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Characterization of a *Bacillus amyloliquefaciens* fermentation for a fed-batch strategy using the Bionet bBreath exhaust gas analyzer

ABSTRACT

For aerobic fermentation processes, oxygen and carbon dioxide off–gas measurement allows a better characterization and a tool for process optimization, enabling new methods for the control process. In the present work, a fermentation process in fed-batch mode at bench–top scale for the production of the bacteria *Bacillus amyloliquefaciens* was performed using a Bionet F0–BABY and the exhaust gas analyzer bBreath to obtain valuable information of the culture.

1 INTRODUCTION

Bacillus amyloliquefaciens is a widely used bacteria for the production of various products, such as industrial enzymes, biostimulants for plants, or biopesticides, among others.
Online measurement of the respiratory activity of microbial cultures in fermenters with exhaust gas analysis is a powerful tool to control and optimize the process. It includes measurements of oxygen uptake rate (OUR), carbon dioxide evolution rate (CER), and respiratory quotient (RQ) of the fermentation process.
Herein, we describe the characterization of a fermentation process at a bench-top scale using Bionet's exhaust gas analyzer (bBreath) coupled to Bionet's F0-BABY lab scale fermenter.

KEYWORDS

Bacillus amyloliquefaciens, fermenter, fed-batch, exhaust gas, OUR, CER, RQ

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2 MATERIALS AND METHODS

Media and solutions

- Inoculum: NB Medium 8 g·L^{-1} Nutrient Broth.
- The media composition for the Bioreactor (Production Media, PM) was 10 g·L^{-1} glucose, 1.7 g·L^{-1} KH_2PO_4, 4.53 g·L^{-1} K_2HPO_4, 5.88 g·L^{-1} $(NH_4)_2SO_4$, 4.53 g·L^{-1} Yeast Extract, and 100 g·L^{-1} Stock 10X Salts Solution.
- The 10X Salts Solution was 2.4 g·L^{-1} $MgSO_4{\cdot}7H_2O$, 0.04 g·L^{-1} $MnSO_4{\cdot}4H_2O$, 0.13 g·L^{-1} $FeCl_2$, 0.14 g·L^{-1} $ZnCl_2$, 0.88 g·L^{-1} $CaCl_2{\cdot}6H2O$.

Cell propagation

For the preparation of the inoculums for the bioreactor, 1-mL long-term stock vials of *B. amyloliquefaciens* spores from the Working Cell Bank were seeded in 100 mL of Nutrient Broth medium, grown for 12 h in a shaker at 30 °C and 250 rpm. The cell cultures were used to seed a Bionet F0-3MB bioreactor with an initial working volume of 2-L according to an inoculum relation of 5 % v/v.

Fermenter

Cells from propagation were seeded into a 3-L working volume Bionet's F0-BABY bioreactor containing 2-L of the PM media (Fig. 1), and connected to the Bionet's exhaust gas analyzer bBreath (Fig. 2), and ROSITA® SW. The main specifications of the bioreactor are shown in table 1, and table 2 summarizes the culture conditions.

Table 1: Bionet's F0-BABY fermenter configuration

ELEMENT	DESCRIPTION	
Application	Microbial (MB)	
Model	Bionet F0-BABY 3-MB; non-jacketed glass vessel. Geometry H:D 3:1	
Cooling water (Chiller)	Cool water supply @ 10°C. Min/Max Pressure: 0.4/2 barg. Min water flow: 3 L/min	
Bioreactor working volume	3 L	
Gas supply	Air @ 2 bar	Flow range 0.2-10 slpm
	O2 @ 2 bar	Flow range 0.1-5 slpm
Sparger	Ring-sparger (pore Ø 1mm)	
Agitation	80–1800 rpm	
Impeller	Rushton type turbines; 2 units. Ratio d/D=0.4	
External variable speed pump	30 rpm	
Accessories	Condenser at exhaust gas	
On-line sensors	pH, DO_2, and Optical Density	
External modules	Bionet bBreath (Exhaust Gas Analyzer)	

Table 2: Process Parameters

PARAMETER	SP VALUE
Working Volume	2 L
pH	6.8±0.2
Temp	30±0.1°C
DO2 (cascade)	30±1% controlled by automatic regulation of: • Position 1. Agitation: 100-1200 rpm • Position 2. Air: 1-3 slpm
Time (h)	36

