CHAPTER EIGHT

Method for high-efficiency fed-batch cultures of recombinant *Escherichia coli*

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Contents

Abstract

Fed-batch processes are commonly used in industry to obtain sufficient biomass and associated recombinant protein or plasmids. In research laboratories, it is more common to use batch cultures, as the setup of fed-batch processes can be challenging.

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This method outlines a robust and reliable means to generate *Escherichia coli* biomass in a minimum amount of fermentation time using a standardized fed-batch process. Final cell densities can reach over 50 g dry cell weight per liter (g dcw/L) depending on the strain. This method uses a predefined exponential feeding strategy and conservative induction protocol to achieve these targets without multiple trial and error studies. If desired, productivity can be optimized by balancing the induction time and feed rates. This method utilizes cost-efficient defined media, minimizes process control complexity, and potentially aids downstream purification.

1. Introduction

Escherichia coli is the workhorse host organism used in research laboratories to generate recombinant proteins and plasmids (Grijalva-Hernandez et al., 2019; Kodym, Calkins, & Story, 1999; Krause, Neubauer, & Neubauer, 2016; Pósfai et al., 2006; Siurkus et al., 2010; Wurm, Hausjell, Ulonska, Herwig, & Spadiut, 2017; Xu, Jahic, Blomsten, & Enfors, 1999). *E. coli* can be cultivated on inexpensive defined media to high cell densities and can achieve high growth rates (Korz, Rinas, Hellmuth, Sanders, & Deckwer, 1995; Sharma, Campbell, Frisch, Blattner, & Harcum, 2007). Further, the genetics of *E. coli* are well-characterized and many strains exist with deletions of proteases and prophages (Pósfai et al., 2006; Swartz, 2001). To maximize the capabilities of *E. coli* to generate high cell densities, computer-controlled fed-batch processes are required (Beckmann et al., 2017). Unfortunately, the setup of fed-batch processes can be challenging for research scientists (Korz et al., 1995). Several fed-batch strategies exist, such as pH-stat, DO-stat and exponential feeding (Chen, Graham, & Ciccarelli, 1997; Chi et al., 2020; Garcia-Arrazola et al., 2005; Johnston, Cord-Ruwisch, & Cooney, 2002; Konstantinov, Kishimoto, Seki, & Yoshida, 1990; Xu, Jahic, Blomsten, et al., 1999); however, the outcomes from these fed-batch processes vary depending on the metabolic state of the *E. coli* (Lee, 1996; Pepper, 2015; Soini, Ukkonen, & Neubauer, 2008).

The basic theory behind a fed-batch process is to minimize overflow and anaerobic metabolism (Krause et al., 2016; Xu, Jahic, Blomsten, et al., 1999; Xu, Jahic, & Enfors, 1999). Both of these metabolic states result in high waste product accumulation, which in turn inhibit growth and recombinant protein production (Luli & Strohl, 1990). Overflow metabolism can be mitigated by controlling the substrate feeding to rates lower than the tricarboxylic acid (TCA) cycle turnover rate (Kitamura, Toya, & Shimizu, 2019; Korz et al., 1995; Ponce, 1999; Vasilakou, van Loosdrecht, & Wahl, 2020).

Some alternatives to controlled feeding are to use substrates with low transport rates, for example, glycerol (Kwon, Kim, & Kim, 1996; Sharma, Blattner, & Harcum, 2007). The most robust fed-batch protocols minimize overflow metabolism by controlled feeding of a limiting substrate to levels that only allow sub-maximum growth rates. Further limiting substrate feed rates can avoid anaerobic conditions by reducing the oxygen uptake rate of the culture, which can be critical at high cell densities.

The objective of this work is to outline a high-efficiency fed-batch process to reliably produce *E. coli* biomass to over 50 g dcw/L (over 160 g wet cell weight per liter). This robust and reliable method uses conservative values for the TCA cycle uptake rate and accounts for the metabolic burden encountered under recombinant protein expression. This method is outlined in several phases to assist new researchers through the fed-batch process setup: (1) overnight culture, (2) preculture, (3) fermenter conditioning, (4) inoculation, (5) fed-batch culture for biomass generation, (6) induction, and (7) harvest. Critical to this method is a strategy for maintaining the cells in an oxidative metabolic state, similar to the exponential phase prior to inoculating the fermenter. Example culture profiles are provided, as well as sample calculations, to assist with these preplanning steps.

2. Before you begin

- Ensure the strain can grow in minimal media, otherwise trace amounts of supplement can be used to account for auxotrophic behavior. The lowest level of supplement that results in acceptable exponential growth should be selected, at least $0.4\,h^{-1}$
- Determine the growth rate of the strain in minimal media. *E. coli* typically can grow in minimal media with growth rates between 0.40 and $0.65\,h^{-1}$ at 37 °C
- Determine induction level needed. Common induction strengths are between 10 μM and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.1 Timing: 2 days before the fermenter is to be inoculated

1. Prepare minimal media for the overnight and preculture shake flasks (see Section 4.1 for overnight and preculture media preparation)
2. Determine the feed pump settings based on growth rate of strain in minimal media (see Section 4.5 for calculations). This feed rate should result in an exponential growth rate that is about half the normal batch growth rate.

2.2 Timing: 1 day before inoculating the fermenter

1. Thaw working cell bank into the minimal batch media for the overnight culture
2. Prepare fermenter.

2.3 Timing: Morning before fermenter will be inoculated in late afternoon

1. Titrate the overnight culture into the preculture media such that the cells will be in the exponential growth phase at the time of fermenter inoculation
2. Finalize fermenter setup including probe calibration.

3. Materials and equipment

3.1 Equipment

1. Air, clean, dry compressed supply regulated at 10 psi (check with fermenter manufacturer for air pressure specifications)
2. Autoclave
3. Balances (analytical and top loader (2 kg capacity))
4. Beakers (4-L (glass), 2-L (glass), 100-mL (plastic))
5. Erlenmeyer flasks (Sterile, 1-L or 2-L)
6. Feed caps (4) (Duran GL 45)
7. Fermenter and fermenter control unit with at least two controlled pumps that can be variable speed or fixed speed operating at fractional duty cycles (Eppendorf, Sartorius, Applikon, etc.)—review manufacturer instructions
8. Filters for sparge gas and vents—Acro 50 Vent Devices with PTFE Membrane—0.2 μm, 1/8 in MNPT hose barb (Pall 4400), or similar
9. Funnels
10. Glucose quantification instrument (Glucose 201 Analyzer (Hemocue), YSI 2900D Biochemistry Analyzers (Yellow Springs Instruments), Cedex Bioanalyzer (Roche), or similar)
11. Graduated cylinders (2-L, Sterile 500-mL and 100-mL)
12. Hemostats or tight clamps (5)
13. Hot mill gloves
14. Microwave (can be replace by the stirred hot plate)
15. Oxygen and nitrogen gas tanks with appropriate gas regulators
16. Peristaltic pump for harvesting

