{dt} = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (1)$$

$$\tau_d = \frac{\ln 2}{\mu_{max}} \quad (2)$$

where μ is specific growth rate (h^{-1}) between two time points t_1 and t_2 , and X_1 and X_2 are biomass dry weight (g/L) at time points t_1 and t_2 , respectively. During exponential growth, *E. coli* cells are growing at their maximum specific growth rate, μ_{max} . The time frame between t_1 and t_2 defines this exponential growth phase, and τ_d is the biomass doubling time (h).

We then calculated the cell doubling time for the two fastest growth rates observed in this study. During fed-batch fermentation, the maximum specific growth rate occurred during the time

frame between 9 and 10 h, during which the steepest slope in the growth curve was seen. We calculated the μ_{max} to be $0.86 h^{-1}$ and doubling time τ_d to be 1.16 h, meaning that during this time frame, *E. coli* dry mass doubled every 1.16 h.

During continuous fermentation, as with fed-batch fermentation, the time frame between $t = 9$ h and 10 h was chosen for calculation. The maximum specific growth rate μ_{max} was $0.46 h^{-1}$ and doubling time τ_d was 2.17 h.

Comparison of process performance

We calculated the volumetric biomass productivity based on cell dry weight over the entire culture period (**Table 6**). Fed-batch fermentation had the highest volumetric biomass productivity at $6.27 g/(L \times h)$ among all culture modes. We also calculated the total glucose consumption and biomass yield on glucose for a more thorough comparison among batch fermentation in chemically defined medium, fed-batch, and continuous fermentation (**Table 7**). The biomass yield on glucose shows the effectiveness of each culture method

“During continuous fermentation, as with fed-batch fermentation, the time frame between $t = 9$ h and 10 h was chosen for calculation.”



Table 7: Biomass yield on glucose in different operation modes

Operation mode	Total glucose consumption (g)	Biomass yield on glucose (g CDW/g glucose consumed)
Batch fermentation, chemically defined medium	273.6	0.282
Fed-batch fermentation	713.7	0.316
Continuous fermentation	846.0	0.248

In batch and fed-batch fermentations, the biomass is collected from the 3 L working volume of the BioBLU 3f vessel. In continuous fermentation, the biomass is collected from the culture in the vessel (3 L) and harvest (1.3 L).

on converting glucose into bacterial biomass, and such effectiveness is an important factor to consider, both technically and economically, when evaluating a fermentation process.

According to **Table 7**, the total glucose consumption in fed-batch and continuous fermentation was 2.6 to 3-fold higher than in batch fermentation. When comparing biomass yield on glucose, fed-batch was the highest, and batch fermentation with chemically defined medium was only 11% less, indicating its effective glucose-to-biomass conversion.

Cost analysis

We calculated the net cost for growing *E. coli* in BioBLU 3f vessels under different fermentation modes based on the cost breakdown for chemicals, consumables, and labor from preparation to the real run and cleanup (**Table 8**). Energy, equipment, and rent are not included here since they vary significantly based on the actual laboratory setup. Regarding the total cost, batch fermentation is the cheapest, especially the batch fermentation in complex medium, which costs 25% less than fed-batch. However, when considering the unit cost per *E. coli* cell dry weight, fed-batch is the most economic fermentation mode, costing 4% less than continuous fermentation, 64% less than batch fermentation in chemically defined medium, and 94% less than batch fermentation in complex medium.

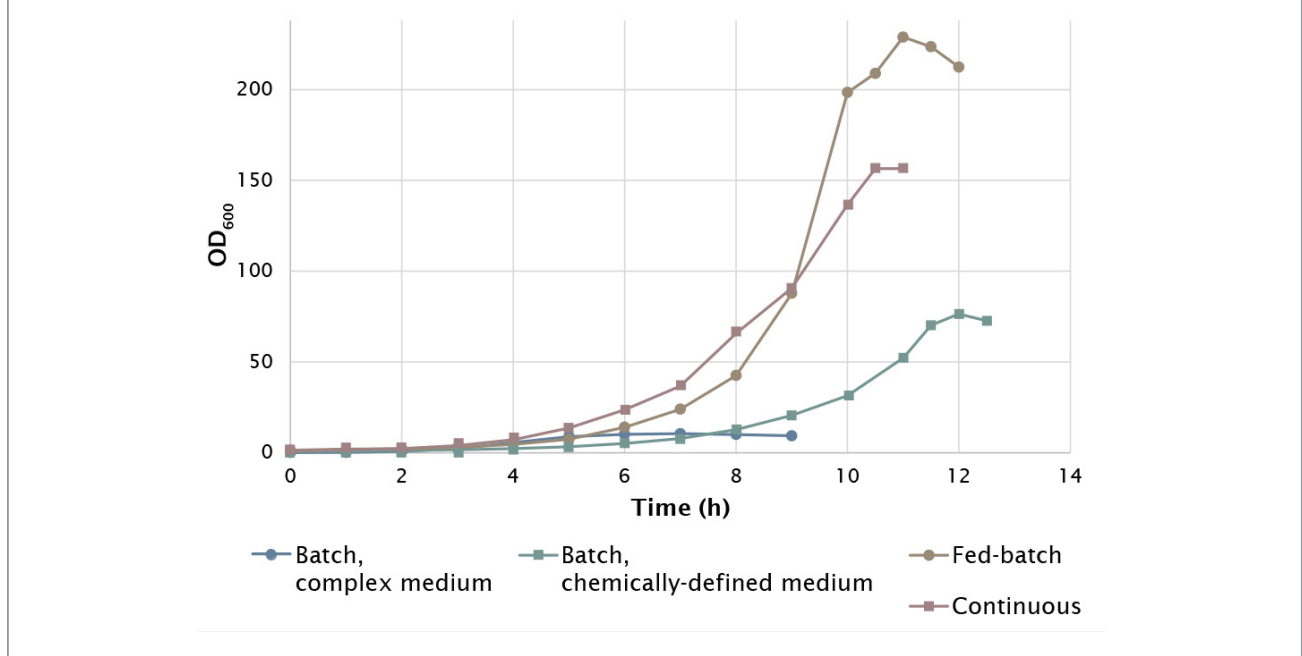
Table 8: Cost analysis of each operation mode for growing *E. coli* in a 3 L working volume in a BioBLU 3f Single-Use Vessel

