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Microaerobic Fermentation of *Lactobacillus acidophilus* within Gut Microbiome Physiological Conditions by BioFlo® Bioprocess Control Stations

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Abstract

There is growing interest across the food and feed and biofuel industries in microaerobic fermentation, a process occurring at close to anaerobic conditions, but still requiring small amounts of oxygen. The dissolved oxygen level is quite low, often less than 5 %. There is much interest in production of probiotics, due to their great health benefits. Many probiotics are beneficial bacteria that naturally thrive in the human gut microbiome under microaerobic conditions. In the experiment described in this application note we successfully performed microaerobic fermentation of a probiotic strain, *Lactobacillus acidophilus*, at a very low oxygen level (< 1 %, representing the natural physiological condition of human gut microbiome) using BioFlo® 120 and 320 control stations. The microaerobic fermentation was carried out in BioBLU® 3f Macrosparge Single-Use Vessels. Through automatic gas mix and control, we demonstrated the feasibility of precisely controlling the dissolved oxygen (air saturation) at 4 % throughout the fermentation process using both the BioFlo 120 and 320 control stations. We showed robust growth of *Lactobacillus acidophilus*, which reached biomass concentrations of 3.13 x 10^9 CFU/mL and 3.73 x 10^9 CFU/mL using the BioFlo 120 and 320, respectively. This application note can serve as a good reference for probiotic and microbiome studies under microaerobic conditions.

Introduction

Lactic acid bacteria (LAB) have probiotic benefits and lactic acid production capability, which make them very important microorganisms [1]. *Lactobacillus*, a typical LAB, can be found in the oral cavities, gastrointestinal tracts, and vaginas of humans and animals [2]. *Lactobacillus* is a major genus in the human gastrointestinal microbiome with health-promoting benefits [2]. So LAB fermentation, though a relatively old technology, is facing brand new opportunities. *Lactobacillus acidophilus*, the LAB strain used in this study, can convert glucose to lactic acid through homolactic fermentation, and is widely used as a probiotic to boost the human microbiome [3].

Oxygen concentration plays a critical role in growing *Lactobacillus acidophilus*, as the strain is microaerophilic and even moderate oxygen concentrations can lead to a decrease in lactate production [4]. It is found that in the healthy mucosa of the small and large intestine, the PO_2 is lower than 10 mmHg (1.4 % O_2 concentration) [5]. Another study shows that the human luminal oxygen level is below 0.5 % [6]. However, currently there is a lack of studies on microaerobic fermentation, especially on how to keep the dissolved oxygen (DO) at a fixed level lower than 10 %. Therefore, the objectives of this study are: (1) to test the feasibility of growing *Lactobacillus acidophilus* at 4 % DO (i.e. 4 % air saturation), representing an environment of ~0.8 % oxygen level) through automatic gas mix and control by Eppendorf BioFlo

Bioprocess Control Stations 120 and 320; and (2) to demonstrate the suitability of BioBLU 3f Single-Use Vessel for microaerobic fermentation. This application note can serve as a good reference for probiotic and microbiome studies in both academia and industry.

Material and Methods

Bacterial strain

The bacterial strain *Lactobacillus acidophilus* (ATCC® 4356™) was purchased from ATCC. We resuspended the pellet in the ATCC vial using 1 mL sterile MRS broth (see below for medium preparation), then transferred the entire suspension to a 15 mL sterile Eppendorf conical tube containing 10 mL sterile MRS broth. The conical tube was loosely capped and put into an anaerobic jar (BD GasPak® 100 System, Becton Dickinson®, USA) with a fresh Thermo Scientific® Oxoid® AnaeroGen® 2.5 L sachet. We placed the jar in a New Brunswick™ Excella® E24 Incubator Shaker at 37 °C [7].

After 24-48 h incubation, we observed that the broth turned turbid and a pellet of microbial biomass settled at the bottom of the conical tube. We prepared ten 2 mL sterile cryogenic vials (BioCision, USA), transferred 1 mL of the suspended broth to each of the vials, and stored them in the -80 °C freezer (New Brunswick Innova U360, Eppendorf) for future use.

