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Study of the effect of the substrate addition in *Pichia pastoris* cultures according to the evolution of exhaust gases as indicators of metabolism

ABSTRACT

The methylotrophic yeast *Pichia pastoris* is one of the most widely used recombinant protein expression systems, with applications from food to the pharmaceutical industry. It is capable of growing using methanol as the only carbon source, for which it uses alcohol oxidase (AOX) promoter, a strong inducer for recombinant protein expression. One of the critical aspects of methanol addition strategies in these cultures is the methanol induction moment and avoiding its excessive addition that leads to its accumulation, making it toxic for the yeast when above certain concentration values in the medium. There are limited on-line monitoring sensors for methanol levels at the process level in cultures with *Pichia*, which can also be scaled and integrated with the process control software and the substrate addition pumps. In this work, the evolution of exhaust gas composition in O2 and CO2 has been used as an alternative to on-line methanol sensors, mainly according to the evolution of exhaust CO2, to establish optimal strategies for the methanol feeding and induction moment. For this purpose, benchtop and pilot Bionet fermenters have been used with external modules for continuous analysis of exhausted O2 and CO2 integrated into the equipment software. This preliminary work will make it possible to implement future controls that optimize the substrate addition strategies in fermentation processes according to a direct measurement of cellular metabolism.

1 INTRODUCTION

The *Pichia pastoris* methylotrophic yeast is an increasingly used recombinant protein expression system, it is a workhorse to produce proteins for the biopharmaceutical industry, industrial enzymes or special proteins for food (Fickers, 2014; Karbalaei et al., 2020). One of the strategies for cultures with *P. pastoris* is to generate high cell densities with fed-batch modes for limiting substrate addition and, in the case of strains with the AOX promoter that use MeOH as a carbon source, there are two critical stages, (1) the induction moment of the recombinant protein expression by the addition of MeOH, and (2) the optimal flux of MeOH addition after the induction phase and adaptation to MeOH, with fluxes that are not at very low (under 0.3 % v/v) or very high (over 1.0 % v/v) levels to avoid the MeOH accumulation and yeast intoxication (Cos et al., 2006; Looser et al. 2015). There are different on-line tools to analyze the concentration of carbon sources used as limiting substrates in *P. pastoris* cultures, such as MeOH sensors, or glucose analyzers. As it is a metabolic reaction with a direct relationship between carbon source consumption, biomass formation and CO2 as shown in the simplified metabolic pathways during glycerol and MeOH growth phases (Chun, 2000; Niu et al., 2013; Levisauskas et al., 2013), a Bionet's bBreath exhaust gas analyser has been used in this work to correlate the optimal stages for the start of the fed-batch mode, the induction stage and adjust the MeOH flow according to the exhaust CO2 values as carbon dioxide evolution rate (CER) measured on-line.

KEYWORDS
Pichia pastoris, fed-batch, exhaust gas analyzer, Carbon dioxide evolution rate (CER)

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Glycerol + ATP → Glycerol-3-phosphate → **3 CO2** + 6 NADH + 2 ATP + GTP + FADH2

NADH + ½ **O2** → P/O (Gly) ATP

MeOH + O2 → Formaldehyde + ½ O2 + H2O

Formaldehyde → **CO2** + 2NADH

Fig. 1: Simplified *P. pastoris* metabolic pathways during glycerol and MeOH growth phases.

2 MATERIALS AND METHODS

2.1 Strain

A strain of *Pichia pastoris* with the AOX promoter has been used to express C. rugosa lipase 1 (CRL1), provided by the Bioprocess Engineering and Applied Biocatalysis Group of the Universidad Autónoma de Barcelona (UAB).

