A step forward to improve recombinant protein production in *Pichia pastoris*: from specific growth rate effect on protein secretion to carbon-starving conditions as advanced strategy

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Abstract

The recombinant protein production platform based on the *GAP* promoter and *Pichia pastoris* as a host has become a very promising system from an industrial point of view. The need for highly productive bioprocesses gives grounds for the optimization of fermentation strategies maximizing yields and/or productivities, which are often associated with cell growth. Coherent with previous studies, a positive effect of high specific growth rate (μ) on the productivity was observed in carbon-limited chemostat cultivations secreting an antibody fragment. Notably, no significant impact of this factor could be observed in the balance intra- and extracellular of the product. Accordingly, fed-batch cultures operating at a constant high μ were conducted. Furthermore, short carbon-starving periods were introduced along the exponential substrate feeding phase. Strikingly, it was observed an important increase of specific production rate (q_P) during such short carbon-starving periods in relation to the exponential substrate feeding intervals. Therefore, the application of carbon-starving periods as an innovative operational strategy was proposed, resulting into increments up to 50% of both yields and total production. The implementation of the proposed substrate feeding profiles should be complementary to cell engineering strategies to improve the relation q_P vs μ, thereby enhancing the overall bioprocess efficiency.

Keywords

Antibody fragment, carbon starvation, fed-batch, GAP promoter, heterologous protein production, optimized feeding strategy, *Pichia pastoris,* protein secretion.

1. Introduction

Currently, a wide range of products such biopharmaceuticals, organic acids, antibiotics, enzymes or amino acids are industrially produced in biological systems using bioprocess technology [1]. Over the last two decades, bioengineering has made a significant progress on the production of heterologous proteins of both therapeutic and industrial interest, being one of the most successful and profitable bioprocesses [2].

Among all the suitable host organisms commonly used for its production, the methylotrophic yeast *Pichia pastoris* is considered one of the most effective and versatile expression system [3,4]. The combination of traits that makes *P. pastoris* a very interesting cell factory for recombinant protein production has been extensively described in several reviews [5,6]. Although this yeast is mainly known for its strong methanol-inducible *AOX1* promoter (P_{AOX1}) [7,8], during the last years, the constitutive production driven by the *GAP* promoter (P_{GAP}) has been perceived as an efficient alternative production strategy to avoid the use of methanol in the bioprocess [9,10]. A comparison between the advantages and drawbacks of both production alternatives can be found in the literature, in which P_{GAP}-based processes offer important advantages from an industrial point of view, such important decreases on heat production and oxygen requirements of the processes [11,12]. Hence, several alternative fermentation strategies have been extensively studied for this expression system [13,14].

A key advantage of *P. pastoris* as a host in front of other alternatives, especially the prokaryotic systems, is its ability to secrete the product to the cultivation broth, which facilitates importantly the downstream processes [15]. In addition, the passage of proteins through the secretory pathways permits posttranslational events that usually are essential for the biological activity of the proteins [16]. Nevertheless, high levels of heterologous protein expression can lead to saturation or overloading of the secretory pathways, where the product is accumulated intracellularly and often also degraded, resulting into an important decrease of the production yield. This fact is often considered a major bottleneck of great importance for this biotechnological process development [17–19].

To study the effect of the secretory pathway saturation on the bioprocess efficiency, it is of capital interest the reliable quantification and recovery of the total amount of product accumulated intracellularly along a cultivation [20,21]. Some previous studies focused on the efficacy of high-pressure homogenization disruption procedures on methanol-based cultivations, as it is known that cells growing on this substrate present a significant widening of the cell wall thickness [22,23]. In addition, since an important amount of the protein of interest is expected to be retained through the secretory pathway, besides the soluble part of the cell lysates, the insoluble fraction must be taken into account in order to avoid an underestimation of the target product, as it contains the cell membranes, endoplasmic reticulum (ER), Golgi and other organelles where the protein of interest may be retained [24]. A reliable quantification of the product present in the insoluble fraction requires an extraction procedure that involves the use of detergents, which its efficiency is protein-dependent.

Previous studies concerning the P_{GAP}-based expression system described an important effect of the specific growth rate (μ) on the bioprocess productivity in both chemostat and fed-batch cultivations. These studies conclude that a high μ positively affects the production rates of protein [25,26]. The most commonly used cultivation strategies for this system are relatively simple, these are basically based on the implementation of feeding rate profiles for the substrate addition that maintain the desired specific growth rate; constant feeding rate for chemostat operations, and pre-programmed exponential feeding profiles for fed-batch cultivations [11,13]. On the other hand, Kern et al. [27] described an important productivity increase of proteins driven by P_{GAP} upon short-time depletion of glucose. This effect was observed in shake-flask cultures, but it has not been reported for high-density fed-batch cultures.

The aim of the present work is to systematically elucidate the effect of the specific growth rate on protein secretion capacity by studying the balance intra- and extracellular of product in carbon-limited chemostat cultures of *P. pastoris* growing in a wide range of dilution rates. Based on these studies, high-cell density fed-batch cultures at high specific growth rate were

conducted to both study the effect of carbon source starvation periods on the secretory efficiency of the recombinant protein and in the overall process productivity and yields, all together as an innovative operational strategy.

A strain expressing the human 2F5 antigen-binding fragment (Fab), has been used as model protein in this work. Fabs have a wide range of applicability as therapeutic agents [28] and are complex proteins composed by different domains connected via disulfide bonds [29], which makes them a suitable model protein for studying the efficiency of recombinant protein production processes.

2. Materials and methods

2.1. Strain

A *P. pastoris* strain X-33 expressing both light and heavy chain genes of the human Fab 2F5 under the control of the constitutive *GAP* promoter was used in this study. This yeast strain is able to secrete the Fab to the medium by means of the *Saccharomyces cerevisiae* α-mating factor signal sequence. The details of the strain construction were described previously [29].

2.2. Fermentation

The preparation of the inoculum cultures for the cultivations in bioreactors were performed as described by Garcia-Ortega et al. [11].

2.2.1. Chemostat cultivation

Chemostat cultivations were performed in a 2 L Biostat B Bioreactor (Braun Biotech, Melsungen, Germany) at a working volume of 1 L. Cells were grown under carbon-limiting conditions at wide range of dilution rates (D) from 0.025 to 0.15 h^{-1}. The cultivation were carried out using the batch and chemostat medium compositions detailed elsewhere [13]. Minor differences were applied to the cited compositions, which are detailed below. Glucose concentration was 50 g L^{-1}, Biotin 0.02% (1 mL), PTM_1 (1.6 mL) trace salts stock solution (also described by Maurer et al. 2006) and antifoam Glanapon 2000kz (0.2 mL; Bussetti & Co GmbH, Vienna, Austria) were added per liter of chemostat medium.

Culture conditions were monitored and controlled at set points: temperature, 25 °C; pH, 5.0 with addition of 15% (v/v) ammonium hydroxide; culture vessel pressure, 1.2 bars; pO_2, above 20% saturation by controlling the stirring rate between 600 and 900 rpm during the batch phase, in the continuous phase it was kept constant at 700 rpm; air gas flow, 0.8 vvm by means of thermal mass-flow controllers (TMFC; Bronkhorst Hi-Tech, Ruurlo, The Netherlands). An exhaust gas condenser with cooling water at 4ºC minimizes mass loses by water evaporation and other volatile compounds. In all the experiments, the continuous cultivations were carried out for at least for five residence times (τ□ to reach steady state conditions before taking samples.

2.2.2. Fed-batch cultivation

Fed-batch cultivations were performed aiming to achieve pseudo-steady-state conditions for specific rates during carbon-limiting growth as previously described [11]. In brief, cells were grown at 25 ºC, pH=5 by adding ammonium hydroxide (30%, v/v) and pO_2 above 20% of saturation by controlling the stirring speed between 600 and 1200 rpm and using mixtures of air and O_2 at total aeration within 1.0 and 1.25 vvm. All the fed-batch cultivations were carried out at the same specific growth rate, 0.15 h^{-1}, by means of the implementation of a pre-programmed exponential feeding rate profile for substrate addition derived from mass balance equations. In addition, determined stops in the feeding profiles were scheduled in order to study the effect of controlled carbon-starving conditions.

2.3. Cell disruption and protein extraction

2.3.1. High-pressure homogenisation

Fermentation broth samples corresponding to an initial OD_{600} ≈ 125 were harvested by centrifugation (4500 *g*, 3 min, 4 ºC) and pellets were washed twice in cold PBS (pH 7.0) in order to remove all media components and other contaminants. Cells were then resuspended in 8 mL of cold breaking buffer (PBS, pH 7.0, 1mM PMSF, phenylmethylsulfonyl fluoride) and disrupted by high-pressure homogenization using a *One-Shot Cell Disrupter* (Constant Systems Ltd, Deventry, UK). Once disrupted, homogenates were clarified by centrifugation (15000 *g*, 30 min, 4 ºC). Supernatants were collected and stored as soluble cytosolic fraction

158 (SCF) while pellets were kept as the insoluble membrane fraction (IMF). The whole
159 disruption process was carried out at low temperature in order to preserve protein properties
160 as well as to avoid possible protease activity. In addition, the PMSF added in the breaking
161 buffer was used as a protease inhibitor.