Growth media

We used both agar and broth media in this study. The agar medium was prepared by dissolving 70 g Lactobacilli MRS Agar (BD 288210) per 1 L DI water by boiling, then autoclaved at 121 °C. The broth medium was prepared by dissolving 55 g Lactobacilli MRS Broth (BD 288130) per 1 L DI water, then autoclaved at 121 °C.

Inoculum preparation

Every time for inoculum preparation, we took three 50 mL sterile Eppendorf conical tubes each containing 35 mL autoclaved MRS broth. We then removed one *Lactobacillus acidophilus* cryogenic vial from the freezer and thawed it at ambient temperature. We added 330 µL of the bacterial suspension from the vial to each of the three conical tubes, which were then loosely capped and transferred into the AnaeroGen sachet equipped anaerobic jar. After 48 hours incubation at 37 °C, we picked the one conical tube showing the densest precipitation of actively growing bacteria at the bottom of the tube and used it as the inoculum for bioreactor. Since the inoculation ratio was 1:100 (v/v) for a 3 L working volume, 30 mL of *Lactobacillus acidophilus* broth was needed.

Standard curve for CFU vs. OD_{600}

Forty-eight hours post inoculation, two of the tubes used for inoculum preparation were concentrated by removing approximately 30 mL supernatant without touching the cell pellets. The cell pellets were resuspended by vortex mixing and combined into one tube.

A serial dilution of the concentrated *Lactobacillus acidophilus* broth was then performed. The experimental procedures are illustrated in Fig. 1. We prepared and autoclaved 300 mL 0.1 % (w/v) peptone (Bacto® Proteose Peptone No. 3, BD Biosciences, USA) solution as the diluent. Meanwhile, 200 mL MRS agar solution was prepared, sterilized, and poured into eight Eppendorf cell culture dishes for cooling and solidifying before use. We took five 15 mL Eppendorf conical tubes, prefilled one with 6 mL concentrated *Lactobacillus acidophilus* broth and the remaining four with 3 mL peptone solution. We transferred 3 mL of the well suspended broth from the first tube to the second one with 3 mL peptone solution, thoroughly mixed, and transferred 3 mL mixture to the third tube. We repeated the same dilution procedure up to the fifth tube, which ended up with a 16-fold dilution. The broth suspension in these tubes was further diluted x10^6 times by transferred 20 µL of each to another tube containing 1,980 µL peptone solution to reach a 1:100 dilution, and repeated the dilution 3 times to reach a final 1:10^6 dilution.

Then from each of the conical tubes containing the final diluted samples, we loaded 100 µL of the broth onto the surface of the agar plate, and spread the sample evenly over the surface. For each dilution rate, we prepared two agar plates as duplicates. All agar plates were then put upside down (agar up) into the AnaeroGen sachet equipped anaerobic jar, and incubated at 37 °C for 48 hours when distinct colony forming units (CFU) became available. Based on the number of CFU (N) on each agar plate, the volumetric CFU can be calculated using the equation: colony-forming units per milliliter = N x 10^6 / 0.1 mL = N x 10^7 CFU/mL.

The four original *Lactobacillus acidophilus* broth samples before the 1:10^6 dilution were saved for optical density measurement at 600 nm (OD_{600}) using an Eppendorf BioSpectrometer®. The fresh MRS broth was used to set blank and as the diluent to keep the direct OD readings below 2.0. After data collection, we can correlate OD_{600} with volumetric CFU to create a standard curve for this specific *Lactobacillus acidophilus* strain.

