ONLINE MONITORING OF *Pichia pastoris* METABOLISM THROUGH RESPIRATION

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RESUMO – *The online respiration monitoring is a useful tool to have insights about the cell culture. Furthermore, it allows modifications during the process to improve yields. Cultures of Pichia pastoris modified to produce L-asparaginase, a chemotherapy enzyme, were evaluated by their oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER). The results indicated which process condition was beneficial to cell growth and that the production phase can be improved by increasing the inductor feed.*

1. INTRODUCTION

As a limiting nutrient that performs an important role in metabolic pathways, oxygen can be a controlling agent of cell growth, substrate consumption and product formation (Schmidell 2001; Garcia-Ochoa et al. 2010). Therefore, online monitoring of respiration in aerobic cultures provides insights about cell metabolism that allows interferences in the culture to achieve better yields. This is especially important in high-density cultures that aims the production of a high added value product, as a biopharmaceutical. This study aims to analyse the parameters associated with respiration in cultures of the yeast *Pichia pastoris* producing L-asparaginase (ASNase), a chemotherapy enzyme, to a future optimization.

2. METHODOLOGY

2.1. Strain and media

The studied strain was *Pichia pastoris* GS115 Glycoswitch® SuperMan® his’ (Biogrammatics Inc.) modified with the gene asnB (GenScript) from *E. chrysanthemi* to express the enzyme L-asparaginase (Effer et al. 2017). Pre-inoculum was performed in the Buffered Complex Glycerol Medium (BMGY) and bioreactor cultures in a modified Basal Salt Medium (BSM), both described in Invitrogen Corporation (2002), with additions of a histidinle solution at 600mg/L (culture A) and 480mg/L (culture B), and antifoam Y30 as necessary. Antibiotic G418 was used at the final concentration of 400µg/mL in all cultures to avoid contamination.
2.2. Pre-inoculum

The pre-inoculum was performed in Erlenmeyer flasks of 500mL Pyrex® (Sigma – Aldrich®) with 100mL of BMGY at pH 6.0 and 30°C during 20h in a temperature-controlled benchtop shaker (New Brunswick Scientific™ Excella® E24). Pre-inoculum provides cells to start bioreactor runs at 1g/L initial cell concentration.

2.3. Benchtop bioreactor cultures

The cultures were performed in the bioreactor Biostat B (Sartorius®, Braun Biotech International, Germany) with 1L working volume, controlled pH at 5.0, 30°C, dissolved oxygen kept above 20% air saturation by cascade controlling (700 – 1000 rpm, 1 lpm.). The O₂ and CO₂ from the exhausted gas were analysed by BioPAT® Xgas (Sartorius®). During the first 24h, it was performed a glycerol batch with 4% v/v; following, a methanol pulse-feed of 1% v/v at each 24h (culture A) and each 12h (culture B) that induc the production of ASNase.

2.4. Oxygen Uptake Rate (OUR) determination

The values of OUR can be determined by the mass balance in the gas phase (Schmidell 2001). The oxygen balance in the gas phase is given by equation 01. The outlet gas flow rate is determined by the nitrogen balance in the gas phase.

\[
\frac{dC}{dt} V = x_{O_2i} \varphi_{gi} - x_{O_2s} \varphi_{go} - OURV \tag{1}
\]

2.5. Determining of \( \mu_{\text{max}} \) through CO₂ balance

In a microbial culture, the CO₂ balance is given by equation 02. It was assumed that during the exponential phase the CO₂ consumption and the respiratory quotient are constant. Moreover, it was considered that the gases behaviour as an ideal gas and the volume is constant. The specific growth rate can be determined graphically by applying the logarithm of equation 2, which is shown in Buba (2018), and it is correlated to lnCO₂ over time.

\[
CER = \frac{\varphi_{go} x_{CO_2o} - \varphi_{gi} x_{CO_2i}}{V} = Xq_{CO_2} \tag{2}
\]