17. Pipettes (Variable 1–10, 10–100, and 100–1000 μL) with sterile tips
18. Pyrex bottles (one 4-L or 5-L, two 1-L, one 500-mL, and one 100-mL)
19. Recirculating chiller—compatible with fermenter control units (consult with fermenter control unit manufacturer for flow rates and pressure limits). Many fermenter manufacturing allow for municipal water, if pressure stepped down. Also, municipal water source should be at least 15 °C below the fermenter temperature setpoint
20. Serological pipettes (10-mL and 25-mL) and pipet aid
21. Shaker incubator (up to 250 rpm and clamps to hold 1-L and 2-L Erlenmeyer flasks)
22. Spectrophotometer (600 nm) and cuvettes (1-mL)
23. Stirred hot plate, 3-cm, 5-cm, and 8-cm stir bars
24. Tubing for additions—four sets 1.2 m LS16 tubing (Cole-Parmer 06508-16) with male Luer-Lok fittings (Cole-Parmer 30800-24) on both ends of the tubing. For these particular feeds, cured silicone (Cole-Parmer 96410-16) or PharMed (Cole-Parmer 06508-16) grade tubing is acceptable. However, for the base control addition line using ammonia, the PharMed grade tubing is recommended
25. Tubing for interior bottle lines—LS16 tubing (Cole-Parmer 06508-16) to attach to interior of feed bottles
26. Tubing for fermenter and bottle ports—LS16 tubing (Cole-Parmer 06508-16) with female Luer-Lok fittings (Cole-Parmer 45508-16) for all ports (at least 12). Length approximately 20 cm
27. Tubing for harvest—LS17 tubing (Cole-Parmer 96410-17) with male Luer-Lok fitting (Cole-Parmer 30800-22). Length approximately 2.0 m
28. Vortex mixer
29. Washcloth or soft brush.

3.2 Chemicals

ACS grade or better for all chemicals

1. Ammonium hydroxide aqueous (28–30%)
2. Ammonium phosphate dibasic anhydrous
3. Antibiotic (depends on strain and plasmid)
4. Antifoam (Sigma SE-15 or similar)
5. Bleach, greater than 10%
6. Boric acid
7. Citric acid
8. Cobalt chloride hexahydrate
9. Copper chloride tetrahydrate

10. D-glucose (dextrose)
11. Detergent, concentrated (Phosphate-free, Decon Labs Contrad 70 or similar)
12. Ethylenediaminetetraacetic acid (EDTA), molecular weight 292.2 g/L
13. Ferric citrate
14. Isopropanol (70%)
15. Isopropyl β-D-1-thiogalactopyranoside (IPTG)
16. Magnesium sulfate anhydrous (≤99% purity)
17. Manganese chloride tetrahydrate
18. Potassium chloride
19. Potassium phosphate monobasic anhydrous
20. Sodium hydroxide (10 M or solid)
21. Sodium molybdate dihydrate
22. Zinc acetate dihydrate.

3.3 Strain and plasmid

Escherichia coli containing an inducible plasmid, expressing a desired protein.

Examples

- *E. coli* BL21 (DE3) expressing gelsolin (Scarab Genomics)
- *E. coli* BL21 T7 Express *lysY/I*^{q} (New England Biolabs) expressing beta-glucuronidase
- *E. coli* MDS42 *lacT7* expressing gelsolin (Scarab Genomics)
- *E. coli* MG1655 (ATCC) expressing TVP1GFP (Garcia-Fruitos, Aris, & Villaverde, 2007).

4. Step-by-step method details

The overall goal of this robust fed-batch method is to permit highly reproducible biomass generation for a variety of recombinant *E. coli* strains expressing soluble and inclusion body prone proteins. Fig. 1 highlights the reproducibility to obtain high cell density cultures within 24 h for four different *E. coli* strains. *E. coli* MG1655 and MDS42 *lacT7* are K-12-derived, while *E. coli* BL21 (DE3) and BL21 T7 Express *lysY/I*^{q} are *E. coli* BL21-derived strains. Gelsolin is a soluble protein, while the TVP1GFP (Garcia-Fruitos et al., 2007) and beta-glucuronidase are expressed mainly as inclusion bodies. The induction times are indicated in Fig. 1A by the arrows and boxed time points. *Note*: The induction times varied from 9 to 17.7 h and cell densities, represented by optical density (OD), ranged from 9.6 to 62 OD_{600}.

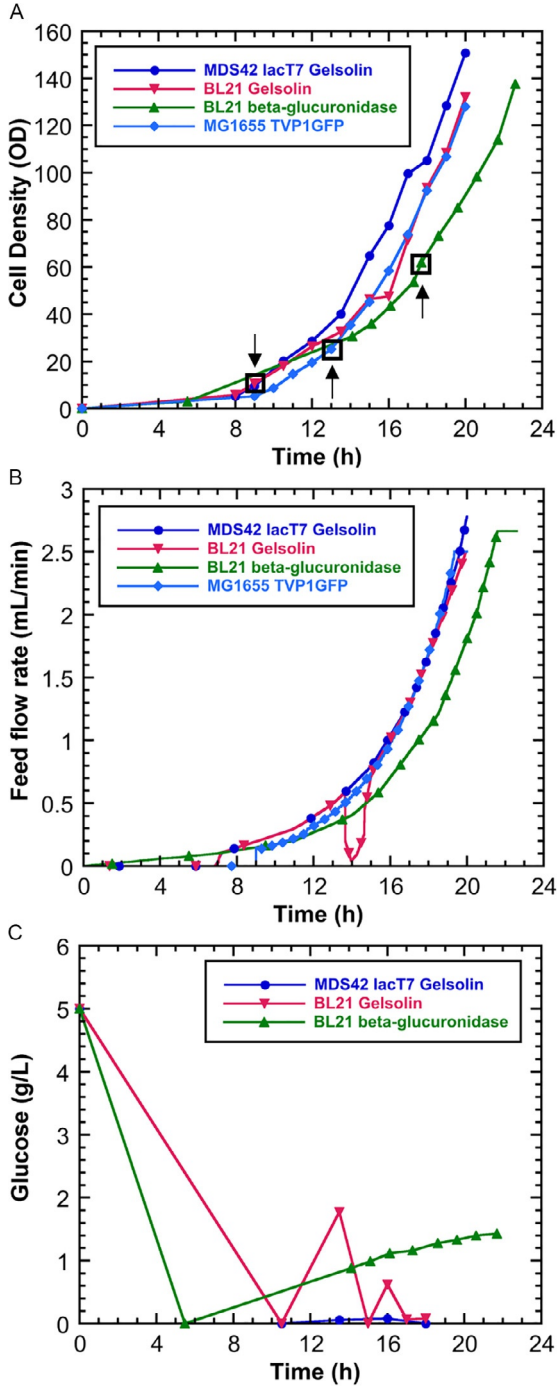

Fig. 1 See figure legend on next page.

4.1 Stock solution preparation

These stock solutions will be used to prepare the batch buffer solution (Table 1), trace metal solutions for the batch and feed (Table 2), batch shake flask and fermenter media (Table 3), and, except for the batch buffer solution and 50% glucose solution, these solutions will be used to prepare the feed solution (Table 4).

