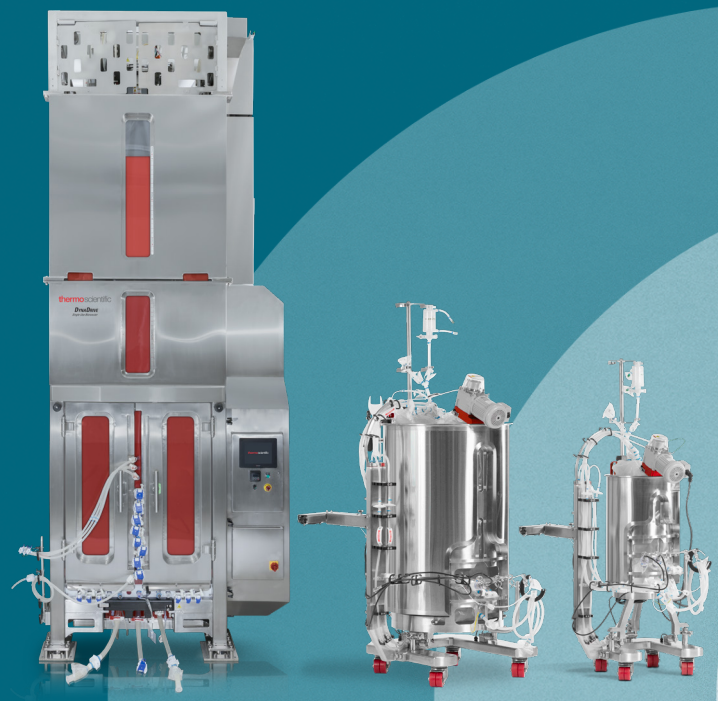
Application note



Single-use technologies

Scale-up of anaerobic bacteria cultivation from 50 L to 5,000 L in single-use bioreactors

Keywords
Single-use bioreactor, anaerobic bacteria, redox

Abstract

The demand for anaerobically produced products has increased, which has created a need for rapid scale-up with single-use systems in contract manufacturing operations. In collaboration with Arranta Bio, we cultured strict anaerobic bacterial strains in a 50 L Thermo Scientific™ HyPerforma™ Single-Use Bioreactor (S.U.B.). We then scaled up three similar oxygen-sensitive processes in a 250 L HyPerforma S.U.B. and scaled up again with a single strain in the 5,000 L Thermo Scientific™ DynaDrive™ Single-Use Bioreactor (S.U.B.). Because the Thermo Scientific™ HyPerforma™ Single-Use Fermentor (S.U.F.) and the subsequently enhanced S.U.F. are designed for aerobic cultures, we propose using S.U.B.s for anaerobic cultures. Our S.U.B.s have been shown to be gentler and less demanding for mammalian cell culture with homogenous mixing at high rates and best-in-class, durable films with a gas-impermeable design. Our S.U.B.s have smaller inlet and outlet filter lines and lower gassing rates, which is optimal for anaerobic bacterial cultures with the added advantage of proven scalability up to 5,000 L.

Background

To manufacture products using multiple strains of anaerobic bacteria in the same production space and reactors, it is essential to use single-use systems from inoculation through final fill and finish. This is because anaerobic strains are typically challenging to cultivate and challenging to demonstrate sterility of shared bioprocessing surfaces between cultures. With stainless steel systems, changing from strain to strain is a time-consuming process that requires extensive cleaning followed by media sterilization, cultivation, and downstream processing. If a quality validation test shows the system does not pass, the batch is discarded, and cleaning must be repeated. Even with all these precautions, there is a chance a spore will survive the process and cause failure of the subsequent batch. Implementation of single-use systems is essential for a CDMO to obtain products from multiple anaerobic bacterial strains.

To date, Arranta Bio has cultivated a large variety of facultative and obligate anaerobes including, but not limited to, *Oxalobacter formigenes*, *Bifidobacterium* sp., *Roseburia* sp., *Lactobacillus* sp., etc. The first strain, *Oxalobacter formigenes* is an obligate anaerobe for which anaerobic conditions are critical, as the presence of any oxygen will slow growth and may cause death. Most films and tubing are permeable to gases like oxygen, so it is important to use low-permeability tubing and film with a gas-impermeable layer. Thermo Scientific™ Aegis™5-14 and CX5-14 films have very low 0.491 cc/m^2 per day permeability to oxygen. Silicone is highly permeable to oxygen gas at about 287 cc/m^2 per day, while most thermoplastic elastomer (TPE) tubing has low oxygen permeability at about 1.29 to 5.4 cc/m^2 per day [1]. Standard Thermo Scientific™ bioreactors, fermentors, mixers, and bioprocess containers (BPCs) are made mostly with TPE tubing with very little surface area of silicone parts. Hence, our S.U.B.s, S.U.F.s, and associated BPCs are excellent for anaerobic applications.

