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Recombinant Protein Expression in *Pichia pastoris* in a bioreactor via a semi-continuous 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 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 $mg{\cdot}mL^{-1}$, taking 650 h to obtain 6±0.5 g of total target protein. On the contrary, the obtention of the equivalent amount of protein with a fed-batch mode would 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 scalable semi-continuous fermentation strategy. These results suggest there is potential 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

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1 INTRODUCTION

Pichia pastoris has emerged as an efficient expression system for the production of recombinant proteins [1]. The productivity and economical efficiency of this yeast as an expression system do 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 low flow rates 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 scalable 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 additional 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 10g·L–1 yeast extract and 20g·L^{-1} peptone were purchased from Scharlab (Barcelona, Spain).

- Production media (PM), with the following composition: 13g·L^{-1} KH_2PO_4, 8.75g·L^{-1} $(NH_4)_2SO_4$, 4.5 g L^{-1} $MgSO_4$, 0.5g·L^{-1} $CaCl_2{\cdot}2H_2O$, 5g·L^{-1} urea, yeast extract 2.5g·L^{-1} and 30g·L^{-1} glucose.

- Supplement of 5mL·L^{-1}, consisting of the following solutions:
  - Trace Element Solution (TES) containing: 2.0g·L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.02g·L^{-1} $CuSO_4{\cdot}5H_2O$, 0.08g·L^{-1} KI, 0.3g·L^{-1} $MnSO_4{\cdot}H_2O$, 0.19g·L^{-1} $Na_2MoO_4{\cdot}H_2O$, 0.02g·L^{-1} H_3BO_3, 2.9g·L^{-1} $FeCl_3$. A
  - Vitamin solution (VT) containing 0.4g·L^{-1} calcium pantothenate, 0.4g·L^{-1} tyamine, 0.4g·L^{-1} myo-inositol, 0.1g·L^{-1} nicotinic acid, 0.4g·L^{-1} pyridoxine and 0.4g·L^{-1} biotin was also periodically supplemented to the culture at a rate of 5mL·L^{-1} every 24h.

Cell propagation

Pre-inoculums and inoculums for Erlenmeyer flasks and bioreactor cultures were grown in a shaker at 30°C and 250rpm. Two 100-mL long-term stock vials from the Working Cell Bank were seeded in 1mL YP medium, grown for 12 h and transferred into 4×50-mL tubes containing 5mL of YP medium with 20g L–1 glycerol. After 24h, cultures were mixed again and 5mL was used to inoculate the 2-L Erlenmeyer flasks containing 250mL of YP medium with 20 g L–1 glycerol. Cell growth was resumed 24 h later and cultures were used to seed the Bionet F1 3-L 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 30g·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 H_3PO_4 or NH_4OH, 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 biorreactor 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 liters	
Bioreactor working volume	2 litres	
Gas supply	Air @ 2 bar	Flow range 0.2-10 slpm
	O2 @ 2 bar	Flow range 0.1-5 slpm
Sparger	Ring-sparger (pore ∅ 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 Accesories	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

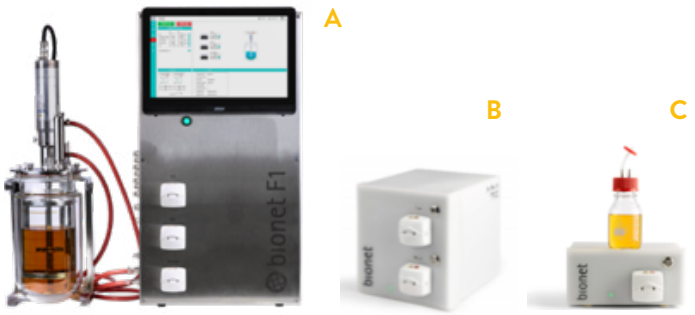

Fig.1: (A) Bionet F1-3 MB single bioreactor, with a jacketed glass vessel of a working volume of 3 liters, (B) Bionet's Continuous Process Module (CPM), and (C) Bionet's External Variable Speed Pump for additions.

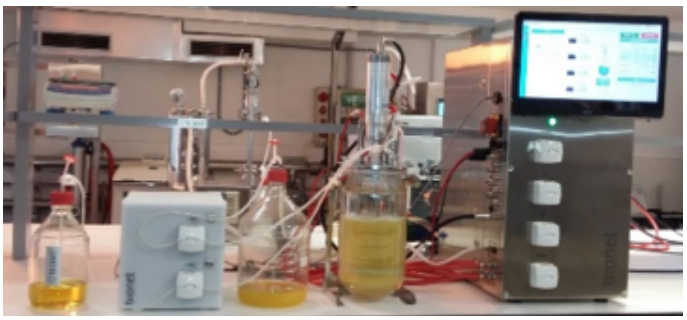

Fig.2: Set-up of the semi-continuous system for the fermentation of *P. pastoris* in Bionet F1 MB Bioreactor model accompanied by the Continuous Process Module (CPM).

