

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

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## Abstract

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.

In batch fermentation, microorganisms are inoculated to a fixed volume of medium in a fermentor. 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.

In continuous fermentation, fresh medium is continuously added to the fermentor, 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 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<sub>4</sub> 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 feeding medium containing MgSO<sub>4</sub>, 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 were 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 <sub>4</sub> , thiamine, and trace elements	n/a
Fed-batch fermentation	Chemically defined medium containing 15 g/L glucose, citrate-phosphate buffer, MgSO <sub>4</sub> , thiamine, and trace elements	Concentrated feeding medium containing 614 g/L glucose, MgSO <sub>4</sub> , thiamine, and trace elements
Continuous fermentation	Chemically defined medium containing 8 g/L glucose, citrate-phosphate buffer, MgSO <sub>4</sub> , thiamine, and trace elements	Concentrated feeding medium containing 614 g/L glucose, MgSO <sub>4</sub> , thiamine, and trace elements

### 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 (OD<sub>600</sub>) 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 OD<sub>600</sub> 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 f Single-Use Vessels have rigid walls that maintain the performance and scalability of traditional stirred-tanks. BioBLU f vessels are specifically designed for microbial applications using bacteria, 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

**Table 2:** Process parameters and setpoints common to all runs

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

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).

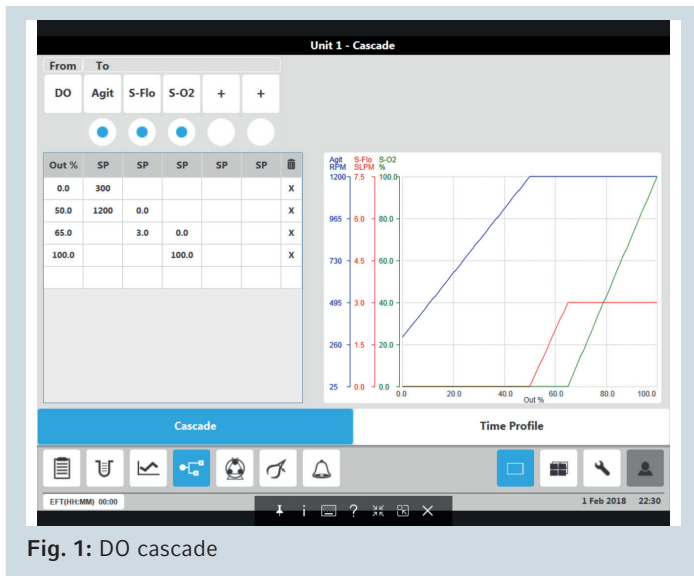


Fig. 1: DO cascade

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

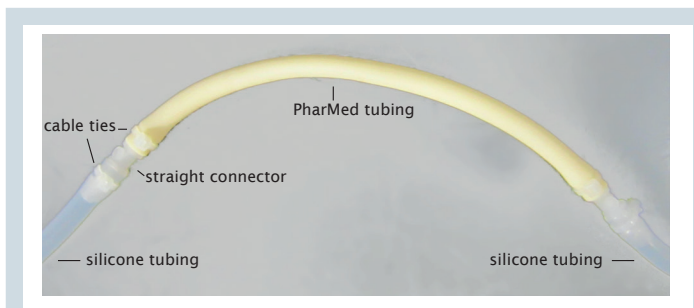


Fig. 2: Silicone tubing connected with PharMed tubing

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.

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 fermentor and addition and harvest bottles can be connected under aseptic conditions, for example in a laminar 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).

