APPLICATION NOTE
July 2025

securecell

PRECISION MONITORING OF OBLIGATE ANAEROBES DURING FERMENTATION

Viable growth assessment using the Numera® automated sampling system

Written by **Pascal Vonlanthen** Securecell AG (pascal.vonlanthen@securecell.ch),
Ahmad Abdulaziz Seres Therapeutics (aabdulaziz@serestherapeutics.com)

Seres Therapeutics' cultivated live biotherapeutic products are formulated from obligate anaerobic bacterial strains that have been isolated and purified from the gut microbiome of healthy human donors. In performing single-strain anaerobic cultivations, it is necessary to control oxygen exposure during all aspects of strain handling, including inoculation, incubation, sampling, and harvesting. As with all bioprocess research, it is important to obtain representative samples during the cultivation process to get accurate information on process conditions and product quality attributes. Seres Therapeutics implemented an automated sampling and sample processing system to obtain samples complementary to existing manual sampling and processing methods. This required the development of new protocols anaerobically manage, handle, and hold samples to ensure analysis results represent the upstream process at the time of sampling. In this application note, we present how Seres Therapeutics developed an innovative implementation of Securecell's automated sampling system Numera® to maintain anaerobic control and viability of samples including during a sample hold time for process development of anaerobic cultivations.

Introduction

In recent years, the knowledge about the human microbiota and its impact on health and disease has improved considerably. Many drug modalities have the aim to manipulate a patient's microbiome through the addition of new microbial strains, the subtraction of deleterious strains, or the rebalancing of the existing microbiome composition (McChalicher & Aunins, 2022). *Lactobacilli* and *Bifidobacteria* are human gastrointestinal (GI) bacteria traditionally used as probiotic dietary supplements and are also under clinical evaluation as pharmaceutical products. For both genera of bacteria, technologically robust strains have been isolated and cultivated on an industrial scale after a long history of use in food processes. However, studies on the human gut microbiota have highlighted other types of commensal bacteria like Akkermansia and Faecalibacterium that are consistently underrepresented in various illnesses (Andrade et al., 2020), and may have dietary or pharmaceutical potential based on cell and animal models (O'Toole et al., 2017). While many aspects of a live microbial drug such as efficacy, safety, and physiological, genomic, and metabolomic properties require considerable research to establish therapeutic potential, other often overlooked but essential aspects such as manufacturing, storage stability, and delivery, also require investigation before widespread practical application (Jimenez et al., 2019).

Many GI commensal microorganisms are strict anaerobes with fastidious nutritional requirements and, therefore, pose significant new challenges for pharmaceutical development, especially during manufacturing, storage, and drug delivery (Andrade et al., 2020). For many GI species of interest, the nutritional requirements for the organisms are initially poorly understood, and therefore significant bioprocessing research must be completed for each new organism to enable cultivation production targets. A key challenge in development and manufacturing is the need to maintain the oxygen-free environment required for viability of obligate anaerobes (Mori et al., 2014). Although

automated systems are highly valuable for bioprocess development, the complexity of anaerobic process controls increases with the additional interconnections associated with automation.

Anaerobic Automated Sampling

The Numera® is a modular sampling system that can be flexibly configured to allow small-volume high-frequency sampling from up to 16 bioreactors or downstream surge tanks, reliable sample processing with a precise dilution (error ≤ 2% SD) and unique tape-filtration technology, cooled sample storage in the Fraction Collector Module and transfer to multiple cell and biochemical analyzers or HPLC systems (Figure 1). Additionally, Numera® comes with the powerful Lucullus® process information management software to digitally integrate unit operations and analyzers, collect and centrally store all process data, and allow process-wide monitoring and control.