Non-induced fed-batch

An exponential fed-batch phase on glucose with a specific growth rate of μ1 = 0.2 h^{-1} was performed adding 500mL of a 40% w/w solution of glucose following the next equation:

$$Fo = \frac{\mu \cdot X \cdot Vo}{Yxs \cdot (Sf - So)} \rightarrow F \cdot GLU = Fo \cdot \exp(\mu 1 \cdot t) \quad [1]$$

Where F is the GLU flow rate in [l.h^{-1}]
- μ_1: The specific growth rate in GLU [h^{-1}]
- **X**: cell dry weight [g/l]
- **V**: Initial Working Volume [l]
- **Yxs**: Biomass yield per gran substrate [g /g GLU]
- **Sf**: GLU concentration in the feed [g/l]
- **So**: GLU concentration in the bioreactor [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:

$$F = \frac{\mu 2 \cdot (0.13 \cdot OD600nm) \cdot V}{Yxs \cdot (Sf - So)} \quad [2]$$

Where F is the MeOH flow rate in [ml.h^{-1}]
- μ_2: The specific growth rate in MeOH [h-1]
- **0,13·OD_{600nm}**: The equivalent of grams of cell dry weight (X) from optical density
- **V**: Working Volume [ml]
- **Yxs**: Biomass yield per gran substrate [g /g MeOH]
- **Sf**: MeOH concentration in the feed [g/l]
- **So**: MeOH concentration in the bioreactor [g/l].

Semi-continuous fermentation process

A standard cultivation protocol was used during the first stage. This protocol included a 24h fed-batch mode fermentation in glucose, followed by a methanol fed-batch process. When cell growth in methanol was culminated between 300 and 350 $O.D._{600nm}$, 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 24h.

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×g. In previous works [5], data of dry weight was measured in a moisture analyzer where wet weight and the $O.D._{600nm}$ 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 styudy, we calculated the dry cell weight (DCW) from our experiments:

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

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 biocon-version for the production of a recombinant protein in *P.pastoris*, a semi-continuous process lasting 660h 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 72–96h. Regarding the semi-continuous mode, five extractions of two-thirds of the culture broth were performed when cell concentration was between 300 and 350 OD_{600nm}.

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 (Yx/s)

CARBON SOURCE	μ (h^{-1})		Yxs (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 Px ($g \cdot L^{-1} h^{-1}$)	Protein (mg/mL)	Protein Pp ($g \cdot L^{-1} h^{-1}$)	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%

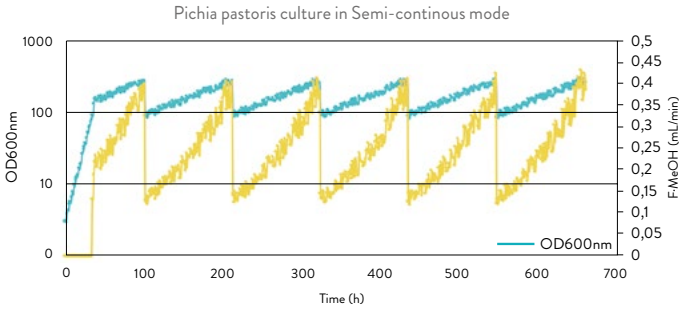

Fig. 3: Time course of the *P. pastoris* semi-continuous process showing biomass production (O.D.600 nm; left Y-axis) and MeOH flow rate (mL/min; right Y-axis). The optical density was monitored on-line with an OD sensor. Data from the SW ROSITA®

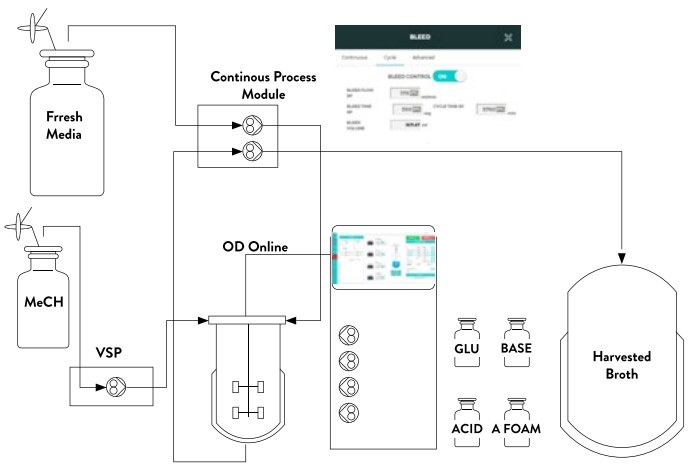

Fig.4: Scheme of the proposed system at laboratory scale for the production of recombinant proteins with the methylotrophic yeast *P. pastoris* in semi-continuous mode using different technologies among Bionet laboratory solutions.

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 efficiency and productivity of the proposed scalable strategy, maintaining 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 cleaning and preparation, inoculums cultivation periods and non-induced stages of the fermentation process.

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