Developing High Cell Density Fed-Batch Cultivation Strategies for Heterologous Protein Production in *Pichia pastoris* Using the Nitrogen Source-Regulated *FLD*1 Promoter

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Abstract: A *Pichia pastoris* strain expressing a *Rhizopus oryzae* lipase gene under the transcriptional control of the promoter from the *P. pastoris* formaldehyde dehydrogenase 1 gene (P*FLD*) was utilized to study the feasibility of this expression system for recombinant protein production using methanol-free fed-batch high cell density cultivations. We have developed a simple and reliable fed-batch strategy using the P*FLD* system based on the use of methylamine and sorbitol as nitrogen and carbon sources, respectively, for the induction phase. Three different fed-batch fermentations were performed at three different constant growth rates, i.e., at a low growth rate (0.005/h), at an intermediate growth rate of (0.01/h), and at a constant residual sorbitol concentration of 8 g/L, i.e., allowing cells to grow at high (near μ_{max}) growth rate (0.02/h). Important differences were observed between the lower and higher growth rate cultivation phases in terms of specific production rate (q_p) profiles. In all three cases, maximum q_p were reached soon after the start of the induction phase; after that maximum, an exponential decrease reaching final values close to zero were observed, except for the cells growing at near μ_{max}. The best results in terms of $Y_{P/X}$, productivity and specific productivity were obtained when the microorganism was growing at the highest growth rate. Furthermore, such results were significantly better in relation to those obtained with the P*AOX*-based system expressing the same protein. © 2005 Wiley Periodicals, Inc.

Keywords: *Pichia pastoris*; fed-batch cultivation; formaldehyde dehydrogenase promoter; *Rhizopus oryzae* lipase

INTRODUCTION

The methylotrophic yeast *Pichia pastoris* is a host system that has become a successful choice for heterologous protein expression, both for academic and industrial purposes. In the recent years, more than 500 proteins have been cloned and expressed using this system (http://faculty.kgi.edu/cregg). Furthermore, it has been selected by several protein production platforms for structural genomics programs (Heinemann et al., 2003; Prinz et al., 2004; Yokoyama, 2003). A key advantage of *P. pastoris* as a host system is that it combines the unique capacity of growing in minimal medium at high cell densities with low levels of endogenous protein secretion and the ability to efficiently secrete heterologous proteins, i.e., simplifying their recovery. Also, it performs many of the higher eukaryotic post-traductional modifications as protein folding, proteolytic processing, disulfide bond formation and glycosylation (Lin Cereghino and Cregg, 2000).

In addition, the availability of the alcohol oxidase 1 promoter (P*AOX*) supports strong and tightly regulated heterologous protein expression. In spite of the high efficiency of this promoter, in some cases its use may be not appropriate, for instance, in microscale or in small cultures such a shake flasks. In these cases, the use of methanol may be a problem due to its volatility. Also, methanol metabolism has a high oxygen demand and, therefore, heterologous protein expression can be affected by the limited oxygen transfer that may take place under these methanol-growing conditions.

In the recent years, some efforts have been made to circumvent the use of methanol. The glyceraldehyde 3-phosphate dehydrogenase promoter (P*GAP*) has been used for constitutive expression of several heterologous proteins (Waterham et al., 1997). This promoter has been successfully used to express some proteins, but its use is not convenient if the expression of the heterologous protein may be toxic for the cells. On the other hand, the cloning and characterization of the formaldehyde dehydrogenase gene and promoter has been reported (Shen et al., 1998). The *FLD1* gene codes for an enzyme that plays an important role in the methanol catabolism as carbon source, as well as in the methylated amines metabolism as nitrogen source.

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In the methanol assimilation pathway (Harder and Veenhuis, 1989), the first oxidation step takes place in the peroxisome, where the alcohol oxidase generates formaldehyde and hydrogen peroxide from methanol. The hydrogen peroxide is degraded by a catalase and a fraction of the formaldehyde is assimilated by the xylulose monophosphate cell biosynthetic pathway. The other portion leaves the peroxisome and it is oxidized in the cytosol via formate to carbon dioxide by a formaldehyde dehydrogenase and a formate dehydrogenase.

In addition to methanol metabolism, FLD is also involved in the assimilation of some C_1-amines such methylamine as nitrogen source (Harder and Veenhuis, 1989). The oxidation of methylamine takes place in the peroxisome by a methylamine oxidase, generating formaldehyde, hydrogen peroxide, and ammonium ions. This formaldehyde can, in principle, be oxidized to carbon dioxide or assimilated to biomass following the same pathways involved in methanol metabolism. However, yeast cannot use methylamine as a sole carbon and nitrogen source, and therefore, a supplementary source of easily metabolized carbon must be provided for sustained growth. The reason for this inability to exploit the methylamine carbon residue is unclear, but is probably related to the observation that the methylamine oxidase synthesis is strongly repressed by ammonia (Harder and Veenhuis, 1989). The presence of an assimilation pathway of the methylamine carbon has been demonstrated in the yeast *Hansenula polymorpha* growing in glucose and methylamine (Jones and Bellion, 1991).