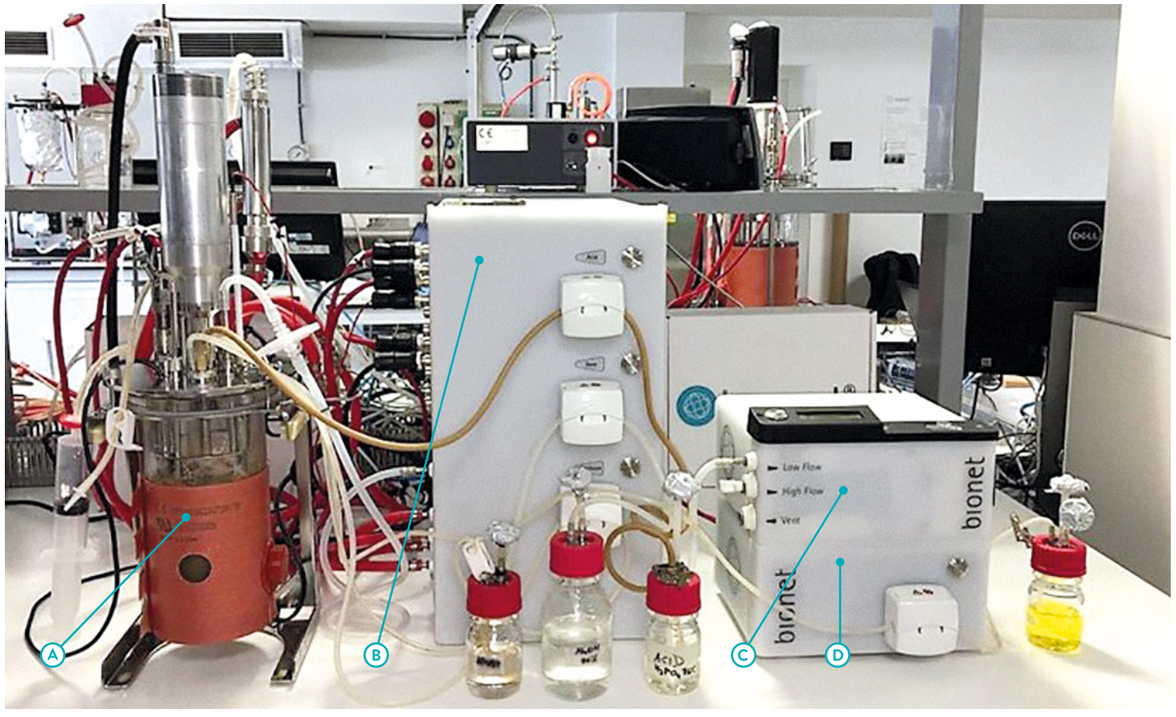

Fig.1: Bionet's F0-BABY Fermenter. Set-up for a fed-batch process with (A) 3L Non-jacketed fermenter, (B) bioreactor control unit (BCU), (C) the bBreath exhaust gas analyzer, (D) external variable speed pump, and monitoring with ROSITA® SW.

Off-Gas analysis

Bionet's bBreath (Fig. 2) was used to analyse the composition of the oxygen and carbon dioxide gases in the exhaust gas from the fermentation process, and to calculate the consumption of oxygen (OUR), the production of carbon dioxide (CER), and the respiratory quotient (RQ) as the ratio of formed CO_2 to consumed O_2.

Fig.2: Bionet's bBreath for exhaust gas analysis.

The formulas for the online calculation were:

- Oxygen Uptake Rate (OUR) in mol/l·h

$$OUR = \frac{Fgas \cdot P}{Vf \cdot R \cdot T} \cdot \left(O2\%in - \frac{1 - O2\%in - CO2\%in}{1 - O2\%out - CO2\%out} \cdot O2\%out\right)$$

- Carbon Dioxide Evolution Rate (CER) in mol/l·h

$$CER = \frac{Fgas \cdot P}{Vf \cdot R \cdot T} \cdot \left(\frac{1 - O2\%in - CO2\%in}{1 - O2\%out - CO2\%out} \cdot CO2\%out - CO2\%in\right)$$

- Respiratory Quotient (RQ):

$$RQ = \frac{CER}{OUR}$$

Where:

- **Fgas** — The inlet gas flow rate in L/h
- **P** — Normal pressure 1.0133 bar
- **Vf** — Working volume in the bioreactor/fermenter in litters (L)
- **R** — Gases constant 8,314.10^{-2} bar·L/K·mol
- **T** — Temperature 273.15 K
- **O_2%$_{in}$** — Oxygen composition in the inlet gas 20.9%
- **CO_2%$_{in}$** — Carbon dioxide composition in the inlet gas 0.05%
- **O_2%$_{out}$** — Oxygen composition in the outlet gas (bBreath)
- **CO_2%$_{out}$** — Carbon dioxide composition in the outlet gas (bBreath)

