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Production in semi-continuous mode with the yeast *Pichia pastoris* using BIONET's F1 bioreactor

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Abstract

The yeast *Pichia pastoris*, has been successfully used as an efficient system to produce heterologous proteins [1]. Herein, we report the development of a semi-continuous process to improve the production of recombinant proteins in *P. pastoris* in BIONET's F1 bench-top 3-liter bioreactor, equipped with a continuous process module (CPM). Six repetitive batches were conducted in a semi-continuous process by replacing 2/3 of the culture every 72-96 h. The overall process in a 3-liter culture yielded a protein concentration of 0.28±0.13 mg·mL^{-1}, where over 650 h were required to obtain 6±0.5 g of the total target protein, while the equivalent amount of protein in a fed-batch would require around 1100 h in total, including inoculums, non-induced stage and expression stage, achieving a reduction in the operation time and cost when compared with the semi-continuous process with fed-batch mode. These results suggest that there are promising possibilities to develop cost-effective processes, even when conditioned by low dilution rates required by the culture, for the expression of recombinant proteins in *P. pastoris* in semi-continuous mode as an approximation to a continuous process.

Objective

The objective is to perform a semi-continuous process production of recombinant protein in P. pastoris using Bionet's F1 bioreactor coupled to a Continuous Process Module (CPM) and two variable speed pumps at low flow rates for additions.

Materials & Methods

Chemicals, media and solutions

- Yeast extract and peptone (YPM) medium having 10 g·L^{-1} yeast extract and 20 g·L^{-1} peptone.
- Production media (PM) with the following composition: 13 g·L^{-1} KH2PO4, 8.75 g·L^{-1} (NH4)2SO4, 4.5 g·L^{-1} MgSO4, 0.5 g·L^{-1} CaCl2·2H2O, 5 g·L^{-1} urea, yeast extract 2.5 g·L^{-1} and 30 g·L^{-1} glycerol; and Methanol.
- Supplemented with 5 mL·L^{-1} of the following solutions:
  - Trace Element Solution (TES) containing: 2.0 g·L^{-1} ZnSO4·7H2O, 0.02 g·L^{-1} CuSO4·5H2O, 0.08 g·L^{-1} KI, 0.3 g·L^{-1} MnSO4·H2O, 0.19 g·L^{-1} Na2MoO4·H2O, 0.02 g·L^{-1} H3BO3, 2. g·L^{-1} FeCl3. A
  - Vitamin solution (VT) containing 0.4 g·L^{-1} calcium pantothenate, 0.4 g·L^{-1} tyamine, 0.4 g·L^{-1} myo-inositol, 0.1 g·L^{-1} nicotinic acid, 0.4 g·L^{-1} pyridoxine and 0.4 g·L^{-1} biotin.

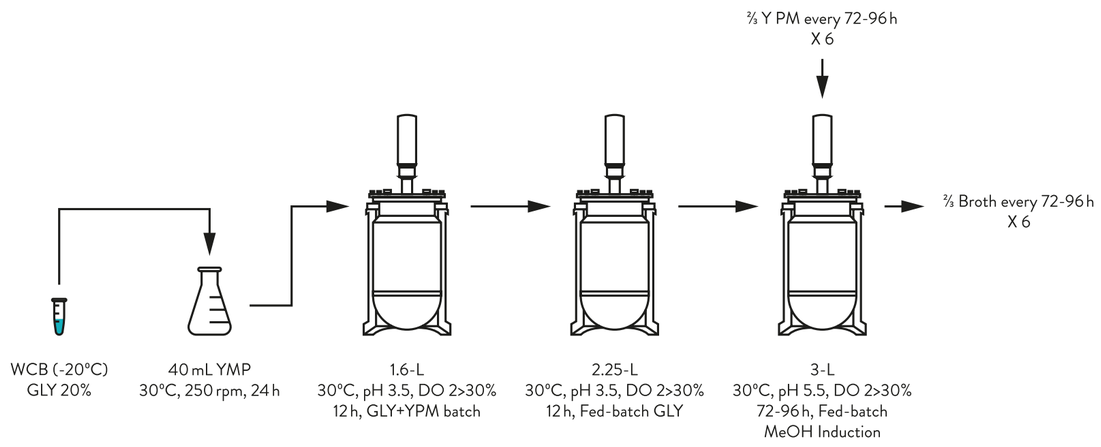

Fig.1: Main steps of the fermentation process. The bioreactor used throughout the process was the same = one unit, but in the flowchart the different units represent the different stages

Bioreactor

- Bionet F1 single, jacketed glass vessel, Microbial configuration (MB)
- Two variable speed pumps
  - External: 4 rpm
  - Integrated: 30 rpm
- Bionet Continuous Process Module (CPM) with two variable speed pumps of 200 rpm