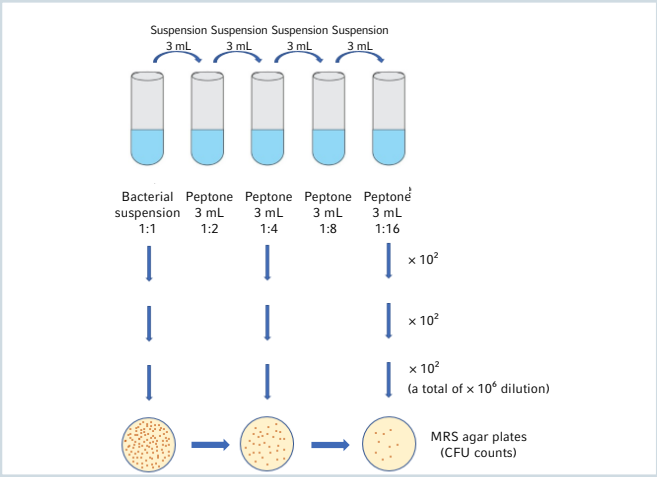

Fig. 1. Serial dilution of the *Lactobacillus acidophilus* suspension for CFU counting.

BioBLU 3f Single-Use Vessel and vessel setup

In this study, we performed all batch fermentations in Eppendorf BioBLU 3f Single-Use Vessels. The vessel has rigid walls with a working volume range of 1.25 – 3.75 L. It has a macrosparger and three Rushton-type impellers specifically designed for microbial applications.

All batch fermentations were carried out at a 3 L working volume. After filling the vessel with 3 L MRS broth as the medium, we added 1.8 mL Antifoam 204 (Sigma-Aldrich®, USA) to the medium to reach an antifoam concentration of 0.06 % (v/v).

We used a pH/Redox ISM® sensor for pH monitoring and an analog polarographic DO sensor for dissolved oxygen measurement (Mettler Toledo®, Switzerland). Both sensors are 12 mm in diameter with 225 mm insertion depth. The two sensors were installed on the head plate of the BioBLU 3f vessel through the two Pg 13.5 ports. A stainless-steel cooling finger was installed through a compression fitting, taking another Pg 13.5 port. We extended the liquid addition ports appropriately for connection with the external bottles later. We then autoclaved the medium-filled vessel, let it cool to 37 °C, and prepared for inoculation. The detailed setup of the vessel can be found in a previously published application note [8].

Process parameter setup in BioFlo 120 and 320

The fermentations were run under microaerobic conditions at 4 % DO. Most process parameters were set identically for the two control stations, the BioFlo 120 and 320, and they are listed in Table 1.

For the BioFlo 120, heating was controlled by an external heat blanket. On the DO control page, in order to maintain an extremely low DO level, which is not typical for fermentation, we changed the "Gains" section from fermentation "Ferm" to cell culture "CC" mode, and further adjusted the P (proportional) value from the default 5.00 to 3.00, and maintained the I (integral) value at 0.30. On the Sparge page, we selected the 4-gas mix option, set the total gas flow rate at 1 SLPM, and changed the O_2 and CO_2 high limit to 0.0 SLPM to prohibit the sparging of these two gases. Therefore, only air and nitrogen were used to control DO at 4 % during fermentation. The detailed DO setup is shown in Fig. 2. Fig. 3 displays an ongoing fermentation controlled by the BioFlo 120.

