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Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive *GAP* promoter

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ABSTRACT

Carbon source and growth rate are two major parameters affecting recombinant protein production in *Pichia pastoris*. The effect of the most commonly used carbon sources (glycerol or glucose) and the specific growth rate (μ) has been studied on the production of a human antigen-binding fragment (Fab) in this cell factory under the constitutive GAP promoter in fed-batch cultures.

Glycerol for batch phase and glucose for fed-batch phase was the most successful carbon source combination. During batch phase, by-products were detected when glucose was used, despite maintaining DO at values higher than 35%. Also, the presence of cell aggregates was detected affecting the reproducibility and operability of the bioprocess.

Conversely, glucose was the best substrate for fed-batch phase. When working at C-limiting conditions, neither by-products nor aggregates were detected and Fab production levels were comparable to those obtained with glycerol. In addition, the lower heat yield ($Y_{Q/X}$) and oxygen to biomass yield ($Y_{O_2/X}$) for glucose-supported cultures made this substrate the best alternative from an industrial operational point of view.

In addition, the effect of specific growth rate on fed-batch Fab production was studied. Medium and high μ (0.10 and 0.15 h^{-1}) set-points showed similar Fab production yield. However, in terms of total and volumetric productivity, higher μ was the best process condition.

1. Introduction

Recombinant proteins, including biopharmaceuticals proteins and industrial enzymes, is a multi-billion dollar market [1,2]. *Pichia pastoris* is currently one of the most effective and versatile systems for the expression of heterologous proteins [3]. This fact is due to the combination of traits of this yeast, such as its accessibility for genetic manipulation including well characterized genetic elements, the ability to grow on defined media at very high-cell densities, its capacity to perform post-translational modifications including glycosylation and disulphide bond formation and the possibility of driving the protein production intracellularly or secreting it to the extracellular medium [4–6].

Different promoters have been successfully employed for recombinant protein production in *P. pastoris* [7]. The alcohol oxidase promoter (P_{AOX1}), which is strongly induced in the presence of methanol, has been extensively used, obtaining very high expression levels [8–10]. Nevertheless, during the last years, the glyceraldehyde-3-phosphate dehydrogenase constitutive promoter (P_{GAP}) has become an increasingly used alternative [11–13]. *GAP* promoter allows the protein expression using glucose, glycerol and other carbon sources as a substrate [14]. Several studies have reported that P_{GAP} is more efficient than P_{AOX1} [15–17], whereas others showed opposite results [18,19]. Thus, it appears that expression levels achieved for a given protein using different promoters vary significantly based on properties of the expressed protein. Additional research would be required to determine which factors impact the efficiency of both promoters [3]. The use of the constitutive *GAP* promoter avoids the use of methanol in the fermentations, which reduces the cell lysis of the cultures, and subsequently, the proteolysis of secreted proteins [20]. From a large-scale processing perspective, the P_{GAP} expression system may be advantageous because it eliminates the hazard and cost associated with the storage and delivery of large volumes of methanol [21] and significantly decreases heat production and oxygen requirements of the processes [22].

Full-size monoclonal antibodies (mAbs) and their fragments are an increasingly class of therapeutic agents. Main characteristics and applicability have been widely described previously [23,24]. Antigen-binding fragments (Fab) are complex proteins composed of the antigen binding regions of an antibody molecule, containing both the heavy chain domains vH–cH and the entire light chain

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Nomenclature

List of symbols

CPR	carbon dioxide production rate (mol CO_2 L^{-1} h^{-1}).
F_i	volumetric feeding rates of the components (L h^{-1})
OUR	oxygen uptake rate (mol O_2 L^{-1} h^{-1}).
P	product concentration (mg Fab L^{-1})
Q_P	volumetric productivity (mg Fab L^{-1} h^{-1})
q_P	specific product formation rate (mg Fab g_X^{-1} h^{-1})
q_S	specific substrate uptake rate (g_S g_X^{-1} h^{-1})
S	substrate concentration (g L^{-1})
V_i	volume (L)
X	dry biomass concentration (g L^{-1})
$Y_{CO_2/X}$	carbon dioxide to biomass yield coefficient (mol CO_2 g_X^{-1})
$Y_{O_2/X}$	oxygen to biomass yield coefficient (mol O_2 g_X^{-1})
$Y_{P/S}$	product to substrate yield coefficient (mg Fab g_S^{-1})
$Y_{P/X}$	product to biomass yield coefficient (mg Fab g_X^{-1})
$Y_{Q/X}$	heat yield coefficient (kJ g_X^{-1})
$Y_{X/S}$	biomass to substrate yield coefficient (g_X g_S^{-1})

Greek symbols

μ	specific growth rate (h^{-1})
ρ_i	density (g L^{-1})
σ	fraction of dry matter in the biomass (g g^{-1})
ΔH_i	combustion enthalpy of the components (kJ g^{-1})

vL–cL chains, which are connected via disulfide bonds [25]. Microbial expressions systems have been investigated for their potential to produce mAbs and different mAbs fragments. The major advantages of these systems lie in their shorter process times and lower production costs as compared to mammalian cell culture [26]. In *P. pastoris*, high expression levels of mAbs and their fragments have been already achieved [27,28].

In this study, the human Fab 2F5 was used as a model protein complex in order to evaluate the effect of the carbon source and the specific growth rate for the production of heterologous protein under the control of the glycolytic *GAP* promoter. Glucose and glycerol were selected and compared as alternative substrates in both phases of the process, batch and fed-batch. Once the best combination of substrates was selected, three different specific growth rates were evaluated and compared. Thorough studies concerning specific rates including cell growth, substrate consumption and production formation have been carried out.

2. Materials and methods

2.1. Strains

The *P. pastoris* strain X-33 pGAPZαA Fab2F5 expressing the human 2F5 Fab under the control of *P. pastoris* constitutive *GAP* promoter was used throughout this study. Briefly, the expression cassettes for the light and heavy chain of the human Fab 2F5 antibody fragment were separately introduced under the control of the *P. pastoris GAP* promoter and combined on one plasmid [25]. This strain expresses the human Fab using the *Saccharomyces cerevisiae* α-mating signal sequence for secretion of the heterologous protein to the extracellular medium.