Costs	Batch, complex medium	Batch, chemically defined medium	Fed-batch	Continuous
Chemicals (\$)	32.7	25.2	47.4	43.4
Consumables (\$)	720.8	740.9	785.1	785.1
Labor (\$75 per hour*)	825.0	1200.0	1275.0	1200.0
Sum (\$)	1578.5	1966.1	2107.5	2028.5
CDW per vessel (g)	9.87	77.16	225.6	210.0
Production cost per CDW (\$/g)	159.9	25.5	9.3	9.7

*from [8]



Figure 14: Growth curve comparison among four different fermentation modes



Conclusion

We averaged the two growth curves for each fermentation mode and presented them in one graph for a clearer comparison (**Figure 14**).

Batch fermentation in complex medium lasted for only 9 hours with the lowest biomass accumulation, and the maximum OD_{600} was 11. Batch fermentation in chemically defined medium, fed-batch, and continuous fermentations had similar durations, between 11 and 12.5 hours. Batch fermentation in chemically defined medium had the longest lag phase before the exponential growth phase to reach a maximum OD_{600} at 77, which might be due to the significant composition change between the complex medium in the

shake flask and the chemically defined medium with high glucose concentration in the fermenter. Fed-batch fermentation provided the highest OD_{600} at 229 on average with the maximum specific growth rate at 0.86 h^{-1} between 9 and 10 hours and a cell doubling time of 1.16 h. Significant biomass accumulation took place early in continuous fermentation after feeding and harvesting were initiated, and the specific growth rate were relatively stable over 4 hours during the exponential growth with an average maximum OD_{600} being 157.

We summarized the key findings and the advantages and disadvantages of each of the four fermentation operations in **Table 9**.



Table 9: Comparison of batch, fed-batch, and continuous fermentation

Operation mode	Maximum OD ₆₀₀	Maximum CDW (g/L)	Cost per unit CDW (\$/g)	Biomass yield on glucose (g CDW/g glucose consumed)	Pros	Cons
Batch, complex medium	11	3.9	159.9	n/a	Easy operation	Limited biomass and yield; long downtime between runs
Batch, chemically-defined medium	77	27.3	25.5	0.282	Easy operation; good biomass accumulation and high biomass yield on glucose	Long downtime between runs
Fed-batch	240	85.0	9.3	0.316	High biomass and yield; limited by-product accumulation (not analyzed in this study); very cost effective per unit biomass	Complex handling; time consuming; timing of feeding start and avoiding glucose depletion can be tricky
Continuous	159	56.3	9.7	0.248	Greatly reduced downtime, if operated in steady state; economically competitive; growth control via control of nutrient supply is possible; constant volume is scale-up friendly	Increased complexity for downstream processing; steady state difficult to reach with fast-growing strains

Batch fermentation in complex medium is a good starting point for fermentation beginners, and it is also the recommended method in the early stages of experimental design for strain selection and culture condition optimization. The growth improvement observed in batch fermentation in chemically defined medium compared to the one in complex medium proves the importance of medium composition in fermentation. Batch fermentation using an optimized medium composition is a feasible approach to achieving decent biomass without complex handling. In addition, batch fermentation in complex medium gave a high biomass yield on

glucose, indicating the robustness of the process in converting carbon source in the medium into bacterial biomass.

When high biomass and product yield are the main goal (if the product yield is positively correlated with biomass), fed-batch fermentation should be considered, and it is also a very cost-effective operation. However, since fed-batch requires complex handling compared to batch fermentation, it poses more challenges for the operator.