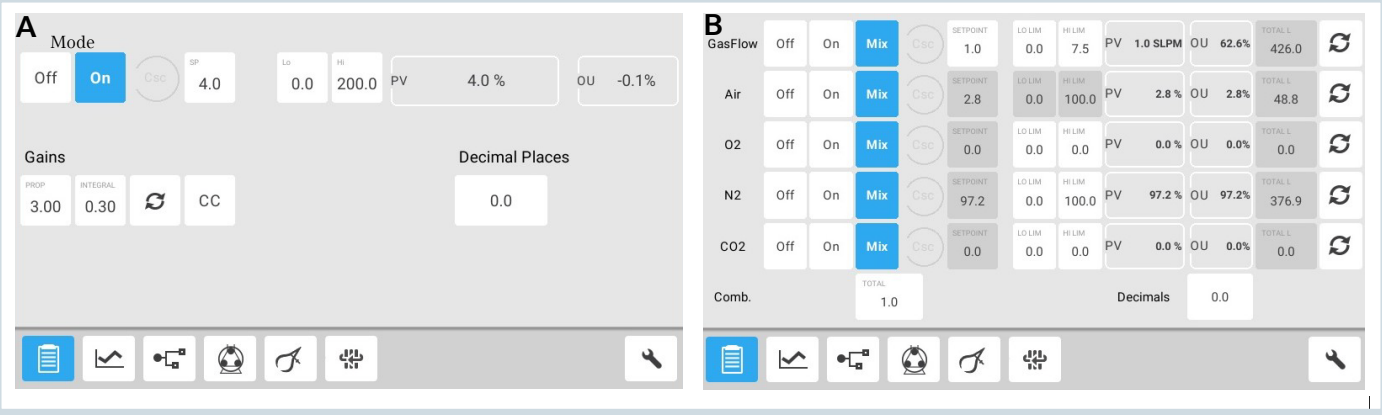

Fig. 2. DO (A) and sparge (B) parameter setup in BioFlo 120.

Fig. 3. *Lactobacillus acidophilus* fermentation in the BioBLU 3f Single-Use Vessel controlled by BioFlo 120.

For BioFlo 320, both cooling and heating were controlled by running water through the stainless-steel cooling finger. On the DO control page, the Gains section was adjusted in the same way as for the BioFlo 120. We further set the DO output between -100 % to 100 %, and built a customized DO cascade using air and nitrogen. In this cascade, the total gas flow was maintained at 1.0 SLPM, and depending on the real-time DO output, the composition of sparged gas was automatically adjusted (Fig. 4). The bioprocess controlled by the BioFlo 320 is illustrated in Fig. 5.

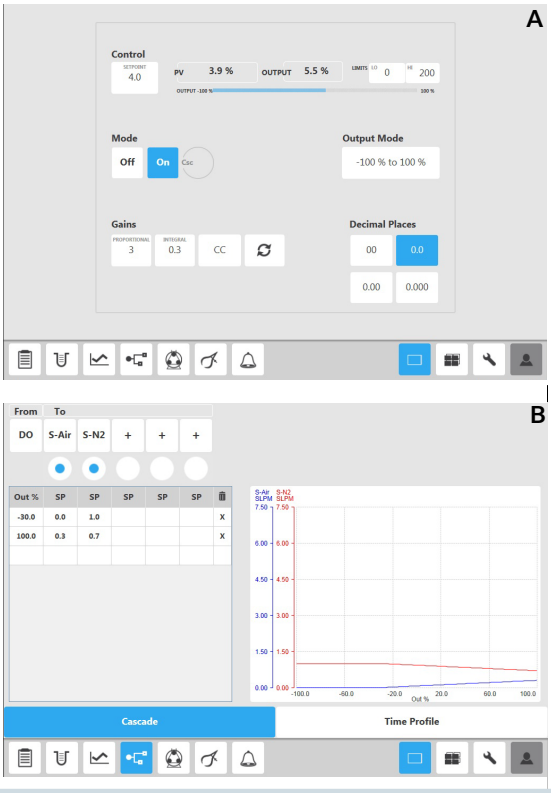

Fig. 4. DO (A) and sparge (B) parameter setup in BioFlo 320.

Table 1. Process parameters applied in this study.

Parameter	Configuration
Vessel	BioBLU 3f
Inoculation density	1 % (v/v), 30 mL inoculum to a 3 L working volume
Dissolved oxygen (DO)	4 % air saturation, ~ 0.8 % oxygen environment
Agitatinon	Magnetic drive, 150 rpm
Gassing	Automatic gas flow and mix
Temperature	37 °C, cooling controlled by a single stainless-steel cooling finger
pH	6.5 ± 0.1 for the first 4 hours, then 5.0 ± 0.1 for the remaining of the culture, controlled by external 4 mol/L sterile sodium hydroxide solution and 2 mol/L sterile hydrochloric acid solution
Impeller	Three Rushton impellers
Sparger	Macrosparger

Sensor calibration

We calibrated the pH sensor outside of the vessel before sterilization. We followed the 2-point calibration method by setting ZERO using buffer at pH = 7 and setting SPAN using the buffer at pH = 4.

