Study of a Single-Use Stirred Tank Bioreactor for Manufacturing a Therapeutic Protein in a High Cell Density Microbial Fermentation

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ABSTRACT

Stirred tank single-use bioreactors (SUBs) have been widely adopted for production of biopharmaceuticals such as monoclonal antibodies in mammalian cell culture. However, they are seldom used for commercial production of biologics with microbial fermentation. SUBs offer time-saving advantages because they do not require significant downtime for cleaning and sterilization, so finding a SUB that can perform well with high cell density microbial fermentation processes has the potential to increase the number of production runs. Therefore, for this study, a His-tagged protease inhibitor was chosen as a model protein to demonstrate that the Sartorius Biostat STR® MO, a SUB recently developed for microbial fermentation, is suited for recombinant protein production by high cell density *Escherichia coli* fermentation processes.

At 50 L scale, the SUB achieved good process control and allowed an oxygen uptake rate (OUR) of up to 240 mmoles/L/h. The fermentation runs produced up to 5.8 g/L of the soluble recombinant protein and a dry cell weight of > 60 g/L at the end of fermentation. Additionally, the SUB showed a similar fermentation profile when compared with data from parallel runs in 15 L sterilise-in-place (SIP) vessels using identical media and process parameters. This study indicates that with a minimum investment of capital resources, stirred tank SUBs could be used in pilot-scale manufacturing with high cell density microbial fermentations to potentially shorten the timelines and costs of advancing therapeutic proteins to clinic.

INTRODUCTION

In the past decade, stirred single-use bioreactors (SUBs) have been successfully developed for commercial mammalian cell culture processes[1] and are commonly used to produce monoclonal antibodies. Since 2013, these types of SUBs have become an essential technology, with biopharmaceutical companies and contract development and manufacturing organizations (CDMOs) establishing SUB facilities for the manufacture of biologics.[2,3] SUBs have become so widely adopted because they are quicker to install than stainless steel vessels, and since they need less complicated fixed pipework and building infrastructure, SUBs require smaller amounts of capital investment. SUBs are also popular as they offer technical benefits including a lower risk of product carryover contamination, reduced clean-in-place (CIP), sterilise-in-place (SIP), and cleaning validation tasks, resulting in shorter batch to batch turnaround times.

Although stirred tank SUBs are widely used for commercial mammalian cell culture processes, they are less frequently used for the operation of rapidly growing microbial fermentation processes. This is because microbial fermentation is often more challenging in commercial-scale SUBs, as microbial cultures tend to grow faster than mammalian cells, produce between 20–75 times more heat[4], demand high volumetric power inputs, and require high gas flow rates.[5] Thus, many commercial biopharmaceuticals generated by microbial fermentation are still produced in traditional stainless steel stirred tank bioreactors.

In this article, we report on our evaluation and development of an intensified *Escherichia coli (E. coli)* fermentation process to produce a soluble recombinant protein at 50 L scale in a stirred tank SUB optimized for microbial fermentation. We will compare process performance with runs performed under identical fermentation conditions in a 15 L SIP stainless steel Biostat® C (Sartorius) vessel.

MATERIALS AND METHODS

Cell Line and Media

A recombinant *E. coli* K12 strain was used to produce a His-tagged protease inhibitor using the pAVEway™ expression system (FUJIFILM Diosynth Biotechnologies, Billingham, UK). This expression system was chosen because it has been successfully used for the commercial manufacture of a variety of therapeutic proteins.[6] The K12 strain was cultured in a proprietary defined, antibiotic-free growth medium, pre-optimized for the pAVEway expression system.

Single-Use Bioreactor

A prototype Biostat STR with a single-use Flexsafe STR MO bag (Sartorius) was used as the pilot-scale stirred tank SUB in this study (**Figure 1**). This SUB was chosen as its design allows consistent mixing and gassing from 50−2000 L bioreactor scales[7] and it has the potential to support high cell density fermentation processes.

The culture bag for each SUB fermentation was equipped with a pair of six-blade Rushton impellers and a disk ring sparger (**Figure 2**). The culture bag had six feed lines with a mixture of tubing diameters and a dip tube to accommodate the fermentation processes.