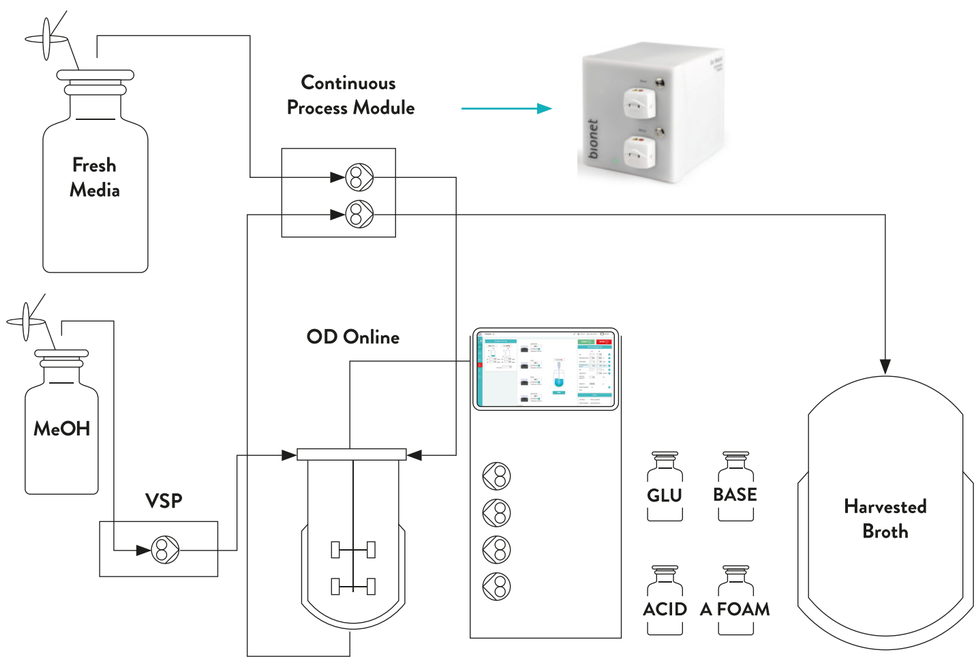

Fig.2: Scheme of the system obtained at bench-top scale for the production of recombinant proteins with the methylotrophic yeast *P. pastoris* in semi-continuous mode using a Bionet laboratory solution

Table 1: Summary of process parameters set points applied

PARAMETER	SP VALUE
Working Volume (Culture)	2-3 L
pH	Growth with glycerol pH @ 3.5±0.2 Growth with MeOH pH @ 5.5±0.2
Temp	30±0.1°C
DO2 (cascade)	30±1% controlled by sparged air and stirring. 1) Agitation: 200–1200 rpm 2) Aeration rate: 0.5–1.5 vvm

Results

Continuous operation and high cell densities are desirable factors for high productivities in fermentation processes. Although several continuous fermentation processes at high cell densities have been reported for the production of recombinant proteins in *P. pastoris* [2], low dilution rates remain a disadvantage for the overall bioprocess performance. As the first step toward an productive, efficient and reproducible continuous fermentation process for the production of a recombinant protein in *P. pastoris*, a semi-continuous process lasting 660 h was established at bench-top scale with six fermentation cycles [3]. The process was started with an initial fed-batch culture, which finalized with the exhaustion of glycerol that was indicated by a spike in the dissolved oxygen concentration. This was followed by repeated methanol fed-batch stages of 72 – 96 h. Regarding the semi-continuous mode, five extractions of two-thirds of the culture broth were performed when cell concentration was between 300 and 350 OD600nm. The addition of fresh media to the bioreactor allowed new MeOH fed-batch stages (Fig.3). When the fed-batch mode was compared with the semi-continuous process, the specific protein productivity in semi-continuous mode was 1.83-fold higher than the fed-batch without cycles. The tables 3 and 4 summarize the yields and productivities obtained.