2.2 Chemicals, media and solutions

- Yeast extract peptone and dextrose (YPD) medium having 10 g·L^{-1} yeast extract, 20 g·L^{-1} peptone, and 20 g·L^{-1} dextrose.
- The composition of the production media (PM) used was 1.8 g·L^{-1} citric acid, 40 g·L^{-1} glycerol, 0.04 g·L^{-1} $CaCl_2 \cdot 2H_2O$, 12.6 g·L^{-1} $(NH_4)_2HPO_4$, 0.9 g·L^{-1} KCl, 0.5 g·L^{-1} $MgSO_4 \cdot 7H_2O$, and 5 g·L^{-1} PTM1.
- PTM1 Trace Salts containing: 6 g·L^{-1} $CuSO_4 \cdot 5H_2O$, 0.08 g·L^{-1} KI, 3 g·L^{-1} $MnSO_4 \cdot 4H_2O$, 0.2 g·L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.02 g·L^{-1} H_3BO_3, 0.5 g·L^{-1} $CoCl_2$, 20 g·L^{-1}, 60 g·L^{-1} $FeSO_4 \cdot 7H_2O$, 5 mL·L^{-1} H_2SO_4 10%.
- Methanol (MeOH) supplemented with 12 g·L^{-1} of PTM1.

2.3 Main Equipment

- Bionet F1-3MB Bench-top Bioreactor with ROSITA Software for control and monitoring, on-line optical density probe, and bBreath-1 for exhaust gas analyzer (Fig. 2).
- Bionet F2-15 Pilot Bioreactor with MARTA Software for control and monitoring, on-line optical density probe, and bBreath-1 for exhaust gas analyzer (Fig. 2).
- Auxiliary: Incubator, Centrifuge, Spectrophotometer.

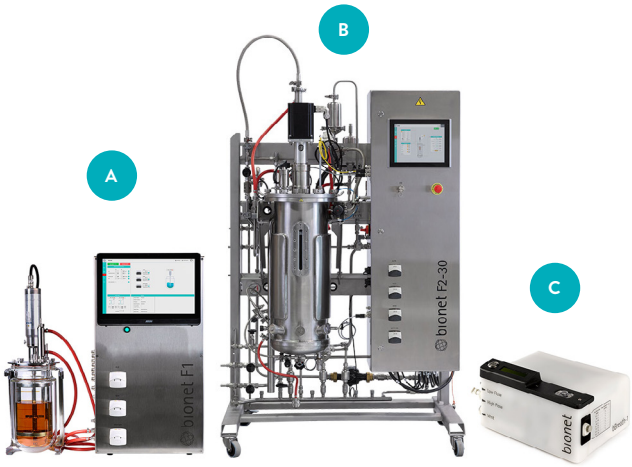

Fig. 2: Bionet's equipment for pilot trials. (A) Bionet F1 for inoculum, (B) Bionet F2 for pilot scale fermentation, and (C) Bionet's bBreath1 exhaust gas analyser for O2/CO2.

2.4 Cell propagation

For the preparation of the inoculums for bioreactor cultures, an inoculum propagation size of 5 % v/v between stages was maintained.

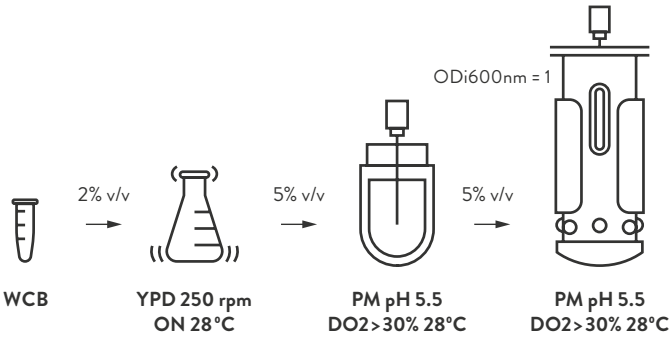

Fig. 3: Cell propagation steps for *P. pastoris* culture.

2.5 Fermentation

For the bench-top scale trials, cells from propagation steps were inoculated into a Bionet's F1-3MB bioreactor containing 1 L of the PM media. For the pilot-scale trials, a Bionet's F2-15 bioreactor containing an initial working volume of 10 L of the PM was used for the final fermentation stage. Table 1 summarises the main process parameters.