1.1. Prepare 2 L of the batch buffer solution (components listed in Table 1). Add ~1 L of dH_2O to a 2-L beaker with an 8-cm stir bar. Begin stirring vigorously with no heating. Weigh out components, add sequentially, and allow each to dissolve between additions. Adjust the pH of the solution to pH 6.9 with 10 M NaOH. Add dH_2O to bring up to 2 L, transfer to an autoclavable bottle, and autoclave. Store at room temperature.

1.2. Prepare 2 L of the 50% (w/v) glucose solution. Add ~1 L of dH_2O to a 2-L glass beaker. Heat the water for 5 min in a microwave (~1500 W). Add 8-cm stir bar to the beaker and begin stirring vigorously. Weigh out 1 kg of anhydrous dextrose. Pour in approximately half of the dextrose. Allow the solution to clear before adding remaining dextrose. Add dH_2O to bring up to 2 L, transfer to autoclavable bottle, and autoclave before storage. The solution may have a yellowish tint after autoclaving. Do not autoclave more than once or the glucose will caramelize. Store at room temperature. *Note*: The glucose solution may also be filter sterilized, if desired.

1.3. Prepare 1 L of the 20% (w/v) magnesium sulfate solution. Add ~500 mL of dH_2O to a 1-L beaker with a 5-cm stir bar. Weigh out 200 g of anhydrous magnesium sulfate. Add slowly to the beaker while stirring. Dissolution is exothermic. Add dH_2O to bring up to 1 L, transfer to autoclavable bottle, and autoclave before storage. Alternatively, the solution can be filter sterilized. Store at room temperature. *Note*: A small

Fig. 1 Cell growth, exponential feed, and glucose profiles for four *E. coli* strains expressing soluble and insoluble proteins. (A) Cell density (OD_{600}), (B) feed profiles (L/min), and (C) glucose profiles (g/L). Two different predefined exponential feed profiles were used 0.25 h^{-1} (blue, magenta, and green) and 0.28 h^{-1} (light blue). *E. coli* MG1655 and MDS42 *lacT7* are K-12-derived, while *E. coli* BL21 (DE3) and T7 Express strains are *E. coli* BL21-derived. Gelsolin is a soluble protein, while the beta-glucuronidase and TVP1GFP were expressed mostly as inclusion bodies. Protein expression induction was with IPTG and indicated by the boxed time points in panel A. The symbols shown on the feed flow rate lines only represent some time point measurements and are provided to aid in visualization of the multiple data sets.

Table 1 Batch buffer (10 ×) for minimal media.

Component	Amount per liter (g)
KH_2PO_4	80
$(NH_4)_2HPO_4$	40
Citric acid	17

Table 2 Trace metal solution (100 ×) components for minimal media.

Component	Amount per liter (g)
$MnCl_2{\cdot}4H_2O$	1.5
$Zn(CH_3COO)_2{\cdot}2H_2O$	1.3
H_3BO_3	0.3
$Na_2MoO_4{\cdot}2H_2O$	0.24
$CoCl_2{\cdot}6H_2O$	0.25
$CuCl_2{\cdot}2H_2O$	0.15
EDTA	0.84

Table 3 Minimal media components.

	Batch volume (L)			
Stock solutions (mL)	**2.0**	**5.0**	**6.0**	**7.0**
Batch buffer (10 ×)	200	500	600	700
Distilled water	1736	4340	5208	6076
50% w/v glucose solution	20	50	60	70
20% w/v $MgSO_4$ solution	4	10	12	14
Trace metal solution (100 ×)	20	50	60	70
Fe^{III} citrate solution (100 ×)	20	50	60	70
Antifoam	0.4	1.0	1.2	1.4

If preparing stock minimal media for shake flasks, all components are combined, minus the antifoam. For fermenters, the first two components are added directly to the fermenter and autoclaved with probes. Remaining components will be mixed and pumped into the cooled fermenter after autoclaving.

Table 4 Feed solution for minimal media.

	Total feed volume desired (mL)		
Sterilize stock solution to add (mL)	**1043**	**2607**	**4171**
50% w/v glucose solution	1000	2500	4000
20% w/v $MgSO_4$ solution	20	50	80
Trace metals solution	18	45	72
Fe^{III} citrate solution	4.8	12	19.2

At least 2.5 L feed is recommended for use with 6.0 L batch media, which results in a final volume of 8.5 L in a 10-L fermenter.

amount of precipitate may form during storage. Avoid adding the precipitate during minimal media preparation, but the precipitate is not a concern for using the solution.

1.4. Prepare 100 mL of the 0.5 M EDTA solution. Add 50 mL of dH_2O to a 100-mL beaker. Add a 3-cm stir bar and begin stirring. Weigh out 14.6 g of EDTA, and add to beaker. Add 10 M NaOH dropwise until all EDTA is dissolved. Use 6 M HCl to adjust to pH 7.0. Add dH_2O to bring up to 100 mL, transfer to autoclavable bottle, and autoclave before storage. Store at room temperature.

1.5. Prepare 1 L of the trace metal solution. Add 500 mL of dH_2O to a 1-L beaker. Begin stirring with a 5-cm stir bar. Add 5.8 mL of the 0.5 M EDTA solution from Section 4.1, step 1.4. Weigh each reagent individually. See Table 2 for the list of trace metal components. Add components sequentially, allow to dissolve prior to addition of next component. Add dH_2O to bring up to 1 L. Sterile filter. Store in dark at room temperature. *Note*: Color will change from blue to purple during storage.

1.6. Prepare a 1 L solution of ferric citrate solution. Add 500 mL of dH_2O to a 1-L beaker. Heat the water in microwave for approximately 2 min (1500 W). Use a 5-cm to stir while heating on the stir plate. Weigh out 10 g of ferric citrate, and add to beaker. Continue to heat and stir until no solids remain. Add dH_2O to bring up 1 L. Filter sterilize. Store in dark at room temperature.

1.7. Prepare a 1000 × antibiotic solution to a final volume of at least 20 mL. *Note*: The working antibiotic concentration will depend on plasmid and strain. For example, plasmids with kanamycin resistance and IPTG-inducible T7 promoter in *E. coli* BL21 strains require a working

concentration of 50 mg/L. To make a 1000× kanamycin stock, add 1.0 g kanamycin to 20 mL dH_2O. This will result in 50 g/L stock solution. To prepare, add the appropriate amount of antibiotic to a 50 mL tube. Add 15 mL dH_2O. Vortex to dissolve. Add dH_2O to bring up to 20 mL. Sterilize using a 0.2 μm syringe filter into a new, sterile 50-mL tube. Aliquot the sterile antibiotic solution into sterile 1.5-mL tubes at 1 mL per tube using sterile techniques. Store tubes at −20 °C for up to 2 years.

1.8. Prepare 2 L of minimal media for the overnight and preculture shake flasks. In a sterile 2-L graduated cylinder, add the component solutions listed in Table 3, in order. Add sterile dH_2O to bring up to 2 L. The complete minimal media will precipitate if autoclaved. If re-sterilization is needed, filter sterilize. Store in dark at room temperature. *Note*: A precipitate may form after several weeks of storage. Good growth results can be obtained with the minimal media for up to 1 year, without redissolving the salts.