We calibrated the pre-polarized DO sensor after autoclave with the sterile MRS broth in the vessel. It is recommended to calibrate DO sensors under the same conditions as the real fermentation. Therefore, we adjusted the pH to 6.5 if needed, set the agitation at 150 rpm, and set the temperature at 37 °C. A 2-point calibration method was also applied here. We sparged pure nitrogen at 1 SLPM until the DO value stabilized to set ZERO at 0 %; then switched the gas supply to air under the same flow rate, waited till the DO value stabilized again, to set SPAN at 100 %. Because we intended to keep the DO value at 4 % (which is very close to 0 %) in this microaerobic fermentation, DO calibration at ZERO was very critical.

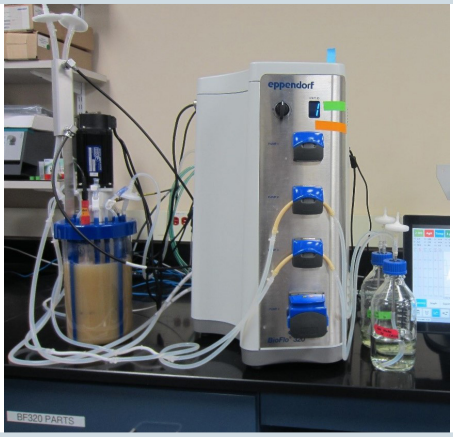

Fig. 5. *Lactobacillus acidophilus* fermentation in the BioBLU 3f Single-Use Vessel controlled by BioFlo 320.

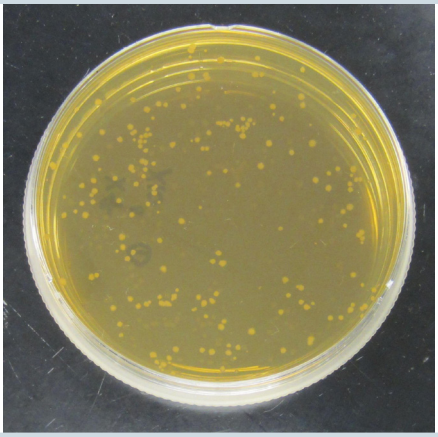

Fig. 6. Colony forming units on MRS agar plate after 48 h incubation.

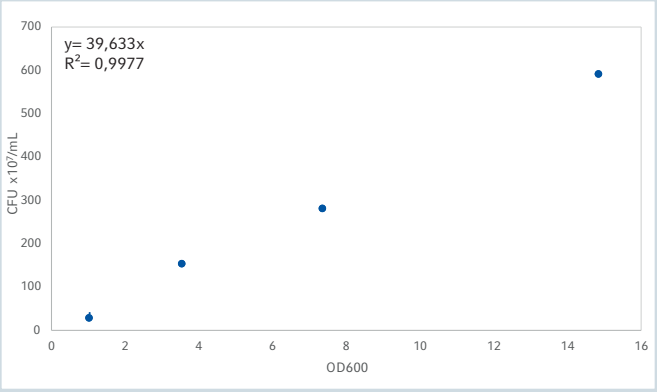

Fig. 7. Standard curve correlating OD_{600} and CFU of *Lactobacillus acidophilus.*

Optical density measurement

Upon completion of DO calibration and right before inoculation, we took a sample of 20 mL fresh MRS broth from the vessel, and used 1 mL of this medium to set blank for optical density measurement at 600 nm. The remaining volume was saved as the diluent for fermentation broth during the run. We took samples for optical density measurement at seven time points: 0, 4, 8, 12, 14, 24, and 30 h after inoculation.

Results

We ran microaerobic *Lactobacillus acidophilus* fermentation twice at a DO level of 4 % in BioBLU 3f vessels, one controlled by the BioFlo 120 and the other by the BioFlo 320. We tracked the real-time DO throughout the run and took intermittent samples for optical density measurement to evaluate the bacterial growth. Finally, based on the linear correlation between optical density and volumetric CFU, we calculated the CFU in each fermentation and estimated the probiotic production potential.