Table 1: Main process parameters

PARAMETER	VALUE
pH	5.5 ± 0.05
Temp (°C)	28 ± 1°C
Aeration (vvm)	1.5 vvm
Agitation (ImpTipSpeed, m/s)	2.5 - 5
DO2 (%)	≥30 ± 1

A standard three-phase cultivation protocol for *P. pastoris* cultures was used during the final fermentation stage. This protocol included a (1) first stage in batch mode, and (2) fed-batch mode with glycerol, followed by (3) a MeOH fed-batch mode.

2.6 Substrate additions triggering

To monitor the presence of carbon source, both glycerol and MeOH, the values of CO2 exhausted using the Bionet bBreath gas analyzer were used. CO2 variations were used as a trigger to initiate the fed-batch stages with glycerol and MeOH, in addition to the induction time with MeOH.

Fig. 4: Example of the scheme of transition configuration between stages using CER to activate an addition pump.

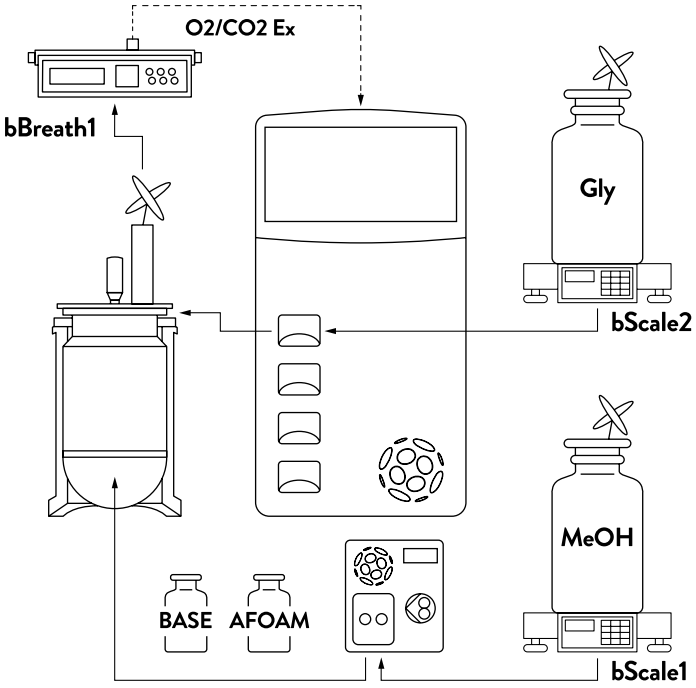

Fig. 5: Set-up of the Bionet system for feeding with 2 substrates for *P. pastoris* cultures using the bBreath exhaust gas analyser to monitor and as a trigger between stages.

2.7 Analysis

Cell growth was monitored on-line by using an optical density probe. The metabolic rate of the culture was monitored according to the carbon dioxide evolution rate (CER) in response to carbon source availability.

3 RESULTS

3.1 The transition from batch to fed-batch glycerol

The first phase transition in the fermentation stage was the transition from batch mode to fed-batch mode with the non-repressor carbon source. To start this first fed-batch stage automatically, a transition was configured using as criteria a time over 18 hours and a reduction in the CER after this time (Fig. 6).

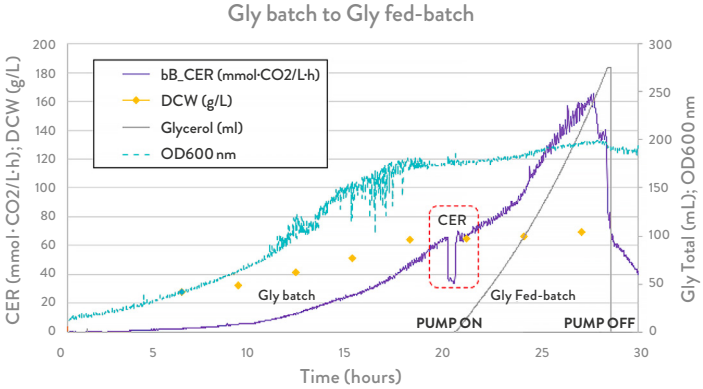

Fig. 6: Evolution of CER and OD600nm in the *P. pastoris* culture to evaluate the response to the use of exhaust CO2 as a trigger to start the fed-batch phase.