Materials and methods

For culturing, MRS medium [2] or one of Arranta's proprietary media blends with animal-free components was used for all strains. Other supplements were added as appropriate per strain requirements. Anaerobic conditions were maintained from inoculation through harvest. Anaerobic 100 mL single-septum bottles and 2,000 mL dual-septum bottles were used for seed cultures. All media was prepared and autoclaved in bottles with the septa in place to bring the solution into anerobic sterile conditions. For the 10 L glass and 400 L steel vessels the MRS medium was heat-sterilized at 121°C for 20 minutes. For the S.U.B.s, the medium was prepared and sterile-filtered through 0.2 µm filters. For the strict anaerobic strain, resazurin dye was included as an oxygen indicator during strict anaerobic culturing.

The sterile-filtered medium was sparged with nitrogen while stirring to bring the solution's redox value to below –200mV. Adding cysteine solution to the medium helped maintain the redox potential needed for strict anaerobic conditions.

Sterile nitrogen was flushed around the bottle tops and connections in a laminar flow hood to help ensure anaerobic conditions were maintained while transferring the inoculum or other solutions. During inoculation or liquid transfer into a bottle, some sterile nitrogen was injected through the septum vented with a 0.2 µm filter to ensure anaerobic conditions were maintained.

Culturing of the obligate anaerobe *Oxalobacter formigenes* was performed in a 50 L HyPerforma S.U.B., which had a worst-case surface-to-volume ratio. The 250 L HyPerforma S.U.B. was tested with two obligate anaerobic strains (*Bifidobacterium* sp. and *Roseburia* sp.) and a facultative anaerobic strain, *Lactobacillus* sp. The 5,000 L DynaDrive S.U.B. (Figure 1) was tested with *Lactobacillus* sp.

When the pre-inoculum cultures reached sufficient growth and the operating conditions in each S.U.B. were achieved (Table 1), the cultures were transferred for production culturing. Samples were taken at time zero to measure the OD_{600}. Fermentation was allowed to continue for 12–24 hours. The OD_{600} was measured throughout culturing with an in-line probe. Redox potential recordings were logged throughout culturing as well.

Figure 1. 5,000 L DynaDrive S.U.B. partially filled during the medium filtration step.

Figure 2. An anaerobic culture in the 50 L HyPerforma S.U.B. just after filtration (left), just after addition of hot L-cysteine (center), and just prior to inoculation with redox reading of about –275 mV (right).

Results and discussion

All S.U.B.s and cultures held anaerobic conditions with less than –200 mV redox potential, which was necessary for obligate anaerobe cultivation. Importantly, the 50 L culture (Figure 2) showed excellent redox conditions for more than 30 hours prior to inoculation, even during pH probe insertion.

The 50 L culture reached its target growth and expression by 16 hours. Monitoring was continued for three more days to ensure that anaerobic conditions were maintained for future longer runs. The final concentration of L-cysteine in the culture solution was 2 mg/L. The N_2 gas was shut off at hour –13, and the pH probe was inserted with an aseptic probe insertion kit. It was noted that the approximately 50 mL of autoclaved air only minimally affected the redox potential. Upon restoring N_2 gas flow, the redox potential was quickly reduced back to less than –300 mV (Figure 3).

Table 1. Bioreactor conditions.

Vessel	50 L HyPerforma S.U.B.	250 L HyPerforma S.U.B.	5,000 L DynaDrive S.U.B.
Culture liquid volume (L)	30	250	5,000
pH	6.8	6.8	6.8
Temperature (°C)	37	37	37
Agitation rate (rpm)	150	125	75
N_2 sparge gas flow (slpm)	1	2	10
N_2 headspace gas flow (slpm)	1	2	20
Redox at inoculation (mV)	≤ –275	≤ –275	≤ –275
Redox at harvest (mV)	≤ –325	≤ –350	≤ –325

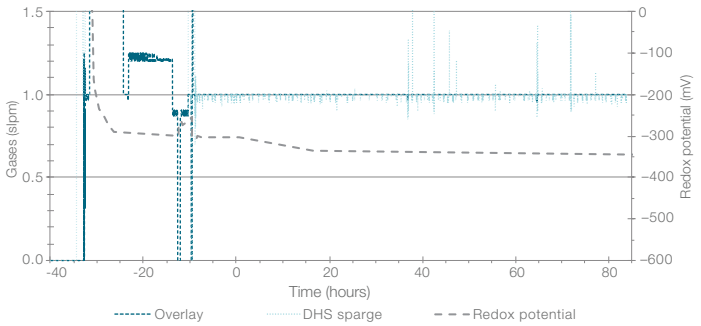


Figure 3. Redox potential and other conditions in the 50 L HyPerforma S.U.B. in preparation for and during anaerobic culturing of *Oxalobacter* sp.

The 250 L S.U.B. cultures grown at Arranta Bio were compared to previous runs in a 400 L working volume stainless steel fermenter (Table 2). The *Bifidobacterium* sp., *Roseburia* sp., and *Lactobacillus* sp. cultures all performed equivalently well in 250 L S.U.B.s. Figure 4 shows the redox potential stayed below –350 mV throughout the 14-hour run.

