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Anaerobic Cultivation and Spore Formation of *Clostridium Sporogenes* Using Ambr® 250 Stirred Tank Vessels and Automation Capabilities

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Abstract

Clostridia are anaerobic bacteria that can form highly resistant endospores as a strategy of survival. These resistant endospores pose significant challenges in the food industry and are further of interest due to their role in the gut microbiome and their potential for cancer treatment. Here we describe the application of the Ambr® 250 Modular benchtop bioreactor system for the optimization of cell growth and spore formation of the anaerobe *Clostridium sporogenes*. We utilized the automation capabilities of the Ambr® 250 Modular to generate and maintain anaerobic growth conditions at an O_2 saturation below 1.5%. In this study, we evaluated pH setpoint values, continuous feeding strategies and stir speed. The output of these experiments led to high growth of *C. sporogenes* and consistent generation of spores, showing that the Ambr® 250 Modular is an efficient system for the cultivation of anaerobic bacteria.

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Introduction

Clostridium sporogenes ATCC 15579 is an obligate anaerobic, spore-forming and non-pathogenic bacterium [1]. Endospores that are formed by *C. sporogenes* are highly resistant to hostile conditions that would otherwise be lethal to the vegetative cells of the bacterium. The dormant spore form of the bacterium can survive chemical, physical and mechanical stresses. These include the presence of oxygen, low levels of nutrients, heat exposure, high pressure, irradiation and toxic chemicals [2]. The ability of the spore to persist in the environment is causing significant challenges for the effectiveness and safety of food processing steps as spores might germinate and grow out in food leading to spoilage and waste. Because *C. sporogenes* is closely related to *C. botulinum*, it is often used in research as a non-toxic surrogate for this common food-borne pathogen [3,4].

C. sporogenes ATCC 15579 has been initially isolated from human faeces [5] and forms a common gut bacterium. For this reason, its spores are also investigated towards their role in the microbiome and their influence on the gut-brain axis [6].

Furthermore, spores of the bacterium *C. sporogenes* are studied with regards to a new cancer treatment delivery system. This is due to the highly efficient tumour colonization and the significant oncolytic effects of the bacterium [7].

Clostridial spores are routinely produced in laboratories under strictly anaerobic conditions. However, due to the highly resistant characteristics of the endospores, production of spores might present some difficulties. These include a high contamination risk and the general availability of fermentation systems for real-time process monitoring under anaerobic conditions.

In this study, we show that the Ambr® 250 Modular is suitable for anaerobic growth of *C. sporogenes*. Furthermore, the real-time process monitoring and automation capabilities of the system allowed optimization of *C. sporogenes* growth towards a consistent and high yielding spore formation. In this study, we used the Whitley DG250, a standard anaerobic workstation, as a control system.

Figure 1. Phase-contrast image of purified *C. sporogenes* spores generated with the Ambr® 250 benchtop bioreactor system.