2.2. Cultivation methods

2.2.1. Inoculum preparation

The inoculum for bioreactor cultures were grown for 24 h in 1 L baffled shake flasks at 25 °C, 130 rpm, in YPG medium (2% peptone, 1% yeast extract, 2% glycerol; pH=7) adding zeocin (100 μg mL^{-1}). The shake flasks containing 150 mL of culture medium were inoculated from cryostocks of the recombinant strain, grown, harvested by centrifugation and re-suspended in batch medium to be inoculated into the bioreactor.

2.2.2. Fed-batch cultivation

Fed-batch cultivations were carried out in a 5 L Biostat B Bioreactor (Braun Biotech, Melsungen, Germany) working at initial volume of 2 L and finishing the process at approximately 4 L. The culture conditions were monitored and controlled at the following values: temperature, 25 °C; pH, 5.0 with addition of 30% (v/v) ammonium hydroxide; pO_2, above 20% saturation by controlling the stirring speed between 600 and 1200 rpm and using mixtures of air and O_2 at aeration within 1.0 and 1.25 vvm. Water evaporation losses were minimized during the processes using an exhaust gas condenser with cooling water at 8 °C.

The standard fermentation strategy consisted of two phases. First, the batch phase starts with a standard carbon source concentration (40 g L^{-1}). It was performed using the batch medium described elsewhere [11]. Glycerol and glucose were alternatively used as sole carbon sources in different fermentations. During this step, yeast grows at maximum specific growth rate until the depletion of the C-substrate achieving a moderate concentration of biomass (≈20 g L^{-1}). Just after that, begins the fed-batch phase, the most important of the process, where the culture reaches high concentrations of biomass and product.

Second, the fed-batch phase was carried out by adding feeding medium, which have similar composition to that described by Maurer et al. [11] with minor modifications detailed below. Glycerol and glucose were alternatively used as carbon source (400 g L^{-1}). Biotin 0.02% (6 mL), PTM_1 (15 mL) trace salts stock solution [29] and antifoam Struktol J650 (0.2 mL) were added per litre of feeding medium.

The fermentation strategy during the fed-batch phase aimed to achieve pseudo-steady-state conditions for specific rates during carbon-limiting growth. A pre-programmed exponential feeding rate profile for substrate addition derived from mass balance equations to maintain a constant specific growth rate (μ) was implemented [30]. This open-loop control structure allows maintaining a constant μ, which enhances process reproducibility and facilitates the systematic study of growth rate-related effects on heterologous protein production [3].

The equations derived from the fed-batch substrate balance used to apply this strategy were described elsewhere [31,32]. A constant biomass to substrate yield ($Y_{X/S}$ = 0.5 g_X g_S^{-1}) was considered to determine the initial feed rate. The feeding medium was added by automatic Crison micro-burettes MicoBU-2031 (Alella, Barcelona, Spain).

2.3. Analytical methods

2.3.1. Biomass determination by dry cell weight (DCW)

Biomass concentration of the samples was determined as DCW using the method previously described [31]. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 4%.

2.3.2. Product quantification

The 2F5 human Fab was quantified by ELISA as previously described [33]. Determinations were performed by triplicate and the RSD was about 4%.

2.3.3. Protein quantification

Extracellular protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce BCA Protein Assay, Prod.

No. 23225, Rockford, IL, USA), according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the protein standard for the calibration curve.

2.3.4. Carbon source and by-products quantification of the cultures

Glucose, arabitol, glycerol and ethanol were determined by HPLC with a HP 1050 liquid chromatograph (Dionex Corporation, Sunnyvale, CA, USA) using an ICSep ICE COREGEL 87H3 column (Transgenomic Inc., Omaha, NE, USA). The mobile phase was 8 mM sulphuric acid. Injection volume was 20 μL. Data was quantified by Chromeleon 6.80 Software (Dionex Corporation, Sunnyvale, CA, USA). The estimated RSD was below 1% for all the analytes.

Acetate was determined by GC with an Agilent 7820-A gas chromatograph (Agilent Technologies, Inc, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a SGE Analytical Science BP21 column (30 m × 0.25 mm × 0.25 μm) (SGE Analytical Science Pty Ltd, Victoria, Australia). Helium was used as carrier gas. Samples were mixed (1:1, v/v) with a 0.2% hexanoic acid solution in acid as an internal standard. Injection volume was 1 μL. Data was quantified by EZChrom Elite Compact Software (Agilent Technologies, Inc, Santa Clara, CA, USA). RSD was about 6%.

3. Theory/calculation

3.1. Dynamic estimation of the working volume during the process

In fed-batch fermentation the determination of culture volume is a key parameter for the precise determination of specific rates and yields of the bioprocess. Applying mass balance equations for the total mass of broth in the reactor, a procedure has been developed to estimate the culture volume throughout the bioprocess. This approximation is composed of different elements: feeding rate of substrate, addition of ammonium hydroxide used in the pH control loop, volume of the samples withdrawn from the bioreactor, water evaporation losses and gaseous exchange term as stated in Eq. (1).

$$\frac{d(V_{broth}\rho_{broth})}{dt} F_{feed}\rho_{feed} + F_{NH_3}\rho_{NH_3} - V_{sample}\rho_{broth} - F_{evap}\rho H_2O + V_{broth}(32\,OUR - 44\,CPR) \quad (1)$$

where V_{broth} is the volume of broth in the bioreactor (L); ρ_{broth}, broth density ($g\,L^{-1}$); F_{feed}, volumetric addition rate of feeding medium ($L\,h^{-1}$); ρ_{feed}, feeding medium density ($g\,L^{-1}$); F_{NH_3}, base feeding rate ($L\,h^{-1}$); ρ_{NH_3}, 30% (v/v) ammonium hydroxide density; V_{sample}, volume of the samples taken out from the bioreactor (L); F_{evap}, water evaporation rate ($L\,h^{-1}$); ρH_2O, water density ($g\,L^{-1}$); OUR, oxygen uptake rate ($mol\,O_2\,L^{-1}\,h^{-1}$); *CPR*, carbon dioxide production rate ($mol\,CO_2\,L^{-1}\,h^{-1}$). Densities, flow rates and V_{broth} were determined gravimetrically at the end of the process. OUR and CPR were estimated according to previously determined observed yields $Y_{O_2/X}$ ($mol\,O_2\,g_X^{-1}$) and $Y_{CO_2/X}$ ($mol\,CO_2\,g_X^{-1}$) [34,35].

At the end of all the fermentations, slight deviations between estimated and measured volumes were observed (3–5%). Consequently, specific correction factors were applied to all the fermentations.