162 ***2.3.2. Evaluation of cell disruption efficacy***

163 Cell number was determined after each disruption pass by means of flow cytometry assays
164 (Guava EasyCyte™ Mini cytometer, Millipore, Hayward, CA, USA). The extent of disruption
165 was expressed as:

166 $$X\ (\%) = 100 \cdot \frac{n - n_i}{n}\ (\%) \qquad (1)$$

167 Where X (%) is the degree of disruption; n, the initial number of cells before disruption; n_i,
168 number of non-disrupted cells after each pass. Determinations were performed by triplicate.
169 Relative standard deviation (RSD) was estimated to be about 2%.

170 ***2.3.3. Protein extraction from insoluble membrane fraction (IMF)***

171 Protein extraction was carried out in chilly conditions. Fraction pellets were resuspended by
172 pipetting in 1 mL extraction buffer supplemented with detergent and then vortexed. Extracts
173 were incubated in gently shaking at 4 ºC, clarified by centrifugation (2300 *g*, 5 min, 4 ºC)
174 and supernatants were stored as IMF extracts.

175 For the initial study focused on a screening for the detergent and buffer optimization, three
176 different buffers were compared for the target protein extraction. Buffer A: 50 mM Tris-HCl
177 pH 7.4, 300 mM NaCl, 5 mM EDTA, 1 mM PMSF; Buffer B: 10% glycerol, 20 mM HEPES
178 pH 7.0, 100 mM NaCl, 1 mM PMSF; Buffer C: 8% glycerol, 10 mM sodium phosphate pH
179 8.0, 5 mM EDTA, 500 mM NaCl, 1 mM PMSF. In addition, all the extraction buffers were
180 also supplemented with 1% of three different detergents, Tween 20, Triton X-100 and
181 CHAPS (Sigma-Aldrich, St. Louis, MO, US) for the detergent-buffer screening.

182 Once the optimal combination of buffer and detergent was selected, different CHAPS
183 concentrations (1-2.5%) and incubation time (0h, 2h and overnight-16h) were tested in order

to elucidate a possible effect of the detergent concentration and incubation time in the Fab extraction efficiency.

2.4. Analytical methods

2.4.1. Biomass determination by dry cell weight (DCW)

P. pastoris biomass concentration of the cultivations samples was determined as DCW using the method described elsewhere [30]. Determinations were performed by triplicate. RSD was estimated to be 3%.

2.4.2. Quantification of the Fab 2F5 antibody

Fab 2F5 was quantified in secreted fractions, as well as in the SCF and the IMF. Fab 2F5 concentration was measured by sandwich ELISA as previously described [31]. Determinations were performed by triplicate. RSD was estimated to be 4%.

2.4.3. Total protein quantification

Total protein 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. Determinations were performed by triplicate. RSD was estimated to be 3%.

2.4.4. Carbon source and by-products quantification

Glucose, arabitol, glycerol and ethanol concentrations 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). Determinations were performed by triplicate. RSD was estimated to be below 1%.

2.4.5. Off-gas analyses

A quadrupole mass spectrometer (Balzers Quadstar 422, Pfeiffer-Vacuum, Asslar, Germany) was used for on-line exhaust gas analysis in chemostat cultivations. Exhaust gas humidity was reduced by using a condenser (water at 4 ºC) and two silica gel columns. The Faraday cup

detector was used for its simplicity, stability, and reliability, determining responses of *m/z* corresponding to the major gas peaks (N_2: 28, O_2: 32, CO_2: 44, Ar: 40).

2.5. Process parameters determination, consistency check and data reconciliation

2.5.1. Mass balance and stoichiometric equations

The oxidative and oxidoreductive growth can be described on a C-molar basis by a single overall reaction, a so-called Black Box model, which is a simplification of all the biochemical reactions involved:

$$S + Y^*_{O_2/s}\, O_2 \rightarrow Y^*_{x/s}\, X + Y^*_{CO_2/s}\, CO_2 + Y^*_{p/s}\, P \qquad (2)$$

where *S* denotes one single limiting substrate as the carbon and energy source; O_2, oxygen; *X*, biomass; CO_2, carbon dioxide; *P*, products. $Y^*_{i/s}$ are stoichiometric coefficients that can also be called overall "i" component-substrate yields.

Specific rates (q_i) typically conversions rates related to the biomass concentration (equation 3). Yields are defined as ratios between rates (equation 4) and positive.

$$q_i = \frac{r_i}{X} \qquad (3)$$

$$Y_{i/j} = \frac{r_i}{r_j} = \frac{q_i}{q_j} \qquad (4)$$

From off-gas results obtained with the mass spectrometer O_2 and CO_2 balances were carried out in order to estimate accurately the oxygen uptake rate (OUR), carbon dioxide production rate (CPR), and respiratory quotient (RQ), as well as their corresponding specific rates for O_2 and CO_2.

For an ideal stirred tank-reactor, considering conversion rates of biomass formation, substrate uptake and product formation, the following mass balance equations for the continuous operation at steady state can be formulated:

$$\begin{bmatrix} \mu \\ q_S \\ q_P \\ q_{O_2} \\ q_{CO_2} \end{bmatrix} XV = \begin{bmatrix} F_{out}\, X \\ -F\, S_0 + F_{out}\, S \\ F_{out}\, P \\ OUR\, V \\ CPR\, V \end{bmatrix} \qquad (5)$$

where μ is the specific growth rate (h^{-1}); q_S, specific substrate uptake rate (g g^{-1} h^{-1}); q_P, specific production rate (µg Fab g^{-1} h^{-1}); q_{O2}, specific oxygen uptake rate (mol g^{-1} h^{-1}); q_{CO2},

specific carbon dioxide production rate (mol g^{-1} h^{-1}); F, substrate feeding rate (L h^{-1}); F_{out}, outlet flow rate (L h^{-1}); V, volume of broth in the reactor (L); S_0, substrate feeding concentration (g L^{-1}); *OUR*, oxygen uptake rate (mol L^{-1} h^{-1}); *CPR*, carbon dioxide production rate (mol L^{-1} h^{-1}). F_{out}, the outlet flow rate can be obtained by the total mass balance for an ideal stirred tank reactor in continuous operation at steady state, as follows:

$$F_{out} = \frac{\rho_{Feed}F - \rho_{H2O}F_{Evap} + \rho_{Base}F_{Base} - \rho_{Broth}F_O + M_{GAS}}{\rho_{Broth}} \qquad (6)$$

where F_{Evap} is the water evaporation rate (L h^{-1}); F_{Base}, base feeding rate (L h^{-1}); F_O, withdrawal rate (L h^{-1}); M_{GAS}, net mass gas flow rate (g h^{-1}); ρ_{Feed}, substrate feed density (g L^{-1}); ρ_{H2O}, water density (g L^{-1}); ρ_{Base}, base density (g L^{-1}); ρ_{Broth}, mean broth density (g L^{-1}). The net mass gas flow rate is calculated with the equation (6):

$$M_{GAS} = -(W_{O2}OUR\ V + W_{CO2}CPR\ V) \qquad (7)$$

where W_{O2} is the oxygen molar mass (g mol^{-1}); W_{CO2}, carbon dioxide molar mass (g mol^{-1}). In case of product stripping for ethanol or any other compound, an additional term is included in equation (4) in order to not underestimate its corresponding specific rate. Substrate and product concentrations were referred to the whole medium, including biomass volume [32]. Corresponding equations to fed-batch culture were previously described [11].

2.5.2. Consistency check and data reconciliation

The consistency of the measurements was checked by standard statistical tests considering elemental balances as constraints [33]. Five key specific rates in the black-box process model: biomass generation (μ), glucose uptake (q_s), product formation (q_p), oxygen uptake (q_{O2}), and carbon dioxide production (q_{CO2}) were measured. Carbon and redox balance were used as constraints and protein production considered negligible within these balances.

The χ^2-test performed (α=0.95) for all the experimental data obtained from chemostat cultivations showed the measurements satisfied mostly the stoichiometric model and hence, both C-balance and e-balance. Data reconciliation procedures were used to obtain the best estimates of reaction rates to fit constraints imposed [34].

3. Results and discussion

Previous studies on recombinant protein production in *P. pastoris* under the control of the constitutive *GAP* promoter have reported that high specific growth rate (μ) positively affects the production rates of secreted proteins [11,13,35]. This important productivity increase has been related to the transcriptional regulation of certain genes [26,36], as well as to the capacity of the secretory pathways to release the recombinant protein to the cultivation broth [25,37]. In fact, although the biological mechanisms that drive the correlation between protein secretion and growth are complex and by far not fully understood, protein secretion has been considered an important bottleneck in the recombinant protein production processes, at least at high μ [18,25,38].

In order to evaluate the impact of growth conditions on the secretion efficiency of the target protein, initially was performed a comparative study of the extracellular/intracellular product ratio among cells growing at different specific growth rates. For this purpose, cell disruption and protein extraction procedures were specifically optimized in terms of cell disruption settings, extraction buffers and detergent choice for our protein of interest (Fab 2F5), host strain and growth conditions.

3.1. Cell disruption and protein extraction procedures

Disruption of *P. pastoris* cells by using *One-Shot Cell Disrupter* was optimized for the recovery of an antibody fragment (human Fab 2F5) produced by *P. pastoris* growing on glucose, which its expression was regulated by the constitutive *GAP* promoter.

Initially, cell number of the fermentation samples were counted by flow cytometry, and then disrupted at 2 kbar or 2.5 kbar for 1 to 8 passes. Cell cytometry was used as direct indicator of cell disruption, since cell counting has been described as the most accurate and reproducible measure of cell rupture [39] (Fig. 1A). From the flow cytometry assays, percentage of disruption was calculated using Eq. 1. Percentage of cell disruption and Fab releasing levels in front of pressure disruption and number of passes is showed in Fig. 1B.