3 RESULTS

Fermentation

Variations in the O_2 and CO_2 composition in the exhaust gas were monitored on-line with ROSITA SW throughout the fermentation. From the graphics, it was notable that the growth curve, the dissolved oxygen consumption according to the cascade control, and the CO_2 percent variation, are related and the different phases of the growth can be identified (Fig. 3). During the exponential phase in the beginning, values of CO_2 in the exhaust gas of up to 4% were reached, followed by a decrease after complete depletion of the limiting substrate, and finally an increase again when the fed-batch initiated the addition of substrate, maintaining CO_2 levels of 2.5 ± 0.5% at the outlet, and an optimal RQ of around 1 with the regulation of the gas flow rate in the inlet.

For aerobic microorganisms that metabolize glucose, for example, six molecules of O_2 are consumed and six molecules of CO_2 are formed during respiration, therefore, maintaining the RQ at around 1, being an optimal indicator of harmonization of the gas flow rate supplied with O_2 consumption and CO_2 evolution rate. Once the growth in the vegetative stage finished, during the stationary phase the CO_2 levels were maintained at around 1.5%, decreasing down to 0.07 ± 0.01% in the spore-forming phase, coinciding with a decrease in oxygen demand (Fig.3).

Fig. 3: The graphic in ROSITA SW from the fed-batch process with *B. amyloliquefaciens*. Note, that the dark-blue line is the dissolved oxygen controlled with the cascade control, the purple line is de CO2 in the exhaust gas, the red line is the RQ, where the relation of the different stages of the growth can be identified.

Monitoring the CER, cell growth was maintained with the addition of substrate, with the objective of maintaining a continuous state of high CO2 emission as a sign of active cellular metabolism related to the presence of substrate. The result was an increase of 1 log in the cellular concentration in the fed-batch mode, comparing to a batch mode (Figures 4, 5, and 6) where the culture was supplemented during 3 hours with a concentrated solution of the limiting substrate after 8 hours from the beginning.

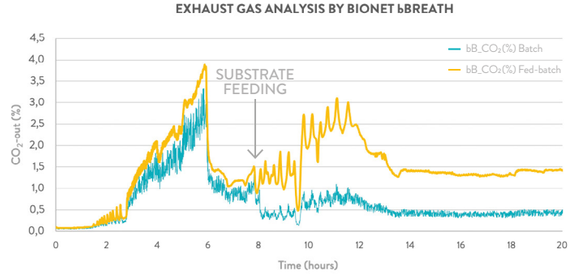

Fig. 4: Graphic comparing the batch and the fed-batch mode process with *B. amyloliquefaciens*, according to the CO_2 percent at the exhaust gas. The arrow shows the point at the fed-batch mode when the feeding of limiting substrate was initiated.

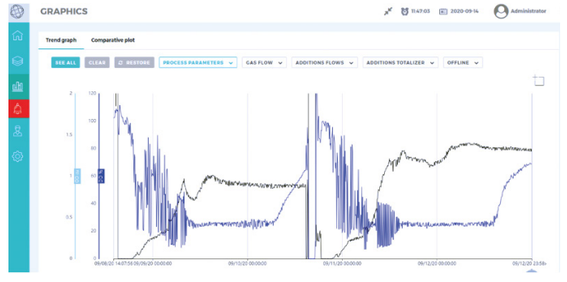

Fig. 5: Graphic comparing the batch and the fed-batch mode process with *B. amyloliquefaciens*, according to the dissolved oxygen (blue line), and optical density (black line).

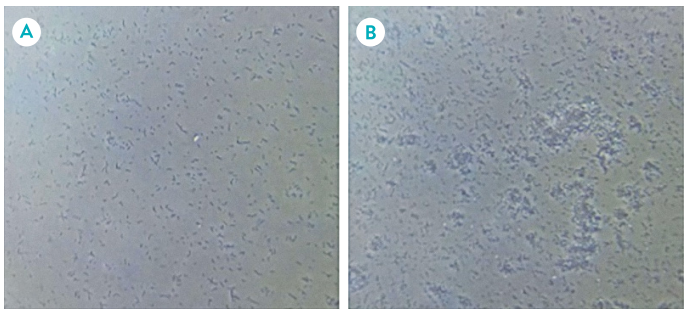

Fig. 6: Samples from the microscope at 40X comparing the production of *B. amyloliquefaciens* in (A) batch mode, and (B) fed-batch mode.