3.2. Correction of substrate and product concentration for total volume

Biomass, substrates and product concentrations were determined as described above, in Section 2.3. Although biomass concentration (X) was related to the total volume (V_{broth}), the substrates (S_{Liq}) and Fab titer (P_{Liq}) were measured on the supernatant, therefore, not considering the volume of biomass. Consequently, substrates (S) and Fab titer (P) were recalculated on the total volume applying Eqs. (2) and (3), as described previously in the literature [36].

$$S_s = S_{Liq,(t)}\left(1 - \frac{X(t)}{\sigma\rho}\right) \quad (2)$$

$$P_{(t)} = P_{Liq,(t)}\left(1 - \frac{X(t)}{\sigma\rho}\right) \quad (3)$$

where ρ is the yeast density and σ is the fraction of dry matter in the biomass. Both were determined experimentally, $\rho = 1068\,g\,L^{-1}$ and $\sigma = 0.304\,g\,g^{-1}$.

3.3. Calculation of specific rates during the fed-batch phase

The estimation procedure for the determination of discrete specific rates: $\mu_{(t)}$, $q_{S_{(t)}}$, and $q_{p_{(t)}}$ was adapted from Cos et al. [37]. Global state variables (*XV*, *SV* and *PV*) were estimated through all the fed-batch phase by applying the smoothing tool (Matlab R2009a Curvefit Toolbox, The Mathworks Inc., Natik, USA) from the off-line data. The first derivatives of the smoothed curves were also obtained. Finally, $\mu_{(t)}$, $q_{S_{(t)}}$, and $q_{p_{(t)}}$ were calculated by using their corresponding mass balances in fed-batch mode. Uncertainties for discrete specific rates were estimated by error propagation from mass balance equations.

Additionally, applying the mass balances mentioned above, mean specific rates of fed-batch cultivations can be calculated by fitting the experimental data to a linear function. Some authors [27] determined q_s and q_p considering the μ value previously estimated, which may lead to a significant error accumulation. The procedure applied in this work determines q_s and q_p independently from the μ estimation. This is an advantage in processes where the relationship between μ and q_s or q_p is not always constant. In this sense, the method used has been based on linear regression as follows:

$$\int_{(XV)_0}^{(XV)} d(XV) = \mu_{mean}\int_{t_0}^{t} XV\,dt \quad (4)$$

$$S_{feed}\int_{t_0}^{t} F_{feed}\,dt - \int_{(SV)_0}^{(SV)} d(SV) = q_{S_{mean}}\int_{t_0}^{t} XV\,dt \quad (5)$$

$$\int_{(PV)_0}^{(PV)} d(PV) = q_{P_{mean}}\int_{t_0}^{t} XV\,dt \quad (6)$$

The standard error of each mean specific rate is estimated as the standard error of the slope from the linear regression data.

3.4. Estimation of process heat production

Heat production has direct consequences on the cooling requirements of the bioprocess for temperature control. Usually, in large-scale cultivations that reach very high-cell densities, this parameter can become an important process bottleneck. The heat generation from microbial cultures (Q) can be estimated with the following equation:

$$Q = \mu\,XV_{broth}Y_{Q/X} \quad (7)$$

where μ is the specific growth rate (h^{-1}); X, biomass of the process ($g\,L^{-1}$); V_{broth}, volume of broth in the bioreactor (L); $Y_{Q/X}$, Heat yield ($kJ\,g_X^{-1}$). This last parameter describes the amount of heat

Table 1
Summary of batch fermentation results using different carbon sources as substrate.

Substrates	Carbon source ($g\,L^{-1}$)	Max. biomass ($g\,L^{-1}$)	Max. Fab concentration ($mg\,L^{-1}$)	Max. specific growth rate (h^{-1})	Overall $Y_{X/S}$ ($g_X\,g_S^{-1}$)	Overall $Y_{P/X}$ ($mg\,Fab\,g_X^{-1}$)	Overall $Y_{P/S}$ ($mg\,Fab\,g_S^{-1}$)
Glycerol	39.5 ± 0.2	19.3 ± 0.2	3.2 ± 0.1	0.197 ± 0.002	0.49 ± 0.01	0.17 ± 0.01	0.081 ± 0.001
Glucose	39.7 ± 0.2	12.3 ± 0.2	2.9 ± 0.1	0.190 ± 0.002	0.31 ± 0.01	0.24 ± 0.01	0.073 ± 0.001

produced per amount of biomass formed, this being independent of biomass concentration as well as growth rate. This yield can be estimated using Eq. (8):

$$Y_{Q/X} = \frac{\Delta H_S - Y_{X/S}\Delta H_X}{Y_{X/S}} \quad (8)$$

where ΔH_S is the combustion enthalpy of substrate ($kJ\,g_S^{-1}$); $Y_{X/S}$, biomass to substrate yield ($g_X\,g_S^{-1}$); ΔH_X, combustion enthalpy of biomass ($kJ\,g_X^{-1}$). The combustion enthalpies of substrates and biomass were estimated from bibliographic data. ΔH_S (glucose): $-15.58\,kJ\,g_S^{-1}$ [38]; ΔH_S (glycerol): $-17.98\,kJ\,g_S^{-1}$ [38]; ΔH_X (yeasts): $-21.21\,kJ\,g_X^{-1}$ [39]. Otherwise, the $Y_{X/S}$ values used were obtained from experimental data.

4. Results and discussion

4.1. Comparison of carbon sources during the batch phase

In P_{GAP}-based recombinant production processes in *P. pastoris*, either glucose or glycerol is conventionally used as the sole carbon source. However, no definitive conclusions have been drawn as to which substrate is the best for recombinant protein production [3]. Several studies have been carried out comparing both substrates showing that glucose is more efficient than glycerol [14,40], whereas others reported opposite results [17,41]. In this work, both alternative substrates were compared at each bioprocess phase, i.e. the batch and fed-batch phases.

The first part of this study was focused on the batch phase. For this step, most of the reported studies use glycerol. Nevertheless, there is a lack of systematic studies of this phase comparing different carbon sources. In the present work, glucose and glycerol were compared as two possible alternatives. As observed in Table 1, the main difference among the substrates was the final biomass reached, and consequently, biomass to substrate yield. Both parameters were 55% higher when cells were grown on glycerol. Nonetheless, no significant difference was observed for the maximum specific growth rate. Total Fab production also presented differences below 4%, although $Y_{P/X}$ was higher for glucose due to its lower $Y_{X/S}$ value. Relevant differences observed in the final biomass obtained should be related to the production of fermentative by-products.