No major differences were observed in terms of cell disruption between using 2 kbar or 2.5 kbar. Otherwise, an important increase in the extent of cell disruption from one disruption pass to two has been observed, while further passes only increased it slightly. In relation to Fab recovery levels, the amounts were similar when using 2 kbar regardless the disruption passes, while a decrease on Fab recovery was observed when using 2.5 kbar as working pressure. Thus, 2 passes at 2 kbar were selected as the optimal working conditions for Fab 2F5 recovery.

In order to achieve a reliable quantification of intracellular proteins and its potential recovery, a protein extraction step is necessary to determine the proteins associated to the membrane fraction and cell organelles present in the IMF. Hence, reaching an accurate determination of the total amount of target protein retained intracellularly allows comparing the effects caused by different cultivation conditions.

Different buffers (A, B, C; described in Materials and Methods section) were selected from the bibliography [40–42] and compared for extraction efficiency when combined with 1% of three different detergents (Tween20, Triton X-100 and CHAPS). Previous studies used SDS as detergent, but this component was discarded as it interferes with the Fab detection system (data not shown). In Fig. 2 can be observed that the best detergent was CHAPS, being Buffer B the one leading to the best levels of Fab extraction. It must be taken into account that the extraction step is a protein dependent process, where the optimal detergent would change depending on protein characteristics.

Different CHAPS concentrations and different incubation times at 4 ºC were tested in order to improve Fab extraction. The amounts of solubilised Fab are shown in Figure 3. It was observed that longer incubation times (overnight incubation) resulted in 2.5-fold increase of Fab determination. The tested concentrations of CHAPS did not have a significant impact on Fab extraction. Thus, the final selected extraction conditions were 1% CHAPS with Buffer B incubated for 16 h (overnight) at 4 ºC.

Thereby, it can be concluded that the developed method improves specifically the overall Fab quantification and recovery by the reliable extraction of protein retained intracellularly, both

from the soluble and the insoluble fraction. Regardless the different culture conditions carried out the IMF fraction represents the 25% of the total protein retained.

3.2. Effect of the specific growth rate on the protein retained intracellularly

A set of carbon-limited chemostat cultivations of the recombinant strain growing at different specific growth rates between 0.025 and 0.15 h^{-1} was carried out. Data was obtained from samples taken after, at least, 5 residence times, from when it is considered that the steady state of the culture is reached. The closure of carbon and redox balances calculated for all the culture conditions compared in the present work were always above 95%. Additionally results were validated using the standard data consistency check and reconciliation procedures described in materials and methods section. These tests confirm the robustness and the reliability of the results obtained from the chemostat cultivations performed.

The main specific rates of the cultivation are plotted in Fig 4A. The parameters related to cell growth (q_{Glu}, q_{O2} and q_{CO2}) presented a similar behaviour; they increased according to the μ. In contrast, RQ remained constant since the proportion between q_{O2} and q_{CO2} was rather constant. The amount of the 2F5 Fab and its distribution in the different fractions studied, as fermentation broth as well as SCF and IMF of the cell lysates, were compared between the different culture conditions tested (Fig. 4B). As described by other authors, q_P presented a rather linear increase as μ was also increasing; for the highest, it was observed up to an 8-fold increase respect to the lowest (47.0 μg Fab g_X h^{-1} for $\mu = 0.15h^{-1}$ respect to 6.3 μg Fab g_X h^{-1} for $\mu = 0.025h^{-1}$). In contrast, no important effect of the specific growth rate was observed in Fab distribution among the different fractions studied; for all the cases, around 90% of the Fab was secreted, 7.5% and 2.5% was detected respectively in the SCF and the IMF. It is also important to mention that, accordingly to q_{Glu} variation on μ shown in Figure 4A, a significant decrease of the overall biomass yield at low μ was observed, which is due to the higher proportion of maintenance energy requirements respect the total energy available for growth [43].

The cause of this important q_P increase have been discussed by other authors, who attributed this effect to several factors. As described Stadlmayr et al. [36], since the expression of the target protein is driven by the glycolytic *GAP* promoter and its transcription levels are directly related with the glycolytic flux, and thus specific growth rate, an important increase of recombinant protein transcription levels takes place at increasing μ. In addition, Rebnegger et al. [26] studied the effect of different specific growth rates on the transcriptome of *P. pastoris*, describing significant changes in the regulation of important groups of genes at high μ that also contributes to the positive effect in the q_P. Specifically, upregulation of translational and UPR genes such those implied in the translocation to ER, the enhance protein folding in ER and cytosolic chaperones; and downregulation of proteolytic degradation of proteins in the secretory pathway and exocytosis related genes. Thus, although important genes of the secretory pathway are upregulated at high μ, the exocytosis processes may constitute, in fact, the real important bottleneck for the protein secretion. However, from the results presented in this section, there is not a significant intracellular accumulation of protein of interest regardless the specific growth rate, even at high μ's, when the secretory pathways are supposed to be saturated. It may be due to proteolytic degradation processes that take place in the proteasome, considered an important sink for recombinant protein in *P. pastoris* [40]. This protein degradation mechanism is regulated by the quality control that takes places in the ER, in which, while the secreted proteins are released into transport vesicles, the misfolded and the excess of proteins that cannot be secreted are degraded by ER-associated degradation (ERAD) [44,45].

Therefore, it can be concluded that the specific growth rate has an important impact on the total recombinant protein production of this expression system. This parameter regulates the transcriptional levels the target protein, as well as several groups of genes that promote the synthesis, processing and secretion of recombinant proteins expressed under P_{GAP} regulation at high μ's. However, no significant effect could be observed in the balance intra- and extracellular of the product. Therefore, the low fraction of Fab retained intracellularly in any

of the conditions tested does not justify the implementation of the recovery of the non-secreted proteins in a real bioprocess in order to improve its efficiency.

3.3. Implementation of carbon-starving periods within carbon-limited fed-batch processes

The previously described correlation between q_P and μ has been widely exploited in order to reach either the maximum protein titres and/or productivities in fed-batch cultures by the implementation of feeding profiles that allows achieving optimal specific growth rates [11,13,46]. From the different strategies proposed, the pre-programmed exponential feeding rate profile for substrate addition to maintain a very high constant μ in carbon-limiting conditions lead to the best results in terms q_P. Furthermore, it has the advantage to be the simplest to carry out in any standard fed-batch cultivation system.

On the other hand, as mentioned in the introduction section, Kern et al. [27] described an important productivity increase of proteins regulated by P_{GAP} for a short-time after the depletion of glucose. However, the work did not hypothesize about the causes that could lead to the rapid increase of protein synthesis during short periods and the potential applications on bioreactor cultures. Thus, this work aimed to study the implementation of short carbon-starving periods in fed-batch cultivations based on pre-exponential feeding rate profiles at high specific growth rates and its effect on the recombinant protein production rates and yields, as well as its distribution among the different cell fractions analysed. From the particular characteristics of this exponential feeding rate profile, no glucose accumulation in the culture broth should be expected. Thus, it can be considered that, rapidly, all glucose fed is totally consumed. Consequently, a period in which the glucose feeding is stopped results into carbon-starving conditions for the cells.

Fig. 5 presents the basic features of the implementation of 30 min carbon-starving periods every 3 h of standard pre-exponential feeding profile. In Fig. 5a the innovative profile is set side by side with the conventional strategy. In addition, q_P profiles are also compared, from which it can be observed that during the periods when the feeding is stopped, and thus the cells are subjected to carbon-starving conditions, there is a very important increase of the

specific production rate, the effect that was aimed to be exploited in this work. In Fig. 5b the time profile of fed glucose and Fab titration are compared showing that the total amount of glucose added in to the system is equivalent as ending criteria used, but in a longer time; in contrast, the production of Fab is significantly higher, which supposes a very important increase in the substrate to product yield.

Therefore, the objective was to compare different combinations of carbon feeding and starvation periods in order to determine the strategy that leads to the maximum increase production at the end of the fed-batch. The different feeding strategies carried out are presented in Table 1, in which were compared feeding periods of 1.5 and 3 h, as well as carbon-starving periods of 0.5 and 1 h. For all the cases, the imposed ending criterion, which allows a systematic comparison among the feeding profiles, was considered the total amount of glucose fed into the system. The different feeding strategies did not affect to the growth parameters of *P. pastoris*. After the starving periods the yeast started to consume the glucose added immediately and the substrate to biomass yield was not affected, thus the final amount of biomass achieved was always around 100 g DCW L^{-1}. In Fig. 6 are plotted the secreted Fab production time profiles of the different strategies tested, the main production parameters of the system are also presented in Table 2. All the new strategies implemented achieved important increases of Fab production in terms of product titration in the broth, as well as in product to substrate ($Y_{P/S}$) and product to biomass ($Y_{P/X}$) yields. The range of increases was between 15% for the strategy A and more than 40% for the strategy C. However, in terms of specific production rate, the results obtained applying the innovative feeding strategy were not always better respect to the conventional pre-exponential feeding profile. The strategies B and C lead to moderate specific production rate enhancements, between 5 and 10%. On the other hand, for the strategy concerning the longest carbon-starving periods (Strategy A, 1 h of starvation), even though the total Fab produced was significantly higher, its productivity rates were significantly lower. This fact is due to increased bioprocess times because of the carbon-starving periods. When cell productivity during the carbon-starving periods is not higher than in the growing periods, the overall bioprocess productivity levels will end up being lower.