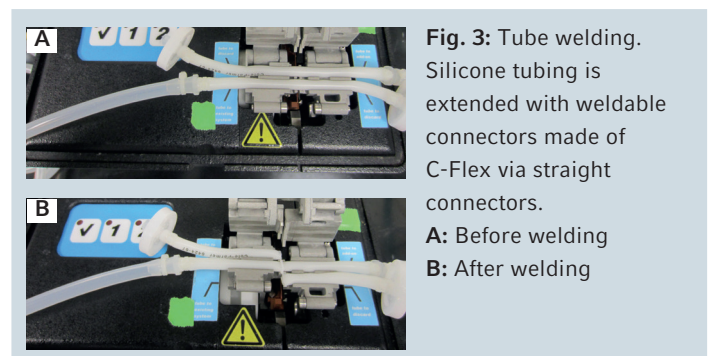
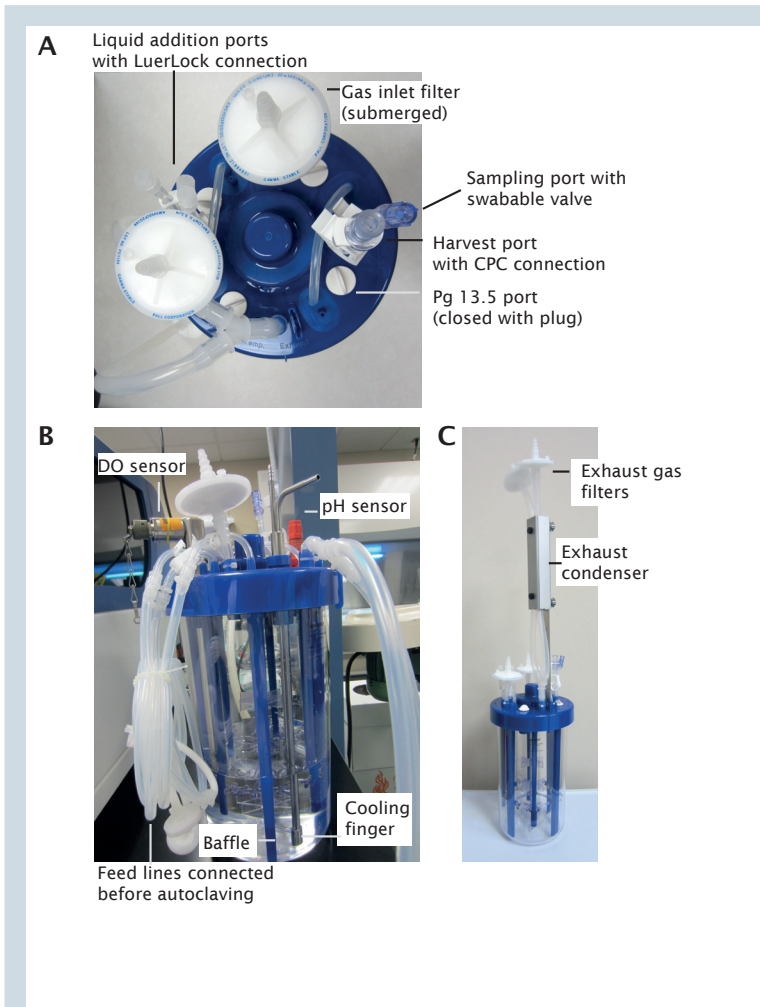


Fig. 3: Tube welding. Silicone tubing is extended with weldable connectors made of C-Flex via straight connectors. A: Before welding B: After welding

### 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) 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



**Fig. 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.

### Technical Features

In this study, we ran all fermentations using the BioBLU 3f Single-Use Vessel with a working volume range of 1.25 – 3.75 L. If you require smaller working volumes, get to know also our BioBLU 0.3f and 1f Single-Use Bioreactors with minimal working volumes of 65 mL and 250 mL, respectively.



[www.eppendorf.group/bioblu-f](http://www.eppendorf.group/bioblu-f)

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 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 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 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 were taken every 0.5 to 1 hours until a decreasing trend of OD<sub>600</sub> 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

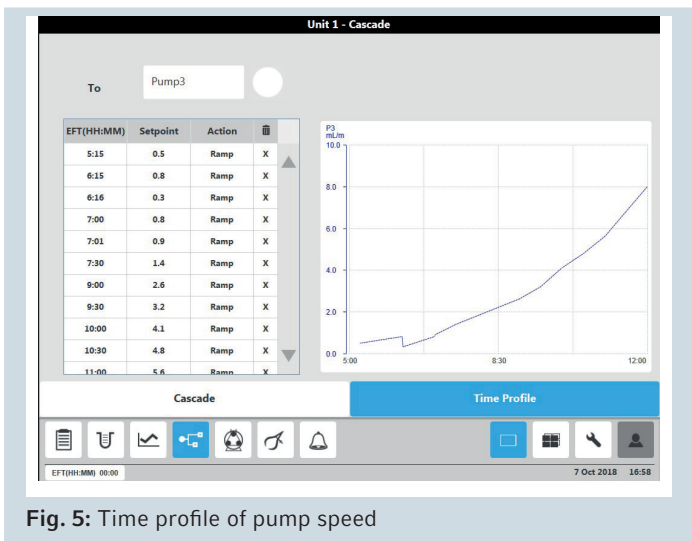


Fig. 5: Time profile of pump speed

Table 3: Time profile of feeding pump speed at different EFT during fed-batch 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