A range of single-use sensors were incorporated into the single-use bag, including an electrochemical pH electrode (Mettler Toledo), an Optisens dissolved oxygen electrode (Krohne), a BioPAT high foam/foam control sensor, and a pressure sensor (Sartorius). There was also a spare port for the connection of auxiliary biomass sensors, such as the BioPAT ViaMass and Fundalux (Sartorius).

Single-Use Bioreactor Setup

PES membrane filter capsules (0.2 µm Sartopore 2 filters) were used to sterilise the fermentation medium components. The filters were aseptically connected to the single-use culture bag using a BioWelder® (Sartorius). Before the culture bags were filled with media, the integrity of the bags was checked by automated pressure hold tests using a Sartocheck® system (Sartorius) according to the manufacturer's instructions. After installation, point-of-use leak tests were also performed using bag fleeces for optimum evaluation of bag integrity.

Sartorius Flexsafe STR transfer set membrane filters (25 cm long, 0.2 µm PTFE) were aseptically connected to the gas supply and exit lines on the culture bag. A single-use exhaust cooler was fitted before the exit gas filter in order to minimize evaporative loss and prevent wetting of the filter, enabling water condensate to return to the bag for further filter protection.

The culture bags were fitted into the Biostat STR vessel via a door on the front of the unit (**Figure 1**).

FIGURE 1. Biostat STR vessel and 50 L single-use Flexsafe MO culture bag integrated to a digital control unit (DCU).
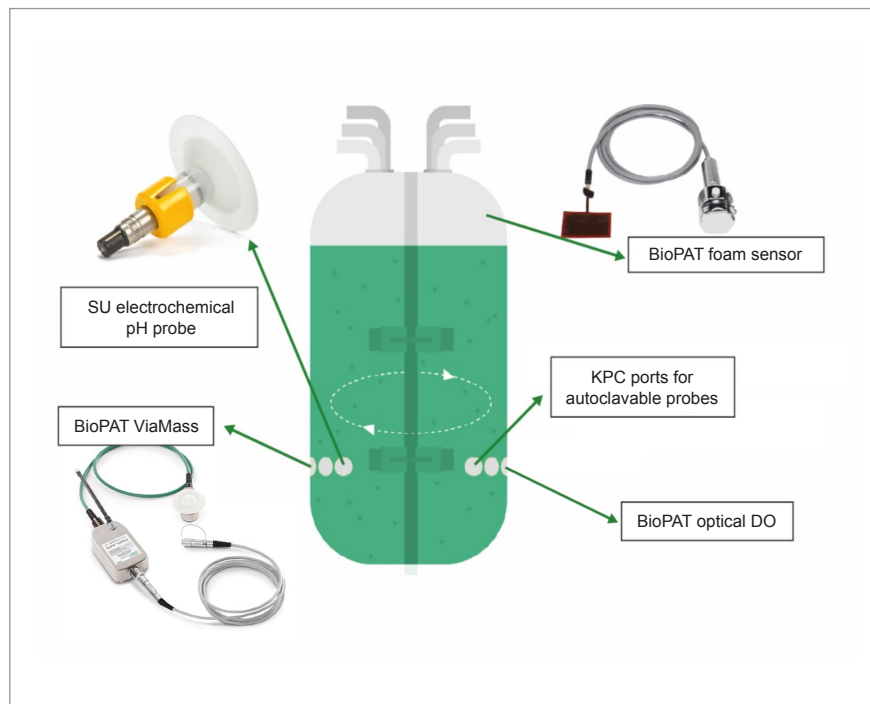

FIGURE 2. Flexsafe STR single-use culture bag configuration for test runs 1 and 2.

Analytical Equipment

A Prima deltaB mass spectrometer (Thermo Fisher Scientific) was used to measure oxygen uptake rate (OUR). OD_{600} was measured with a Spectronic Helios gamma spectrophotometer (Thermo Fisher Scientific).