Standard curve for CFU vs. OD_{600}

After 48 hours of incubation in the anaerobic jar, the distinct colony forming units (CFU) were available, as shown in Fig. 6. Based on the number of CFU (N) on each agar plate, we averaged the counts on the duplicated plates for each dilution, and correlated them with the respective OD_{600} readings to create a standard curve (Fig. 7). The correlation is: CFU/mL = 39.63 x OD_{600} x 10^7.

Performance of DO control by BioFlo 120 and 320

As described previously, the DO was set at 4 % for both runs, but the control was performed differently by the two control stations. We set the sample rate at every 5 seconds for data collection. Here, in Figures 8 and 9, the trends are displayed for the first 24 hours after inoculation, during which the pH was adjusted from 6.5 to 5.0 at t = 4 h. Except for one pH adjustment when acid was called for, base was needed throughout the fermentation to neutralize the lactic acid continuously produced by *Lactobacillus acidophilus*.

As we can see from the trends, in the BioFlo 120 DO stayed at the baseline of 4 % for most of the time, but there were a few intermittent sharp peaks due to the overshoot of air (Fig. 8). In the BioFlo 320, DO was much more stably con-

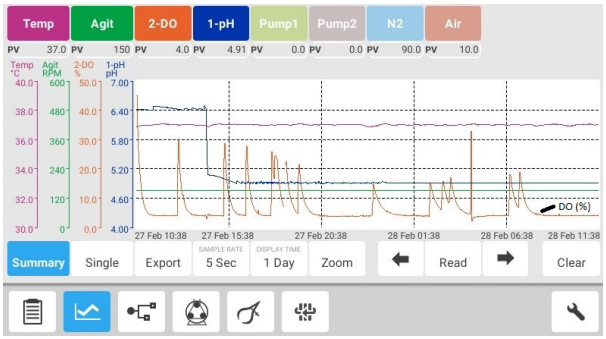

Fig. 8. Trends shown on BioFlo 120 when growing *Lactobacillus acidophilus.*

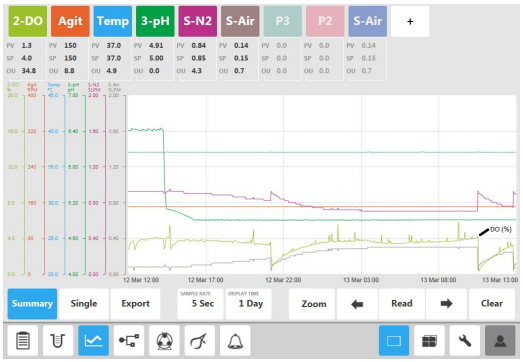

Fig. 9. Trends shown on BioFlo 320 when growing *Lactobacillus acidophilus.*

trolled at 4 % (Fig. 9).

It is more challenging to maintain DO at a very low level (4 %) using the Bioflo 120 during microaerobic fermentation. In order to achieve it, two "contradictory" gases, air and nitrogen, are needed simultaneously. For both control stations, automatic gas flow and mix with a total gas flow of 1 SLPM (0.33 VVM) was applied. For the BioFlo 120, the sparge gas module has 1 TMFC (thermal mass flow controller), which has 4 solenoid valves but allows only one gas to flow through at any given time without pre-mixing. When we chose the 4-gas mix option and further shut off carbon dioxide and oxygen, only nitrogen and air could be called during the process, and the duration of sparging for each gas was dependent on the real-time DO output. The random peaks in the DO trend are due to the overshoot of air at certain times during fermentation. This occurred because the PID (proportional-integral-derivative) algorithm modulates the combined impact of control through a single TMFC of nitrogen and air. The combined PID control is more accurate for nitrogen. Since the DO was very low, nitrogen was called most of the time. When a long period of nitrogen sparging was immediately followed by a switch from nitrogen to air, an overshoot of air could possibly take place. For BioFlo 320, the sparge gas module has 4 TMFCs, meaning that all 4 gases can be called simultaneously at different flow rates, and allowing nitrogen and oxygen to each have its own independent PID control. The DO cascade we built guaranteed a minimum nitrogen flow rate at 0.7 SLPM throughout the fermentation, along with independent PID, greatly reducing the risk of air overshoot for a smoother DO trend at 4 %.