Table 4: Time profile of feeding pump speed at different EFT during continuous fermentation

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

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 OD<sub>600</sub> ranging from 0 – 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**

The growth curve of batch fermentation in complex medium is shown in Figure 6. *E. coli* growth was limited, with the highest OD<sub>600</sub> 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 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 OD<sub>600</sub> 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 OD<sub>600</sub> increase, was accompanied by the drastic consumption of 80 g/L of glucose between 7 and 12 h. After glucose

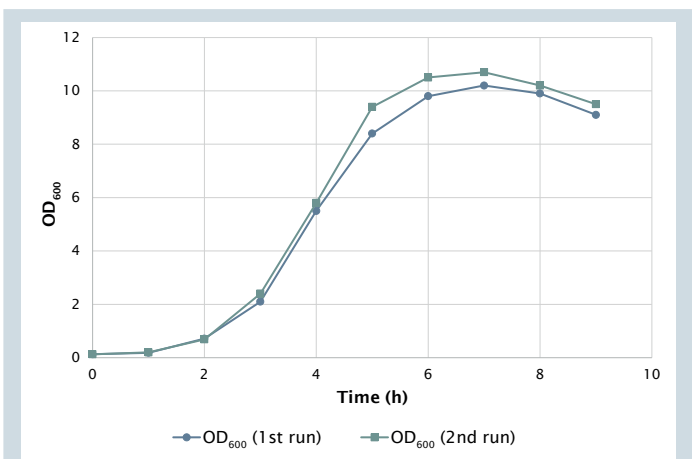


Fig. 6: Batch fermentation in complex medium.

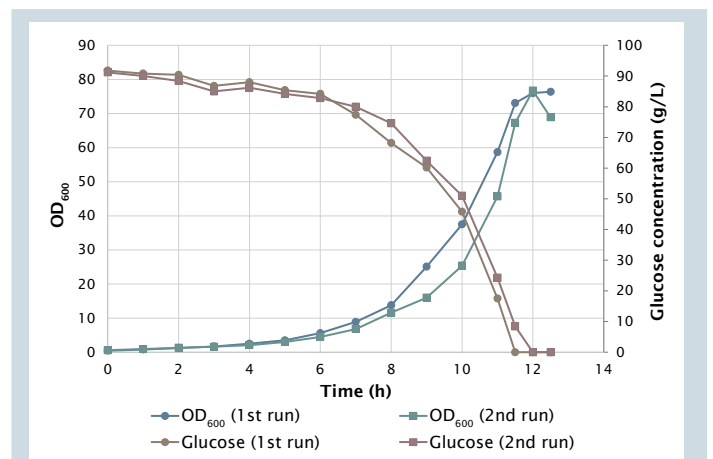


Fig. 7: Batch fermentation in chemically-defined medium

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.

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  $OD_{600}$  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. The detailed growth curve and glucose consumption pattern are shown in Figure 9. We initiated

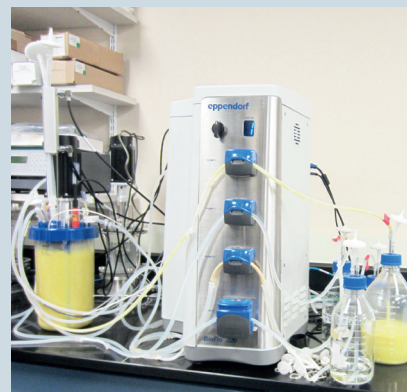
feeding before glucose depletion, which triggered strong exponential *E. coli* growth. With the exponential increment of feeding rate, we avoided early glucose depletion throughout most of the fermentation process and we observed a significant accumulation of biomass corresponding to a more than 150-fold  $OD_{600}$  increase between 8 and 10 h. Glucose was depleted or almost depleted when the maximum  $OD_{600}$  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