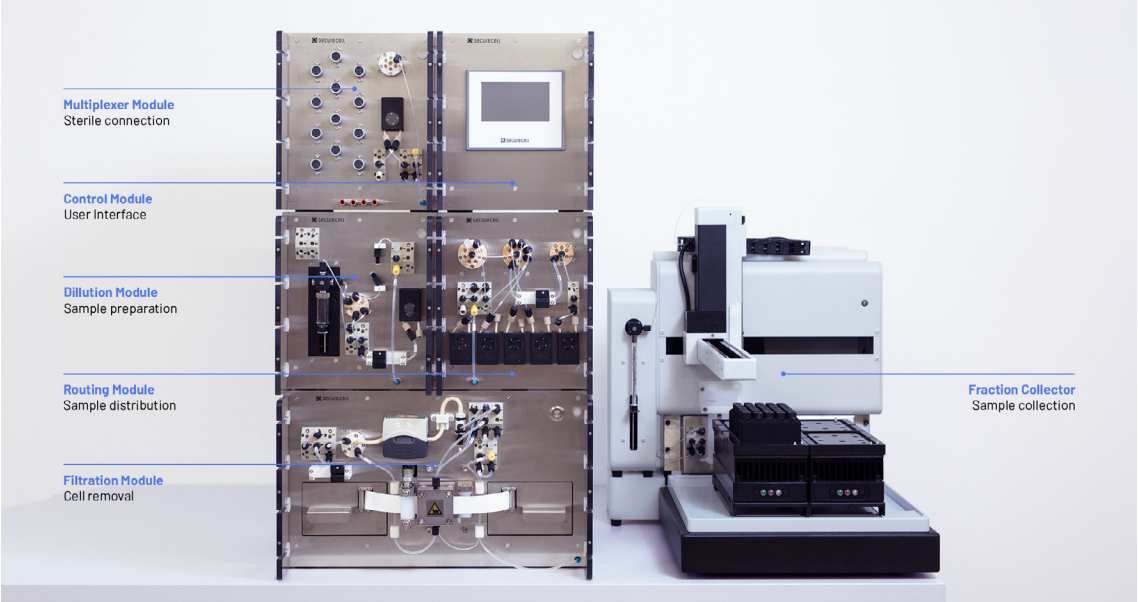

Figure 1: Image of the automated sampling system Numera ®. The Multiplexer Module forms the interface to the bioreactors (in USP) and surge tanks (in DSP). After optional sample processing by the Dilution and/or Filtration Module, the Routing Module directs the sample either to the Fraction Collector Module for sample storage or directly to connected 3rd party analyzers.

The Numera® and Lucullus® technologies are standardly used in upstream bioprocess research to automate and digitalize workflows and support researchers in measuring critical process parameters (CPPs) and critical quality attributes (CQA) automatically to identify nutritional and other organism requirements. Reported here are modifications to the standard Numera® installation to enable compatibility with strict anaerobes. To manage, handle, and hold samples anaerobically, the Numera® system was operated with sterile N_2 instead of sterile air. Furthermore, an oxygen-scavenger substrate solution was added to the vials of the Fraction Collector Module to protect the sample from the presence of environmental oxygen.

Material & Methods

Cultivation set-up: Experiments were executed using a wild-type, human-commensal strain of the *Blautia* species from Seres Therapeutics' proprietary strain library. Cultivations occurred in a single-use 1.0 L DASGIP® Parallel Bioreactor System (Eppendorf). The bioreactor was filled with 1000 mL of sterile-filtered proprietary medium (0.22 µm filter) optimized for the growth of this strain. The filled bioreactor was sparged with nitrogen gas to strip dissolved oxygen from the bioreactor system before inoculation. The bioreactor conditions included a temperature setpoint of 33°C, an agitation rate of 100 rpm, and a sparging rate of 0.003 VVM nitrogen. Two separate sample lines

were present on the bioreactor: one for manual sampling and the other dedicated to the Numera® automated sampling system. An on-line gas analyzer was connected to the bioreactor's off-gas vent filter to measure the output oxygen and carbon dioxide during the cultivation process.

On-line automated sampling set-up: The installed Numera® system uses a pneumatic movement of samples using nitrogen gas (2.0 bar) rather than a typical installation that uses compressed air. The Numera® system was controlled by the software Lucullus® (Securecell AG) and configured with the predetermined sample time points based on elapsed cultivation time. At each timepoint, samples drawn by the Numera® Multiplexer Module were split into two sample fractions: whole broth, 1.0 mL was directly dispensed to the Fraction Collection module into glass vials containing 30 µL of oxygen scavenger substrate (Oxyrase, Oxyrase®Inc.) and clarified broth (1.0 mL) was first prepared using the Filtration Module and then dispensed to glass vials in the Fraction Collector. Both samples vial types are held on a cooled rack at 4°C.

Off-line manual sampling: Off-line samples were collected from the bioreactor. 5ml samples of culture broth were manually drawn at each sampling point and immediately transferred into an anaerobic chamber for processing. Samples were assayed for colony-forming units (CFUs, brain/heart infusion agar), off-line pH, and optical density (600 nm).