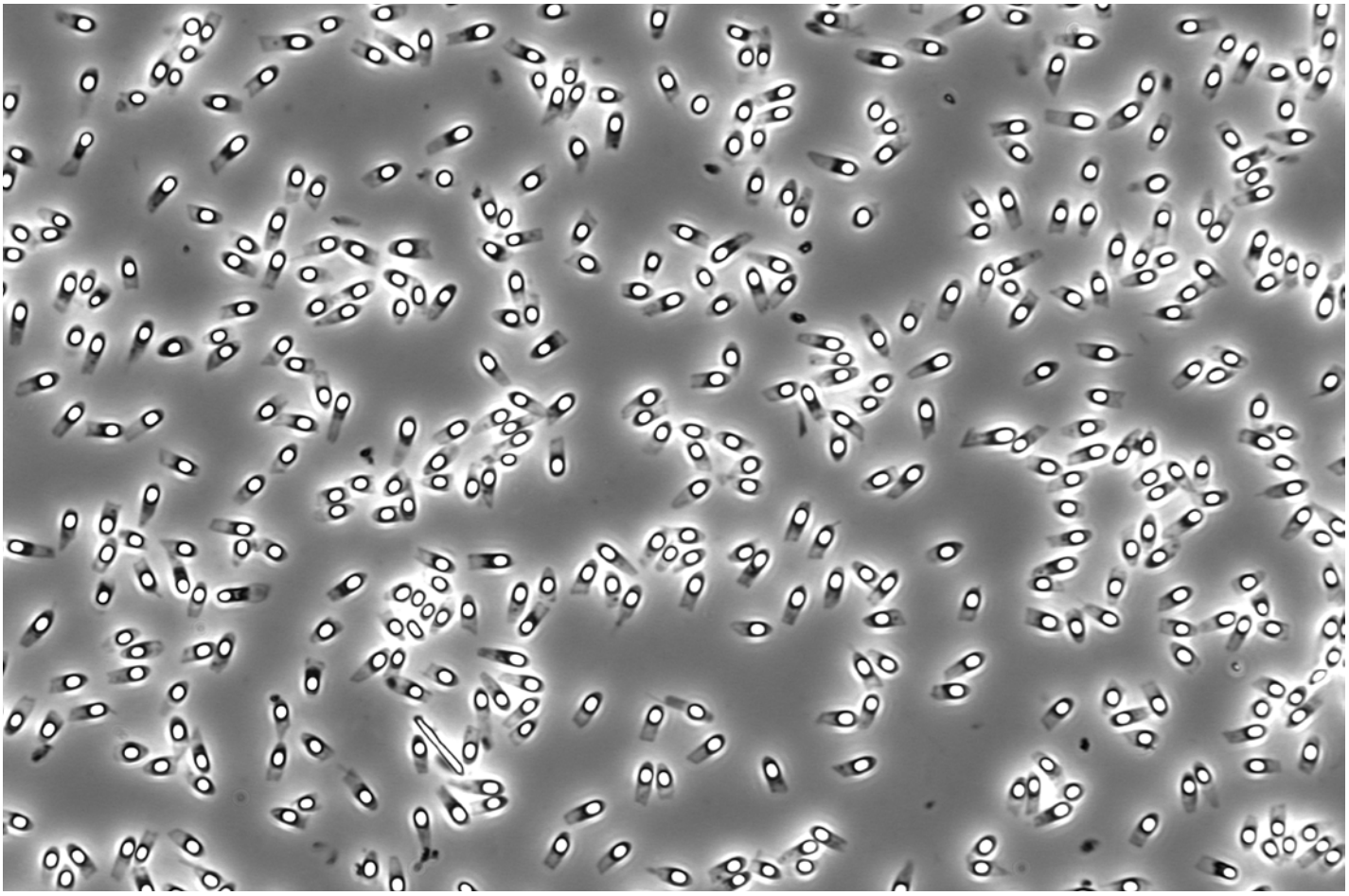


Figure 2. Ambr® 250 single-use bioreactor with integrated fluid supply system.

Materials and Methods

The Ambr® 250 Modular system was selected in this study to achieve real-time monitoring of the growth behaviour of *C. sporogenes,* and furthermore to evaluate the effect of pH, feed and stir speed on the sporulation of the bacterium. The Ambr® 250 Modular is a benchtop bioreactor system for parallel cell culture of up to eight single-use vessels with 100-250 mL working volume. Each single-use bioreactor has integrated sensors for temperature, pH and dissolved oxygen (dO_2), which allows real-time evaluation of these process parameters. An operator module allows the control and full automation of stir sped, feed addition and the above process parameters. Off-line cell culture measurements are made easily available via a Luer lock sampling port, reducing the risk of contamination.

Spore preparation

Cultures were started at 37°C in a Whitley DG250 Anaerobic Workstation under an anaerobic gas mixture of 80% nitrogen, 10% carbon dioxide and 10% hydrogen. To develop the inoculum, the anaerobic Modified Anellis broth [8,9] was inoculated with purified spores which had been stored in water at 4°C. These cultures were grown to an OD_{600} between 1.0 and 1.5 in 20 mL flasks, before being diluted with Modified Anellis broth to an OD_{600} of 0.5. The actively growing cells were aseptically added to the Ambr® 250 vessels via a syringe through a septum cap for a 10% v/v inoculum. Samples for external measurements were taken in defined intervals through the Luer lock connection port of the vessels. Apart from sampling for off-line measurements, the fermentation process required minimal operator interactions due to the automation capabilities of the Ambr® 250 system. Spore formation was evaluated by phase-contrast microscopy and by OD_{600} measurements. The purified spores were stored in water at 4°C until further use.

Set up of the Ambr® 250 for inoculation and cultivation of *C. sporogenes*

Modified Anellis broth was aseptically filled into the Ambr® 250 vessels under aerobic conditions und sparged with a gas mix of 90% nitrogen and 10% carbon dioxide at a flow rate of 40-60 mL/hr until a dO_2 of 2.5% had been reached. This gas mixture did not contain any hydrogen and, therefore, differed to the gas mixture used with the anaerobic workstation. In the workstation, the hydrogen gas mixture is circulated through a catalyst to remove oxygen by forming water. Since hydrogen is categorized as an extremely flammable gas and is not required with the Ambr® 250 system, it was omitted. Before inoculation, the gas flow was switched from sparger to headspace gas flow. During this step, the sparger gas flow rate was set to 0.1 mL/min to prevent backflow into the air filters. The headspace gassing was set under an automated control loop to maintain a dO_2 below 1% through a constant and automated adjustment of gas flow between 5 mL/hr and 60 mL/hr.