3.2 Glycerol depletion and MeOH induction

The next stage after finishing the fed-batch stage with the non-repressive carbon source was MeOH induction adding 2 ml/L after complete depletion of glycerol when CER reached a value below 10 mmol·CO2/L·h (Fig.7).

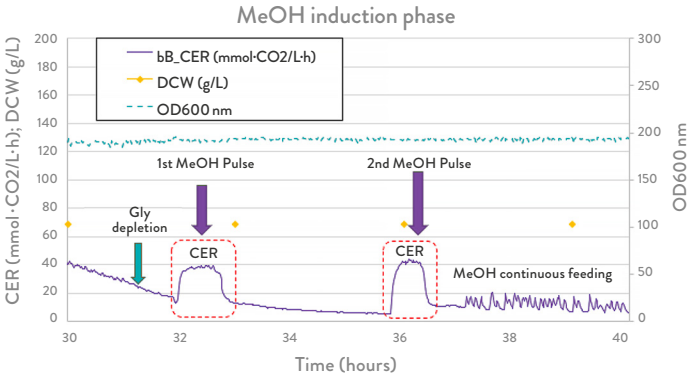

Fig. 7: Evolution of CER and OD600nm in the *P. pastoris* culture after complete depletion of glycerol and during the MeOH induction phase.

3.3 Adaptation phase to MeOH feeding

After the induction stage, an optimal MeOH feed must be adjusted. As shown in the graph in figure 8, (1) after the induction stage, (2) when the continuous MeOH feeding starts, the yeast consumes the MeOH but part of it continues to accumulate. Once the yeast has properly adapted the metabolism to optimally consume the MeOH, (3) the consumption rate increases, observing a drop effect in the CER when all the MeOH is consumed until some substrate accumulates again, so it is advisable (4) to exponentially increase the feed flow to cover the consumption rate of yeast.

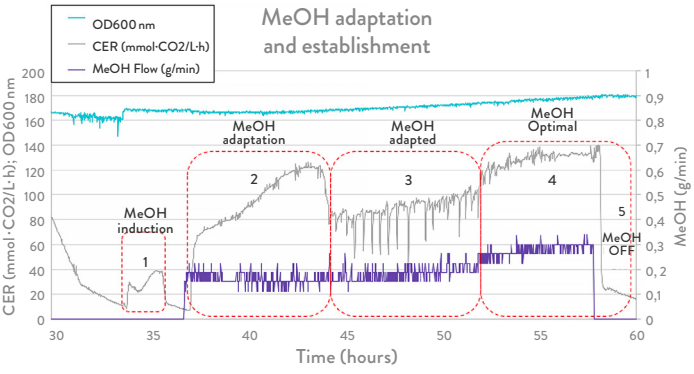

Fig. 8: The evolution of CER and OD600nm in the *P. pastoris* culture after complete MeOH induction and complete adaptation to MeOH consumption.

The sensitivity of the CO2 signal as an indicator of cell metabolism also served to observe non-optimal conditions of MeOH addition (Fig. 9 and 10). The samples were analyzed by HPLC, obtaining MeOH values of around 1.6% v/v, showing a correlation with the gradual decrease trend as a negative effect of MeOH flow, different from the behavior of the peak decrease, which shows rapid consumption and lack of MeOH.