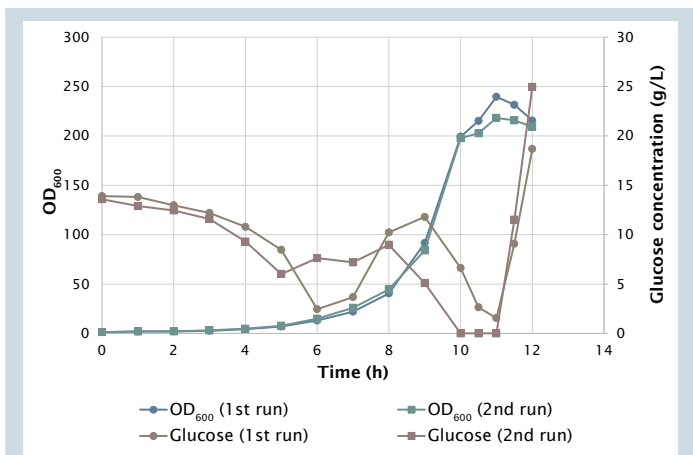
The maximum  $OD_{600}$  observed in continuous fermentation (Figure 10) was 159 at  $t = 10.5$  h in one run. The detailed growth curve and glucose consumption pattern are displayed in Figure 11. As with fed-batch fermentation, we initiated feeding before glucose depletion, and began broth harvest



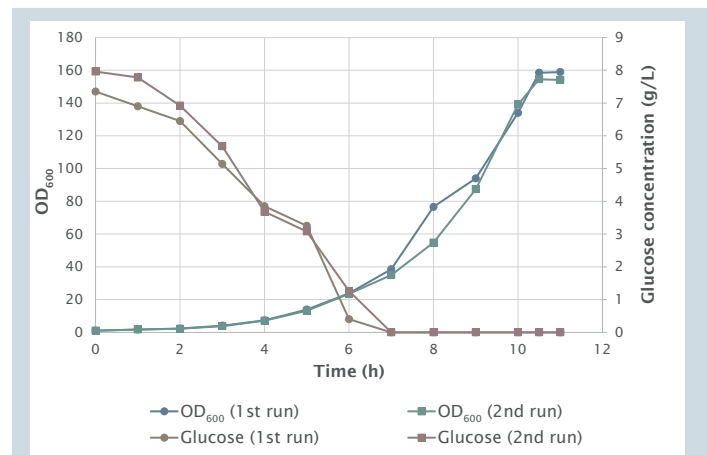
**Fig. 8:** High-density *E. coli* culture in the BioBLU 3f Single-Use Vessel. Fed batch fermentation process 11 hours post inoculation.



**Fig. 10:** Setup of the continuous fermentation process.



**Fig. 9:** Fed-batch fermentation



**Fig. 11:** Continuous fermentation



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  $t = 10.5$  h. The culture volume stayed constant throughout the culture. The  $OD_{600}$  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 $OD_{600}$

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).

**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

### Specific growth rate and doubling time

Specific growth rate  $\mu$  and cell doubling time  $\tau_d$  can be calculated based on biomass concentration in terms of dry weight as shown in equations (1) and (2) [7]

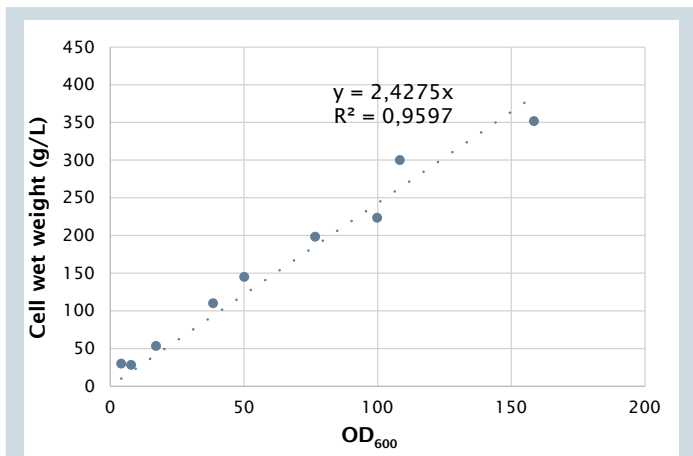
$$\mu = \frac{1}{X} \frac{dX}{dt} = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (1)$$