4.2 Overnight and preculture preparation

These instructions are based on a 10-L working volume fermenter. For a margin of safety, the final designed volume is 8.5 L. If smaller volumes are desired due to smaller available fermenters, scale down all volumes proportionally. Make sure to stay within recommended fermenter working volume (see manufacturer's instruction).

2.1. One day prior to the fermenter inoculation, start the overnight culture from a frozen working cell bank prepared in minimal media. To prepare the overnight culture, add 100 mL of minimal media to a sterile 500-mL Erlenmeyer flask (shake flask). Add 100 μL of the 1000× antibiotic solution. Inoculate the shake flask with one 1 mL working cell bank, thawed. Incubate the overnight culture overnight (~16 h) at 250 rpm and 37 °C in a shaker/incubator.

Tip: Typically, a working cell bank (1 mL) is prepared from a 1 OD culture mixed with an equal volume of 50% glycerol and stored frozen (−80 °C). A working cell bank prepared from exponentially growing cells will have a consistent and robust recovery.

2.2. Prepare the preculture shake flask. Add 200 mL of minimal media to two 1-L sterile shake flasks for a total preculture volume of 400 mL. Add antibiotics as necessary (200 μL per 200 mL for a 1000× antibiotic stock).

2.3. In the morning, for a late afternoon inoculation, measure the cell density of the overnight culture with a spectrophotometer. The overnight culture should be between 0.2 and 5 OD_{600} to indicate good recovery.

2.4. Determine initial cell density of the preculture [$X_{Pre}(\Delta t)$]. For example, if growth rate (μ) in the batch phase in minimal media is 0.55 h^{-1}, and inoculation is planned for 7 h in the future (Δt), the desired cell density of the preculture is 0.053 OD_{600}. This initial cell density will allow the culture to reach 2.5 OD_{600} ($X_{Pre}(t=0)$) by inoculation. For other timings use Eq. (1):

$$X_{Pre}(\Delta t) = \frac{X_{Pre}(t=0)}{e^{\mu \Delta t}} \tag{1}$$

2.5. Determine the volume of the overnight culture (V_{OV}) needed to generate the preculture. Assuming an overnight cell density (X_{OV}) of 3.1 OD_{600}, the overnight culture volume will be 3.5 mL added to 200 mL of preculture media (V_{Pre}). Eq. (2) outlines this calculation.

$$V_{OV} = \frac{V_{Pre} X_{Pre}(t=0)}{X_{OV} - X_{Pre}(t=0)} \tag{2}$$

Tip: For an initial fermenter with 6 L of minimal media, a preculture with 2.5 OD_{600} (X_{Pre}) will require a volume of 123 mL (V_{Pre}). If the cell density of the preculture is lower, the required volume increases. The preculture volume required for the inoculation is given by Eq. (3).

$$X_{Pre} V_{Pre} = X_{Ferm}(V_{Pre} + V_{Ferm}) \tag{3}$$

Where X_{Ferm} is initial cell density, and V_{Ferm} is initial fermenter volume. The equation rearranged to solve for V_{Pre} is given by Eq. (4).

$$V_{Pre} = \frac{X_{Ferm} V_{Ferm}}{X_{Pre} - X_{Ferm}} \tag{4}$$

This approach is robust, since the preculture volume required to inoculate the fermenter is much less than the amount prepared. For example, if the preculture only reaches 1.8 OD_{600} by the time of inoculation (instead of the anticipated 2.5 OD_{600}), only 168 mL is required, which is less than the amount prepared (2 × 200 mL precultures), as calculated by Eq. (4).

Conversely, if a preculture of 200 mL is prepared, the lowest cell density at the end of the preculture phase only needs to be 1.55 OD_{600}. This is obtained as Eq. (5), a rearrangement of Eq. (3) to solve for $X_{Pre}(t=0)$.

$$X_{Pre}(t=0) = \frac{X_{Ferm}(V_{Pre} + V_{Ferm})}{V_{Pre}} \quad (5)$$

This approach provides two margins of safety, a cell density buffer and a volume buffer. If cells were a bit past exponential phase overnight, the preculture can experience a slight lag phase.

Tip: A key to reproducibility in the fermenter is the state of the preculture being mid-exponential phase. Using a Genesys 20 spectrophotometer (Thermo Spectronic), the exponential growth rate range is between 0.5 and 2.5 OD_{600} corresponding to 0.25–1.25 g dcw/L, respectively (a ratio of 0.5 g dcw/L per OD). Spectrophotometer absorbance values vary by spectrophotometer brand and model for the same cell density in g dcw/L. These differences are due to the combination of cell morphology and size, light scatter by the cells, absorbance by the cells, light source to cuvette distance, and the detector to cuvette distance. Confirm absorbance to dry cell weight for any spectrophotometer.

Tip: Absorbance measurements must be in the linear range, typically between 0.01 and 0.5 absorbance units, in order to accurately determine the cell densities. High cell density samples will require dilution to stay in the spectrophotometer linear range, as the fermenter is expected to reach over 100 OD_{600} (50 g dcw/L). For dilutions up to 1:20, a single dilution is adequate. For higher dilutions, serial dilutions will be required. For example, a cell density of 70 OD_{600} should be serially diluted as 1:10 and 1:20 to result in an absorbance reading of 0.35.

Tip: The overnight incubations may be longer than 16 h if: (1) shaker/incubator cannot reach the prescribed conditions; (2) the cells have been stored in the −80 °C freezer for more than 1 year; (3) working cell banks were made at too low of a cell density; or (4) the cells have not been adequately adapted to the fermenter media. Failure to reach the target cell densities will push the inoculation time of the fermenter and/or require a very high inoculation volume.

4.3 Feed preparation

3.1. Prepare the feed glucose solution by heating half the required dH_2O in a microwave (~1500 W) on high for 5 min (see Table 4 for amounts). For example, 1.25 L dH_2O for 2.5 L feed solution. This solution is in addition to the 50% (w/v) glucose solution prepared in Section 4.1, step 1.2. Place the warmed water in a beaker on a stirred hot plate, add a 5-cm stir bar. Stir vigorously, and heat moderately (do not boil).

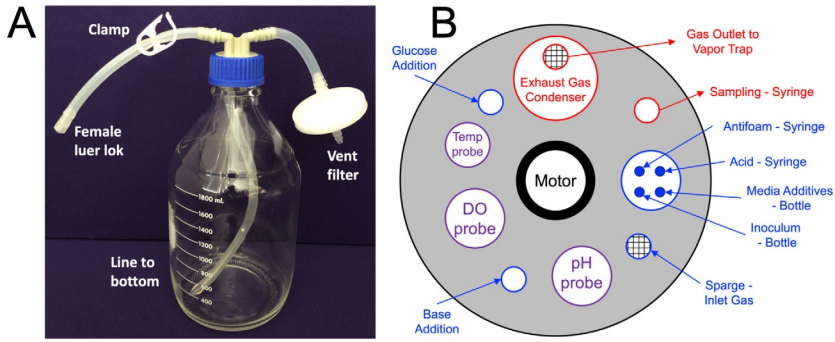

Fig. 2 Feed bottle configuration and headplate schematic. (A) feed bottle with vent, female Luer-Lok and withdrawal tubing (line to bottom). (B) Headplate with the inlets (blue) and outlets (red), probes (purple), and motor (black) shown. The cross-hatched inlet and outlet ports are for gasses. All other ports handle liquids. *DO*: dissolved oxygen; *Temp*: temperature.