With the information provided with Bionet's bBreath exhaust gas analyzer, besides the optimization of the process under fed-batch strategies, or even continuous fermentation processes to maintain the growth according to the cellular metabolism with the addition of substrate, can be used for scale-up process analysis of the oxygen requirements of the microorganism for aerobic processes, where with the oxygen uptake rate (OUR), we can obtain valuable information to determine the oxygen transfer rate (OTR) requirements for our bioreactor, depending on the media and process conditions, to cover the OUR (Figure 7).

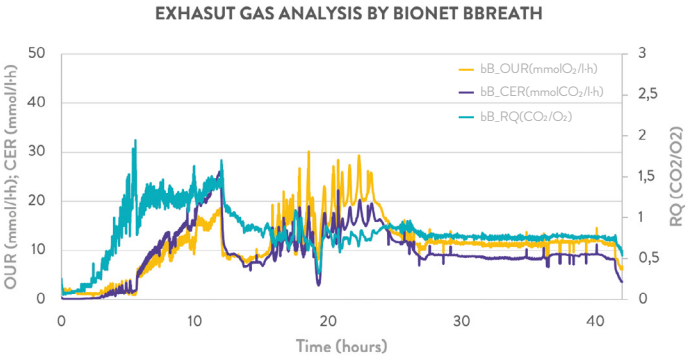

Fig. 7: Exhaust Gas Analysis from the fed-batch process with *B amyloliquefaciens* using the Bionet's bBreath.

The variation of the dissolved O_2 concentration during the time in a fermentation process can be calculated and correlated with the OTR and OUR as follows:

$$\frac{dC \cdot O2}{dt} = OTR - OUR$$

Where:

OTR = KLa · (C^*_{O2} − C_{O2})

- **KLa** — Volumetric oxygen transfer coefficient (h−1)
- **C^*_{O2}** — Saturated dissolved O2 concentration, maximum O_2 carrying capacity of the liquid in mol/L
- **C_{O2}** — Current dissolved O2 concentration in the liquid in mol/L

OUR = qO2 · X

- **qO2** — specific oxygen uptake rate in mol/g·cell·h
- **X** — Biomass concentration in g·cell/L

On the other hand, the biomass formation can be related to the respiration according to the OUR and CER, which is why the CO_2 variation can be directly linked to the biomass formation and the specific growth rate (Figure 8). At the first stage of the culture, corresponding to the first log phase, the trend of increasing CO_2 composition and biomass formation was similar during the first 5 hours of culture. In the second log phase when the substrate addition started, both parameters increased during the time the addition was active, the start of the decrease the CO_2 composition at the exhaust gas being a signal of decrease in the growth after 12 hours of culture. The optical density remained with hardly any variations, as an indicator of constant biomass concentration, and the CO_2 composition varied, remaining constant during the stationary phase and decreasing significantly during the sporulation phase. The possibility to apply the exhaust gas variation to the biomass formation can be an optimal solution for cultures where the measurement of the turbidity is not possible, such as in fungal or pellet forming cultures, or even can be an alternative or complementing tool for on-line optical density probes. This application has been done with a microbial process. In the case of mammalian cells or cell culture the addition of CO_2 to control the pH must be taken into account for the CER.

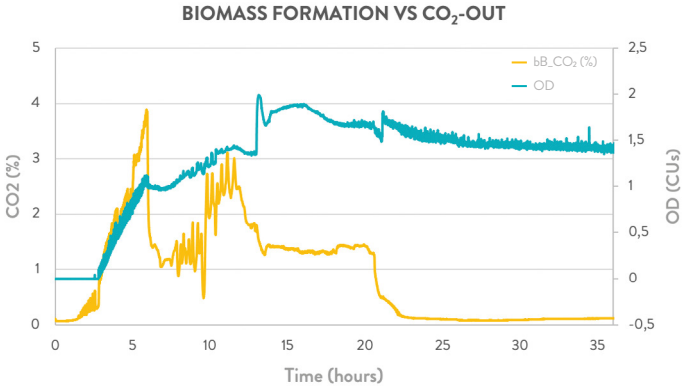

Fig. 8: Biomass formation and CO_2 emission from the fed-batch process with *B. amyloliquefaciens* using the Bionet's bBreath and integrated optical density (OD) on-line from ROSITA® SW.

4 CONCLUSIONS

Off-gas analysis using an exhaust gas analyzer, such as Bionet's bBreath to monitor a fermentation process continuously as a direct measurement of the health, biomass formation, and the different phases of the culture, is a powerful tool for the control and optimization of processes, allowing the obtaining of valuable information and a better knowledge of the cellular metabolism and oxygen requirements of our microorganism, in addition to facilitating the implementation of feeding strategies optimally.