Time profiles are shown in Fig. 1A and B. Ethanol, arabitol and acetate were detected in glucose-supported cultivations (maximum concentrations: ethanol ≈ 4 g L^{-1}, arabitol ≈ 1 g L^{-1}, acetate ≈ 0.5 g L^{-1}). Conversely, concentrations of these by-products were always below 0.1 g L^{-1} when cells were grown on glycerol. Notably, pO_2 and OUR time profiles were significantly different depending on the C-source, reflecting different oxygen consumption rate profiles of the cultures (Fig. 1A). Whereas in cultures using glycerol the oxygen consumption rose exponentially until the depletion of the substrate, an unexpected behaviour of oxygen consumption was observed when using glucose. Specifically, during the first hours, the oxygen consumption also rose exponentially, causing a decrease on pO_2. Afterwards, when pO_2 attained values around 35%, no clear trend was observed. In this case, the appearance of significant amounts of fermentative by-products in the broth was detected, despite the DO level was kept always above 30% for both cases. From this point, the exponential expected behaviour of the OUR time evolution changed, presenting a lower slope. Besides, estimated OUR values showed higher oxygen consumption for the glycerol-supported cultures.

These observations suggest that, although cultivation conditions of both batches were fully aerobic throughout the whole experiment, when growing in excess of glucose at high growth rate, *P. pastoris* appears to show a limited Crabtree effect, that is showing a respirofermentative metabolism. Also, one is tempted to speculate that this yeast could sense the decrease of available oxygen in the broth and switch its metabolism from fully respirative to respirofermentative, even under fully aerobic conditions. Specifically, this metabolic change could be triggered when, in excess of glucose in the broth, the DO level of the cultivation reaches values around 35%. In these cultivation conditions, the metabolic state of cells could be similar to a culture with a lack of oxygen, which could lead to an accumulation of intracellular reduced NADH because of a limitation of the oxidative capacity of the cells and hence to an imbalance in the redox state of the cells [42,43]. This metabolic phenomenon has been already suggested in other works for cultures with oxygen limitation [42]. All these metabolic changes could explain the alteration observed in the oxygen consumption profiles, the important reduction of biomass yield and the production of fermentative by-products mentioned above. The results are in concordance with others published where ethanol [13,43,44] and acetate [13,43] were produced under similar conditions.

The flocculation is a common phenomenon widely described for yeast cell, specially for brewer yeasts [45]. In glucose-supported batches it is important to note that when the substrate was rather depleted the appearance of cell flocs was followed by microscopic observation. The presence of aggregates affected the reproducibility and operability of the bioprocess.

Similar changes observed for the biomass yields were also reported in previous studies comparing glucose and glycerol as a sole carbon sources in batch cultures [41], and comparing among batch and fed-batch cultivations growing on glucose [22]. Some of the observed by-products were also reported with chemostat cultures growing under glucose and oxygen limitation [46].

Based on the presented results, glycerol should be selected as the most suitable carbon source for batch cultivations.

4.2. Comparison of carbon sources for both phases of fed-batch cultivation

Although glycerol seems to be the most promising carbon source for the batch phase, the combination of both carbon sources was tested for the two phases of fed-batch cultivation. In order to avoid the phenomenon's described above, using glucose at the batch phase, DO was kept above 50% throughout the whole experiments. As a result, the production of fermentative by-products was significantly reduced, and consequently, higher biomass yield was reached ($0.43\,g_X\,g_S^{-1}$ instead of $0.31\,g_X\,g_S^{-1}$). Therefore, the differences observed among using both substrates in the batch phase were reduced making easier the comparison. Four different fed-batch cultivation strategies were performed using all the possible combinations of carbon source, glycerol or glucose, within batch and fed-batch phase. All the cultures had the same termination

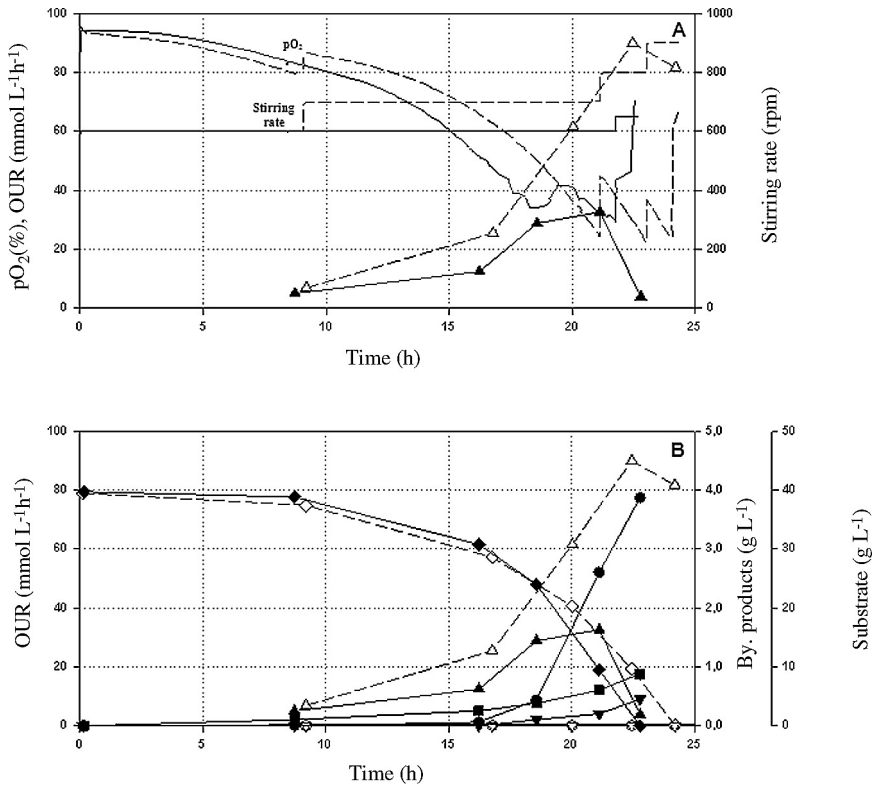

Fig. 1. Comparison of time profiles among the use of glycerol (— — — —) and glucose (————) as a substrate in the batch phase. (A) Glycerol: OUR (△), pO_2 and stirring rate; glucose: OUR (▲), pO_2 and stirring rate. (B) glycerol: OUR (△), glycerol (◊), ethanol (○), arabitol (□), acetate (▽); glucose: OUR (▲), glucose (♦), ethanol (●), arabitol (■), acetate (▼).

criterion: similar values of DCW of about 100 g L^{-1}. Up to this value mass transfer problems are not relevant. All the cultivations were aimed to keep the same set-point of specific growth rate (μ), 0.10 h^{-1}. The set-point was achieved without significant deviations. Physiological and production parameters obtained from the cultivations are shown in Table 2A and B. As example, the time evolution profile of the fermentation for a combination glycerol–glucose is shown in Fig. 2A. Discrete and mean specific rates were calculated as described in Section 3.3 and are presented in Fig. 2B and C.

