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Scale-up of *E.Coli* culture for optimal fed-batch strategy using exponential feeding

ABSTRACT

Escherichia coli is one of the most popular expression systems for the production of recombinant proteins. In the present study, a brief protocol to apply a fed-batch mode at lab scale with *E.coli* and the scale-up to 30 liters was performed. The high cell density of *E.coli* growth was achieved using a fed-batch mode in a Bionet F1 Bench-top bioreactor, after characterization in batch mode by using an online optical density probe to register automatically the growth. A specific growth rate of 0.46h^{-1} was obtained, resulting in a 7-fold biomass concentration yield with the fed-batch strategy, in comparison with the alternative batch mode. The process developed at the bench-top scale was scaled up with a factor of 10, under constant volumetric oxygen transfer coefficients KLa criterion in a Bionet F2-30A bioreactor at pilot scale.

1 INTRODUCTION

Protein expression in the bacteria *E.coli* has been the most popular expression system for over two decades for the production of recombinant proteins, due to the improved ability to quickly reach high cell densities in inexpensive media, with short culturetime, easy genetic manipulation, low-cost media and the approved regulatory status for human and animal applications [1].
For the scale-up of aerobic fermentation, the effect of the gas-liquid mass transport is the most significant factor. Therefore, scale-up in aerobic fermentation is often performed, based on maintaining the volumetric mass transfer coefficient (kLa) constant throughout scales [2].
The success of the scale-up process is usually confirmed by experimental results, showing limited differences between small- and large-scale fermentation, carried out under the same oxygen transfer rate.
In the present work, the growth of an *E.coli* strain was characterized at lab scale using the Bionet F1 bioreactor, by on-line optical density measurements of to establish the optimal feed rate of the substrate in a fed-batch mode in the exponential phase. With the data obtained, the process was scaled-up to a fermenter of 30 liters, using the Bionet F2-30A bioreactor and applying a scale-up ratio of about 1:10.

KEYWORDS

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

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2 MATERIALS AND METHODS

Equipment

- Bioreactors/Fermenters. Table 1 shows a complete description of the equipment:
  - Bionet F1-3MB Bench-top Bioreactor (Fig. 1) with ROSITA® Software for control and monitoring.
  - Bionet F2-30A (Fig.2) with MARTA® Software for control and monitoring.

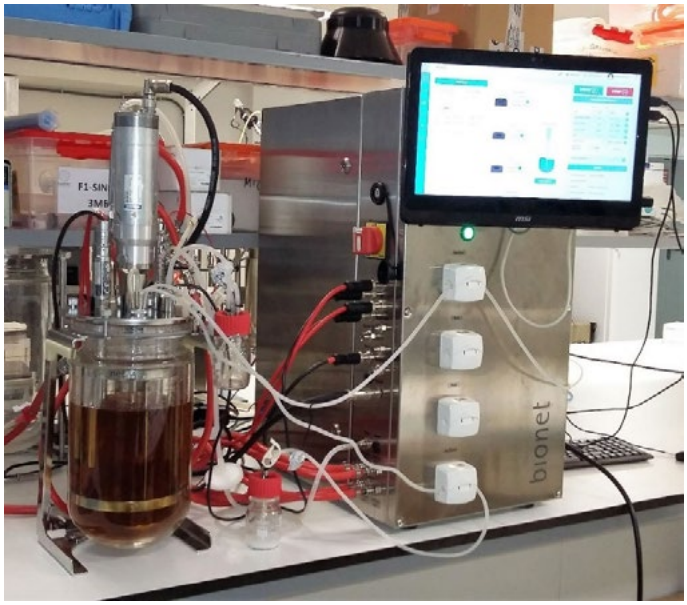

Fig.1: Bionet F1-3MB Bench-top Bioreactor used for process characterization and optimization. This is one of BIONET's entry-level autoclavable solutions for microbiology and cell culture applications in stirred tank bioreactors. Available in 1, 3, 5, 8 and 10L working volume models, and single or twin configurations.