Results and Discussion

Evaluation of anaerobic media conditions under Ambr® 250 self-regulation

In this first step, the Ambr® 250 Modular system was evaluated for its capabilities to set and maintain anaerobic conditions. We assessed the suitability of the Ambr® 250 Modular for culturing *C. sporogenes*, an obligate anaerobe that can only tolerate an O_2 saturation of up to 3% [10]. For this study, we performed three consecutive runs with two bioreactor vessels in parallel.

Prior to inoculation, 180 mL of culture medium was filled into the bioreactor vessels and sparged with an anaerobic gas mix (90% N_2 and 10% CO_2) at a flow rate of 40 mL/min until the setpoint of dO_2 = 2.5% was reached. Following this initial drop in dO_2, the gas delivery was switched from sparge to headspace. This change in gas flow as well as flow rate adjustments of headspace gas to achieve and maintain a dO_2 below 1% was controlled through the automation capabilities of the Ambr® 250.

The adjustment of dO_2 within the initial 10 hours after process initiation is shown in Figure 3. The dO_2 was efficiently dropped below 2%, and thus below the O_2 saturation tolerated by *C. sporogenes*, within the initial 6 hours. This dO_2 adjustment is not adding any extra time to the cultivation process, as it is achieved simultaneously with the calibration of the pH probe that is integrated into the single-use vessel. The dO_2 was further decreased under headspace gasing to below 1.5% for the majority of the cultivation of *C. sporogenes* (data not shown). Due to the reliable automated adjustment and control of dO_2 by the Ambr® 250 system, the process of generating anaerobic conditions was simple and time-saving.

In the following experiments, pH adjustments and feed strategies were tested. The associated addition of acid, base or feed solution from the Ambr® 250 Modular reservoirs, which are set in an aerobic environment, had no impact on the maintenance of low dO_2 (data not shown), which showed the robustness of the Ambr® 250 benchtop system to control low oxygen concentrations.

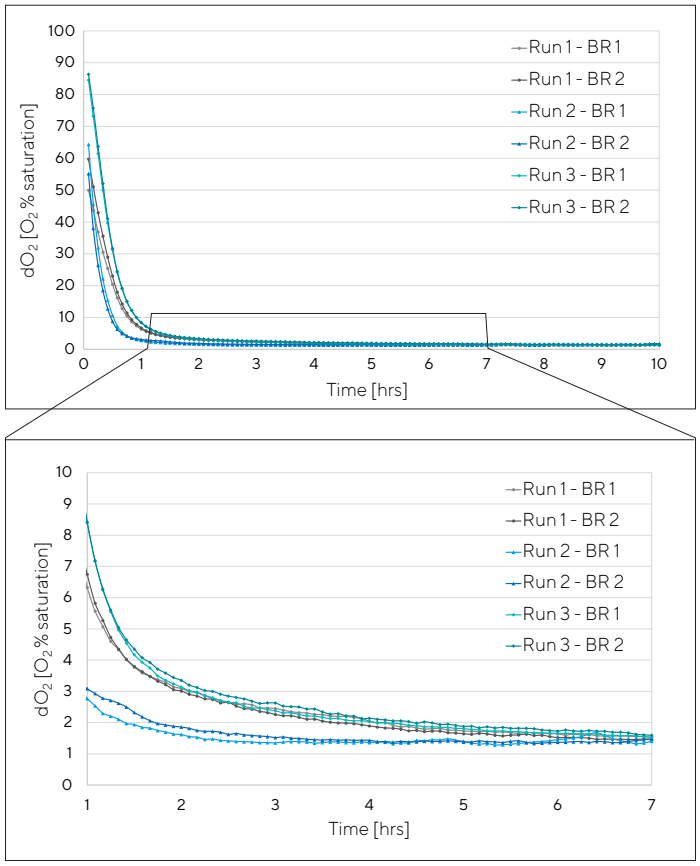


Figure 3. Monitoring of dO_2 values under Ambr® 250 self-regulation using two bioreactors (BR) simultaneously during three consecutive runs.

pH optimization and growth consistency under Ambr® 250 self-regulation

It was noted that during the growth of *C. sporogenes* in Modified Anellis broth (pH 6.3), the pH steadily increased, plateauing at a pH of 7.4 (data not shown). This observation led to the evaluation of the influence of a constant pH on growth and sporulation of *C. sporogenes*. Here, two bioreactor vessels were run in parallel with one bioreactor set to maintain a pH below 6.3 and the other bioreactor set to maintain a pH above 7.4, using the automation capabilities of the Ambr® 250. This automated self-regulation was achieved through control loops by the addition of NaOH or HCl at an adjustable flow rate of 0-10 mL per hour.

