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.

**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
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, 1.7g·L⁻¹ KH₂PO₄, 4.53 g·L⁻¹ K₂HPO₄, 5.88 g·L⁻¹ (NH₄)₂SO₄, 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 MgSO₄·7H₂O, 0.04 g·L⁻¹ MnSO₄·4H₂O, 0.13 g·L⁻¹ FeCl₃·6H₂O, 0.14 g·L⁻¹ ZnCl₂, 0.88 g·L⁻¹ CaCl₂·6H₂O.

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Cell propagation
For the preparation of the inocula for the bioreactor, 1-mL long-term stock vials of B. amyloliquefaciens spores were inoculated by shaking at 30 ºC and 250 rpm. The Working Cell Bank were seeded in 100 mL of Nutrient Broth.

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3 RESULTS

Fermentation
Variations in the O₂ and CO₂ 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 CO₂ 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 CO₂ 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 CO₂ levels of 2.5 ± 0.3% 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 O₂ are consumed and six molecules of CO₂ are formed during respiration, therefore, maintaining the RQ at around 1, being an optimal indicator of harmonization of the gas flow rate supplied with O₂ consumption and CO₂ evolution rate. Once the growth in the vegetative stage finished, during the stationary phase the CO₂ 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).

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 1log 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.

The formulas for the online calculation were:
- Oxygen Uptake Rate (OUR) in mol/l·h
  \[ OUR = \frac{F_{gas} \cdot P}{V_f \cdot T} \]
  - CO₂ Evolution Rate (CER) in mol/l·h
  \[ CER = \frac{F_{gas} \cdot P}{V_f \cdot T} \]
  - Respiratory Quotient (RQ):
  \[ RQ = \frac{CER}{OUR} \]

Where:
- \( F_{gas} \) = The inlet gas flow rate in L/h
- \( P \) = Normal pressure 1.013 bar
- \( V_f \) = Working volume of the bioreactor/Fermenter in liters (L)
- \( T \) = Temperature 273.15 K
- \( Q_{O2} \) = Oxygen composition in the inlet gas (BT reach)
- \( Q_{CO2} \) = Carbon dioxide composition in the inlet gas (BT reach)

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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).

On the other hand, the biomass formation can be related to the respiration according to the OUR and CER, which is why the CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ to control the pH must be taken into account for the CER.

The variation of the dissolved O₂ concentration during the time in a fermentation process can be calculated and correlated with the OTR and OUR as follows:

\[
\frac{dC_{O2}}{dt} = OTR - OUR
\]

Where:
- \( OTR = \text{KLa} \cdot (C_{O2}^* - C_{O2}) \)
- \( \text{KLa} \) — Volumetric oxygen transfer coefficient (h⁻¹)
- \( C_{O2}^* \) — Saturated dissolved O₂ concentration, maximum O₂ carrying capacity of the liquid in mol/L
- \( C_{O2} \) — Current dissolved O₂ concentration in the liquid in mol/L
- \( OUR = qO2 \cdot X \)
- \( qO2 \) — specific oxygen uptake rate in mol/g·cell·h
- \( X \) — Biomass concentration in g·cell/L

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.