Fig.2: Bionet F2-30A stainless steel Bioreactor used for the scale-up, autonomous configuration (no steam installation needed for sterilization). Available in 15 and 30L working volume models, and autonomous or non-autonomous configurations for sterilization.

Table 1: Comparison of Bionet's bioreactors configurations for the *E. coli* fermentation process in fed-batch mode.

ELEMENT	DESCRIPTION	
Image		
Application	Microbiology (MB)	Microbiology (MB)
Model	Bionet F1 single, jacketed glass vessel Total Geometry H:D 3:1 Working H:D 2.3:1	Bionet F2-30A stainless steel. Autonomous Total Geometry H:D 3:1 Working H:D 2.1:1
Working volume (L)	1-3	10-30
Gas supply @ 2 barg (Mass Flow Controllers)	For Air up to 2.5 vvm	For Air up to 1.5 vvm
	For O2 up to 1.25 vvm	For O2 up to 0.5 vvm
Sparger	Ring-sparger (pore ⌀1mm)	Ring-sparger (pore ⌀1mm)
Agitation	80-1800 rpm	160-1200 rpm
Impeller	Rushton type; 2 units Ratio d/D=0.4	Rushton type; 3 units Ratio d/D=0.33
Variable-speed pump for fed-batch (Optional)	Speed 30 rpm Flow rate: 0.3-25.5 ml/min Tube bore ID: 0.8-4.8 mm	Speed 100 rpm Flow rate: 2.8-85 ml/min Tube bore ID: 1.6-4.8 mm
Chilled Water supply	Pressure: 0.6-5 barg Flow rate min: 6 L/min	Pressure: 0.9-5 barg Flow rate min: 15 L/min
Pressure Control	No	Yes; Pressure Control Valve (PCV) Range: 0-2 barg
Accessories & Optional	• Condenser for exhaust gas • Dip tube for sampling (variable height) • Optical Density Probe; On-line turbidity integrated, 0-6 CU	• Condenser for exhaust gas • ADRI-SAV (Sterilizable Addition Valve) for additions • Crane
Roughness (parts in contact with the product)	Standard (Ra ≤ 0,8)	Standard (Ra ≤ 0,8)

Media

Unless otherwise indicated, all reagents used in this work were purchased either from Sigma–Aldrich (St. Louis, MO, USA) or Scharlab (Barcelona, Spain).

Inoculum

- Luria-Bertani broth (LB)
- Terrific broth (TB)
- Glucose (GLU)
- Ampicillin (antibiotic)
- Isopropyl-β-thiogalactopyranoside (IPTG)
- $MgSO_4 \cdot 7H_2O$
- NH_4OH and H_3PO_4
- Antifoam Sigma 204

Recombinant BL21 strains of *E.coli* from a working cell bank (WCB) were propagated in 250 ml flasks, containing 50ml of Luria–Bertani (LB) broth, 50 $\mu g \cdot mL^{-1}$ ampicillin and 4 $g \cdot L^{-1}$ of glucose for 12 h at 37ºC and 250 rpm in an orbital shaker, until reaching an OD_{600nm} of between 0.6-0.9 units for the inoculation of the 3 litters bioreactor. The relation of the inoculums between all steps and scales was 1% v/v, therefore, for the equivalent operation in fermentation process at 30 liters, the vials stock from the WCB were propagated in 1000 ml flasks, containing 200 ml of LB, 50 $\mu g \cdot ml^{-1}$ ampicillin and 4 $g \cdot L^{-1}$ of glucose.

Fermentation

The fermentation trials were carried out in two separate experiments:

i. Characterization and optimization at the lab scale
   - Batch mode to calculate the specific growth rate (μ) of the strain.
   - Fed-batch mode using an optimal feed rate control.

ii. Scale-up from 3 liters to 30 liters
   - Fed-batch mode using an optimal feed rate control from the characterization at the bench-top scale, and under constant volumetric oxygen transfer coefficients KLa criterion.