Sample Analysis of automated samples: Whole broth samples held on the Fraction Collector Module of the autosampler system Numera® were transferred into an anaerobic chamber and analyzed for CFUs as above. Filtered samples were analyzed for in- process sugar utilization via Ion Chromatography System (Thermo Scientific™, Dionex™ ICS-6000).

Results and Discussion

Seres Therapeutics has established a human GI commensal strain library that includes many obligate anaerobes with varying nutritional requirements and environmental sensitivities. Oxygen exposure is often toxic to these isolates, as it is typical for well-studied obligate anaerobes such as acetogenic bacteria, methanogens, and *Clostridia* (Rigottier-Gois, 2013). To challenge the Numera system setup described in this report, a proprietary Blautia strain was selected based on previously established sensitivities to oxygen exposure and internal phenotypic assessment. To establish the oxygen sensitivity of the Blautia strain used in these studies, anaerobically grown culture samples were exposed to two conditions: a hypoxic environment (0.4% oxygen) for 180 minutes or exposed to air (~21% oxygen) for 20 minutes. The exposure to air had a detrimental impact on viability, resulting in a 10,000-fold reduction in viable cell titer for the Blautia strain compared to the exposure to the hypoxic environment (Figure 2). This phenotype represents a typical phenotype amongst the oxygen-sensitive strains in Seres library, and ensures this strain is a suitable model organism for studies verifying the capability of the adapted system to prevent against oxygen exposure. The bars in Figure 2 show the range of phenotypes observed in the Seres library with respect to hypoxic and fully aerobic exposures. These data show it is important to minimize exposure of obligate anaerobes to oxygen during cultivation and to obtain representative samples with limited oxygen exposure to ensure reliable cell viability measurements for bioprocess development studies.

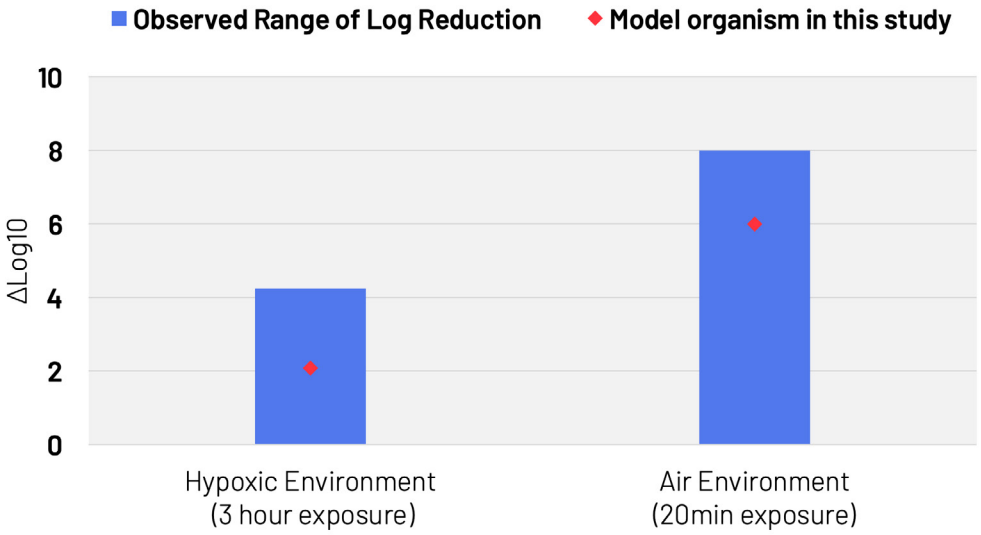

Figure 2: 10,000-fold reduction in the viability of *Blautia* after 20 minutes of air exposure compared to 180 minutes in a hypoxic environment. The ΔLog10 is defined as the common logarithm of the ratio of the levels before and after exposure. An increment of 1 corresponds to a reduction in concentration by a factor of 10.

Because exposure to oxygen can have a profound effect on viability, we investigated whether holding samples in vials that were prefilled with an oxygen scavenger substrate solution would protect viability during hold steps. Table 1 presents the experimental design that tested the effect of holding conditions (Anaerobic vs Aerobic and RT vs 4°C) and holding durations on sample vegetative cell viability (titer). Four strains were tested: two with high sensitivity to oxygen (including the Blautia strain described above) and two with low sensitivity to oxygen. The results compare the titer with no hold and the titer with hold in Log Scale as Log Reduction Factor (LRF).