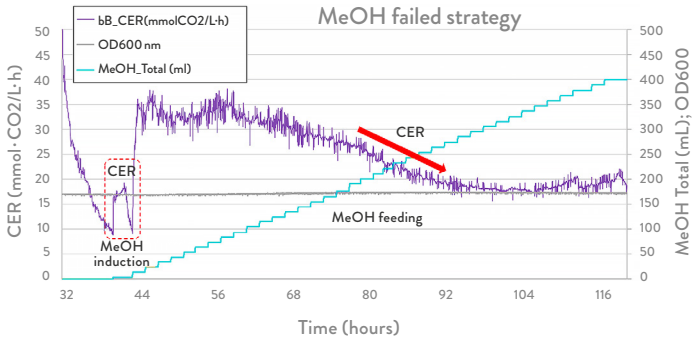

Fig. 9: Negative effect in the CER as a non-optimal MeOH feeding strategy.

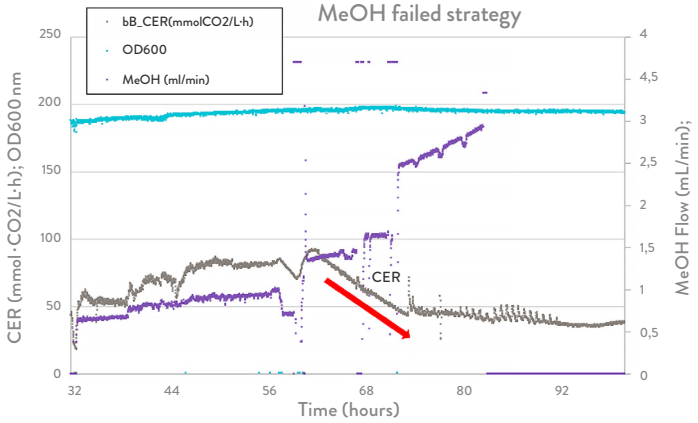

Fig. 10: Effect in the CER at different MeOH feeding strategies increasing flow rate.

3.4. Scale-up trials

Finally, a 1:10 scale-up test was carried out to study the behavior of CER, replicating the bench-top scale tests with a 15-liter pilot fermenter. By using CER instead of CO2 in %, the value of this exhausted gas is normalized per unit of liter and time. The profile results between both scales were very similar in the main fermentation stages: batch and fed-batch with glycerol, induction, and fed-batch with MeOH, and similar values regarding the secreted protein expression, reaching 0.52 g/L and 0.48 g/L for F1-3MB and F2-15, respectively (Fig. 11).

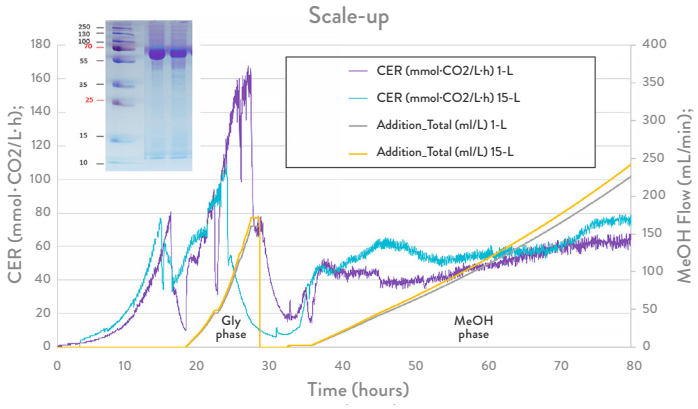

Fig. 11: Comparison of CER profiles according to substrate addition in culture with *P. pastoris*, with a 1:10 scalability.

4 CONCLUSIONS

There is a correlation between the evolution of CO2 during culture with the *Pichia pastoris* yeast and the presence of the limiting substrate. Strategies can be established to optimize limiting substrate feeding, mainly MeOH, according to CO2 evolution. The CO2 value has been optimally used as a trigger to start the induction stage and subsequent start of MeOH feeding. The CER values can be used as a criterion to scale and reproduce fermentation processes in aerobic processes.

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