Single-Use Bioreactor Operation

The fermentation process parameters were automatically controlled in the SUB via a DCU, which was configured for local operation. The DCU provided live process trend plots, alarms for possible excursions, and touch screen mimics to aid intuitive operation. Peristaltic pumps on the DCU were used to control nutrient, base, and antifoam feeding. The full-length window on the vessel was used to help operators visually check the fermentation. Adjustable baffles (three for run 1 and four for run 2) were used to boost the oxygen transfer rate.

PROOF OF CONCEPT STUDY

Performance Test Runs in SUB

Two *E. coli* fed-batch fermentations (36 L initial working volume growth medium) were carried out in 50 L-scale Biostat STR MO SUB with Flexsafe MO culture bags to test equipment performance.

The SUB test fermentations used the same inoculation and fermentation process (**Figure 3**), except for differences in media feeding and the point of induction, which are noted for runs 1 and 2.

SUB Test Run 1

Nutrient feed was added during the fed-batch phase of fermentation at a fixed rate of 11 g nutrient feed/L/h, and protein expression was induced 1.5 h after the start of medium feeding. Total run time was 27 h. When the
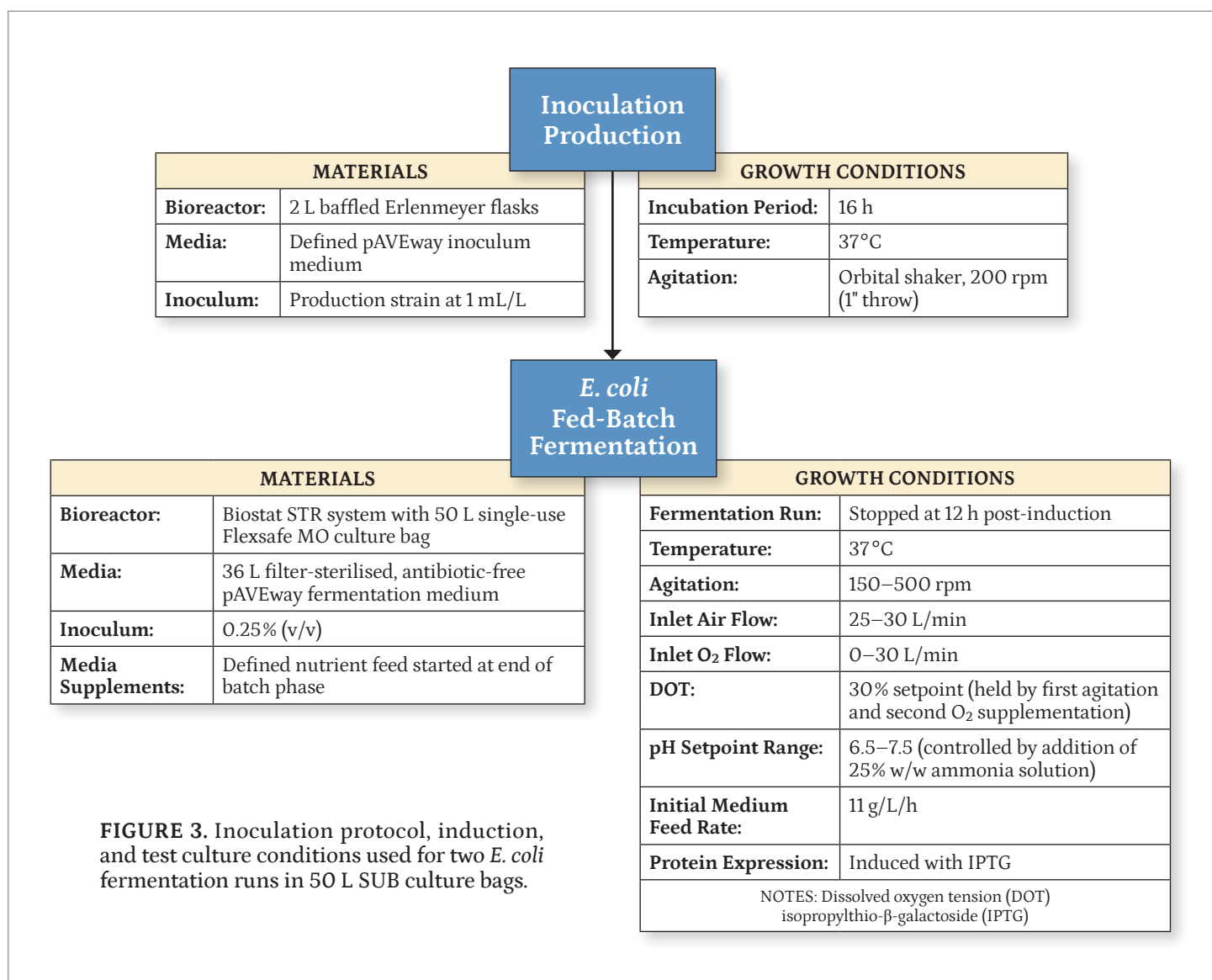