Slight differences were observed in biomass to substrate yields, these were among the range 0.45–0.50 $g_X\ g_S^{-1}$, being faintly higher when glycerol was the carbon source of the fed-batch phase. When comparing these values with those calculated in the batch cultures, significant differences were observed in the biomass yield of glucose-supported cultivations depending on whether the substrate was limiting or in excess, being higher at limiting conditions. Similar differences were also reported and discussed in previous studies [13]. The main differences were found in Fab production among the cultivations growing with glucose or glycerol throughout the batch phase. When using glycerol, final product titers were around 30% higher than in glucose-grown cultures. Consequently, similar increases were observed in other parameters directly related to it such as q_p, Q_p (volumetric productivity), total productivity and product yields. In contrast, when comparing cultivations that used the same carbon source in the batch phase but different substrates in the fed-batch-phase, minor differences were

Table 2
Physiological and production parameters of *P. pastoris* during fed-batch cultivations at nominal specific growth rate $\mu = 0.10$ (h^{-1}), using different combinations of carbon sources as a substrate.

A

Substrates	Max. Biomass (g L^{-1})	Max. Fab concentration (mg L^{-1})	Fed phase μ mean (h^{-1})	Fed phase q_S mean ($g_S\ g_X^{-1}\ h^{-1}$)	Fed phase q_P mean ($\mu g\ Fab\ g_X^{-1}\ h^{-1}$)
Glycerol–glucose	97.0 ± 3.9	24.7 ± 0.9	0.101 ± 0.001	0.225 ± 0.001	25.7 ± 0.4
Glycerol–glycerol	101.7 ± 4.1	23.2 ± 0.8	0.098 ± 0.001	0.185 ± 0.002	23.2 ± 0.3
Glucose–glucose	95.3 ± 3.8	17.7 ± 0.6	0.099 ± 0.003	0.203 ± 0.001	17.4 ± 0.3
Glucose–glycerol	99.6 ± 4.0	18.6 ± 0.7	0.098 ± 0.001	0.206 ± 0.001	19.6 ± 0.3

B

Substrates	Overall $Y_{X/S}$ ($g_X\ g_S^{-1}$)	Overall $Y_{P/X}$ (mg Fab g_X^{-1})	Overall $Y_{P/S}$ (mg Fab g_S^{-1})	Total production (mg Fab)	Total productivity (mg Fab h^{-1})	Q_p, volumetric productivity (mg Fab $L^{-1}\ h^{-1}$)
Glycerol–glucose	0.45 ± 0.02	0.26 ± 0.01	0.113 ± 0.004	89.6 ± 3.1	2.31 ± 0.08	0.64 ± 0.02
Glycerol–glycerol	0.50 ± 0.02	0.23 ± 0.01	0.114 ± 0.004	79.6 ± 2.8	2.13 ± 0.07	0.62 ± 0.02
Glucose–glucose	0.45 ± 0.02	0.19 ± 0.01	0.088 ± 0.003	70.4 ± 2.5	1.73 ± 0.06	0.44 ± 0.01
Glucose–glycerol	0.47 ± 0.02	0.19 ± 0.01	0.087 ± 0.003	72.3 ± 2.5	1.80 ± 0.06	0.47 ± 0.02