Table 1: *List of sample vial holding conditions and type of organism tested based on oxygen sensitivity LRF and holding duration (BSC: Bio Safety Cabinet; TCU: Temperature Control Unit).*

				Observed Log Reduction Factors (ΔLog10)			
High Sensitivity to Oxygen Presence Organisms	**Strain 1 & Strain 2**	Sample vial held for up to ½ (h)	Anaerobic Chamber	Aerobic @ RT (BSC)	Aerobic + Substrate @ RT (BSC)	Aerobic @ 4°C (TCU)	Aerobic + Substrate @ 4°C (TCU)
		Sample vial held for up to 1.5 (h)					
		Sample vial held for up to 3.5 (h)					
		Sample vial held for up to 6 (h)					
		Sample vial held for up to 24 (h)					
Low Sensitivity to Oxygen Presence Organisms	**Strain 3 & Strain 4**	Samplevial held for up to 1.5 (h)	Anaerobic Chamber	Aerobic @ RT (BSC)	Aerobic + Substrate @ RT (BSC)	Aerobic @ 4°C (TCU)	Aerobic + Substrate @ 4°C (TCU)
		Sample vial held for up to 3.5 (h)					
		Sample vial held for up to 6 (h)					
		Sample vial held for up to 26 (h)					

To enable reliable decision-making during future bioprocess research and development, an upper limit log reduction factor of 0.5 log10 was selected as the design criteria for the storage condition. To enable convenient equipment design and operation, it was further desired to store samples in a fully aerobic environment. Based on the results of the designed study (data not shown), storage in an aerobic environment for at least 3 hours is possible for even the most sensitive strains when samples are held at 4°C and with the presence of the oxygen scavenging substrate. For strains with high sensitivity to oxygen exposure, longer hold times increase the log reduction factors beyond 0.5 log10, and a higher LRF was observed in samples pulled earlier in the growth phase compared to samples pulled at later growth phase of the cultivation process. Further investigation is required to be able to make a general conclusion for a wider range of obligate anaerobes. For later experiments reported in this study, the storage vials on the Numera® Fraction Collector included a 30 µL of Oxyrase substrate solution per 1 mL sample size based on the experimental results.

The performance of the Numera® system for monitoring the oxygen-sensitive *Blautia* strain during cultivation in bioreactors was evaluated during both exponential growth and stationary phases. Manual and automated Numera® sampling was scheduled after 12, 14, 16, 18, and 20 hours of anaerobic cultivation (see Material and Methods section) to enable head-to-head comparison of the two methods. The manual samples were analyzed directly after sampling for titer (CFU/mL)(Reported in Log Scale), while the automated Numera® samples were held on the fraction collector at 4°C in vials that contained Oxyrase and analyzed after a hold of either 2 or 4 hours after sampling (Figure 3).