Inoculation Production

MATERIALS		GROWTH CONDITIONS	
Bioreactor:	2 L baffled Erlenmeyer flasks	**Incubation Period:**	16 h
Media:	Defined pAVEway inoculum medium	**Temperature:**	37°C
Inoculum:	Production strain at 1 mL/L	**Agitation:**	Orbital shaker, 200 rpm (1" throw)

E. coli Fed-Batch Fermentation

MATERIALS		GROWTH CONDITIONS	
Bioreactor:	Biostat STR system with 50 L single-use Flexsafe MO culture bag	**Fermentation Run:**	Stopped at 12 h post-induction
Media:	36 L filter-sterilised, antibiotic-free pAVEway fermentation medium	**Temperature:**	37°C
		Agitation:	150–500 rpm
Inoculum:	0.25% (v/v)	**Inlet Air Flow:**	25–30 L/min
Media Supplements:	Defined nutrient feed started at end of batch phase	**Inlet O_2 Flow:**	0–30 L/min
		DOT:	30% setpoint (held by first agitation and second O_2 supplementation)
		pH Setpoint Range:	6.5–7.5 (controlled by addition of 25% w/w ammonia solution)
		Initial Medium Feed Rate:	11 g/L/h
		Protein Expression:	Induced with IPTG
		NOTES: Dissolved oxygen tension (DOT) isopropylthio-β-galactoside (IPTG)	

FIGURE 3. Inoculation protocol, induction, and test culture conditions used for two *E. coli* fermentation runs in 50 L SUB culture bags.

run was stopped, the culture was cooled immediately afterward to <10 °C in 2 h. Then it was ready for harvest.

SUB Test Run 2

In this run, the medium feed rate was increased exponentially at 0.15/h for 4 h from 11 g nutrient feed/L/h, then protein production was induced, and the medium feed rate was fixed at 18 g nutrient feed/L/h. Total run time was 32 h. The culture was cooled using the same conditions as test run 1 in preparation for harvest.

The similarities between the two SUB test fermentations allowed assessment of the reproducibility of the SUB fermentations during the 15 h batch phase of these test fermentations.

Control Runs

In parallel with the two SUB test fermentations, two control fed-batch fermentations (initial working volume 12 L) were also run in a 15 L-scale Biostat C stainless steel SIP fermenter vessel, which had different aspect ratios (H:D) to the SUB. The same process conditions used for SUB test run 1 were replicated for control run 1, and the same process conditions used for SUB test run 2 were used for control run 2.

Analytical Methods

On-line off gas analysis via mass spectrometer was used to measure OUR in SUB run 2 and SIP control runs 1 and 2. To determine cell density, 10 mL samples were taken at multiple timepoints during the runs, and the OD_{600} of those samples was measured with the gamma spectrophotometer. Each sample was centrifuged to produce a cell pellet, and the supernatant was removed. Since the His-tagged protease inhibitor being produced is a soluble protein produced in the cytoplasm, protein analysis was carried out using SDS-PAGE (4–12% bis-tris gel) on the cells to determine protein yield. Cell pellet samples were dried and weighed to determine dry cell weights in each fermentation batch.

RESULTS AND DISCUSSION

Temperature and pH Control in SUB Test Runs

Results showed that there were no deviations in process control during the two SUB test fermentation runs. The culture temperature was maintained at a constant 37°C during the fermentations over 27 h for run 1 and 32 h for run 2, as indicated by the flat line in **Figure 4.**

Furthermore, accurate pH control was demonstrated with the single-use electrochemical pH probes maintaining the pH to within 0.02 units of the setpoint in both SUB test fermentations (**Figure 5**).