2.6. Respiratory Quotient (RQ) as a metabolic clue

The RQ is a relationship between produced CO₂ and consumed O₂ that is given by equation 3 (Schmidell 2001). Theoretically, the RQ value reflects the ratio CO₂:O₂ when the substrate is completely oxidized. In case of consuming glucose, RQ value is 1 (C₆H₁₂O₆+6O₂=6CO₂+6H₂O) (6CO₂:6O₂). In the studied cultures, the substrates were glycerol and methanol, therefore, the expected RQ values are 0.86 (C₃H₈O₃+3.5O₂=3CO₂+4H₂O) and 0.67 (CH₄O+1.5O₂=CO₂+2H₂O) respectively.
\[ RQ = \frac{CER}{OUR} \]  

2.7. Cell concentration

Cell concentration was determined by optical density at the wavelength of 600nm. Absorbance was related to dry mass cell concentration, measured by 0.45µm membrane filtration, through equation 4.

\[ X_{DO} = 0.556Abs_{600nm} \]  

3. RESULTS

3.1. OUR, growth and production analysis

During the growth phase, up to 24h in figure 1.1, culture A showed longer exponential growth and higher OUR values than B. However, in B, the population was higher than in A after 12h, figure 1.2. Therefore, the higher histidine concentration did not benefit the cell growth, as lnCO₂ confirms. In addition, we can conclude that the oxygen specific consumption rate was higher in A. During the production phase, after 24h, average OUR was kept higher in B, therefore the cells were not inhibited by the higher frequency of methanol pulses.

![Figure 1](image1.png)

Figure 1 – Comparison of: 1: OUR profile obtained by mass balance in the gas phase and 2: cell concentration obtained by optical density in the cultures A and B.

3.2. Determining \( \mu_{\text{max}} \) through CO₂ balance

The logarithm of CO₂ concentration showed to be a good predictor of the specific growth rate, the highest difference was 14% between \( \mu_{\text{max}} \) and the predicted value. For the culture A, the values were \( \mu_{\text{max}X} = 0.1792 \text{ h}^{-1} \) and \( \mu_{\text{max}CO2}=0.1531 \text{ h}^{-1} \); and, for culture B, \( \mu_{\text{max}X} = 0.2023 \text{ h}^{-1} \) and \( \mu_{\text{max}CO2}=0.188 \text{ h}^{-1} \). Moreover, it was also a good predictor of the period of exponential phase. Therefore, it can be used to online monitor cell growth when it is not possible to calculate cell concentration.

3.3. RQ as a metabolic clue
Experimental RQ values were very similar to the theoretically predicted values during the substrates consumption in some intervals, signalized by the blue arrows in figure 2. In culture A, RQ values were next to glycerol predicted value until methanol was accidentally fed (9h), leading to values near to methanol theoretical RQ (red arrow in fig. 2). Soon after methanol pulses, the RQs in both cultures were near to the theoretical value of the alcohol. Hence, it was possible to monitor substrate consumption indirectly online.

**Figure 2 – RQ over time. Glycerol consumption during first 24h followed by methanol pulses at each 24h (A) and 12h (B). Orange line represents the theoretical value for RQ during glycerol consumption; grey line the theoretical RQ for methanol consumption; and black line the experimental data.**

4. CONCLUSIONS

Respiration analysis showed to be a robust system to monitor cell formation and substrate consumption. This can be useful during cultures where it is not possible to measure cell or substrate concentration, allowing for the operator to act over the process parameters to improve cell metabolism. In this case, the analysis showed that the lower histidine concentration is better because the specific growth rate and the maximum cell concentration were higher (less limited), and the methanol feed can be increased to keep cells in the highest metabolism levels (higher OUR values), which, probably, will result in higher productivity of L-asparaginase.

5. NOMENCLATURE

| \( \phi_g \) | Mass Flowrate (g/t) |
| C | Volume (L) |
| \( \mu_{\text{max}} \) | Maximum specific growth rate (1/h) |
| t | Time (h) |
| x | Volumetric fraction |
| i | inlet |
| o | outlet |

6. REFERENCES

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