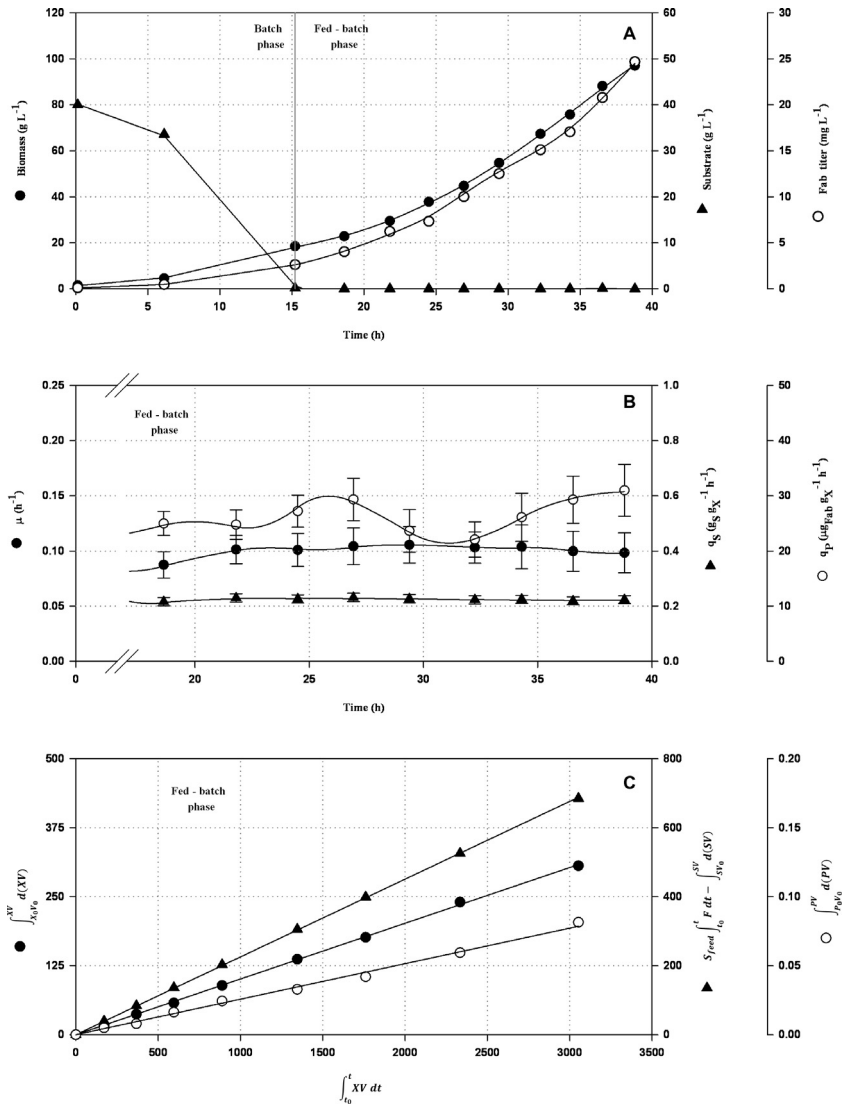

Fig. 2. Standard fed-batch culture with a combination of glycerol–glucose at nominal $\mu = 0.10\ h^{-1}$: (A) biomass (●), substrates (▲), and Fab titration (○) time profiles; (B) specific growth rate (μ) (●), specific substrate uptake rate (q_s) (▲) and specific Fab production rate (q_p) (○) time evolution, error bars indicate the uncertainty of the discrete estimated specific rates; (C) Determination of the mean specific rates: specific growth rate (μ) (●), specific substrate uptake rate (q_s) (▲) and specific Fab production rate (q_p) (○).

found in Fab production and related parameters mentioned above. These differences were quantified around 5–6%. The only exception to this trend was observed in the total Fab production in fed-batch cultivations grown on glycerol during the batch phase. Total Fab production was 13% higher in the glucose-supported fed-batch phase.

The results obtained in fed-batch cultures combining different carbon sources indicate that the batch phase has a strong influence on the overall process yield and productivity, being significant higher when the substrate used for this first phase was glycerol. These differences could be related to the metabolic changes described in the previous section. Specifically, during the last hours of the glucose batch and the first hours of the fed-batch phase, when the metabolic changes are taking place in the cells, the productivity of heterologous proteins lowers until the metabolic quasi-steady state is reached. Conversely, the results of production parameters (q_P and Q_P) and biomass yields did not differ significantly among cultures using glycerol or glucose as alternative carbon sources throughout the fed-batch phase. Since glucose and glycerol are both limiting substrates during the fed-batch phases, one can hypothesize that both metabolic pathways are fully respirative, opposite to the batch phase using glucose, where the respirofermentative metabolism can be suggested.

Thus, the selection criterion between both alternatives must be based on other parameters than the mentioned above. From an industrial point of view, heat production and oxygen consumption could become bottlenecks in large-scale processes. Therefore, both parameters have been used to compare different carbon sources in order to identify the best alternative for industrial *P. pastoris* fed-batch cultivations. The comparison of these parameters among glucose-based processes and methanol-based processes has been already reported in previous works, showing prominent advantages for glucose-supported cultivations [22].

In the present study a comparison of estimated heat production and oxygen requirements for either glucose or glycerol supported cultivations of *P. pastoris* were carried out. In order to identify which is the most suitable carbon source in terms of heat production, equivalent bioprocesses using glucose or glycerol were compared applying the estimation methodology described in Section 3.4. All the terms of the heat production Eq. (7) are identical except $Y_{Q/X}$. Therefore, this is the key factor that was used as a selection criterion. Applying Eq. (8) with the bibliographic data shown in Section

3.4, the estimated value of $Y_{Q/X}$ for glycerol-supplied cultivations was 10% higher than growing on glucose. It must be remarked that this heat yield is highly sensitive to $Y_{X/S}$ changes.

On the other hand, in aerobic systems, oxygen is a key substrate employed for growth, maintenance and in other metabolic routes including product synthesis. Due to its low solubility in cultivation broths and the important requirements of it in fed-batch cultivations at very high cell densities, the oxygen transfer to the cells is crucial, especially in large-scale aerobic bioprocesses [47].

In *P. pastoris* fed-batch cultivations, the maximum oxygen transfer capacity of the reactor can be the limiting factor that determines the maximum amount of biomass that can be reached. Air supplementation with pure oxygen is possible at small scale but it presents important drawbacks at large-scale production in terms of cost and safety risk [48]. Assuming as equivalent the rest of system parameters that affects to the oxygen consumption, oxygen yield ($Y_{O_2/X}$) must be taken into account as a key factor for the comparison between both substrates. Literature values of this parameter for *P. pastoris* growing in different carbon sources have been compared: glucose, 0.022 mol O_2 g_X^{-1} [35]; glycerol, 0.043 mol O_2 g_X^{-1} [34]; methanol (P_{AOX1} system, Mut^+ strain), 0.185 mol O_2 g_X^{-1} [49]. The biggest difference of these estimations is observed among methanol-based and glucose or glycerol-based cultivations. Comparing glucose and glycerol a clear difference is also observed, that is, using glycerol oxygen requirements are two-fold higher.

In summary, glucose was the carbon source selected for fed-batch phase. The lower $Y_{Q/X}$ and $Y_{O_2/X}$ have important advantages from an industrial application point of view.

4.3. Analysis of the effect of the specific growth rate in fed-batch cultivations

Specific growth rate is a key parameter for the production of heterologous protein. It affects directly to the productivity of the bioprocess. As a result, the impact of this parameter has been comprehensively studied for different yeast hosts using chemostats [11,50–52]. However, to our knowledge, there are no equivalent studies using fed-batch cultivations, due the higher complexity of the experimental set up.

In this work, once glycerol was selected for batch phase and glucose for fed-batch phase as best substrates, a study of the influence of specific growth rate on Fab production, using this substrate combination, was made. A pre-programmed exponential feeding rate profile for substrate addition to maintain constant μ was the operational strategy selected. The selected set-points of specific growth rate in fed-batch phase growing on glucose were 0.05, 0.10 and 0.15 h^{-1}. The maximum selected set-point of μ was lower than the μ_{MAX} obtained in batch experiments (0.19 h^{-1}) in order to avoid a possible glucose accumulation. Again, fermentations were stopped when DCW values reached around 100 g L^{-1}. All fermentation parameters and shown in Table 3A and B, and the evolution of biomass and specific growth rate during the fermentation are presented in Fig. 3A and B. As can be seen in Fig. 3B and the calculated values of μ mean, one can conclude than the pre-programmed exponential feeding rate was accurately implemented. As observed in Fig. 3C, the evolution of specific glucose consumption rates in each growth rate condition is consistent with the corresponding specific growth rates selected. The evolution of Fab production and their specific production rates are presented in Fig. 4A and B. The worst condition was the lowest specific growth rate tested (0.05 h^{-1}). Not only the production is virtually stopped after 40 h of operation, but also the maximum Fab production was 60% lower than for the best condition. This fact is also reflected in the evolution of specific production rate (Fig. 4B), decreasing until five fold lower than the maximum.