Dissolved Oxygen Control in SUB Test Runs

In both SUB test runs, agitation rate changes followed by oxygen enrichment was used to control DOT at 30%. The results showed that
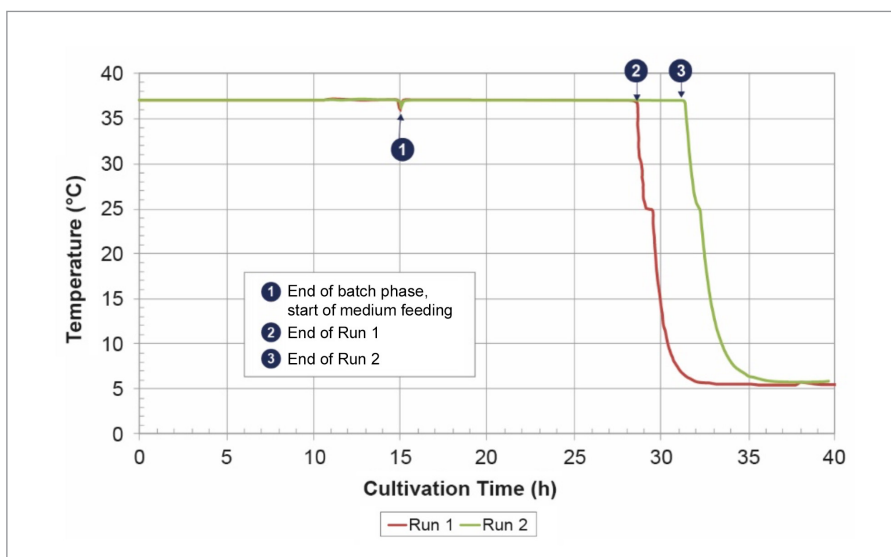

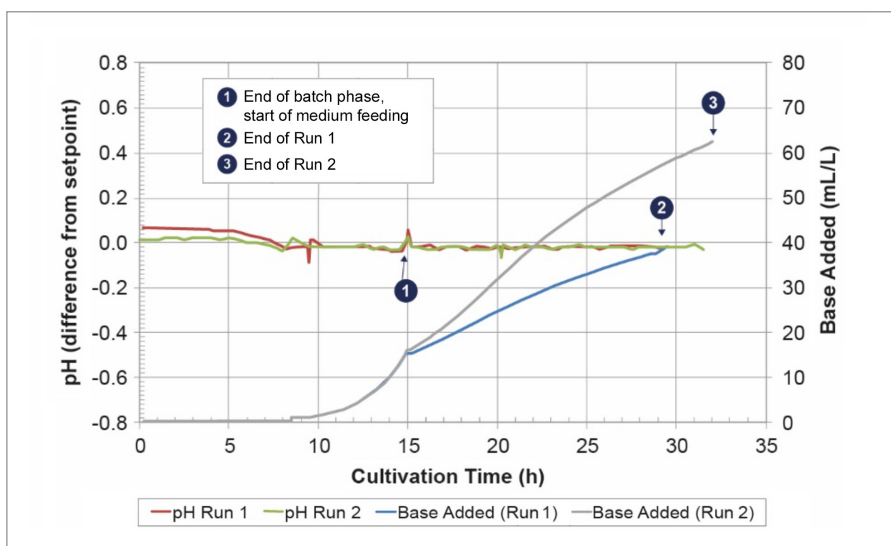**FIGURE 4.** Temperature control profile in SUB test fermentations.

FIGURE 5. Control of pH in SUB test fermentations.

DOT was sufficiently controlled throughout the SUB test fermentations to promote high cell density culture. The results also demonstrate that DOT fluctuated more when oxygen enrichment was used, after 11 h (**Figure 6**).

The maximum level of oxygen enrichment used in the SUB fermentations was 57% and the maximum total gas flow was 60 L/min (**Figure 7**). Backpressure was always <10 mBar during the SUB fermentations, as the exit gas filters were well-protected by the gas exhaust cooler. In this study, no added antifoam was needed throughout the runs. However, the single-use foam sensor provided good results for monitoring foam production in both runs.