Considering all the compared production parameters, strategy C, based on the combination of 1.5 h of feeding followed by 0.5 h of starvation, lead to the best results. The production increment presented can be considered as an important operational improvement for the P_{GAP}-based expression system. A similar intermittent feeding strategy was previously proposed by Heo et al. [47] for fed-batch cultivations of the yeast *Hansenula polymorpha* presenting positive results respect to the conventional feeding profiles.

In addition, the amount of Fab secreted to the cultivation broth during the carbon-starving periods was monitored in order to be able to describe the evolution of product increase along the period within glucose is depleted. This monitoring was performed during all the starving periods of the feeding strategy A, therefore non-fed periods of 1 h. The average of the time-evolution increase of Fab secreted is presented in Fig. 7, where the increment every 20 min is represented in percentage respect to the total increase of secreted Fab during the studied period. The observed curve can be fitted to a first-order kinetics in which an important fraction of the total secreted Fab during the whole period is released during the first 20 min, corresponding to the time constant of the first-order system. Consequently, in order to achieve the best results for all the productivity rates, the carbon-starving periods longer than 30 min should be avoided since the increase of production after 30 min of no feeding is relatively small.

The distribution of the Fab expressed by the recombinant *P. pastoris* strain among the different cell fraction mentioned above in previous sections (SCF and IMF) was also determined for fed-batch cultures implementing this innovative strategy. In this case the aim was to determine the cause of the Fab increment observed in order to identify if the Fab increase detected in the broth during the non-fed period is due to the synthesis of new protein, or to the release of protein previously expressed during the growing phase but retained intracellularly as a result of a possible saturation of the secretory pathways. The distribution of Fab among the different cell fractions and the gradual increase of Fab levels along the successive carbon-starving periods of the fed-batch cultivation performed applying the feeding strategy C is shown in Fig. 8. Although moderate increases of Fab levels were

detected after each starving period, no significant differences were observed on the extra/intracellular distribution of Fab.

Therefore, it could be concluded that the increase of secreted Fab is due to the fact that during the non-fed period the yeast is still able to synthetize recombinant proteins driven by P_{GAP} upon short-time depletion of glucose. It has been widely reported that yeasts are sensitive to the level of nutrients such carbon and nitrogen sources, which allows them to adapt readily to changing nutritional states [48,49]. Early studies in the field described an overall decrease of protein synthesis levels for carbon-starving conditions due to a global inhibition of the translational capacity of the cells [50]. Nevertheless, recent works have concluded that, although the overall expression levels of proteins is reduced, combined complex mechanisms of transcription and translation regulation lead to an expression increase of many genes whose products promote adaptation to low glucose environments [51,52]. Thus, even though cells starving are likely to have lower flux through the glycolysis due to the lack of glucose in the medium, the transcription and translation levels of the glycolytic genes could be upregulated in order to be able to give a fast and effective adaptive response to the low glucose environment. This mechanism could be understood as a cell response to preserve the capacity for rapid resumption of growth due to a possible glucose return or the starvation signal turn out to have been a false alarm [51]. Accordingly, since the glycolytic *GAP* promoter regulates the expression of the product, the productivity of the recombinant protein could be also upregulated in carbon-starving conditions. Similar production increases of P_{GAP}-regulated proteins were described by Baumann et al. under hypoxic conditions [53,54],

As a conclusion, the implementation of carbon-starving periods in fed-batch cultivations can be considered as a truly step forward in the optimization of recombinant protein production processes using *P. pastoris*. In general, the optimization of these bioprocesses depends mainly on the relationship between product formation and biomass growth. The interdependence of these factors is of key importance regardless of the selected criterion for the optimization, both maximizing yield and productivity [7]. In fact, in large-scale industrial fermentation processes, biomass is frequently considered as an unavoidable waste product but essential,

due to most of microbial production processes are growth-associated. Furthermore, as in the case described in this work, very often the higher specific production rates are obtained at higher specific growth rates [55]. In contrast, it must also be taken into account that the biomass growth in a bioprocess is limited, which may be due to biological and different physical restrictions, mainly heat and mass transfer. Often oxygen availability is the most important limitation for aerobic processes [56]. Thus, lower growth rates lead to longer fermentation times, and therefore, more product may be generated before the process is stopped due to system limitations, whenever the specific production rate do not decrease drastically within the operation range for μ. In fact, a compromise between higher q_P and lower μ arises when productivity and/or product yield are the performance indexes to be optimized. High values for these indexes result in the reduction of capital and operating costs. The trade-off between yield and productivity is key in the design of a bioprocess and its optimal performance.

Consequently, some authors have proposed different approaches to deal with this scenario. For the system based on P_{GAP}, Maurer et al. [13] implemented an optimal trajectory for feeding rate that, by controlling the specific growth rate, maximizes the volumetric productivity with also suitable product yields. Alternatively, Buchetics et al [25], aimed to improve the ratio between q_P and μ, especially for lower μ, by engineering the producer strain. On the other hand, for processes based on the *AOX1* promoter, which have been extensively used, different alternatives for optimizing the production of some recombinant proteins can also be found in the literature [57–59].

In the present work it has been proposed a non-complex fermentation strategy that makes possible at the same time the growth of the yeast at very high specific growth rates and to prolong the bioprocess. Initially, significant increase of the q_P has been described during short carbon-starving periods respect to fed phases. Therefore, by exploiting this effect, important overall increases both in productivity and, especially, in total amount of product formation and yields ($Y_{P/S}$, $Y_{P/X}$) have been described for the bioprocess. This innovative strategy can be considered as an important operational improvement for the P_{GAP}-based expression system. In

addition, it has been proposed that the rise of recombinant protein production achieved with this novel strategy is due to the yeast adaptive response to the environmental stress that leads to higher synthesis rates of proteins regulated by the *GAP* promoter during short periods of carbon starvation, and not to effects related with the saturation of the secretory pathways.

Acknowledgements

This work was supported by the project CTQ2013-42391-R of the Spanish Ministry of Economy and Competitiveness and the grant FPU (X.G.) of the Spanish Ministry of Education and Science. The group is member of 2014-SGR-452 and the Reference Network in Biotechnology (XRB) (Generalitat de Catalunya).

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Figure legends

Fig. 1. Cell disruption of *P. pastoris* cells by high-pressure homogenisation. **A:** Cell counting by flow cytometry used to determine the extent of cell disruption after different passes; ellipse gate differentiates the whole undamaged cells from damaged and broken cells. **B:** Comparison between extent of cell disruption and amount of 2F5 Fab released after different passes. Error bars indicate standard deviation.

Fig. 2. Release of 2F5 Fab obtained by using the buffers and detergents compared. Error bars indicate standard deviation.

Fig. 3. 2F5 Fab obtained by using different concentrations of CHAPS and incubation times. Error bars indicate standard deviation.

Fig. 4. Steady states of chemostat cultures at different specific growth rates. **A:** Main growth parameters and rates: glucose uptake rate (q_{Glu}; **O**); oxygen uptake rate (q_{O2}; △); carbon dioxide production rate (q_{CO2}; ▲); respiratory quotient (RQ; □) and total Fab production rate (q_P, ●). **B:** Fab distribution among the different fractions studied: Total 2F5 synthetized (●); 2F5 Fab secreted to the broth (**O**); 2F5 present in the soluble cytosolic fraction (SCF; ▲); 2F5 present in the insoluble membrane fraction (IMF; △).

Fig. 5. Main features of the implementation of 30' carbon-starving periods every 3 h of exponential feeding respect to the standard exponential feeding profiles in fed-batch cultivations at nominal specific growth rate μ= 0.15 (h^{-1}). **A**: Time evolution of the specific production rate of 2F5 Fab (q_P) versus the feeding rate time profile. Solid lines indicate mean specific rates calculated within feeding and starving periods. **B:** Comparison of total 2F5 Fab produced and fed glucose time profile.

Fig. 6. Time profile of the total amount of 2F5 Fab secreted to the fermentation broth by using the different feeding strategies compared. Standard strategy (△); Strategy A (**O**); Strategy B (▲) and Strategy C (●).

Fig. 7. Time evolution average of the 2F5 Fab secreted during the successive carbon-starving

periods. Error bars indicate standard deviation.

Fig. 8. Cell fraction distribution of the 2F5 Fab produced before and after 30 min carbon-starving periods.

Table 1: Summary of the different feeding strategies implemented in fed-batch cultivations grown at nominal specific growth rate $\mu = 0.15$ (h^{-1}).

Strategy	**Feeding time periods** (h)	**Starving time periods** (h)	**Total fed-batch time** (h)
Standard	-	.	15.5
A	3	1	20.4
B	3	0.5	18.1
C	1.5	0.5	20.4

Table 2: Main production parameters obtained by applying the different feeding strategies compared.