The redox potential, pH, temperature, agitation rate, added antifoam, and OD_{600} of the 5,000 L culture were monitored. The data suggested anaerobic conditions were easily maintained, as the redox potential was well below –300 mV through the entire culturing run (Figure 5). The final density at hour 12 was more than 16 g/L wet cell weight (WCW).

The cell mass in the 5,000 L DynaDrive S.U.B. (Figure 6) and the equivalent culture in the 250 L S.U.B. were twice as dense as the cell mass in similar 10 L benchtop glass vessels and a 400 L stainless steel fermentor. This may be attributable to the use of heat-sterilized media in the 10 L and 400 L reactors or using three times as much $MgSO_4$ in the DynaDrive S.U.B. than was used in the smaller cultures. This was previously shown to result in more productive cultures.

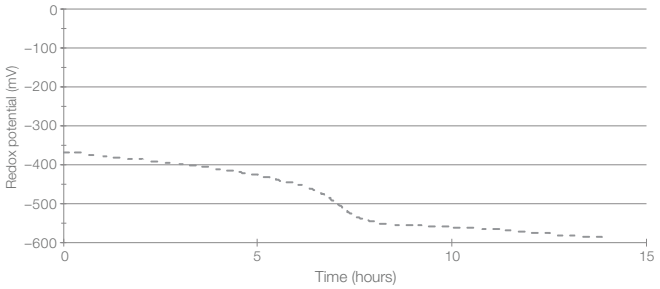


Figure 4. Redox potential during *Roseburia* sp. anaerobic culturing in the 250 L S.U.B.

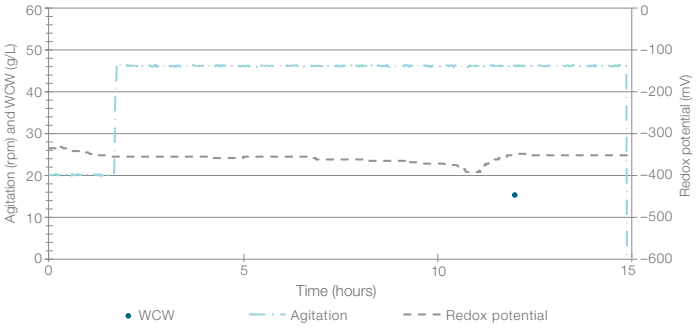
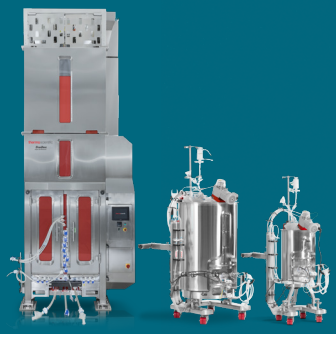


Figure 5. Conditions during *Lactobacillus* sp. anaerobic culturing in the 5,000 L DynaDrive S.U.B.

Table 2. Comparison of culturing of anaerobic production strains in the 250 L HyPerforma S.U.B. and previous runs in a stainless steel (SS) vessel.

Strain type	Bioreactor	Harvest OD_{600}	CFU/mL	CFU/g drug substance powder
Obligate	SS	3.9	0.54×10^{10}	1.3×10^{10}
	S.U.B.	4.5	0.61×10^{10}	2.6×10^{10}
Facultative	SS	3.5	0.49×10^{10}	3.7×10^{10}
	S.U.B.	3.7	0.53×10^{10}	6.3×10^{10}
Obligate	SS	2.4	0.12×10^{10}	24×10^{10}
	S.U.B.	3.5	0.28×10^{10}	19×10^{10}



Conclusions

This study was undertaken to prove the feasibility of anaerobic culturing at the 50 L, 250 L, and 5,000 L scale in Thermo Scientific S.U.B.s. To show that anaerobic conditions could be maintained for the duration of longer culturing runs, the 50 L test culture was held under anaerobic conditions for an additional three days. All of the required titers were met, and anaerobic conditions were maintained in each S.U.B. with standard TPE tubing line sets and BPCs. A key to maintaining a redox value of –300 mV or less is having few, if any, silicone parts. The S.U.B.s were shown to be able to handle most any anaerobic culture.

The HyPerforma S.U.B.s and BPCs maintained the required conditions to enable manufacturing of multiple strains of anaerobic bacteria. Using S.U.B.s for this type of process saves time, labor, and resources by making the extensive cleaning processes, equipment, and space required for stainless steel manufacturing unnecessary. Single-use products are considered essential to CDMOs for anaerobic upstream production lines.

Recommendations

Anaerobic applications are feasible in standard Thermo Scientific S.U.B.s, S.U.F.s, and related BPCs from 30 L to 5,000 L when utilizing CX5-14 or Aegis5-14 films and standard low-permeability tubing like C-Flex™, AdvantaPure™, neoprene, or similar TPE tubing. Most applications require stirring in the BPC to adequately mix solutions, deliver nutrients, and maintain homogenous mixtures.

Figure 6. Anaerobic *Lactobacillus* sp. culture in the 5,000 L DynaDrive S.U.B. just before harvest.

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Acknowledgements

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