Comparison of SUB Test Runs with Control Fermentations

Oxygen Uptake, Cell Density, and Dry Weight

On-line off gas analysis results of SUB test run 2, and the control fermentation vessel showed that the maximum OUR occurred at the end of batch phase, 15 h into the runs for both types of fermentation. The oxygen transfer rate provided by the SUB was sufficient to accommodate an OUR of at least 240 mmoles/L/h, which is comparable to the OUR of 251 mmoles/L/h seen in control fermentation run 2 (**Figure 8** and **Table 1**). This result indicates that the SUB can support the oxygen demands of a fed-batch high cell density fermentation.

The SUB test runs and the control fermentations in the SIP vessel grew consistently in batch phase (**Table 1**). Analysis showed that the SUB can support cell densities with an OD_{600} of 200 (**Figure 9**) and a dry cell weight of >60 g/L (**Figure 10**). Biomass concentration at the end of fermentation was greater in SUB test run 2 than SUB test run 1, (**Figure 10**) where extra nutrient feed was added to SUB test run 2, indicating that higher biomass yields can be achieved using the fed-batch approach in run 2.

Additionally, OD_{600} analysis and dry cell weight growth profiles of the SUB test runs were similar to those of the control
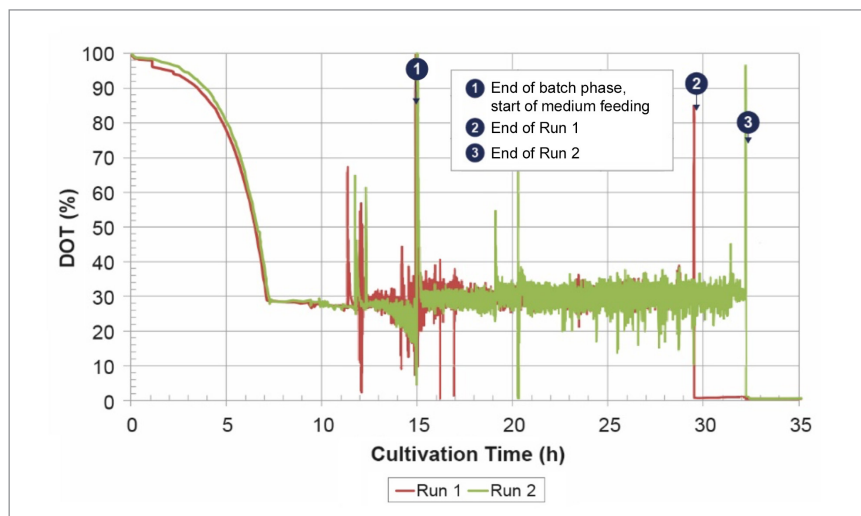
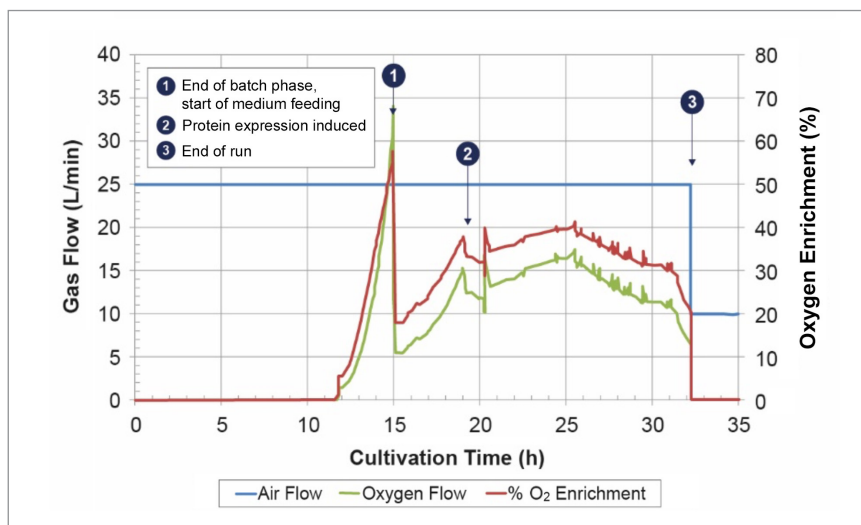

FIGURE 6. DOT profiles of SUB test fermentations.