Strategy	Standard	A	B	C
Feeding time (h)	15.5	20.4	18.1	20.4
Fab production (mg Fab)	90.4	105	110	131
Fab production increase (%)	-	15.7%	21.8%	44.8%
$\mathbf{q_P}$ (µg Fab g_X^{-1} h^{-1})	35.2	31.4	37.1	36.8
$\mathbf{q_P}$ **increase** (%)	-	-10.9%	5.40%	4.55%
$\mathbf{Q_P}$ (mg Fab V^{-1} h^{-1})	0.73	0.69	0.83	0.95
$\mathbf{Q_P}$ **increase** (%)	-	-6.20%	12.73%	29.53%
$\mathbf{Y_{P/S}}$ (mg Fab g_S^{-1})	0.122	0.146	0.152	0.173
$\mathbf{Y_{P/S}}$ **increase** (%)	-	19.6%	24.7%	41.9%
$\mathbf{Y_{P/X}}$ (mg Fab g_X^{-1})	0.245	0.282	0.294	0.359
$\mathbf{Y_{P/X}}$ **increase** (%)	-	15.0%	20.0%	46.6%

Figure 1a

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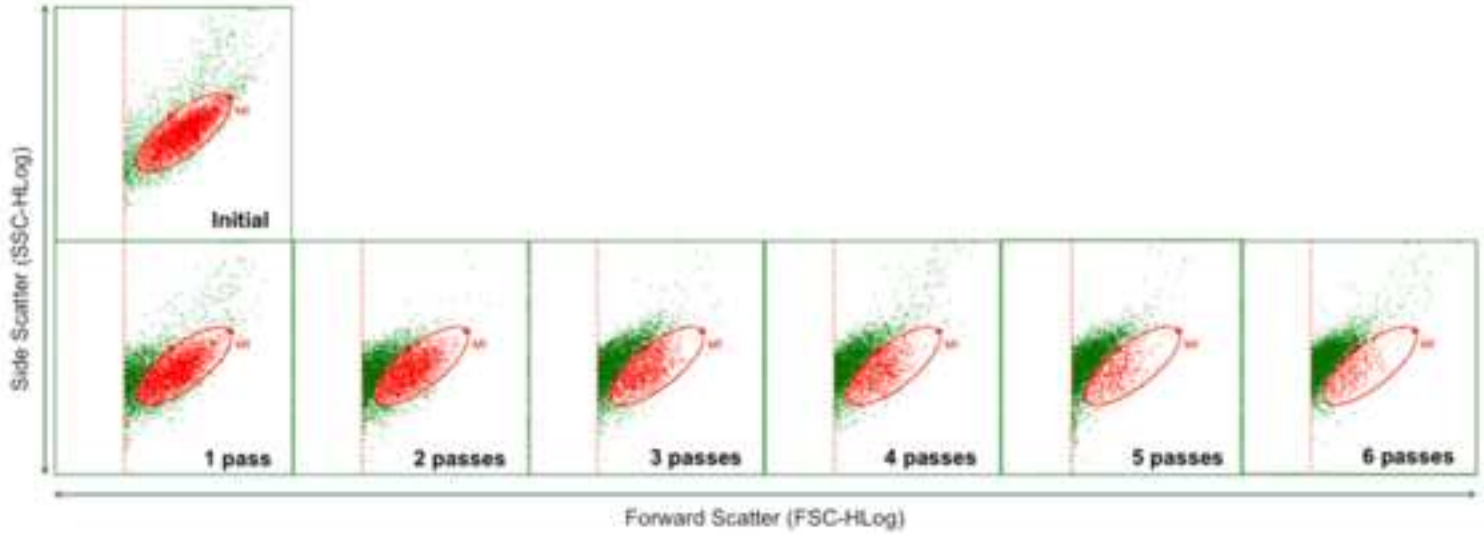

Figure 1b

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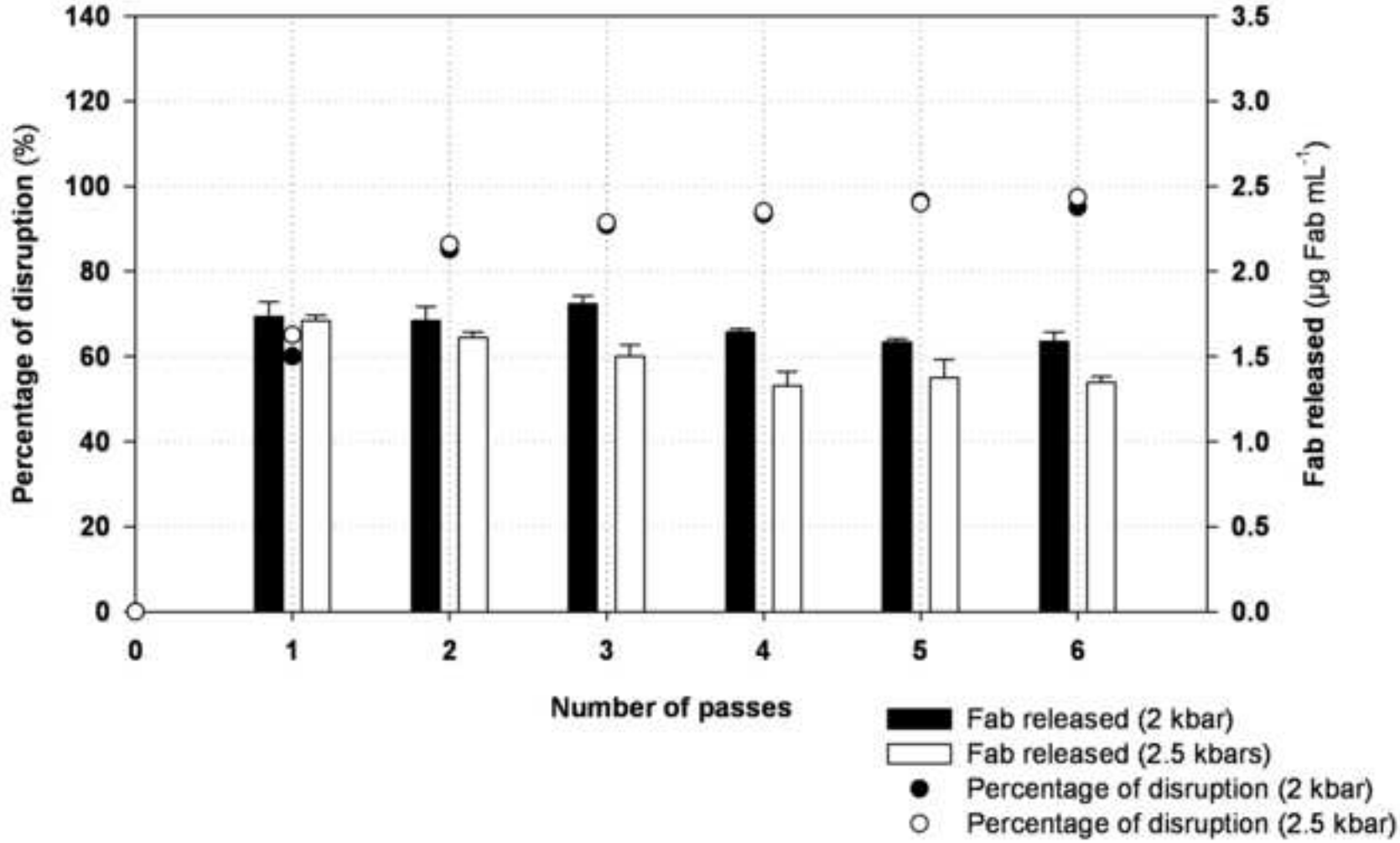

Figure 2

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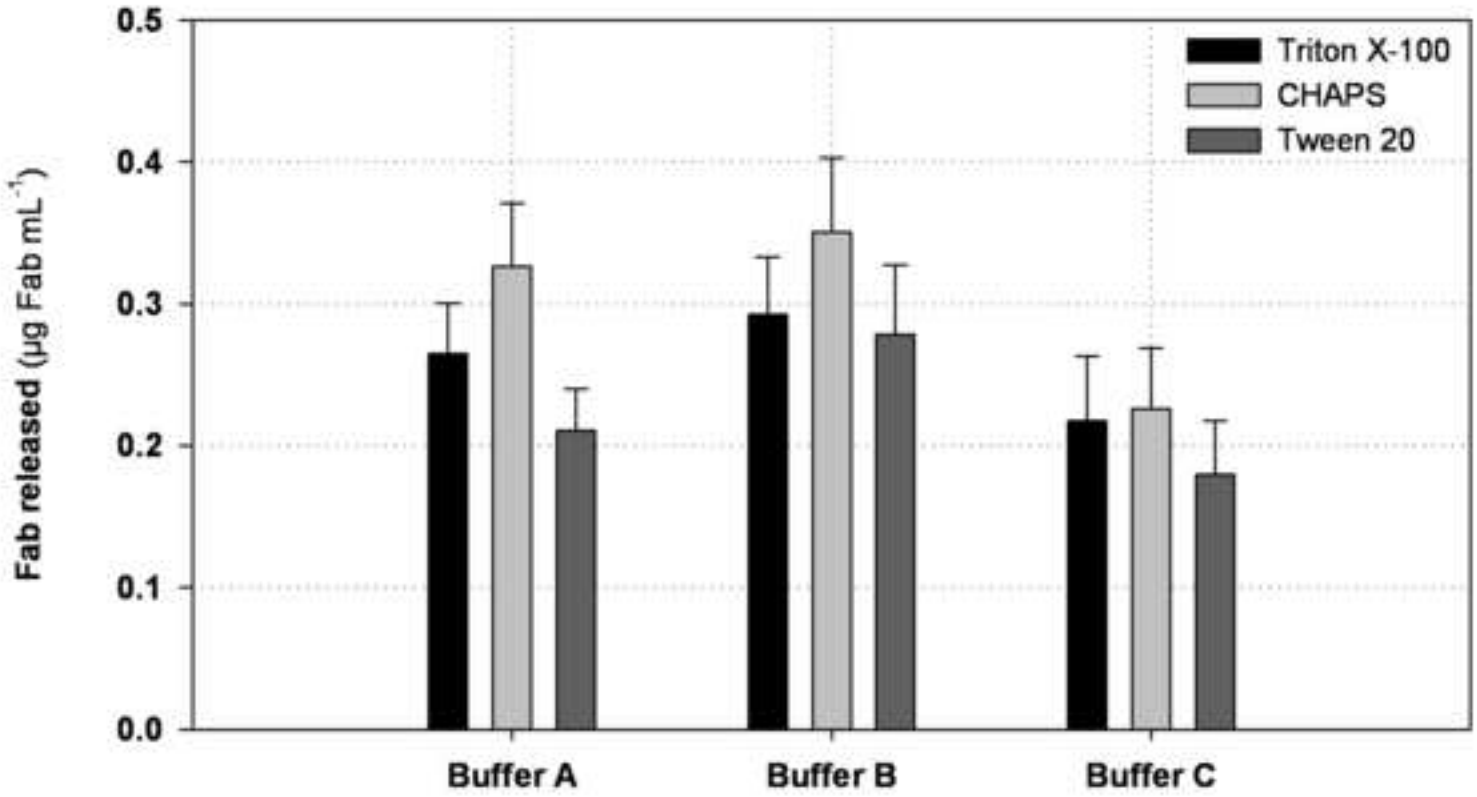