Alternatively, the dH_2O could be heated entirely on the stirred hot plate. Add the glucose slowly to the dH_2O in the beaker until dissolved. Add dH_2O to bring up to 2.5 L.

3.2. Prepare the feed solution bottle. Add the glucose solution from Section 4.3, step 3.1 to a 5-L Pyrex bottle. Attach the feed cap (Duran GL 45) with LS16 tubing such that the tubing inside reaches the bottom of the bottle. See Fig. 2A for an image of the feed cap with vent and tubing. The external feed barb needs 12 cm of LS16 tubing with male Luer-Lok end. Attach an autoclavable inline filter to the other barb as a vent.

3.3. Connect the glucose feed solution to the fermenter for autoclaving. Clamp any line entering the liquid prior to autoclaving. The other components will be added with sterile technique after the autoclave step.

4.4 Fermenter preparation

4.1. Prepare the fermenter for autoclaving. Add 600 mL of the batch buffer solution (described in Table 1) to a clean 10-L fermenter. Using the same graduated cylinder, add 5.2 L of dH_2O to the fermenter. Use dH_2O to ensure complete rinsing of the batch buffer solution from the graduated cylinder into the fermenter.

Tip: For ease of use, the connection port tubing lines on the fermenter and bottles should have female Luer-Loks, and the connection lines (tubing)

should have male Luer-Loks. The only exception is the sparger inline filter. This connection should have one end with a male Luer-Lok and one end with a female Luer-Lok. This enables connection from the gas source (male Luer-Lok) to the fermenter (female Luer-Lok).

4.2. Seal the headplate of the fermenter. Review the manufacturer's instruction for preparing the vessel, as needed. Add clean pH, dissolved oxygen (DO), and temperature probes, and any other desired probes. Confirm that there are at least five ports on the headplate with LS16 tubing and female Luer-Loks connected, beyond the sample and gas sparge and exhaust lines. See Fig. 2B for a schematic of a typical headplate arrangement. An adequate length of tubing is needed for these lines to be clamped with a hemostat or similar clamp. *Note*: The five ports are: (1) feed solution (glucose), (2) pH control (base addition), (3) inoculation, (4) acid addition, and (5) antifoam. If the fermenter vessel does not have five ports beyond the sample line, a Y-connector can be used to split the acid and inoculum or antifoam and media addition lines. Do not split the glucose or base addition lines, as these will be under pressure during the run.

4.3. Attach the inline filter to the exhaust port using LS16 tubing with a male Luer-Lok. Leave the inline filter attached to the exhaust port unclamped and uncovered. This will allow the fermenter to aseptically vent during autoclaving and the cooling cycle.

4.4. Attach a second inline filter to the sparging port using LS16 tubing with a male Luer-Lok on one end and a female Luer-Lok on the other end. Both sides of the inline filters should have tubing such that it can be connected to the gas supply. As the sparge line is below the liquid level in the vessel, make sure this line is clamped tight prior to the inline filter to protect it from liquid during the autoclave step. Two clamps should be used if both clamps are plastic. Fig. 2A shows a plastic clamp that should be used cautiously on the fermenter lines entering the liquid during autoclaving.

4.5. Attach 1.2 m of tubing to each of the pH control and feed ports on the headplate. Both ends of the tubing should have male Luer-Loks.

4.6. Leave an addition port on the headplate unclamped, uncapped, but covered with aluminum foil as a backup to prevent over pressure in the autoclave.

4.7. Clamp any lines that go into the liquid on the fermenter. Cover the drive shaft per manufacturer's instruction. Cap probe electrical connectors.

4.8. Prepare 2.0 m of LS17 tubing with male Luer-Loks for harvest tubing and two additional 1.2 m of LS16 tubing with male Luer-Loks as backup. Do not attach these to the fermenter.

4.9. Cover all tubing ends with aluminum foil. This will maintain sterility post-autoclave.

4.10. Autoclave the fermenter, connected feed bottle, extra tubing sets, and any other freshly made stock reagents that are autoclavable. Ensure lines that go into liquid are clamped. Use an autoclave on liquid cycle with a 1 h sterilization phase at 121 °C. For fermenters larger than 5-L, consider having two people to move the fermenter.

4.5 Fermenter setup

5.1. Program the feed pump. Eq. (6) can be used to determine the feed solution flow rate with time (relative to the beginning of the fed-batch):

$$F = \frac{\mu_{set} S_0 V_0}{S_F} e^{\mu_{set} t_{fb}} \tag{6}$$

where F is the feed flow rate in L/h and a function of time relative to the fed-batch time (t_{fb}) in hours, μ_{set} is the desired exponential growth rate (h^{-1}), S_0, and S_F are the substrate concentrations in the minimal media and the feed solution, respectively, and V_0 is the initial volume of the inoculated fermenter (not the same as V_{Ferm}). Depending on the fermenter manufacturer, these flow rates may need to be entered as percentage of pump speed, fraction of duty cycle, segments approximating the exponential profile, or as an equation. For a feed flow rate corresponding to an exponential rate of $0.25\,h^{-1}$ for 6 L of batch media, an example flow profile is shown in Table 5. Fig. 1B shows predefined exponential feed profiles for three of the four different *E. coli* strains. *E. coli* BL21 gelsolin accumulated glucose post induction, and the feed pump was decreased from approximately 13.5–15.5 h.

Note: The predefined exponential growth rate was $0.28\,h^{-1}$ for the *E. coli* MG1655 TVP1GFP fermentation. Also, for the *E. coli* BL21 T7 Express beta-glucuronidase fermentation, the feed pump was started at fermentation time 0, such that antifoam was always added. The amount of feed (V_{fb}) required at any fed-batch time and for the total time can be determined by Eq. (7).

Table 5 Feed solution flow rates for an exponential growth rate of 0.25 h^{-1}.