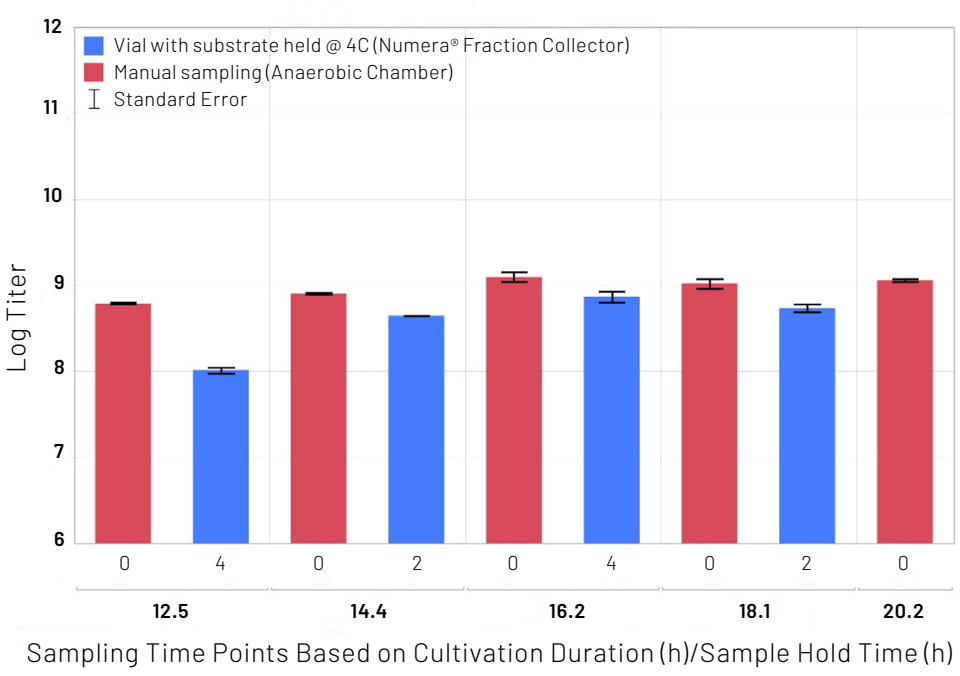

Figure 3: Log titer of model organism vs. Sampling Time Points based on cultivation duration. For each sampling time point except the 20-hour time point, one manual sample and one automated Numera® sample were taken an analyzed for viable titer. The sampling time points were approximately 12, 14, 16, 18, and 20 hours post-inoculation. The red bars represent manually drawn samples analyzed immediately after collection (Sample Hold Time = 0), while the blue bars represent automated Numera® samples analyzed after 2 or 4 hours of sample hold time.
Each error bar is constructed using 1 standard error from the mean.

The automated Numera® samples showed less than half Log10 drop after a 2 or 4-hour hold time during late exponential towards stationary phase (Figure 4). At all timepoints other than the initial mid-exponential growth phase timepoint at 12.5 hours, the difference between the sampling methods was within the 0.5 log10 log reduction factor targeted during the storage condition development study above. At the 12.5 hour timepoint, the difference was larger with an observed log reduction factor of 0.8 log10 but after a 4 hour hold time rather than the intended storage limit of 3 hours.

In addition to the viability analysis, the Numera® system collected filtered samples at cultivation durations of 6, 8, 9, 10, 11, and 12 hours. These samples were subsequently used to analyze the sugars consumption rate estimation during exponential growth (Figure 5). These filtered samples were held at 4°C on the fraction collector throughout the cultivation time before analysis.

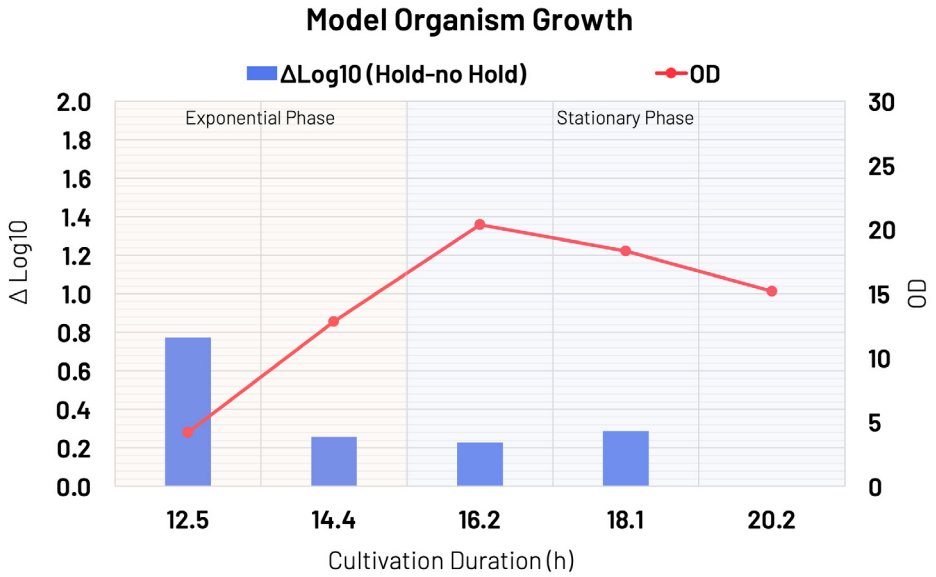

Figure 4: ΔLog10 of model organism andOptical Density at 600 (OD) vs. Sampling Time Points based on Cultivation Duration. For each sampling time point, except the 20 hour time point, one manual sample and one automated Numera® sample were taken, analyzed and the ΔLog10 calculated. The sampling time points were approximately 12, 14, 16, 18, and 20 hours post-inoculation. The orange line shows the OD and represents the stage of growth of a bacterial culture.

