

Recombinant Protein Expression in *Pichia pastoris* in a bioreactor via a semicontinuous mode

ABSTRACT

Pichia pastoris has been successfully used as an efficient expression system for heterologous protein production. Herein, we report the development of a semi-continuous process to improve the production of recombinant proteins in *P. pastoris* by its cultivation in a BIONET's F1 bench-top bioreactor of 3 L 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 volume of 3 L of culture yielded a protein concentration of $0.28 \pm 0.13 \text{ mg} \cdot \text{mL}^{-1}$, taking 650 h to obtain $6 \pm 0.5 \text{ g}$ of total target protein. On the contrary, the obtention of the equivalent amount of protein with a fed-batch mode require around 1100 h of total time, including the inoculum preparation, the non-induced fermentation stage and the expression stage. Therefore, a reduction of the operation time and of the operation cost is evident when using a semi continuous fermentation strategy. These results suggest the interesting gap for the development of cost-effective processes for the expression of recombinant proteins in *P. pastoris* in semi-continuous mode as an alternative to the productive continuous process mode that is not available when low dilution rates are required (due to the lack of available pumping technology).

KEYWORDS

laboratory bioreactor, yeast, *Pichia pastoris*, recombinant protein, semi-continuous

CONTACT DETAILS

info@bionet.com
sales@bionet.com

www.bionet.com

1 INTRODUCTION

Pichia pastoris has emerged as an efficient expression system for the production of recombinant proteins [1]. The productivity and economics of this yeast as an expression system does not only depend on several genetic and physiological factors of the host cell but also on the optimization of the fermentation process. The fermentation process including a glycerol or glucose batch phase followed by a methanol induction throughout a fed-batch stage has been accepted as a standard protocol to produce recombinant proteins in *P. pastoris* [2]. In general, this strategy allows for high protein expression levels and high cell densities [3]. However, these fermentation processes are time-consuming and require a low flow rate of additions due to the low *P. pastoris* growth rate in methanol. It has been previously demonstrated that the productivity of fermentation processes with *P. pastoris* can be improved by using a semi-continuous system as an approximation to a continuous system [4]. Herein, we report the development of a semi-continuous process production of recombinant protein in *P. pastoris* using a Bionet's F1 bioreactor coupled to a Continuous Process Module (CPM) and two extra variable speed pumps at low flow rates for additions.

2 MATERIALS AND METHODS

Chemicals, *P. pastoris* Culture Media and Solutions

Yeast extract and yeast extract peptone (YP) medium having 10 g·L⁻¹ yeast extract and 20 g·L⁻¹ peptone were purchased from Scharlab (Barcelona, Spain).

The composition of the production media (PM) used was 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 glucose;

Supplemented with 5 mL·L⁻¹ of next 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, 2.9 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 was also periodically supplemented to the culture at a rate of 5 mL·L⁻¹ every 24 h.

Cell propagation

Pre-inoculums and inoculums for Erlenmeyer flasks and bioreactor cultures were grown in a shaker at 30 °C and 250 rpm. Two 100-mL long-term stock vials from the Working Cell Bank were seeded in 1 mL YP medium, grown for 12 h and transferred into 4×50-mL tubes containing 5 mL of YP medium with 20 g·L⁻¹

glycerol. After 24 h, cultures were mixed again and 5 mL was used to inoculate the 2-L Erlenmeyer flasks containing 250 mL of YP medium with 20 g·L⁻¹ glycerol. Cell growth was resumed 24 h later and cultures were used to seed the 3-L Bionet's F1 bioreactor with an initial working volume of 2-L.

Fermentation

Cells from propagation steps in 2× 250-mL YPD medium were seeded into a 3 L Bionet's F1 bioreactor (Bionet, Spain) containing 2 L of the PM media supplemented with 30 g·L⁻¹ glucose. A complete description of the bioreactor configuration is explained in table 1. During the whole process, pH was kept at 3.5 and 5.0 by adding H₃PO₄ or NH₄OH, the temperature was kept at 30 °C and dissolved oxygen was maintained at 30% saturation by regulating agitation and aeration rates. Table 2 summarizes the culture conditions.

Table 1:
Bionet's F1 bioreactor configuration

Element	Description	
Application	Microbial (MB)	
Model	Bionet F1 single, jacketed glass vessel Geometry H:D 3:1	
Cooling water	Cool water supply @ 10°C Min/Max Pressure: 0.6/5 barg, Min water flow: 6 L/min	
Bioreactor working volume	3 litres	
Bioreactor working volume	2 litres	
Gas supply	Air @ 2 bar	Flow range 0.2-10 slpm
	O ₂ @ 2 bar	Flow range 0.1-5 slpm
Sparger	Ring-sparger (pore Ø 1mm)	
Agitation	Max speed 1500 rpm	
Impeller	Rushton type turbines; 2 units Ratio d/D=0.4	
Two Variable speed pump for fed-batch	Speed 4 rpm (External) Flow rate: 0.1-3.4 ml/min Tube bore ID: 1.6-4.8 mm	
Continuous Process Module (CPM) Two variable speed pumps for Addition/Bleeding Accessories	Speed 200 rpm (External) Flow rate: 5.6-170 ml/min Tube bore ID: 1.6-4.8 mm	
Accessories	Condenser at the exhaust gas Dip curve tube for harvesting Dip tube for sampling (variable height) On-line Optical Density sensor	
Roughness (in parts in contact with the product)	Ra ≤ 0,8	