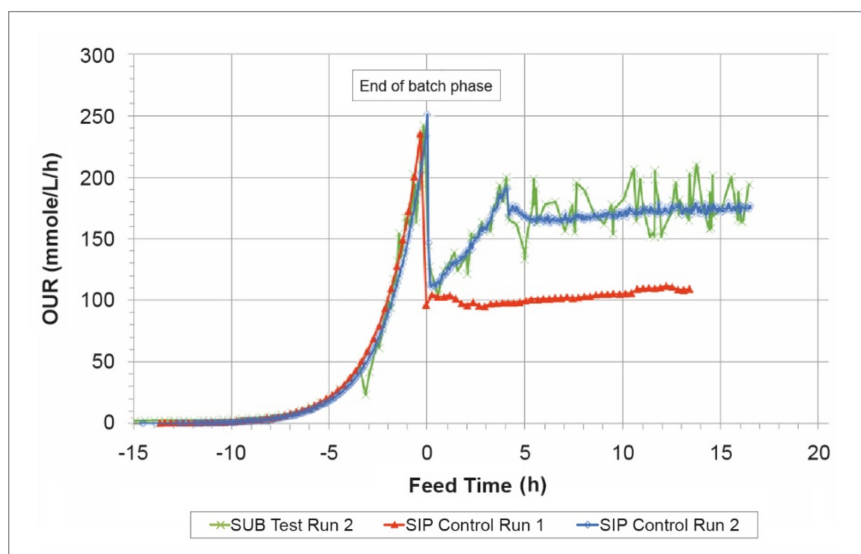**FIGURE 7.** Gas flow profile of SUB test run 2.

FIGURE 8. OUR in SUB test run 2 compared with SIP control runs 1 and 2. (NOTE: There was no gas analysis for SUB test run 1.)

TABLE 1. Comparison of OUR, OD_{600}, and dry cell weights measured at the end of the batch phase from *E. coli* fermentation runs in SUB and SIP control vessels.