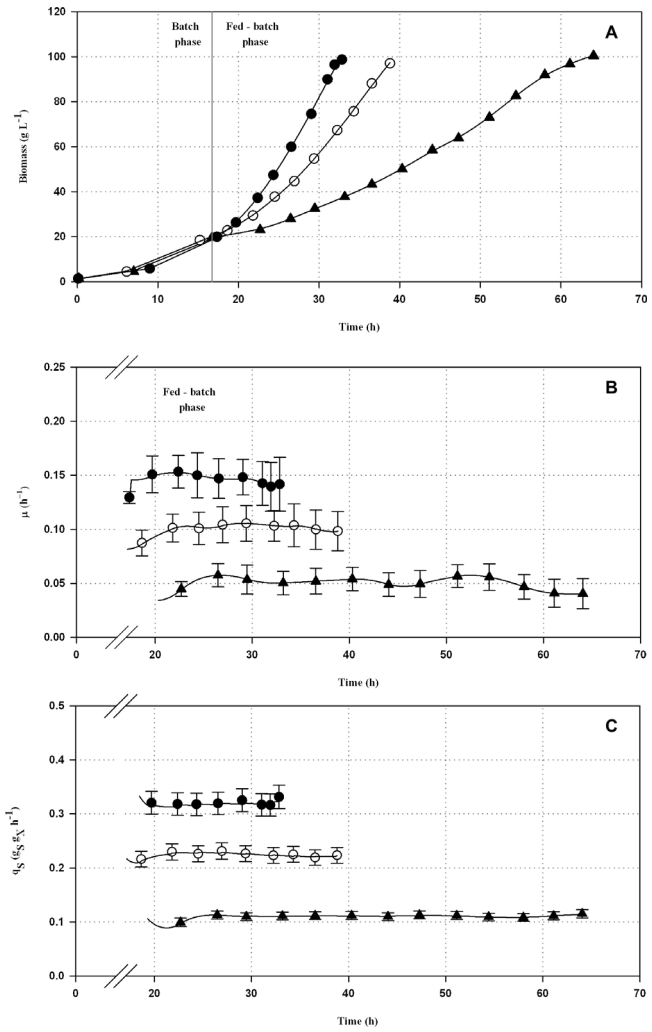

Fig. 3. Biomass, μ and q_s time evolution in fed-batch cultures at different nominal specific growth rates, (▲) μ = 0.05 h^{-1}, (○) μ = 0.10 h^{-1}, (●) μ = 0.15 h^{-1}; (A) biomass concentration profiles; (B) discrete specific growth rate (μ); (C) discrete specific substrate uptake rate (q_s). Error bars indicate the uncertainty of the discrete estimated specific rates.

The maximum Fab production was reached at the nominal μ set-points of 0.10 and 0.15 h^{-1}. However, although in terms of $Y_{P/X}$ the values are quite similar (Table 3B) (0.25 mg Fab g_X^{-1}), total and volumetric productivity was higher at the maximum specific growth rate tested.

Although the total Fab produced for 0.10 and 0.15μ set-points (h^{-1}) is similar, the different fermentation time employed made the highest specific growth rate selected as the strategy to maximize the productivity.

For all the μ tested the amount of extracellular Fab titered represents about 10–15% of the total extracellular protein produced.

Relationships between specific rates are presented in Fig. 5. From the slope of the q_S variation against the μ, one can conclude that the overall substrate to biomass yield is independent from the μ applied. Also, a typical specific growth rate monotonic increasing pattern for the specific production rate is depicted. The higher the μ, the higher the q_P is. However, $Y_{P/X}$ is dependent on the specific growth rate attained.

These results are consistent with previous works that studied the impact of the specific growth rate in the production of Fab in *P. pastoris* under the control of glycolytic promoter P_{GAP} [11,52]. Specifically, the production of the human Fabs 3H6 and 2F5 were studied in chemostat under glucose limitation using the

Table 3
Physiological and production parameters of *P. pastoris* during fed-batch cultivations with glycerol (batch phase) and glucose (fed-batch phase) as substrates at different nominal specific growth rates.

A

Nominal μ (h^{-1})	Max. biomass ($g\,L^{-1}$)	Max. Fab concentration ($mg\,L^{-1}$)	Fed phase μ mean (h^{-1})	Fed phase q_S mean ($g_S\,g_X^{-1}\,h^{-1}$)	Fed phase q_P mean ($\mu g\,Fab\,g_X^{-1}\,h^{-1}$)
0.05	100.5 ± 4.0	15.1 ± 0.5	0.050 ± 0.001	0.111 ± 0.001	7.7 ± 0.4
0.10	97.0 ± 3.9	24.7 ± 0.9	0.101 ± 0.001	0.225 ± 0.001	25.7 ± 0.4
0.15	98.7 ± 3.9	24.1 ± 0.8	0.146 ± 0.001	0.312 ± 0.002	35.2 ± 0.7

B

Nominal μ (h^{-1})	Overall $Y_{X/S}$ ($g_X\,g_S^{-1}$)	Overall $Y_{P/X}$ ($mg\,Fab\,g_X^{-1}$)	Overall $Y_{P/S}$ ($mg\,Fab\,g_S^{-1}$)	Total production ($mg\,Fab$)	Total productivity ($mg\,Fab\,h^{-1}$)	Q_P, Volumetric productivity ($mg\,Fab\,L^{-1}\,h^{-1}$)
0.05	0.46 ± 0.02	0.15 ± 0.01	0.069 ± 0.002	55.0 ± 1.9	0.86 ± 0.03	0.24 ± 0.01
0.10	0.45 ± 0.02	0.25 ± 0.01	0.113 ± 0.004	89.6 ± 3.1	2.3 ± 0.08	0.64 ± 0.02
0.15	0.45 ± 0.02	0.24 ± 0.01	0.110 ± 0.004	90.41 ± 3.2	2.75 ± 0.10	0.73 ± 0.02