Bacterial growth comparison

Based on the OD_{600} we collected and the conversion between optical density and volumetric CFU, a growth curve was drawn for both fermentations using BioFlo 120 and 320 (Fig. 10). As we can see from the figure, a distinct exponential growth phase was observed for both runs between 8 and 12 hours and the maximum OD_{600} and CFU occurred at t = 14 h. For the BioFlo 120 and 320 cultures, maximum OD_{600} reached 7.9 and 9.4, and maximum biomass was 3.13 x 10^9 CFU/mL and 3.73 x 10^9 CFU/mL, respectively.

Considering the probiotics available on the market, which usually include between 20 and 40 billion CFU per capsule, if we assume 30 billion *Lactobacillus* CFU per capsule and *Lactobacillus acidophilus* is the only strain, based on the maximum biomass obtained we produced 313 capsules per BioBLU 3f vessel controlled by BioFlo 120 and 373 capsules with the BioFlo 320.

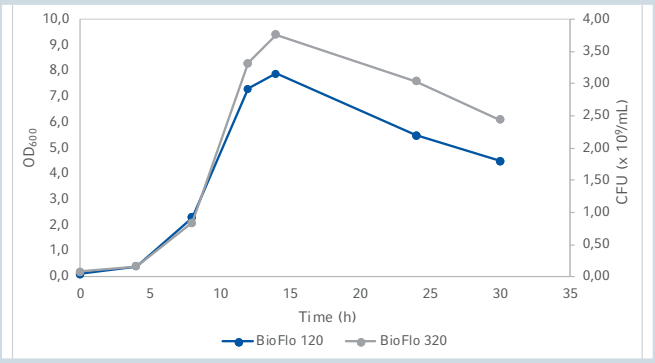

Fig. 10. Growth curves of *Lactobacillus acidophilus.*

Conclusion

In this study, we demonstrated the feasibility of precisely controlling the dissolved oxygen level at 4 % which is equivalent to an oxygen concentration of 0.8 %, representing the physiological conditions of the gut microbiome. Microaerobic fermentation was carried out in BioBLU 3f Single-Use Vessels to grow a probiotic LAB strain, *Lactobacillus acidophilus*, controlled by Eppendorf BioFlo 120 and 320 bioprocess control stations; we showed robust growth under each control station, which produced 3.13 x 10^9 CFU/mL and 3.73 x 10^9 CFU/mL probiotic *Lactobacillus*, respectively.

With a rapid growth of the probiotic and microbiome markets, better control of the microaerobic bioprocess is critical and urgent. This application note shows a detailed example of precisely controlling the DO at 4 % to support microaerobic fermentation, and will be very helpful for peers in the field of microbiome research to apply to their specific bioprocesses.

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Ordering information

Description	Order no.
BioFlo® 120	
Advanced control station bundle	B120ACS000
BioFlo® 320	
Base control station.	1379963011
BioBLU® f Single-Use Vessels	
BIOBLU® 3 f	1386000900
System Accessories	
Heat blanket adapter for BioBLU®	1386811900
Heat Blanket, for DASGIP® vessel 0.5 – 3L, with Pt100, 95 x 260 mm, 100 W, 230 VAC, CE/UL certified	78525275
Cooling Finger, stainless steel, complete, O.D. 12 mm, L 295 mm, Li 240 mm	77102037
pH/Redox sensor, Mettler Toldedo®, InPro 3253i, ISM®	P0720-6657
DO sensor, Mettler Toldedo® InPro 6820, 225 mm, straight T-82 connector	P0720-6526

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