For all experiments, cells from inoculum in LB medium were propagated into Bionet bioreactors, containing at the beginning for the batch phase 65% of the final fermentation volume, the initial media composition being 47 g·L^{-1} Terrific broth (TB), supplemented with 10 g·L^{-1} of glucose and containing 0.5 g·L^{-1} of antifoam. Before the inoculation with cells, the bioreactors were sterilized with the media for 30 mins at 121°C, using an autoclave for the glass-vessel bioreactor (Bionet F1-3MB) and sterilization in place (SIP) for the stainless bioreactor (Bionet F2-30A). Glucose was sterilized in an autoclave and added to the medium after bioreactor sterilization. 50 mg·L^{-1} of ampicillin as an antibiotic was added after sterilization and just before cell inoculation. During all experiments, pH was kept at 7±0.2 by adding NH_4OH or H_3PO_4, the temperature was kept at 37°C for the first phase and 30°C for the protein expression phase, and dissolved oxygen was maintained at 30±1% saturation by regulating agitation and aeration rates with DO_2 cascade control.

Batch mode in 3 litters

Cultures in Bionet's F1-3MB bioreactor were grown to OD_{600nm} of 0.6-0.9, after which IPTG was added to 0.5 mM final concentration for the induction of the recombinant protein expression. Incubation continued for 8 h for production of recombinant protein. The optical density was monitored online with a turbidity sensor connected to the bioreactor and integrated into the ROSITA® control SW.

Fed-batch mode in 3 litters

The first stage before induction was equal to a batch mode of OD_{600nm} of 0.9, with the addition of IPTG to 0.5 mM to the final concentration for induction of recombinant protein expression. Then, when the dissolved oxygen spiked up, reaching a value of over 90%, the fed-batch mode was initiated automatically, activating a variable speed pump for the addition of a 100 g·L^{-1} glucose solution, previously sterilized, containing a 18.7 g·L^{-1} $MgSO_4 \cdot 7H_2O$ solution. This has been previously sterilized by microfiltration with a pore size of 0,2 µm, starting with an initial feed rate and increasing exponentially with the exponential function of the control SW (Fig. 2), according to the simple version of the next equation for exponential feeding [3]. The specific growth rate was controlled to 0.2 h^{-1}. The glucose feeding was maintained until an OD_{600nm} of 80 ±10 was achieved:

$$Q(t) = \frac{Xo \cdot Vo \cdot \mu}{Yxs \cdot So} \cdot \exp(\mu \cdot t) = Qo(to) \cdot \exp(\mu \cdot t)$$

- **Q(t)**: Flow rate of the feed solution (L·h^{-1})
- **Xo**: Initial cell density in g·L^{-1} of dry cell weight (DCW)
- **Vo**: Initial volume (L)
- **µ**: Specific growth rate (h^{-1})
- **Y·xs**: Biomass yield coefficient (gDCW/g·glucose)
- **So**: Concentration of substrate in the feed solution (g·L^{1})
- **t**: time (h)

Fig.2: Integration software capabilities. Example of the configuration of the addition pump with exponential function in ROSITA® SW.

Scale-up from 3 liters to 30 liters

In this study, a scale-up, under constant volumetric mass transfer coefficient KLa criterion with a combination of a range of constant impeller tip speed, was applied. The KLa was calculated using the physical dynamic method [4], according to the equation for the determination of the oxygen transfer rate (OTR).

Table 2: Summary of criteria for fermentation process scale-up used in this study.

METHOD	CALCULATION	PARAMETERS
Constant impeller tip speed (V_{tip})	Tip seed (m/s) **$V_{tip} = \pi \cdot N \cdot D$**	**N** = Agitation speed (s^{-1}) **D** = Impeller Diameter (m)
Oxygen transfer rate (OTR)	Oxygen transfer rate, OTR (mmol O2/m3/h) **$OTR = KLa \cdot (C^*_G - C_L)$**	**C^*_G** = Oxygen saturation concentration in the gas phase **C_L** = Measured oxygen saturation concentration in the liquid phase

Centrifugation

To calculate the final biomass yield on glucose as $Y_{x/s}$, a sample of the harvested broth was centrifuged at 4500 × g at RT for 15 minutes, using an OrtoAlresa centrifuge model Dicigen 21, with an RT 138 rotor. The pellet obtained was dried at 100°C for 1 hour, in order to measure the dry cell weight (DCW).