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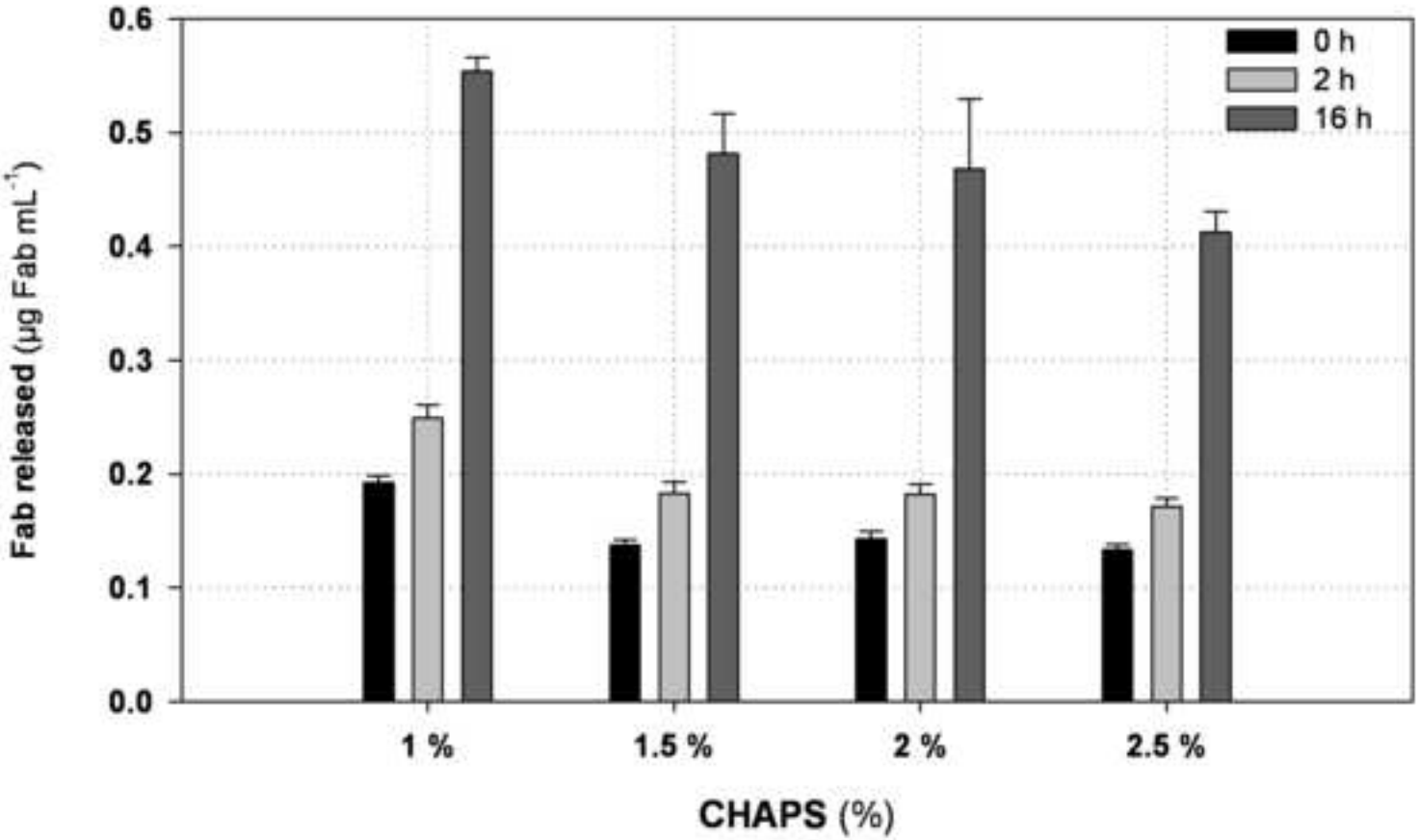

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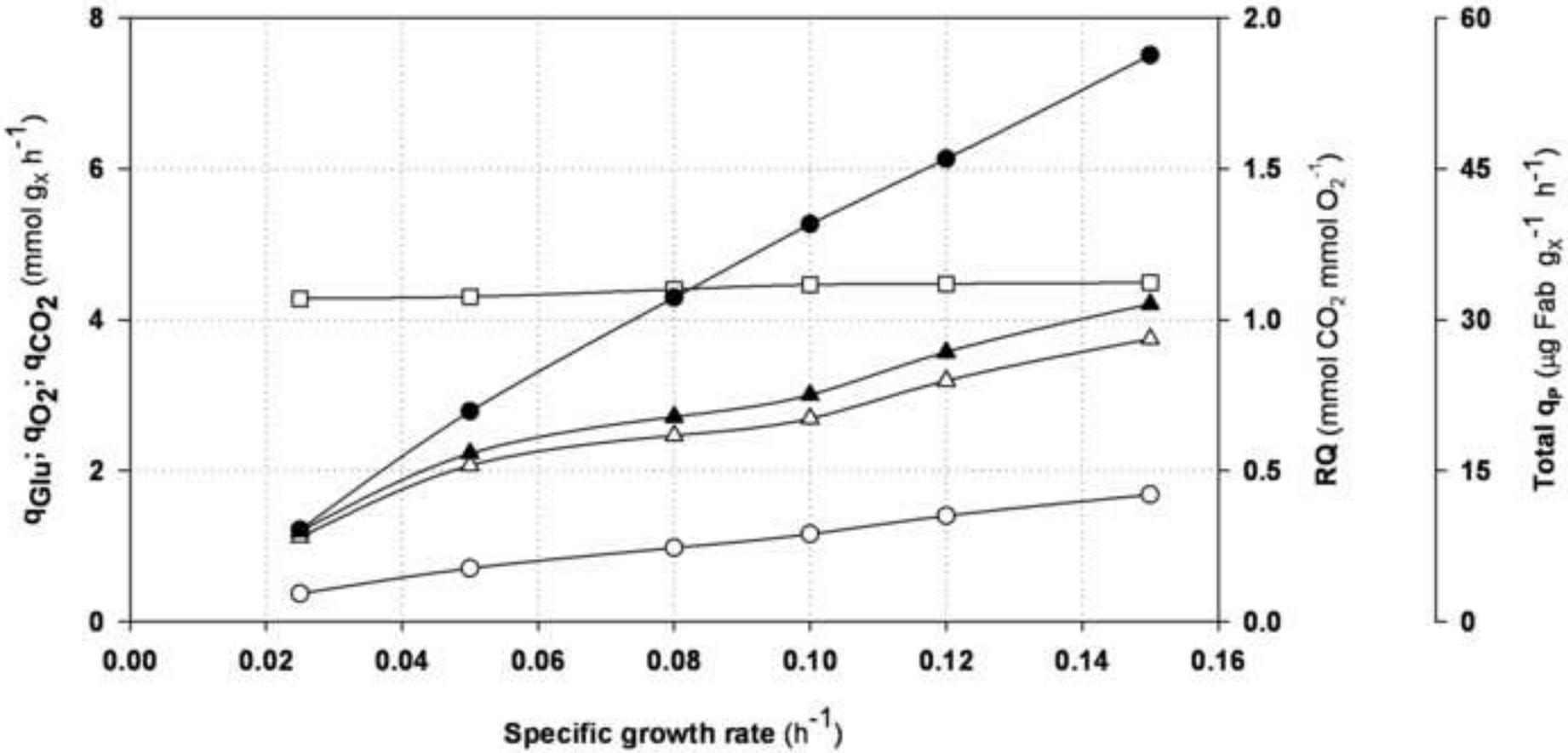