Fermentation Run	At End of Batch Phase		
	OUR (mmoles/L/h)	OD_{600}	Dry Cell Weight (g/L)
SUB Test Run 1	—	38.3	17.3
SUB Test Run 2	242	38.5	17.6
SIP Control Run 1	235	39.5	ND
SIP Control Run 2	251	37.5	16.8
Mean of these runs	243	38.5	17.2
NOTE: There was no gas analysis for SUB test run 1.			
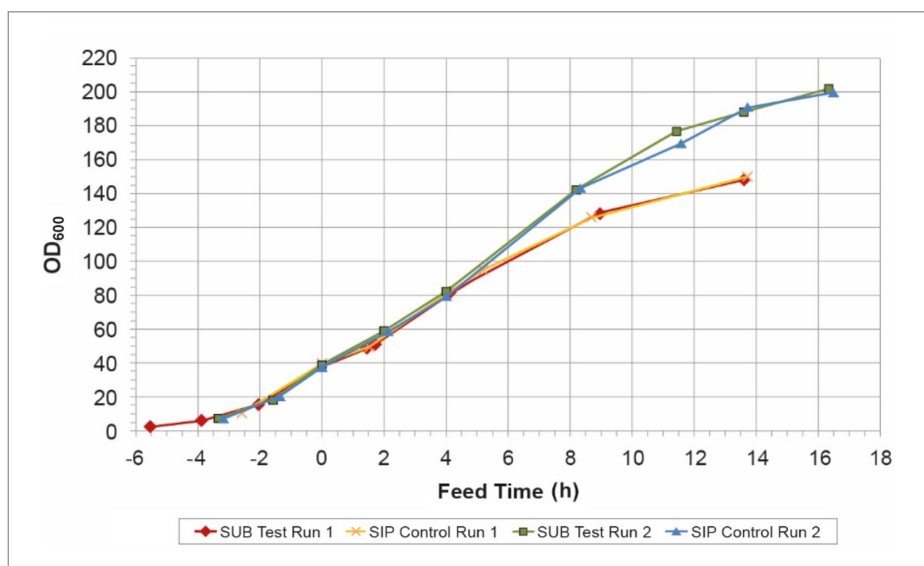
FIGURE 9. OD_{600} profiles of *E. coli* during fermentation runs in SUB and SIP vessels.
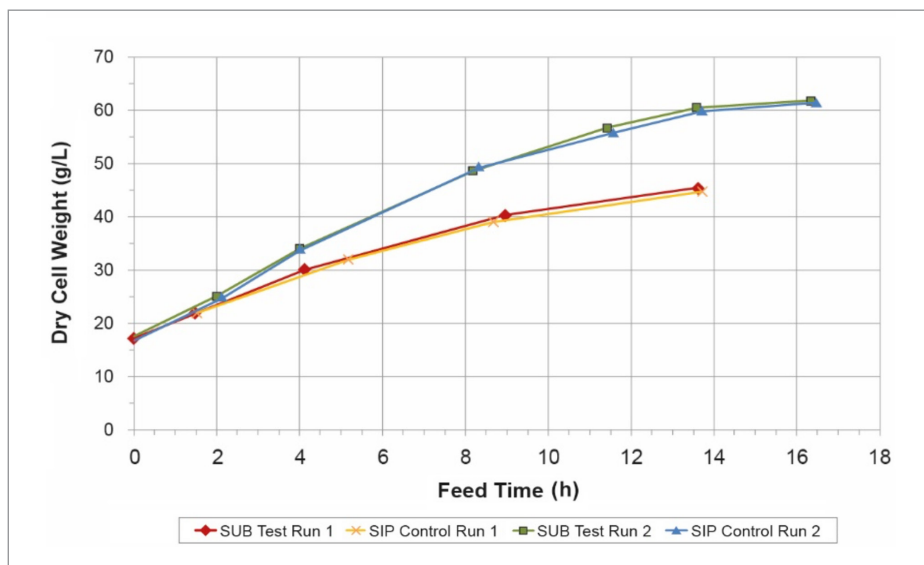
FIGURE 10. Dry cell weight profiles of *E. coli* from fermentation runs in SUB and SIP vessels.

fermentations cultured in the SIP vessels (**Figures 9** and **10**, respectively). These results indicate that this SUB can support the oxygen demands of a high cell density fed-batch fermentation to produce comparable dry cell weights to a fermentation using a traditional SIP vessel.

Protein Titer

As seen in **Table 2**, in both SUB test fermentation runs, the recombinant His-tagged protease inhibitor protein was successfully produced at levels which were comparable to product titers expressed in the corresponding SIP control fermentation vessels. Run 2, in both the SUB and SIP control, produced >30% more protein. These results demonstrate that the SUB can successfully accommodate high cell density *E. coli* fermentation processes for the manufacture of therapeutic proteins.

TABLE 2. Soluble His-tagged protease inhibitor protein yields from fermentation runs in SUB and SIP vessels.

Fermentation Run	Product Titre (g/L)	
	SUB Test	SIP Control
Run 1 (first process)	4.3	4.5
Run 2 (second process)	5.8	5.8

CONCLUSIONS

The high cell density SUB test microbial fermentations in the 50 L-scale Biostat STR MO described in this study were operated without any process deviations by scientists who had not previously used the equipment. The SUB was found to be quicker to set up than the SIP fermentation vessel and suitable for the processes assessed. Fermentation process performance in the SUB matched that in a traditional SIP vessel with differences in the SUB

and SIP vessel aspect ratios having no apparent impact on process performance.

The SUB provided sufficient mixing and mass transfer, operated successfully at high gas flow rates, and had enough cooling capacity to allow effective fermentation temperature control.

In summary, the combination of the Biostat STR MO SUB, E. coli pAVEway strain, and fermentation process provided a highly effective strategy for production of a model His-tagged protease inhibitor protein. The results from this study suggest that this type of SUB can operate with high cell density fermentation processes and could be substituted for a SIP vessel to potentially reduce the costs and timelines for manufacturing therapeutic proteins using E. coli fermentations.

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