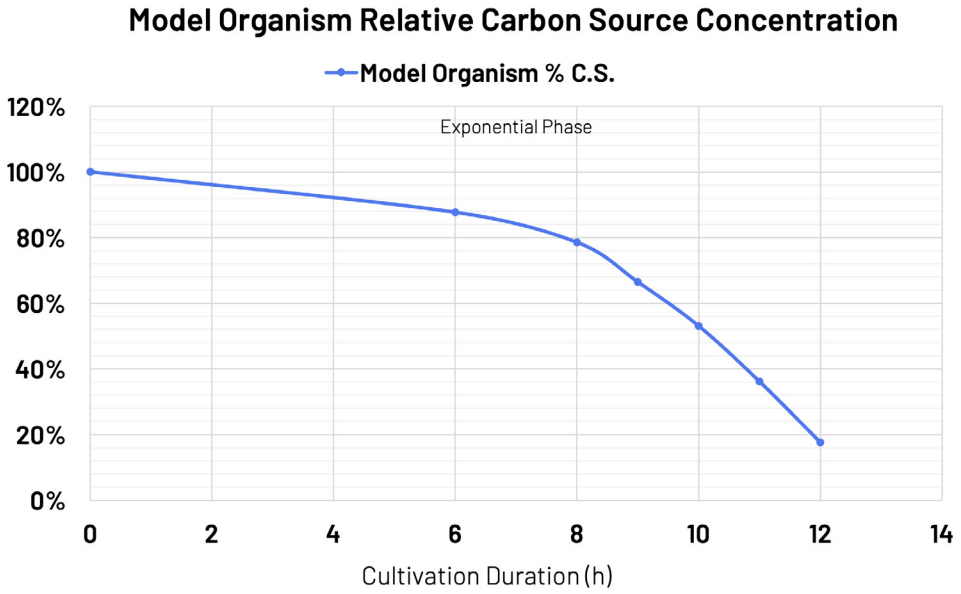

Figure 5: Relative Carbon Source Concentration vs. Cultivation Duration. The Carbon source concentration at 6, 8, 9, 10, 11, and 12 hours of cultivation time samples collected by automated Numera® sampling through the Filtration Module. (Carbon source concentration is determined relative to time 0)

The suitability of the Numera® system, including the developed sampling method, was evaluated for full-day sampling, including unstaffed overnight periods, using the model Blautia strain. Automated Numera® samples were pre-scheduled based on elapsed fermentation time at 3, 4, 5, 6, 7, and 8 hours after inoculation of the bioreactor. Additionally, manual samples were taken at 11, 14 and 16 hours post-inoculation. The holding time of the automated Numera® samples varied depending on the sample time point, with a maximum hold time of six hours for the initial 3-hour sample and shorter hold times for each incremental sample. All automated collected samples were analyzed at nine hours post culture initiation for viability and residual carbon source. Additionally, off-gas data was collected, and the carbon dioxide transfer rate (CTR) was calculated from the carbon dioxide content in the off-gas and the sparging rate. Figure 6 presents result of the viability measurements, showing an exponential growth rate of 0.87 hr-1 during the overnight sampling period through the 8-hour sample point. Viability measurement of samples after 10 hours post-inoculation show on-set of stationary phase. These observations are confirmed by the CTR and residual carbon source measurements in Figure 7. The CTR data shows exponential growth from approximately 5 to 9 hours after inoculation and then the onset of stationary phase, corresponding to the full consumption of the carbon source.

The automated Numera® system allowed for the reliable collection of a comprehensive set of samples during time periods outside of normal staffing times. Analysis of these samples, including for cell viability analysis, have been shown to complement online cultivation data. These data sets are sufficient to enable further development of optimized medium recipes for continued process improvement, implementation of fed-batch strategies to further increase production, or demonstrate robustness of process controls across a series of replicate experiments.