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

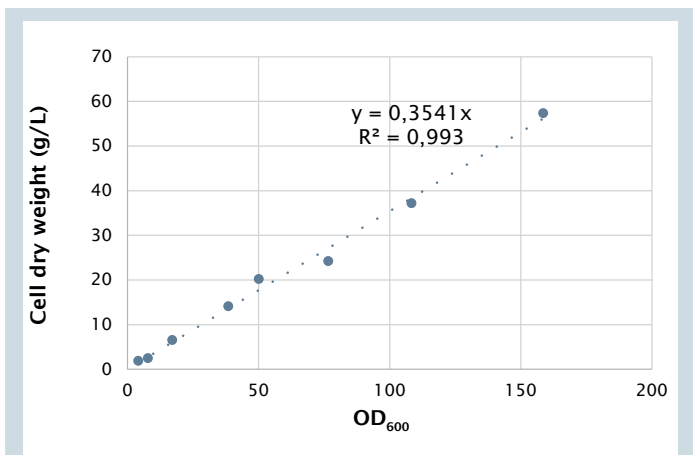
where  $\mu$  is specific growth rate ( $h^{-1}$ ) between two time points  $t_1$  and  $t_2$ , and  $X_1$  and  $X_2$  are biomass dry weight (g/L) at time points  $t_1$  and  $t_2$ , respectively. During exponential growth, *E. coli* cells are growing at their maximum specific growth rate,  $\mu_{max}$ . The time frame between  $t_1$  and  $t_2$  defines this exponential growth phase, and  $\tau_d$  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  $\mu_{max}$  to be  $0.86 h^{-1}$  and doubling time  $\tau_d$  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



**Fig. 12:** The correlation between *E. coli* cell wet weight and  $OD_{600}$



**Fig. 13:** The correlation between *E. coli* cell dry weight and  $OD_{600}$

$\mu_{\max}$  was 0.46 h<sup>-1</sup> and doubling time  $\tau_d$  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).

**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 3f vessel. In continuous fermentation, the biomass is collected from the culture in the vessel (3 L) and harvest (1.3 L).

**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).

**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]

Fed-batch fermentation had the highest volumetric biomass productivity at 6.27 g/(L·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 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**

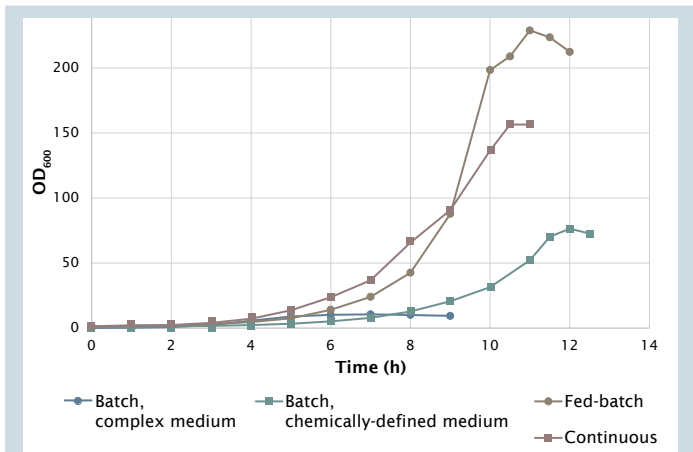
We calculated the net cost for growing *E. coli* in BioBLU 3f vessels under different fermentation modes based on the cost breakdown for chemicals, consumables, and labor from preparation to the real run and cleanup (Table 8). Energy, equipment, and rent are not included here since they vary significantly 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.

## 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 fermentor. Fed-batch fermentation provided the highest  $OD_{600}$  at 229 on average with the maximum specific growth rate at  $0.86\text{ h}^{-1}$  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



**Fig. 14:** Growth curve comparison among four different fermentation modes

**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

OD<sub>600</sub> being 157.

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

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 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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## Appendix

**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 <sub>4</sub> 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 <sub>4</sub> 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 <sub>4</sub> 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 <sub>4</sub> 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 $\text{KH}_2\text{PO}_4$ , 40 g $(\text{NH}_4)_2\text{HPO}_4$ , and 17 g citric acid per 1 L DI water
100 x trace element solution	10 g iron (III) citrate, 0.25 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.5 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.15 g $\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.3 g $\text{H}_3\text{BO}_3$ , 0.25 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 1.3 g $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$ , and 0.84 g EDTA per 1 L DI water solution, sterilize by 0.2 $\mu\text{m}$ membrane filtration
Thiamine (vitamin B1) stock solution	20 g thiamin per 1 L DI water solution, sterilize by 0.2 $\mu\text{m}$ membrane filtration
Magnesium sulfate stock solution	240 g $\text{MgSO}_4$ per 1 L DI water solution, sterilize by 0.2 $\mu\text{m}$ membrane filtration
50 x ampicillin stock solution	0.5 g ampicillin per 100 mL DI water solution, sterilize by 0.2 $\mu\text{m}$ membrane filtration
25 % (v/v) ammonium hydroxide solution	250 mL $\text{NH}_4\text{OH}$ (Thermo Fisher Scientific) per 1 L DI water solution, sterilize by 0.2 $\mu\text{m}$ membrane filtration

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