3 RESULTS

Bench-top Fermentation

The cell growth data was collected for process analysis from a CSV file generated from Bionet's ROSITA® SW, for bench-top scale. The growth rate (µ) in batch mode with glucose as a limiting substrate was calculated from the growth curve plotted on a log scale (fig. 3). The specific growth rate obtained for this strain with glucose in the log phase was µ=0.46h^{-1}.

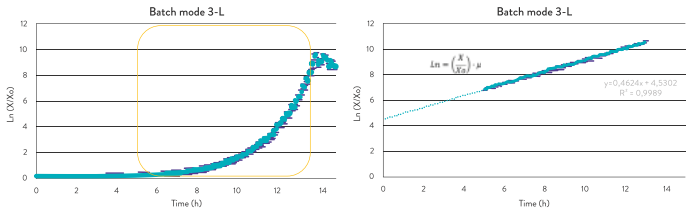

Fig.3: Time course of On-line data of optical density extracted from ROSITA® SW to calculate the specific growth rate (µ).

The optimal feed rate conditions of the fed-batch strategy were established 40% lower than the maximum specific growth rate calculated in batch mode, to ensure the complete consumption of the glucose. When the culture changed the trend of the DO_2 being an indicator of completely depleted of limiting substrate 3 h post-induction, the addition of the concentrated glucose solution was started automatically from the recipe programmed in the Bionet's SW.

The final biomass concentration in fed-batch mode was about 25 g·L^{-1} of DCW, and biomass yield coefficient YX/S was 0.63 g/g·glucose. These were similar to results reported from other authors [5].

Scale-up to 30 liters

The most commonly used criteria for scale-up from lab to pilot scale and from pilot to production or plant scale fermentation is to keep one or more parameters similar between various scales. Usually it is impossible to maintain all the parameters in the same ratio to one another. These parameters include impeller tip speed, oxygen transfer rate (OTR), oxygen mass transfer coefficient (kLa), power input per unit bioreactor volume, mixing time and impeller's Reynold's number. Constant DO_2

concentration is also used as a scale-up parameter to produce recombinant proteins [6]. For the scale-up of aerobic fermentation, the effect of gas-liquid mass transport is the most significant factor.
The impeller tip speed -criteria has some advantages in the case of bioprocesses with sensible microorganisms over shear stress produced by a stirrer, because it determines the maximum shear stress in the tank, determining the possible cell damage [2]. Table 3 shows a comparison of the impeller tip speed calculated for the Bionet's F1-3MB and F2-30 at different agitation regimes (N) correlated according to the diameter of the impeller (D).

Table 3: Correlation between tip speeds (m/s) and agitation speeds (rpm) for the two scales.

TIP SPEED (M/S)	AGITATION (RPM)	
	F1 3MB	F2-30A
0.71	250	153
1.41	500	305
2.26	800	490
2.83	1000	612
3.39	1200	735
4.24	1500	918
5.08	1800	1100

A range of KLa's were calculated using the physical dynamic method [4], according to the equation for the determination of the oxygen transfer rate (OTR) for a minimum and maximum tip speed of 1.41 m/s and 4.24 m/s, respectively, the airflow rate in the range of 0.5-1.5 vvm and the temperature at 37°C with the medium without microorganism (Fig. 4). The process was established to maintain a KLa value in the range of 50-120 h^{-1}, varying the agitation with the DO_2 cascade control with an equivalent impeller tip speed in the range of 1.41-3.39 m/s and maintaining constant the airflow rate at 1 vvm. As a reference value, the preliminary characterization of the Bionet's F1-3MB bioreactor as a maximum KLa value of 300 h^{-1} was determined, corresponding to the maximum OTR of the equipment of around 350 mmol·O_2·$L^{-1}h^{-1}$ (unpublished data).