Continuous fermentation is an operation which is also economically competitive. Once well established with a constant



Table A1: Medium preparation for different modes of fermentation in a vessel with a 3 L working volume

Medium	Recipe	Use
Complex medium	300 mL DI water solution containing 14.28 g Terrific Broth Modified (Thermo Fisher Scientific) and 1.2 mL glycerol. After autoclave sterilization, transfer 150 mL medium to each of the two 500 mL sterile Erlenmeyer flasks, and add 3 mL 50 x ampicillin stock solution to each flask.	Overnight inoculum preparation
Complex medium	2,800 mL DI water solution containing 142.8 g Terrific Broth Modified, 12 mL glycerol, and 0.9 mL Antifoam 204 (Sigma-Aldrich, A6426). Autoclave at 121 °C for 20 minutes and cool to room temperature. Dissolve 0.3 g ampicillin in 50 mL DI water, sterilize through 0.2 µm membrane filtration, and aseptically add it to the medium.	Batch fermentation in complex medium
Chemically defined medium	1,650 mL DI water solution containing 225 mL 10 x citrate phosphate buffer and 0.9 mL antifoam. Autoclave at 121 °C for 20 minutes and cool to room temperature. Then aseptically add 24 mL MgSO ₄ stock solution, 24 mL 100 x trace element solution, and 0.5 mL thiamine stock solution to the vessel. Another 1 L solution containing 270 g glucose and 0.3 g ampicillin is sterilized through 0.2 µm membrane filtration into a 2 L bottle, and pumped into the vessel through aseptic connection before inoculation.	Batch fermentation in chemically defined medium
Chemically defined medium	1,250 mL DI water solution containing 225 mL 10 x citrate-phosphate buffer and 0.9 mL antifoam. Autoclave at 121 °C for 20 minutes and cool to room temperature. Then aseptically add 24 mL MgSO ₄ stock solution, 24 mL 100 x trace element solution, and 0.5 mL thiamine stock solution to the vessel. Then 25.2 g glucose and 0.3 g ampicillin are dissolved in 36 mL DI water, sterilized through 0.2 µm membrane filtration into a 50 mL sterile bottle, and aseptically transferred to the vessel as well.	Fed-batch fermentation
Chemically defined medium	2,500 mL DI water solution containing 450 mL 10 x citrate-phosphate buffer and 0.9 mL antifoam. Autoclave at 121 °C for 20 minutes and cool to room temperature. Then aseptically add 24 mL MgSO ₄ stock solution, 24 mL 100 x trace element solution, and 0.5 mL thiamine stock solution to the vessel. Then 25.2 g glucose and 0.3 g ampicillin are dissolved in 36 mL DI water, sterilized through 0.2 µm membrane filtration into a 50 mL sterile bottle, and aseptically transferred to the vessel as well.	Continuous fermentation
Feeding medium	Mix 135 mL MgSO ₄ stock solution, 0.5 mL thiamine stock solution, 45 mL 100 x trace element solution, and 1,315 mL 70 % (w/v) freshly made and 0.2 µm membrane filtration-sterilized glucose solution containing 920.5 g glucose to make a total of 1.5 L DI water solution.	Fed-batch and continuous fermentation

Table A2: Recipes for stock solutions

Solution	Recipe
10 x citrate-phosphate buffer	133 g KH ₂ PO ₄ , 40 g (NH ₄) ₂ HPO ₄ , and 17 g citric acid per 1 L DI water
10 x trace element solution	10 g iron (III) citrate, 0.25 g CoCl ₂ •6H ₂ O, 1.5 g MnCl ₂ •4H ₂ O, 0.15 g CuCl ₂ •6H ₂ O, 0.3 g H ₃ BO ₃ , 0.25 g Na ₂ MoO ₄ •2H ₂ O, 1.3 g Zn(CH ₃ COO) ₂ •2H ₂ O, and 0.84 g EDTA per 1 L DI water solution, sterilize by 0.2 µm membrane filtration
Thiamine (vitamin B1) stock solution	20 g thiamin per 1 L DI water solution, sterilize by 0.2 µm membrane filtration
Magnesium sulfate stock solution	240 g MgSO ₄ per 1 L DI water solution, sterilize by 0.2 µm membrane filtration
50 x ampicillin stock solution	0.5 g ampicillin per 100 mL DI water solution, sterilize by 0.2 µm membrane filtration
25 % (v/v) ammonium hydroxide solution	250 mL NH ₄ OH (Thermo Fisher Scientific) per 1 L DI water solution, sterilize by 0.2 µm membrane filtration



volume, continuous fermentation can reach a state when the specific growth rate is purely controlled by the feeding rate, which is very useful for growth control in both academic research and industrial production. It requires a thorough understanding of the bioprocess before one can successfully carry out continuous fermentation, and the operator needs to deal with the challenges in maintaining sterility and productivity throughout the run which can last for weeks and months.

Overall, depending on the experimental needs and the laboratory settings, and with a brief estimate of the process budget and scheduling, we hope that this application note will help fermentation scientists to choose the ideal fermentation method to meet their unique needs.

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