Table 2
Summary of process parameters set points applied

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

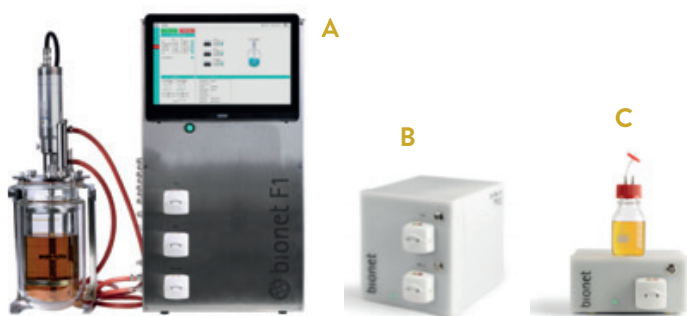


Fig.1. (A) Bionet F1-3 MB single bioreactor, with a jacketed glass vessel of a working volume of 3 liters, (B) Bionet's Continuous Process Module (CPM), and (C) Bionet's External Variable Speed Pump for additions.

Non-induced fed-batch

An exponential fed-batch phase on glucose with a specific growth rate of $\mu_1 = 0.2 \text{ h}^{-1}$ was performed adding 500mL of a 40% w/w solution of glucose following the next equation:

$$F_0 = \frac{\mu \cdot X \cdot V_0}{Y_{xs}(S_f - S_0)} \rightarrow F \cdot GLU = F_0 \cdot \exp(\mu_1 \cdot t) \quad [1]$$

Where F is the GLU flow rate in $[\text{l} \cdot \text{h}^{-1}]$

- μ_1 : The specific growth rate in GLU $[\text{h}^{-1}]$
- X: cell dry weight $[\text{g/l}]$
- V: Initial Working Volume $[\text{l}]$
- Y_{xs} : Biomass yield per gram substrate $[\text{g/g GLU}]$
- S_f : GLU concentration in the feed $[\text{g/l}]$
- S_0 : GLU concentration in the bioreactor $[\text{g/l}]$.

Induced fed-batch

Upon exhaustion of glucose, indicated by a sharp increase in dissolved oxygen, methanol induction was made following the *P. pastoris* Fermentation Process Guideline [3]. The flow rate at which methanol (MeOH) was added responded to the next equation:

$$F = \frac{\mu_2 \cdot (0.13 \cdot OD_{600nm}) \cdot V}{Y_{xs}(S_f - S_0)} \quad [2]$$

Where F is the MeOH flow rate in $[\text{ml} \cdot \text{h}^{-1}]$

- μ_2 : The specific growth rate in MeOH $[\text{h}^{-1}]$
- $0.13 \cdot OD_{600nm}$: The equivalent of grams of cell dry weight (X) from optical density
- V: Working Volume $[\text{ml}]$
- Y_{xs} : Biomass yield per gram substrate $[\text{g/g MeOH}]$
- S_f : MeOH concentration in the feed $[\text{g/l}]$
- S_0 : MeOH concentration in the bioreactor $[\text{g/l}]$.

Semi-continuous fermentation process

A standard cultivation protocol was used during the first stage. This protocol included a 24 h fed-batch mode fermentation in glucose, followed by a methanol fed-batch process. When cell growth in methanol was culminated between 300 and 350 O.D.600 nm, two-thirds of the culture broth were withdrawn from the bioreactor and replaced with sterile fresh media to carry out the semi-continuous fermentation process periodically using Bionet's Continuous Process Module (CPM). Figure 2 shows the set-up of the experiment at a laboratory scale. Throughout the entire fermentation process, supplements of TES and VT

solutions were added to the culture medium every 24 h.



Fig.2. Set-up of the semi-continuous system for the fermentation of *P. pastoris* in Bionet F1 MB Bioreactor model accompanied by the Continuous Process Module (CPM).