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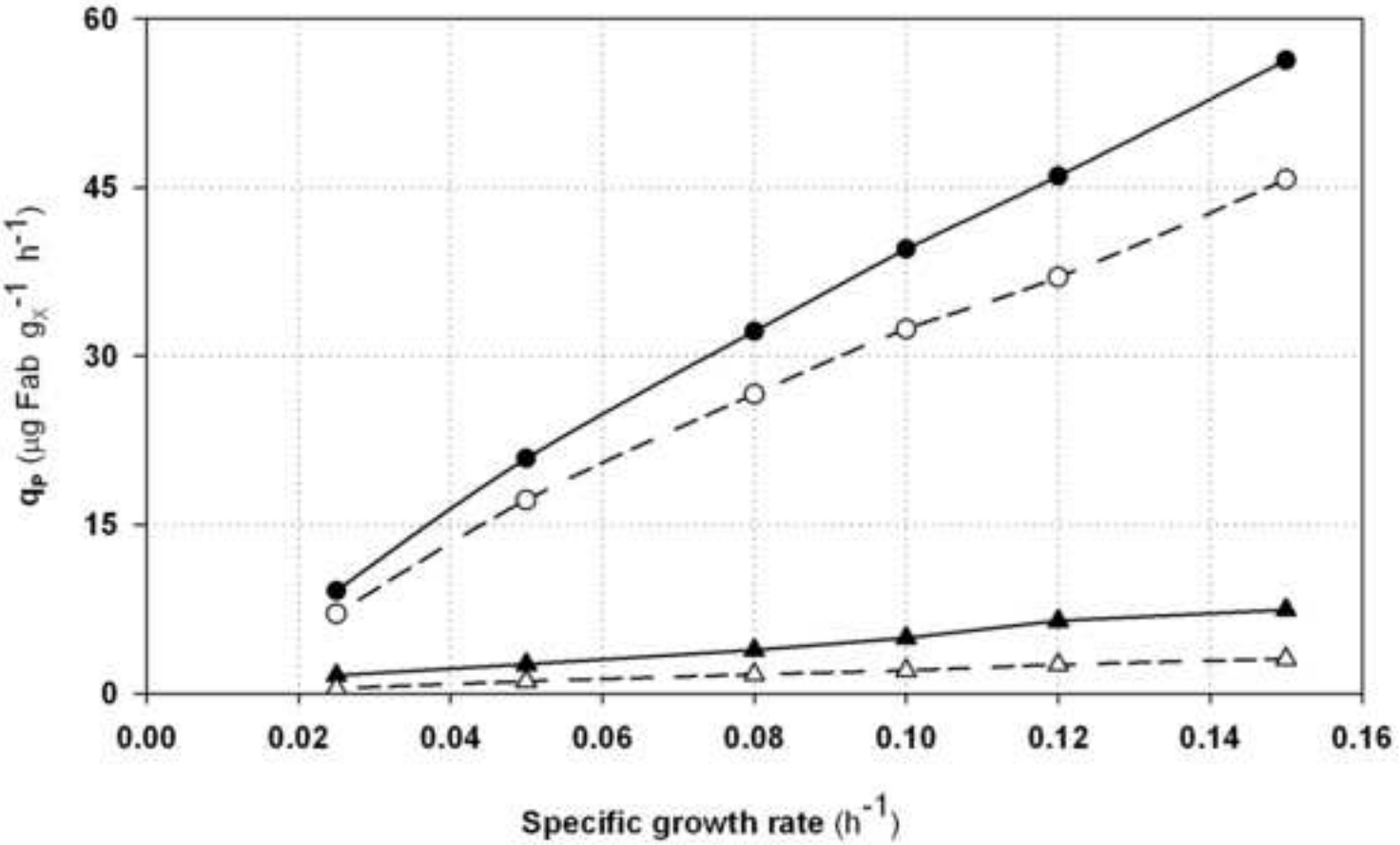

Figure 5a

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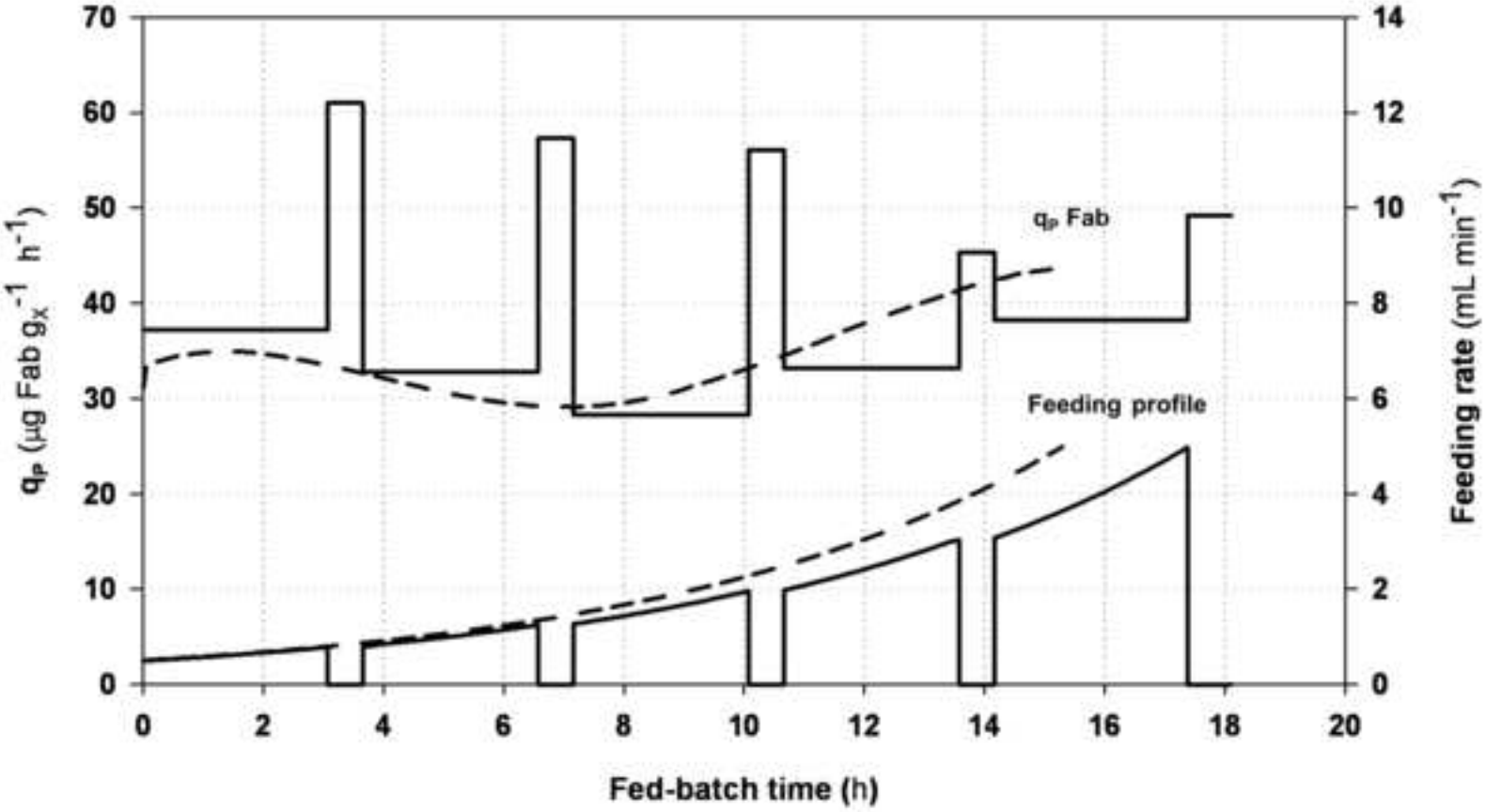

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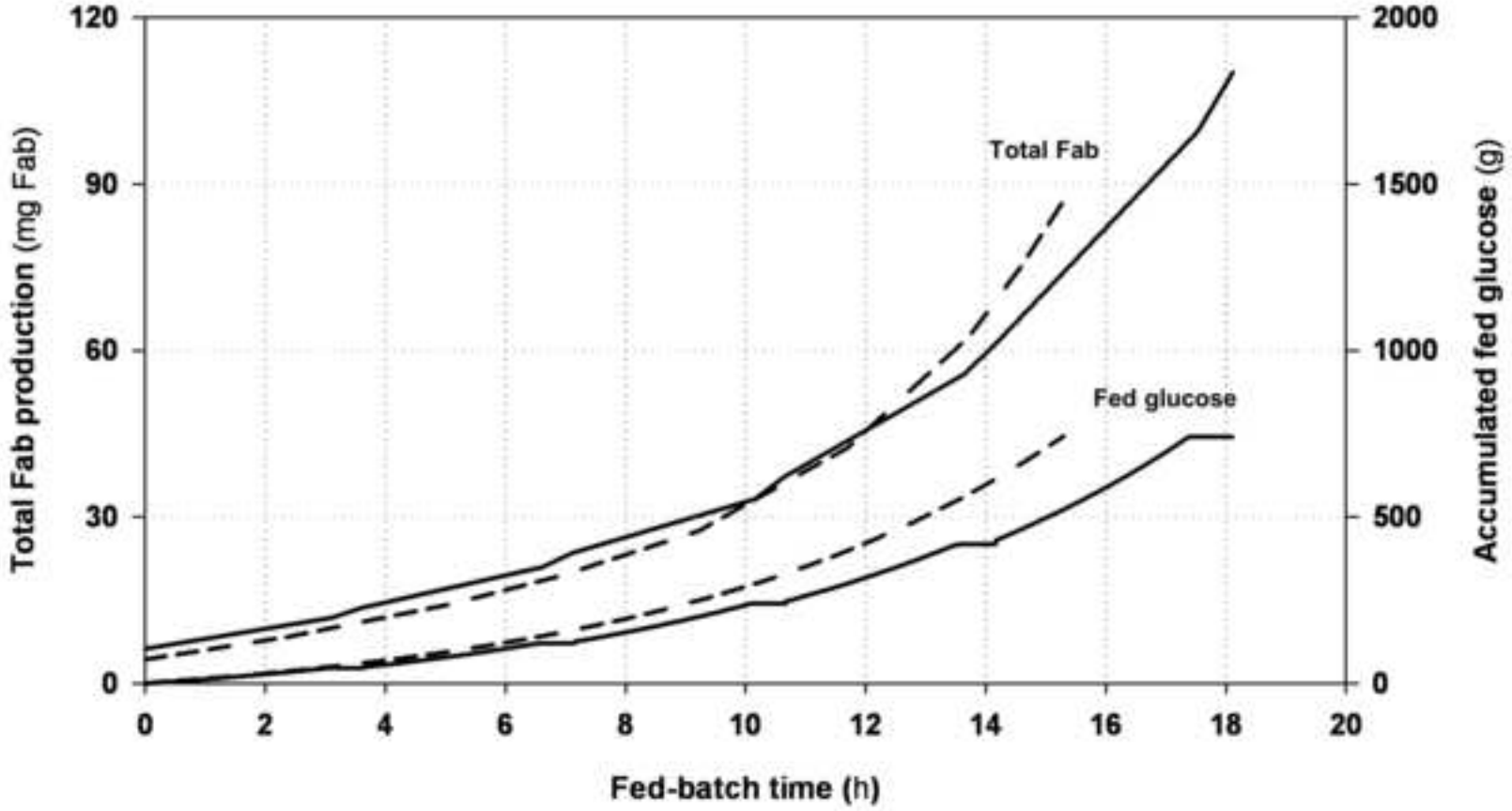