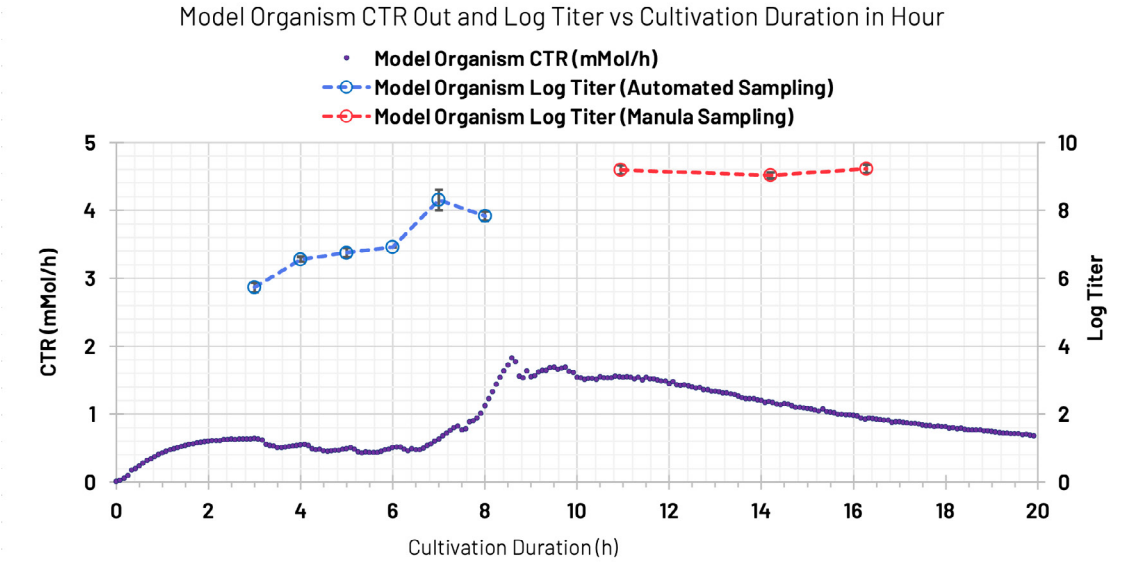

Figure 6: Log titer of model organism and Carbon dioxide transfer rate (CTR) vs. Cultivation duration in hours. The blue line represents the Log titer measured for the automated Numera® samples scheduled 3, 4, 5, 6, 7, and 8 hours post-inoculation, while the red line represents the log titer measured for the manually drawn samples taken approximately after 11, 14 and 16 hours post-inoculation. The CTR is based on in-line measurement.
Each error bar is constructed using 1 standard error from the mean.

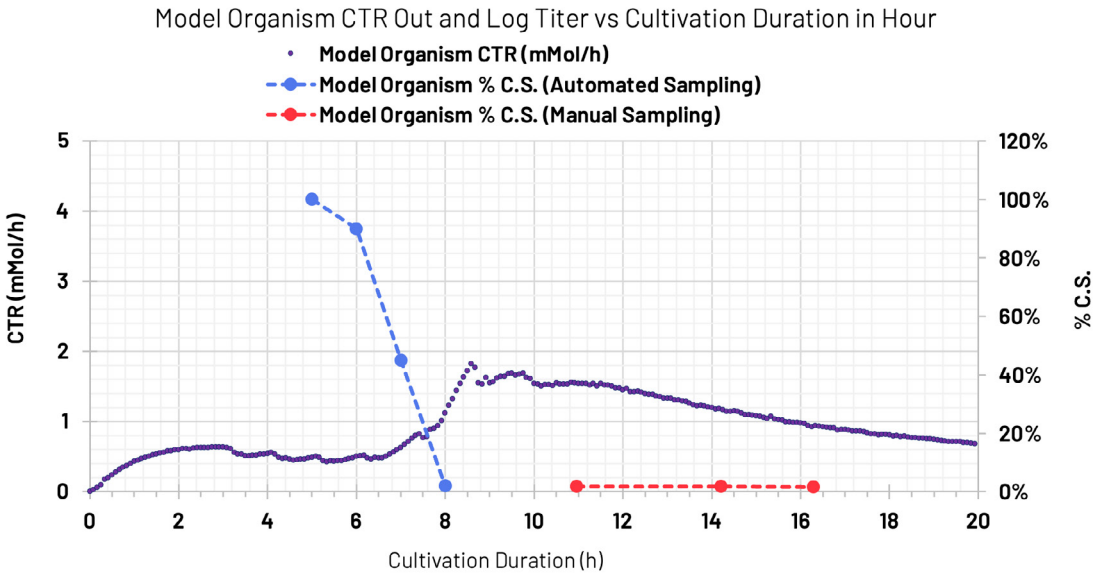

Figure 7: CTR and Relative Carbon Source Concentration (% C.S.) vs. Cultivation duration in hours. The % C.S. 5, 6, 7, and 8 hours after cultivation (automated Numera® sampling) and 11, 14 and 18 hours after cultivation (manual sampling). (Carbon source concentration is determined relative to time 0)