The impact on the growth curve is shown in Figure 4. A pH of 7.4 lead to an increased growth with a ~25% higher OD at the end of the culturing process. Phase contrast microscopy indicated that a pH of 7.4 is more beneficial for growth than a pH of 6.3 (see Figure 5). Growth and sporulation at pH 7.4 resulted in almost 3 times more purified spores than at pH 6.3. This experiment was run in duplicate and resulted in consistent and repeatable growth and sporulation behaviour of *C. sporogenes*.

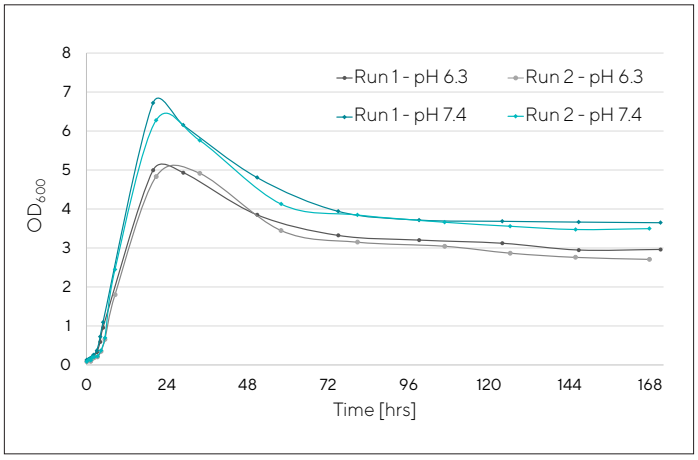
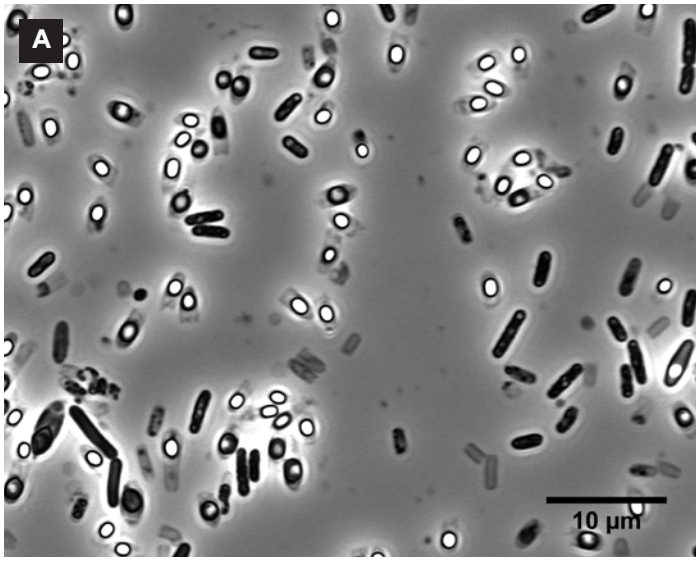
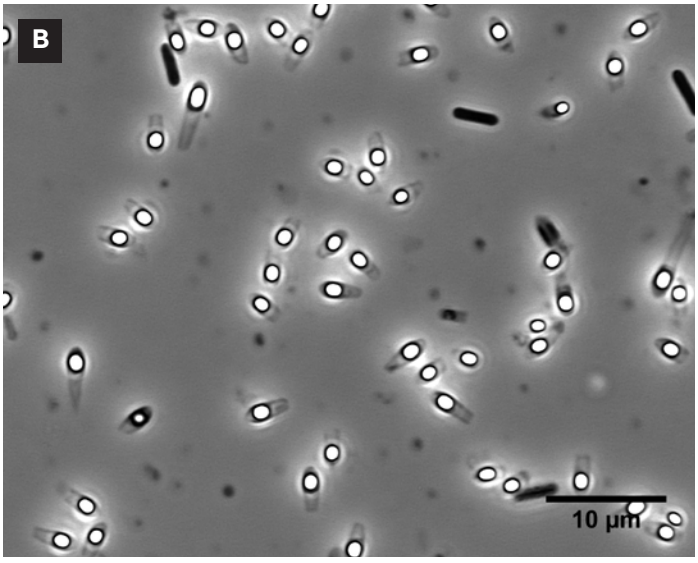


Figure 4. Growth curve of *C. sporogenes* simultaneously cultured at pH 6.3 and at pH 7.4 in two Ambr® 250 bioreactor vessel. The experiment was run in duplicate.