Figure 6

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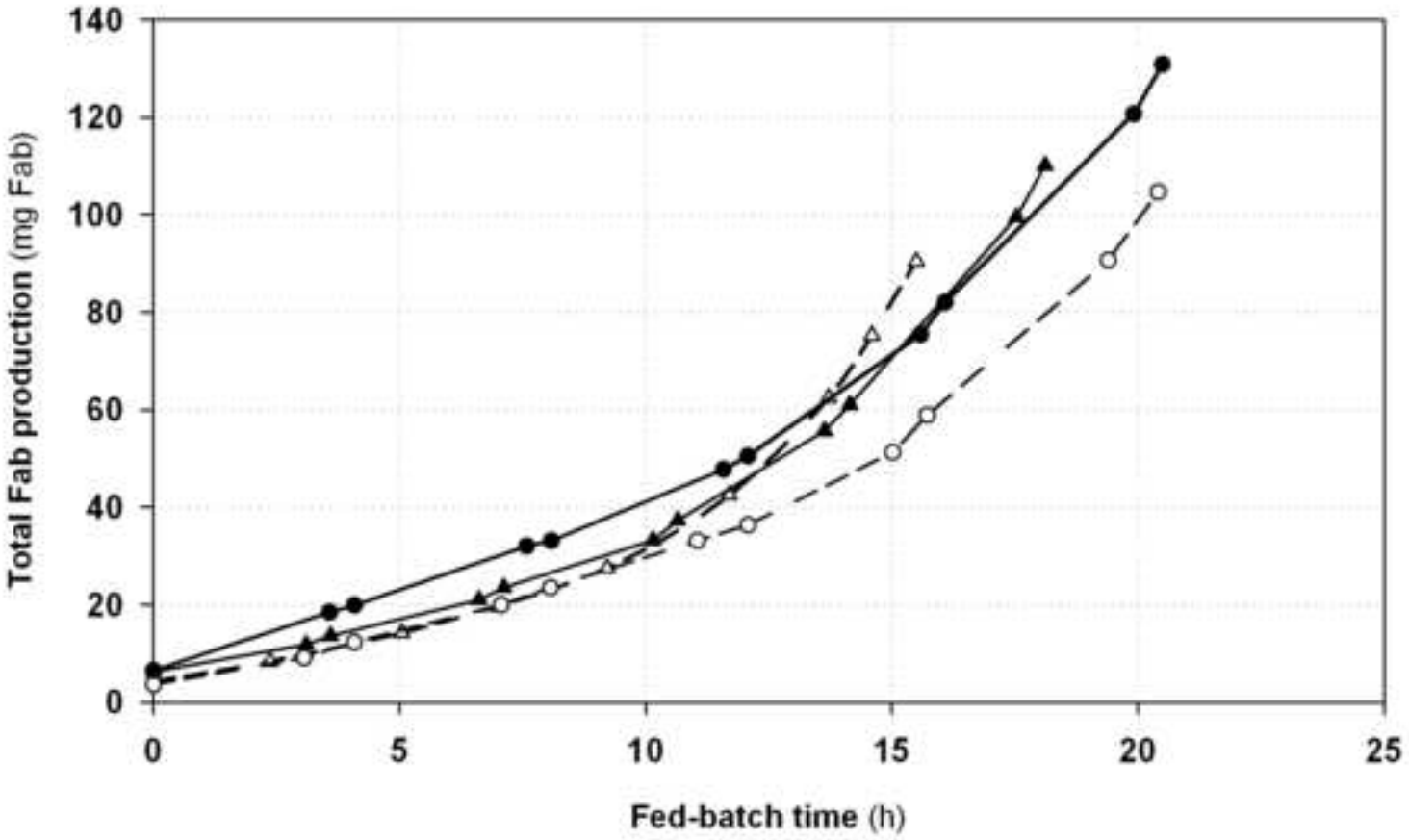

Figure 7

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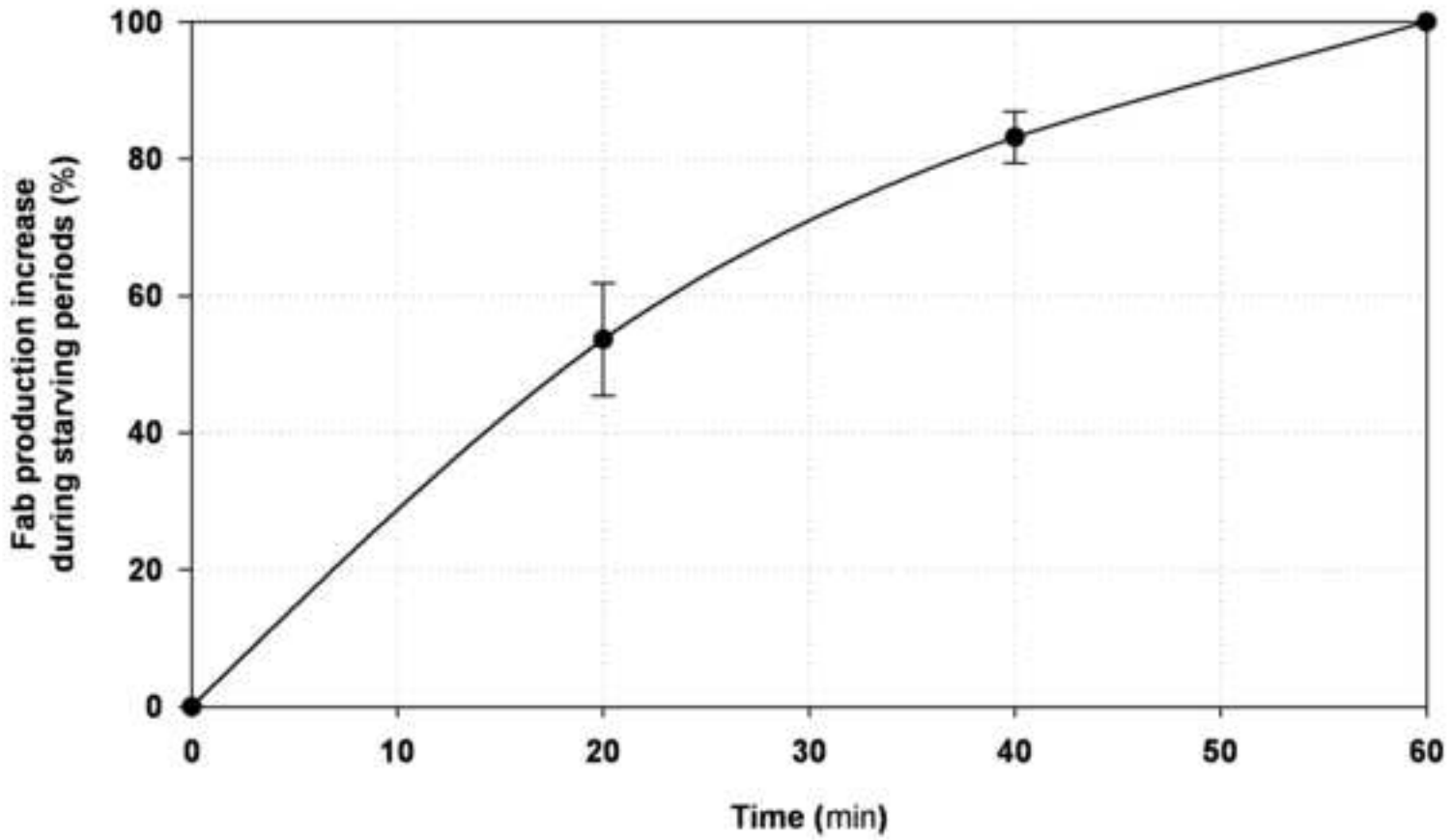

Figure 8

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