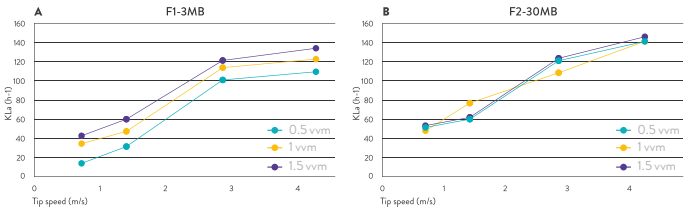

Fig.4: Volumetric mass transfer coefficient (KLa) as a function of stirrer speed at 37°C with the media used, and different airflow rates performed in the (A) F1-3MB and (B) F2-30A Bionet's bioreactors.

On average, E. coli cultures at an OD600nm of 75 with a DCW of 25 g/L were achieved in both scales for 20 hours of the process, as shown in the figure 5. A brief description of the process parameters configuration is shown in the recipe described in Table 4 for both scales.

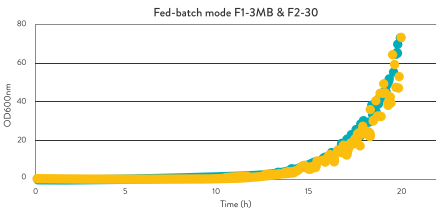

Fig.5: Comparison of the time course of On-line data of optical density exported from ROSITA® SW and MARTA® SW from Bionet's F1-3MB and F2-30A, respectively, from the scale-up trials at fed-batch mode.

Table 4: Configuration of the recipe for E. coli fermentation process in fed-batch mode using Bionet bioreactors.

PARAMETER	STAGE 1	STAGE 2	STAGE 3	STAGE 4
Temp (°C)	37	37	30	12
pH	7±0.2	7±0.2	7±0.2	7±0.2
Agitation (rpm)	• F1-3: 250 • F2-30: 150	Cascade • F1-3: 500-1200 • F2-30: 300-735	Cascade • F1-3: 500-1200 • F2-30: 300-735	• F1-3: 250 • F2-30: 150
Gas (vvm)	0.5	Cascade • F1-3MB: 0.5–1.5 vvm • F2-30A: 0.5–1 vvm	Cascade • F1-3MB: 0.5–1.5 vvm • F2-30A: 0.5–1 vvm	0.5
O_2-Enrichment (%)	OFF	Cascade • F1-3MB: 0–0.2 vvm • F2-30A: 21% – 34%	Cascade • F1-3MB: 0–0.2 vvm • F2-30A: 21% – 34%	OFF
DO_2 cascade	OFF	ON: DO_2>30±1%	ON: DO_2>30±1%	OFF
Addition	OFF	OFF	Exponential • Q_0 = 0.32 mL/min·L • μ = 0.2 h^{-1}	NO
Pressure (barg)*	0.5	0.5	0.5	0.5
Transition	Time>3 h	DO>90% Y Time>3 h	Time>18 h *OR* OD_{600nm}>75	OFF

*Pressure only controlled in F2-30A

CONCLUSIONS

The high cell density of a *E. coli* process was achieved using a fed-batch mode in the Bionet's F1 Bench-top bioreactor, and the scale-up to the F2-30 pilot bioreactor, with the automation of the critical stages of the process programmed with ROSITA® SW and MARTA® SW, respectively. The strain was previously well characterized in batch mode, by collecting on-line data and simplifying the calculations of growth, in order to obtain the specific growth rate. By applying this growth rate in an optimal manner as an exponential feeding rate in fed-batch, a biomass yield, 7-fold compared to the batch mode, was achieved. A successful scale-up to the factor of 10, was achieved in the Bionet F2-30 bioreactor. In addition to a good characterization of the strain used and well-defined scaling criteria, good technical knowledge of the equipment, in terms of process characterization in all production scales, is also of key importance, to ensure the reliability and reproducibility of the results of process scaling.

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