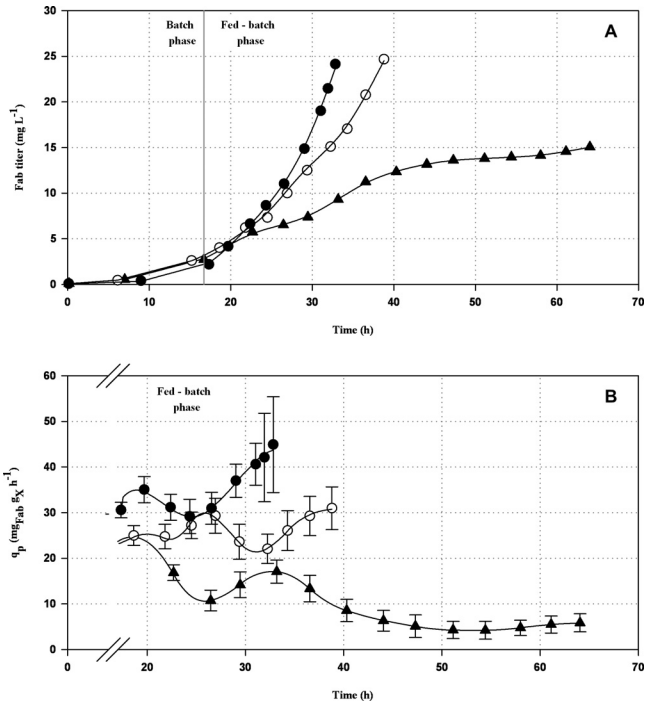

Fig. 4. Product time profiles in fed-batch cultures at different nominal specific growth rates: (▲) $\mu = 0.05\,h^{-1}$, (○) $\mu = 0.10\,h^{-1}$, (●) $\mu = 0.15\,h^{-1}$: (A) 2F5 Fab concentration; (B) discrete specific Fab production rates (q_p), error bars indicate the uncertainty of the discrete estimated specific rates.

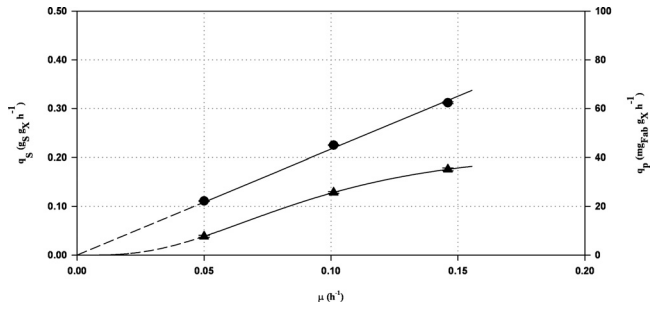

Fig. 5. Relationship between specific rates (q_S, q_P) and specific growth rate (μ). Specific substrate uptake rate (●) and specific Fab production rate (▲).

same expression system, presenting also the best production rates at the highest specific growth rate.

The best results of Fab production in fed-batch cultivations obtained in this study can be also compared with previous works where the same Fab was produced in fed-batch process using the same strain. In these studies, different feeding strategies were applied in the fed-batch phase, namely, a constant feed rate [25] and an optimized feeding profile that aimed to maximize the volumetric productivity [11]. In order to compare the results obtained with different operational strategies mentioned above, mean specific production rates (q_P) were estimated as time-averaged values from discrete available literature data.

Thus, the q_P value reached in this work, 35.2 $\mu g\,Fab\,g_X^{-1}\,h^{-1}$, was about four-fold higher than using a constant feed rate, 8.2 $\mu g\,Fab\,g_X^{-1}\,h^{-1}$, and 25% higher than applying an optimized profile, 28.0 $\mu g\,Fab\,g_X^{-1}\,h^{-1}$. To maximize the volumetric productivity, Q_P, can be also a criterion to evaluate different operational strategies, but always considering biomass produced within the processes. Focusing on this performance parameter, the results presented in this work are slightly higher than the reported applying an optimized profile, 0.73 vs. 0.67 ($mg\,Fab\,L^{-1}\,h^{-1}$). Nevertheless, both strategies increased about 3.5-fold the values obtained using a constant feed rate, 0.21 ($mg\,Fab\,L^{-1}\,h^{-1}$), in which it is also important to note that the biomass reached was 50% higher than in the other fed-batch cultivations.

The major advantage of the strategy described in this work is the simplicity of the whole system based on an exponential feed compared to the application of optimal trajectories, somewhat more complex.

5. Conclusions

Glucose and glycerol have been used as alternative carbon sources in *P. pastoris* fed-batch bioprocesses producing recombinant proteins under the constitutive *GAP* promoter. In this work, both options have been compared producing the human Fab 2F5 as a protein model.

For the first step of the bioprocess, the batch phase, maximum specific growth rate and total yield attained was quite similar for both substrates. Nevertheless, when glucose was used as C-source the production of fermentative by-products and the observed cell aggregates affected the reproducibility and operability of the batch phase.

In fed-batch cultivations using both substrates, an increase of DO set-point until 50% for glucose-supported cultures in the batch phase reduced the fermentative by-products, but the overall Fab

production was still negatively affected. Thus, glycerol was selected as the best substrate at the batch culture phase.

When glycerol and glucose were compared in the fed-batch phase, yields and productivities did not differ significantly. Nonetheless, the carbon source selected for the fed-batch phase was glucose, due to provide lower heat yield ($Y_{Q/X}$) and oxygen to biomass yield ($Y_{O_2/X}$) comparing with glycerol. Hence, minor cooling and oxygen requirements would be great advantages from an operational point of view. Other authors also recommended the use of glucose only under substrate limited growth conditions [13].

Finally, the effect of the specific growth rate on Fab production was also evaluated for the constitutive Fab 2F5 production. The lower the μ implemented, the lower $Y_{P/X}$, $Y_{P/S}$ and final production were. The maximum values of these parameters were obtained from medium to high μ set-point. This fact seems to be a common characteristic for the constitutive Fab production and general for this constitutive P_{GAP}-based expression system of *P. pastoris* [11,52]. Exponential feeding at high specific growth rates would be the most suitable strategy due to its simplicity and to provide similar productivities than those previously reported for the production of the same Fab in fed-batch cultivations [11].

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