Fermentation time (h)	Fed-batch time (h)	Flow rate (L/h)	Cumulative feed volume (L)	Fermenter volume (L)	Predicted cell density (OD_{600})
9	0	0.016	0.00	6.0	5
10	1	0.020	0.02	6.02	6.4
11	2	0.026	0.04	6.04	8.2
12	3	0.033	0.07	6.07	10.5
13	4	0.042	0.11	6.11	13.4
14	5	0.054	0.16	6.20	17
15	6	0.070	0.22	6.22	22
16	7	0.090	0.30	6.30	27
17	8	0.115	0.40	6.4	35
18	9	0.148	0.53	6.5	44
19	10	0.190	0.70	6.7	55
20	11	0.244	0.91	6.9	68
21	12	0.313	1.19	7.2	84
22	13	0.402	1.55	7.5	103
23	14	0.516	2.00	8.0	124
24	15	0.663	2.59	8.6	149

Batch volume: 6 L; inoculum cell density: 0.05 OD; batch initial glucose: 5 g/L; feed solution: 481 g/L glucose. The batch phase is predicted to be 9 h, where the fed-batch phase then starts. The predicted cell densities are based off an uninduced culture, without metabolic burden.

$$V_{fb} = \frac{S_0 V_0}{S_F}\left(e^{\mu_{set} t_{fb}} - 1\right) \tag{7}$$

5.2. Cool the fermenter after autoclaving. Remove the fermenter from the autoclave, clamp the backup inline filter, so that air only goes through the inline filter on the exhaust condenser during cooling. Do not connect the chiller to the fermenter until cooled <60 °C. Turn on the chiller and set to 12 °C. Connect the exhaust condenser, not the fermenter, to the chiller to prevent excess evaporation of the media while cooling. All probes can be connected to the control unit. *Note*: Some DO probes require polarization of least 1 h prior to calibrating the probe. Confirm times for DO probe by manufacturer.

5.3. Begin sparging the vessel by connecting the gas supply to the sparger inline filter.

Tip: Quickly complete this step. These steps maintain a positive pressure inside the fermenter.

Use Luer-Lok connections, but do not unclamp the tubing yet. Clamp the exhaust tubing with the inline filter using a hemostat or similar clamp. Remove the inline filter from the exhaust port at the Luer-Lok connection. Connect the exhaust line to approximately 0.7 m LS16 tubing (does not need to be sterile). Place the 0.7 m tubing into a 1-L graduated cylinder with approximately 700 mL of water. This vapor trap reduces aerosols. Turn on a small amount of air (~1 L/min), unclamp the sparger, and allow bubbles to form in the fermenter. Once bubbles form in the fermenter, unclamp the exhaust hemostat. The exhaust line inside the 1-L graduated cylinder should start to bubble. Set the airflow to 3 L/min. This corresponds to 0.5 vvm (volume air per volume liquid per minute).

5.4. Attach the motor stirrer to the fermenter. Set the stir speed low to around 200–400 rpm.

5.5. When the fermenter's temperature cools to <60 °C, the fermenter may be connected to the chiller, and a set point of 37 °C entered into the controller.

Tip: Major temperature differences between the fermenter temperature and chilled water can rupture the glass vessel.

Tip: Do not stir the fermenter too rapidly prior to inoculation as this can entrain air, which will skew the initial cell density measurement.

4.6 Fermenter inoculation

6.1. Prepare the complete feed solution. Place the cooled feed bottle from Section 4.3, step 3.3 inside of a biosafety cabinet. Use serological pipettes or sterile glass graduated cylinders to transfer the remaining components into the feed bottle (listed Table 4).

Precaution: For an *E. coli* BL21-derived strain, it is recommended to use antifoam in the feed. Stir feed bottle on a stir plate with a rare earth magnetic stir bar in order to completely resuspend the antifoam. *E. coli* BL21 strains harbor numerous prophage (Campbell, 1996; Kolisnychenko et al., 2002; Studier, Daegelen, Lenski, Maslov, & Kim, 2009), which can result in cell lysis at high cell densities. In contrast, reduced genome strains such as *E. coli* MDS42, lack all prophage and thus are much less prone to lysis. Further, *E. coli* BL21 strains require higher DO setpoints (Hausjell, Weissensteiner,

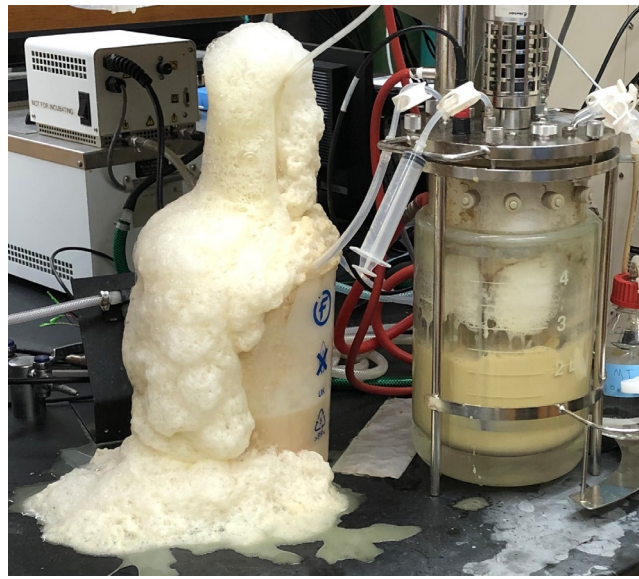

Fig. 3 Example of a foam-out in a 5-L fermenter due to the cell lysis not being controlled adequately by antifoam. An *E. coli* BL21 strain was being cultured.

Molitor, Halbwirth, & Spadiut, 2018; Marisch et al., 2013; Wurm et al., 2017), which can result in higher stir speed for the same cell density. These factors contribute to foaming. Fig. 3 shows an example of a "foam-out" for an *E. coli* BL21 fermentation. To prevent excessive foaming, the antifoam amount should be increased to between 10 and 30 mL per 2.5-L feed for *E. coli* BL21-derived strains. As the foaming can occur prior to 9 h, it is recommended to initiate the feed pump at a low rate (20 mL/h) to provide antifoam overnight. Fig. 1B shows this modified feed flow rate for *E. coli* BL21 T7 Express *lysY/I*^{q} expressing beta-glucuronidase. This feed modification will slightly delay the start of the fed-batch phase due to the extra glucose added to the batch phase.

6.2. Prepare the remaining minimal media components. Mix the remaining components in a sterile 500 mL bottle with a feed bottle connector. Table 3 lists these components. Ensure the tubing reaches the bottom of the bottle.

6.3. Connect the antifoam. In a biosafety cabinet, transfer 10 mL of sterile antifoam into a sterile 10-mL syringe. Using sterile technique, connect the antifoam syringe to an addition port.

6.4. Prepare the sampling device. Connect a sterile 20-mL syringe to the sample port on the headplate.

6.5. Prepare the base addition solution in a chemical hood. Fill a 500-mL bottle with 28–30% ammonium hydroxide. Approximately 300 mL will be required per fermenter run. Attach a feed cap to the bottle and clamp shut.

6.6. Add the remaining minimal media components to the fermenter (Table 3) through an addition port via LS16 tubing (see Fig. 2B). Insert the tubing through either an external pump or one on the fermenter control unit pumps. Unclamp the lines, turn on the pump. Add all the remaining components. Re-clamp the tubing, remove the tubing from the pump, and leave the tubing connected to the vessel until the fermentation is complete.

6.7. Make sterile connections for feed and base. Using the LS16 tubing, connect both the feed and base addition solutions to respective addition ports on the headplate via the Luer-Lok connections. Do not unclamp, load the tubing inside the pumps, or prime the lines yet.

6.8. Prepare the feed line. Load the feed tubing into the fermenter control unit pump designated for feed (or substrate addition). Unclamp both ends of the tubing. Prime the line until the liquid level reaches the addition port on the headplate. Reset the totalizer for the feed on the fermenter control unit.