Conclusion

The work presented here confirmed that the Numera® system can be adapted to be a suitable tool for automated sampling of cultivations of obligate anaerobes, including accurate determination of viable titers for samples after a hold time of up to 3 hours in aerobic environment. During initial sample method development, comparable sample measurements to manual sampling were achieved with the combined effect of the use of nitrogen gas rather than air for system pneumatics, the use of an oxygen-scavenging substrate in collection vials for sample preservation, and cooling of collection vials to 4°C. While in general hold times of up to 6 hours for samples collected during the cultivation process were deemed to be suitable for the model obligate anaerobe tested here, optimal conditions may vary amongst strains and may need to be empirically determined. Larger differences in viability measurements were observed for exponential-phase cultures with subsequent narrowing of the gap as cultures progress to late exponential and stationary phase, and systematic differences between manual and automated samples may be observed depending on inherent strain characteristics including cell growth cycle. Further research is required to determine if these differences arise from differing oxygen sensitivity, metabolic activity, or some other physiological characteristic of these cells at each respective growth phase. The quality of data achieved in this study is suitable for many bioprocess research and development activities, including early medium development studies, optimization of fed-batch process control, or identification of growth-limiting nutrients. Further system configuration improvements, such as placing the Fraction Collection Module inside an oxygen-free isolator, may continue to improve sample preservation for oxygen-sensitive microbes. Configuration of the Numera® system with online measurements of viability could additionally reduce the impact of sample hold times.

While there are opportunities for continued research and improvements, the setup as described here enables automated sampling of obligate anaerobe cultivations and delivered comparable results for viability measurements in addition to preservation of samples suitable for measurement of substrate concentration and optical density. Importantly, the hold times are sufficient to allow extended times without operator involvement. When combined with other online measurements, like carbon dioxide production, data collected from these samples enable a consistent interpretation of the profile of the cultivation to enable further optimization or robustness analysis.

KEY RESULTS

- Oxygen exposure is often detrimental to human-commensal bacteria, including *Firmicutes*, leading to a 10,000-fold reduction in cell concentration after 20 minutes, under the tested condition
- The combined effects of N2 gas for pneumatics, pre-loading oxygen scavenger substrate solution addition into fraction collection vials, and holding vials at 4°C in the Fraction Collector Module enabled the stable hold of viable obligate anaerobe samples
- The stability of samples was determined to be maintained for up to 6 hours as measured by viable cell titer measurements
- Numera® allowed for reliable sampling during times without operator coverage. The combination of stable samples for viable cell number measurements, and use of clarified broth samples for measurement of residual substrates. In addition to online measurement of carbon dioxide evolution will enable robust investigation of culture performance and provide sufficient data for continued optimization and robustness assessments

References

- Andrade, J. C., Almeida, D., Domingos, M., Seabra, C. L., Machado, D., Freitas, A. C., & Gomes, A. M. (2020). Commensal Obligate Anaerobic Bacteria and Health: Production, Storage, and Delivery Strategies. *Frontiers in Bioengineering and Biotechnology, 8*. https://www.frontiersin.org/journals/bioengineering-and-biotechnology/articles/10.3389/fbioe.2020.00550
- Jimenez, M., Langer, R., & Traverso, G. (2019). Microbial therapeutics: New opportunities for drug delivery. *Journal of Experimental Medicine*, *216*(5), 1005–1009. https://doi.org/10.1084/jem.20190609
- McChalicher, C., & Aunins, J. (2022). Drugging the microbiome and bacterial live biotherapeutic consortium production. *Current Opinion in Biotechnology, 78*, 102801. https://doi.org/10.1016/j.copbio.2022.102801
- O'Toole, P. W., Marchesi, J. R., & Hill, C. (2017). Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nature Microbiology*, *2*(5), 17057. https://doi.org/10.1038/nmicrobiol.2017.57
- Rigottier-Gois, L. (2013). Dysbiosis in inflammatory bowel diseases: the oxygen hypothesis. *The ISME Journal*, *7*(7), 1256–1261. https://doi.org/10.1038/ismej.2013.80

SECURECELL AG

In der Luberzen 29
CH – 8902 Urdorf
+41 44 732 91 00
contact@securecell.ch