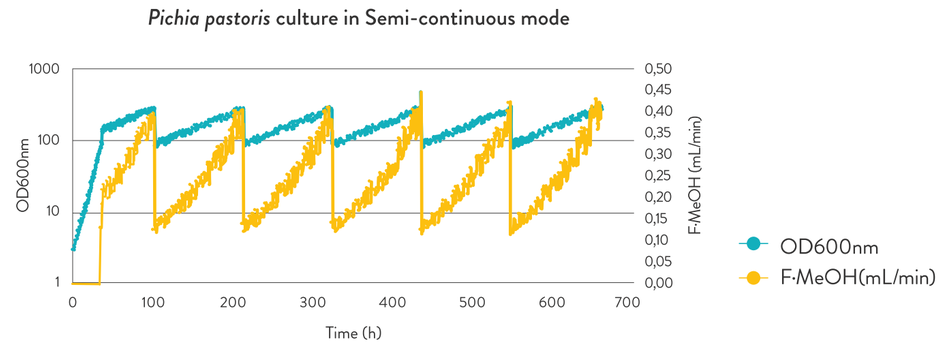

Fig. 3: Time course of the *P. pastoris* semi-continuous process showing biomass production (O.D.600 nm; left Y-axis) and MeOH flow rate (mL/min; right Y-axis). The optical density was monitoring on-line using an OD sensor. Data is from the SW ROSITA®

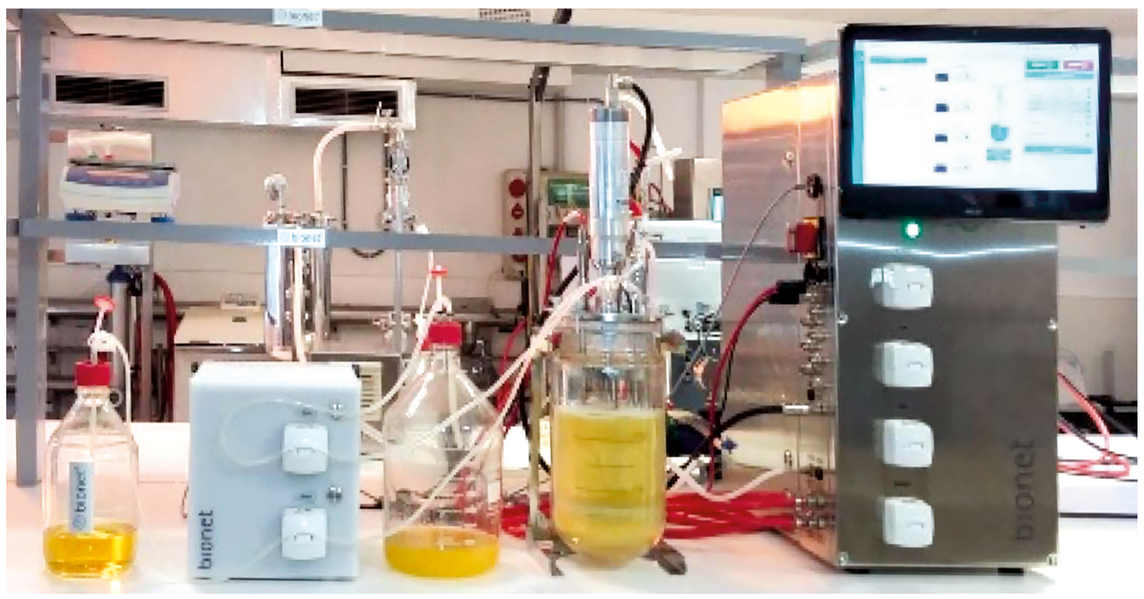

Fig. 4 - Scheme of the system obtained at bench-top scale for the production of recombinant proteins with the methylotrophic yeast *P. pastoris* in semi-continuous mode using the Bionet laboratory solution.

Table 2: Results of specific growth rate (μ) and Biomass yield (Yx/s).

CARBON SOURCE	μ (h-1)		Yxs (g/g·S)	
	Fed-Batch	Semi-Cont	Fed-Batch	Semi-Cont
Glycerol	0.18	0.19	0.45	0.44
Methanol	0.01	0.01	0.28	0.29

Table 3: Productivities obtained in fed-batch and semi-continuous mode.

OPERATION MODE	Biomass Px (g·L^{-1} h^{-1})	Protein (mg/mL)	Protein Pp (g·L^{-1} h^{-1})	Protein purity (%)
Fed-batch	0.042	0.29±0.10	0.27±0.07	37±5%
Semi-continuous	0.076	0.28±0.13	0.51±0.05	35±6%

Conclusions

The main advantages of the semi-continuous process for the production of recombinant protein with the yeast *P. pastoris* were the easy and productive establishment of a semi-continuous process as an approximation to a continuous process, a scalable strategy maintaining the culture in protein expression stage using MeOH as carbon source continuously and solutions to problems related to limitations due to the low specific growth rate of the *P. pastoris* when grown using MeOH, mainly for strains Mut·s. Furthermore, this process improves efficiency in terms of operation, by reducing non-productive time periods during the non-induced stage, such as the preparation of the bioreactor, inoculums and cleanings, achieving thus a higher recombinant protein productivity when compared to the process with fed-batch.

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