6.9. Prepare the base addition line by inserting the LS16 tubing into the pump designated for base addition on the control unit. Unclamp both ends of tubing and begin priming the base line. Prime the base just past the pump head, but not all the way to the fermenter. *Note*: Ammonium hydroxide forms a gas at room temperature and expands inside the tubing. Priming to the fermenter port will cause base to leak into the vessel and lead to increased pH above the desired set point.

6.10. Measure the cell density of the preculture. Determine the preculture volume required for an initial cell density in the fermenter of 0.05 OD_{600} (X_{Ferm}) using Eq. (4).

6.11. Turn on control parameters and data recording on the fermenter control unit. This will initiate the control loops for the feed profile and DO control. Fig. 4 shows the online data corresponding to the *E. coli* MDS42 lacT7 gelsolin fermentation shown in Fig. 1.

6.12. In a biosafety cabinet, measure out the calculated volume of preculture using a sterile 500 mL graduated cylinder. Add the preculture volume to a sterile 1-L bottle with a feed cap. Connect the preculture bottle with LS16 tubing to the vessel. Pump the inoculum into the

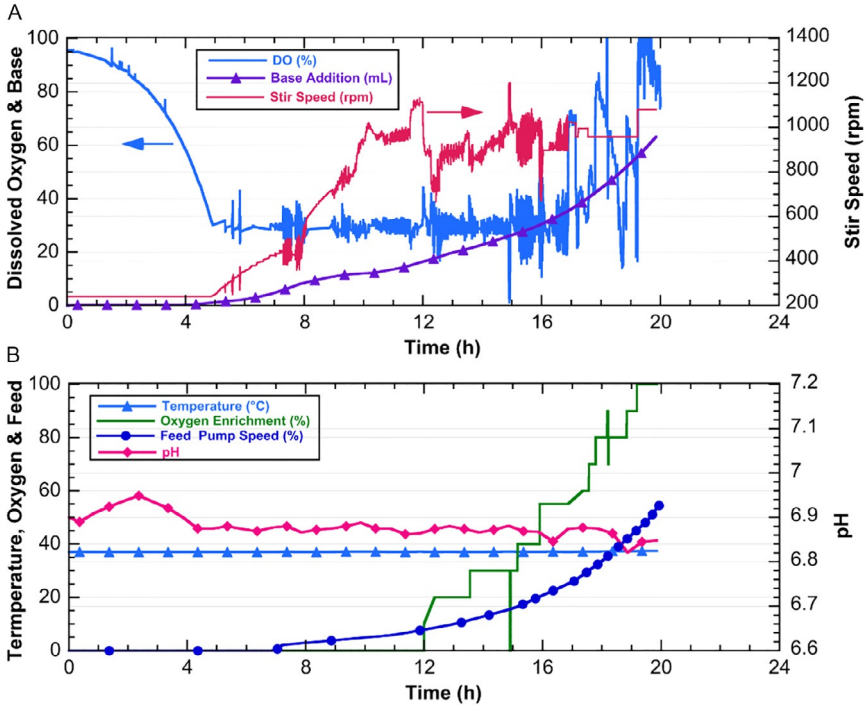

Fig. 4 Typical online data from computer-controlled fermenters. These data correspond to the *E. coli* MDS42 *lacT7* gelsolin cultures shown in Fig. 1. (A) Dissolved oxygen-DO (light blue), stir speed (red), and base addition (purple). (B) Temperature (°C) (light blue), oxygen enrichment of the air stream with oxygen (green), feed pump speed (blue), and pH (magenta). Online data is captured as frequently as every 6 s, therefore the symbols shown only represent some time point measurements and are provided to aid in visualization of the graphs with multiple data sets.

fermenter using the same technique used for the minimal media additives. Clamp the tubing and leave the tubing attached to the vessel.

6.13. Take the first cell density sample using the sample port and the sterile 20-mL syringe. Flush the line multiple times with the syringe to replace the sample line contents with vessel contents. Withdraw ~1 mL from fermenter and add 1 mL of sample to a spectrophotometer cuvette. Reconnect the syringe to the sample port or replace on the sample port with a new syringe. If the initial cell density is between 0.05 and 0.10 OD_{600}, the culture will take approximately 17 h to reach the induction cell density of 50 OD_{600} (see Eq. 1).

6.14. Measure the glucose concentration.

6.15. Record all cell density and glucose measurements in a spreadsheet. Fig. 1C demonstrates the need to measure glucose, as some strains when induced have decreased growth rates due to the metabolic burden (Bentley, Mirjalili, Andersen, Davis, & Kompala, 1990). In the case of glucose accumulation, the flow rate can be adjusted to allow the cells to consume the excess glucose, then the flow rate can often be resumed (Fig. 1B).

Tip: To simplify tracking of culture progress and health during the run, it is ideal to create an electronic spreadsheet for recording in parallel with handwritten values. Useful information to record includes sample time/date, elapsed fermentation time, absorbance, dilution factor, calculated cell density (OD_{600}), calculated growth rate (μ), glucose (g/L), notes, and list of samples saved. If preserving samples for SDS-PAGE analysis or other assays, it is also useful to calculate volumes for samples in terms of constant cell mass.

6.16. After inoculation, if the pH is significantly above the set point of pH 6.9, such as pH > 7.2, one may use 6 M HCl to return the pH to the set point. Fill a 20-mL syringe with 6 M HCl, connect to an unused addition port, and slowly add the acid until the pH reaches the set point.

Tip: Starting the pH lower than pH 7.0, will cause the base addition pump to actuate sooner. The movement of the pump can help prevent the development of pinches in the tubing. The use of very durable tubing, such as Cole-Parmer 06508-16, can also help.

6.17. Before leaving the culture overnight, place the exhaust cylinder into secondary containment (>5-L beaker or equivalent).

4.7 Induction phase

7.1. Once the culture has reached the fed-batch phase around 9 h, the feed solution will be added based on the feed equation (Eq. 6). Fig. 1 shows the cell density and feed flow rate for several *E. coli* strains using this standardized approach. Induction times were varied from 9 to 17.7 h and cell densities between 9.6 and 62 OD. This robust fed-batch method recommends induction at a cell density of 50 OD_{600}. This allows for 4–6 h of induction prior to the recombinant protein metabolic burden significantly inhibiting growth, as the goal is to reach 100 OD_{600} over the next 6 h.