Figure 5. Phase contrast images of *C. sporogenes* 7 days into the cultivation process at a constant pH of (A) 6.3 and (B) 7.4. The pH of 6.3 lead to suboptimal growth and sporulation which can be noted in the shape of the vegetative cells and in the ratio of vegetative cells to spores in comparison to a pH of 7.4.





Feed optimization and stir speed evaluation using the Ambr® 250 self-regulation

This part of the study aimed to evaluate how continuous feeding and different stir speed rates effect the growth and spore formation of *C. sporogenes*.

The automation capabilities of the Ambr® 250 were used to set up a continuous feed which started 12 hrs after inoculation, adding 100 mL of medium at a rate of 8.3 mL/hr over 12 hrs. One bioreactor vessel was run at 150 rpm, whereas the second vessel was run at 300 rpm. The effect of feed addition and different stir speed on *C. sporogenes* growth is shown in Figure 6. The results indicate that additional feed leads to a prolonged growth and less steep OD decline, after peaking at around 21 hrs into the fermentation process. This influence of feed on the growth curve and the growth cycle of *C. sporogenes* resulted in a higher OD at the end of the culturing process than in previous runs without feed (compare Figure 4). However, due to a higher ratio of vegetative cells to spores at the end of the cultivation, the quantity of purified spores remained similar to that of culturing runs without feeding.

The stir speed was evaluated with regards to the impact of different agitation and shear forces on the growth behaviour of the bacterium. However, there were no significant differences noted between the stir speed of 150 rpm and 300 rpm. These results suggest that *C. sporogenes* can withstand a wide range of agitation. Further studies with different stir speed rates could be of interest.

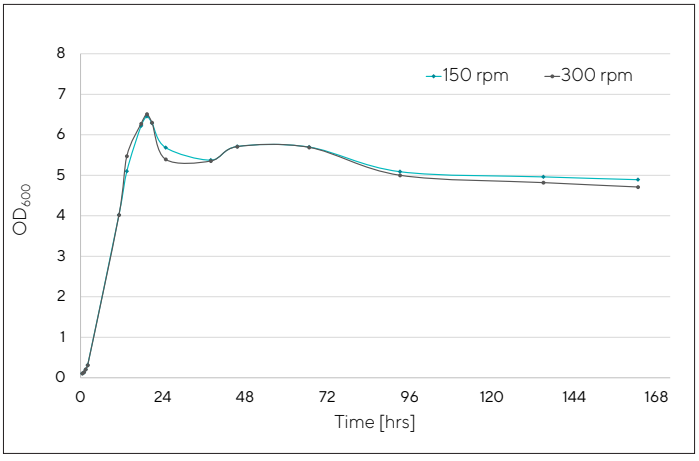


Figure 6. Growth curve of *C. sporogenes* cultured at pH 7.0 with continuous feeding and a stir speed of 150 rpm or 300 rpm.

Conclusion

This study shows that the Ambr® 250 benchtop bioreactor is capable to generate and maintain anaerobic conditions below 1.5 % and is therefore an efficient system for culturing anaerobic bacteria, such as *C. sporogenes*. The system presents efficient control of dO_2, pH, stir speed and feed as part of its automation features, enabling specific culturing strategies. These include pH adjustments and feed additions via high precision syringe pumps as well as gas flow strategies, through sparge or headspace gassing, as shown in this study.

The Ambr® 250 Modular which can run up to eight vessels in parallel poses an optimal system for growth behaviour studies and process optimization. The easy vessel "engage/disengage" functionality and the short amount of time needed to reach appropriate dO_2 levels, enable rapid turnover between experiments. Due to its single-use bioreactor vessels, the Ambr® 250 Modular does not require any sterilization processes between runs and efficiently prevents cross-contamination. In this study, these features significantly simplified the generation of anaerobic medium as well as the culturing process of *C. sporogenes*.

The Ambr® 250 automation furthermore enables standardization between runs, which resulted in repeatable growth behaviours of *C. sporogenes* and in spore batches of consistent quality and high quantity. The Ambr® 250 bioreactor vessels are furthermore fully scalable towards a range of larger stirred tank bioreactors such as the Biostat STR®.

For future work, it may be of interest to test different substrate media and stir speed rates to further determine process influence on spore morphology. Utilizing a design of experiments (DoE) software such as Modde®, would also enable a better understanding of how varying process parameters can impact on metabolic pathways and products. Further optimization enabled by the Ambr® 250 automation capabilities may lead to even higher growth and better standardization for spore formation.

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