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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.

1. Introduction

Currently, a wide range of products such as 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,

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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 cultivations 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., [13]) 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 bar; 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 4 °C 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 °C, 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 $OD_{600} \approx 125$ were harvested by centrifugation (4500 *g*, 3 min, 4 °C) 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, 1 mM 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 °C). Supernatants were collected and stored as soluble cytosolic fraction (SCF) while pellets were kept as the insoluble membrane fraction (IMF). The whole disruption process was carried out at low temperature in order to preserve protein properties as well as to avoid possible protease activity. In addition, the PMSF added in the breaking buffer was used as a protease inhibitor.