7.2. In the morning, about 14 h after inoculation, take cell density and glucose measurements. Continue sampling every hour. To sample,

withdraw ~1 mL from the fermenter, and place on ice in a tube. Measure the OD_{600} using serial dilutions. The absorbance of the diluted sample should be between 0.1 and 0.5 OD_{600}. Dilutions 1:100 or greater should be created by two serial dilutions of 1:10 each. The final dilution can be directly mixed in the spectrophotometer cuvette. Vortex the sample after each dilution, including the cuvette. If the cell density is 50 ± 5 OD_{600}, move to the next step to induce the fermenter. If the culture is not within this range continue measuring the cell density every 30–60 min. To predict when the culture will reach a cell density of 50 OD_{600}, consecutive samples can be used to estimate the growth rate using Eq. (8):

$$\mu = \frac{\ln\left(\frac{X_2}{X_1}\right)}{t_2 - t_1} \tag{8}$$

Where the optical cell densities, X_1 and X_2, correspond to times t_1 and t_2, respectively. The doubling time (t_D) can be found using Eq. (9):

$$t_D = \frac{\ln(2)}{\mu} \tag{9}$$

If more accurate growth rate calculations are desired, use Eq. (10), which accounts for the volume change, V_1 and V_2, corresponding to times t_1 and t_2, respectively.

$$\mu = \frac{\ln\left(\frac{V_2 X_2}{V_1 X_1}\right)}{t_2 - t_1} \tag{10}$$

7.3. While waiting for the culture to reach 50 OD_{600}, prepare the inducer, up to two hours prior to induction. Remove the IPTG chemical bottle from the −20 °C freezer. Allow the IPTG to warm for 10 min before removing seal and lid. This will prevent water condensation on the powdered IPTG. For a 1.0 M IPTG solution, weigh out 2.0 g of IPTG. Pour the IPTG powder into a sterile 15-mL tube. Add 8.5 mL of sterile dH_2O to the IPTG powder, then vortex to mix. This amount of IPTG solution will be sufficient to induce 8.5 L to 1.0 mM IPTG, the total volume in the fermenter and feed bottle.

7.4. Record the volume in the feed bottle. Add the IPTG solution to the feed bottle using the ratio of 1 mL IPTG solution to 1 L feed to achieve a 1.0 mM IPTG solution. For example, if there is 800 mL of feed

remaining, add 800 μL of IPTG to the feed. Add the remaining IPTG to the fermenter using a port and syringe. *Note*: The IPTG does not require sterilization for two reasons, (1) the time remaining in the culture is short, and (2) the cell density is very high, and *E. coli* are the most prolific, thus unlikely to be outcompeted at this point. If inducing at much lower cell densities, sterile IPTG could be used. Stock solutions of 1 M IPTG can be filtered sterilized and stored frozen (−20 °C), if preferred.

7.5. Continue sampling the fermenter every hour for six hours after induction. Both the cell density and glucose should be measured. See Fig. 1A and C for example profiles. The glucose reading should be zero during exponential feed. If glucose begins to accumulate, especially above 5 g/L, it is advisable to slow the feed pump, or even stop the pump. See Fig. 1B for the modified feed profile used for *E. coli* BL21 gelsolin. In this case, glucose accumulation was observed, and the pump speed was reduced for several hours.

Tip: It is advisable to save cell pellets prior to induction and at each hourly sample post induction to characterize protein production.

7.6. This process can be modified if inclusion bodies or toxic effects need to be mitigated. For example, the culture temperature can be decreased at induction. Preliminary shake flasks studies can be used to determine alternative induction conditions.

4.8 Harvesting the fermenter

8.1. Approximately 30 min prior to harvest (6 h post induction), cool the centrifuge that will be used to pellet the cells to 4 °C. Weigh the empty centrifuge bottles and record masses. Place all centrifuge bottles into a tray of ice water. These actions will slow metabolism during the harvest.

8.2. Begin the harvest by turning down the fermenter setpoint temperature to 16 °C. Turn off the feed pump, feed profile, and base pump. Clamp the feed and base tubing at both ends. Remove the tubing from the pumps.

Tip: Do not turn off the gas flow as the positive pressure is required to prevent water from entering the vessel from the exhaust cylinder.

8.3. Attach LS17 tubing to the sample port. Load the LS17 tubing into a fast offline pump. Unclamp the sample port and begin pumping slowly to ensure there are no leaks or blockages in the system. Once the fluid is

flowing well, the speed of the offline pump can be increased to expedite harvest. Pump the culture into the iced centrifuge bottles to the desired level and cap. Once the culture is harvested, weigh the bottles to balance for centrifugation. Keep the centrifuge bottles on ice to slow the culture metabolism.

8.4. Once all of the culture has been harvested, turn off the gas supply and any remaining control loops. Stop data logging, and turn off the controller, following manufacturer instructions. Turn off the chiller and close gas supplies.

8.5. Centrifuge the cultures for 30 min at 4 °C and 5000 × *g*.

8.6. After centrifugation, decant centrifuge bottles into an autoclavable container. Weigh the centrifuge bottle again to obtain the wet pellet weight in each centrifuge bottle. The pellet may be removed by scraping or stored frozen (−20 °C or −80 °C) in the bottles until needed. Alternatively, a lysis buffer may be added to the cell pellet of a ratio of 1:4 (v/v) or greater. Resuspend the cell pellet in the lysis buffer by vortexing, pipetting, or whisking. Freeze bottle with pellets or lysed cell pellets at −80 °C.

8.7. Archive the online data. Fig. 4 shows an example set of online data for the *E. coli* MDS42 *lacT7* gelsolin fermentation shown in Fig. 1.

4.9 Fermenter clean-up

9.1. Review the manufacturer's instructions. Briefly, disconnect all electrical cables from the headplate: pH, DO, ground, temperature probes, and any other probes. Disconnect the water lines to condenser, water jacket, sparging tubing, and any other lines that would prevent the fermenter from being transported. Remove the probes from the fermenter and place in a 5-L plastic beaker. Rinse the probes with 70% isopropanol and allow to soak for at least 30 min. Then rinse the probes with dH_2O and wipe clean with a delicate task wiper. Do not wipe the optical sensor on any of the optical probes. Return the probes to the storage solution, as per manufacturer instructions.

9.2. Decontaminate the vessels with 10% bleach for at least 30 min. Use 10% bleach to wash out any tubing that contacted cells. Decontaminate any work stations that contacted cells with 70% isopropanol.

9.3. Wash the fermenter with water and concentrated soap after decontamination. Thoroughly rinse the fermenter and the headplate with water. Wipe off the headplate O-ring, apply a small amount of silicone grease

to the O-ring, and insert the O-ring into the headplate, as needed. Reassemble the headplate. Visually inspect the entire vessel for cleanliness before storage.

5. Expected outcomes

The expected outcomes include biomass concentrations in excess of 50 g dcw/L, corresponding to a wet weight of at least 160 g wet cell/L. The amount of recombinant protein produced will be similar to the levels obtained in shake flasks on a per cell basis. This protocol has been used to obtain over 30 g/L of soluble protein from about 80 g dcw/L cultures.

6. Advantages

The advantage of this method is a straightforward approach to reach high cell densities within 24 h. The work load is mainly in the setup and maintaining operations during the induction period.

7. Limitations

Strains that cannot be cultured in minimal media due to auxotrophic requirements may require preliminary experiments to determine the minimum level of supplement to support a specific final cell yield. The supplement can be added at low levels to the feed. If too rich a media is used, glucose will not be the limiting substrate and the exponential feed calculation assumption will not be valid. Other limiting carbon sources, such as xylose, have been used in this protocol to reach over 100 OD_{600} (Harcum & Caldwell, 2020).

8. Safety considerations and standards

As recombinant *E. coli* are being cultured, one must follow NIH guidelines for working with recombinant organisms.

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