Analysis

Time-course samples were withdrawn from the bioreactor at regular intervals to determine dry cell weight (DCW) and protein concentration. Cell density was measured as grams of wet weight per liter of broth, which was obtained by centrifuging the samples for 10 min at $12,500 \times g$. In previous works [5], data of dry weight was measured in a moisture analyzer where wet weight and the O.D.600 nm of samples from more than 35 fermentation processes in PM medium were statistically analyzed by simple regression analysis. A linear correlation between parameters was confirmed, with regression coefficients higher than 0.99. By using the following experimental equation that resulted from said study, we calculated the dry cell weight (DCW) from our experiments:

$$DCW (\text{g} \cdot \text{L}^{-1}) = 0.18 \cdot \text{wet weight} (\text{g} \cdot \text{L}^{-1}) - 3.79 \quad [3]$$

Recombinant protein characterization

Total protein concentration was quantified using the Bradford method with BSA as standard [6]. The expression of the recombinant protein was determined by densitometric analysis of Coomassie based Instant blue-stained SDS-polyacrylamide gels using the SW ImageJ 1.44p (National Institute of Health, USA).

3 RESULTS

Semi-continuous production of recombinant protein in *P. pastoris*

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* [7], the requirement for low dilution rates remain a disadvantage for the overall bioprocess performance. As the first step towards an economic and reproducible extractive bioconversion 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 (Fig. 3). The process involved an initial fed-batch culture, ending with the exhaustion of glucose that was indicated by a spike in the dissolved oxygen

concentration, followed by repeated methanol fed-batch addition 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 starting new MeOH fed-batch stages (Fig.3). When the fed-batch mode is compared to the semi-continuous process, the specific protein productivity in semi-continuous mode is 1.83-fold higher than the fed-batch without cycles. The tables 3 and 4 summarize the yields and productivities obtained:

Table 3
Results of specific growth rate (μ) and Biomass yield ($Y_{x/s}$)

Carbon Source	μ (h^{-1})		$Y_{x/s}$ (g/g-S)	
	Fed-Batch	Semi-Cont	Fed-Batch	Semi-Cont
Glucose	0.18	0.19	0.45	0.44
Methanol	0.01	0.01	0.28	0.29

Table 4
Productivities obtained in fed-batch and semi-continuous mode

Operation Mode	Biomass P_x ($g \cdot L^{-1} \cdot h^{-1}$)	Protein (mg/mL)	Protein P_p ($g \cdot L^{-1} \cdot h^{-1}$)	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%

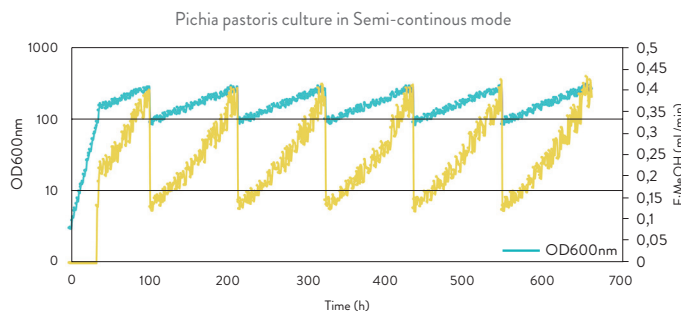


Fig. 3. Time course of the *P. pastoris* semi-continuous process showing biomass production (O.D.600 nm; left Y-axis) and MeOH flow rate (mL/min; right Y-axis). The optical density was monitored on-line by the OD sensor. Data from the SW ROSITA®

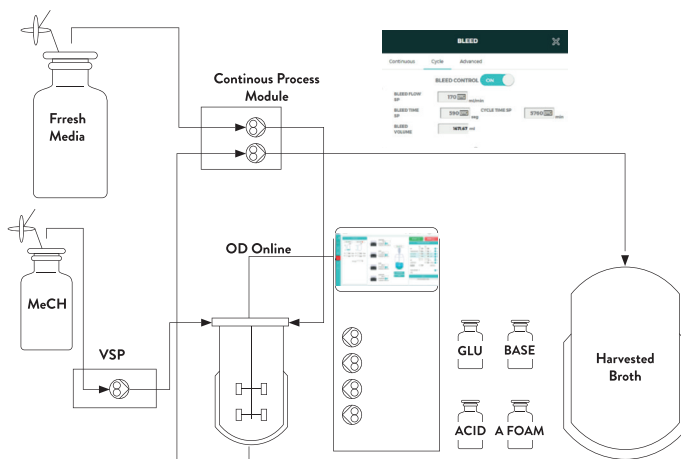


Fig.4. Scheme of the proposed system at laboratory scale for the production of recombinant proteins with the methylotrophic yeast *P. pastoris* in semi-continuous mode using different technologies among Bionet laboratory solutions.

CONCLUSIONS

In summary, the main advantages of the semi-continuous process as an approximation to a continuous process for the production of recombinant protein in *P. pastoris* were the ease and effectiveness by the proposed scalable strategy, which maintains the culture in protein expression stage using MeOH as carbon source continuously. The semi-continuous mode solves the limitations regarding the low specific growth rate of the *P. pastoris* when growing using MeOH, and reduces non-productive times over the batch mode by avoiding several cycles of bioreactor cleanings and preparation, inoculums cultivation periods and non-induced stages of the fermentation process.

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