Initial characterization studies showed that the *FLD1* promoter (P*FLD*) from *P. pastoris* was strongly and independently induced either by methanol as carbon source or methylamine as nitrogen source (Shen et al., 1998). Recently, Resina et al. (2004) performed batch cultivation experiments in a bioreactor using a *P. pastoris* strain expressing a *Rhizopus oryzae* lipase gene under the transcriptional control of the P*FLD*. Sorbitol and methylamine were used as carbon and nitrogen sources, respectively. Levels of the heterologous lipase secreted into the extracellular medium were comparable to those obtained when the lipase gene was expressed under the classic P*AOX*. These results strongly indicated that sorbitol, a carbon source that does not repress the synthesis of methanol metabolism enzymes (Thorpe et al., 1999), also allows induction of the P*FLD* by methylamine. This suggested that the use of sorbitol as carbon source combined with methylamine as nitrogen source could be the basis for the development of methanol-free fed-batch fermentation processes for heterologous protein production in *P. pastoris* based on the P*FLD*.

Although several fed-batch cultures control strategies for P*AOX*-based *P. pastoris* systems have been described, e.g., those based on the on-line measurement of the residual methanol concentration in the cultivation broth (Chung, 2000; Trinh et al., 2003; Zhang et al., 2002), or those based on the dissolved oxygen (DO) spike method or preprogrammed linear feed rates (Rodríguez-Jiménez et al., 1997), to our knowledge, there are no substrate addition strategies for heterologous protein production fed-batch processes using P*FLD*-based *P. pastoris* systems.

Hence, the major objective of this study has been the development of a simple, methanol-free, fed-batch strategy based on a pre-programmed exponential feeding of sorbitol and methylamine solution at a stoichiometrically fixed ratio aiming to maintain the specific growth rate constant during the induction phase. By using this strategy, we have studied the behavior of the system in terms of productivity and overall efficiency under different growth conditions. In addition, this study allowed us to assess the feasibility of our methanol-free fed-batch strategy and compare it to the conventional one used for P*AOX*-based systems.

MATERIALS AND METHODS

Strains

A *P. pastoris* X-33-derived strain containing the expression vector pPICZFLDαROL (Resina et al., 2004) integrated in its genome *FLD1* locus was used throughout this study.

Culture Maintenance

P. pastoris strains were grown on YPD agar medium plates containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2%(w/v) agar and stored at 4°C. Long term stocks were prepared as recommended by Invitrogen (http://www.invitrogen.com) and deep frozen at −80°C.

Inoculum Preparation

Pre-inocula for shake-flask and bioreactor cultures were grown for 24 h in baffled shake flasks at 30°C, 250 rpm, in BMGY (buffered glycerol-complex medium) containing 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate, pH 6.0, 4×10^{-5} % (w/v) biotin, 1% (w/v) glycerol in 50 mL of final volume. Cells were centrifuged at 4,000*g* and resuspended in the required fresh medium to inoculate shake flask cultivations. Alternatively, the 50 mL culture was used to inoculate a 500 mL (final working volume) of BMGY in a 1-L top-bench bioreactor (Braun Biotech, Melsungen, Germany). The culture was grown overnight at 30°C and subsequently was centrifuged at 4,000*g*. Harvested cells were resuspended in bioreactor culture medium and used to inoculate a 5-L Biostat ED bioreactor (Braun Biotech, Melsungen, Germany).

Fed-Batch Cultivation Set Up and Operational Conditions

Bioreactor fed-batch cultivations were carried out using a mineral medium with the following basal composition per liter: for the batch phase, KH_2PO_4 12.0 g, $MgSO_4 \cdot 7H_2O$ 4.70 g, $CaCl_2 \cdot 2H_2O$ 0.36 g, 0.1 mL of antifoam, alkoxylated ester JG73 (Strucktol, Hamburg, Germany), 1 mL of a biotin solution (400 mg/L), and 1 mL of trace salts solution (0.2 mM

$CuSO_4 \cdot 5H_2O$, 1.25 mM KI, 4.5 mM $MnSO_4 \cdot 4H_2O$, 2 mM $Na_2MoO_4 \cdot 2H_2O$, 0.75 mM H_3BO_3, 17.5 mM $ZnSO_4 \cdot 7H_2O$, 44.5 mM $FeCl_3 \cdot 6H_2O$). The biotin and trace salts components were sterilized separately by microfiltration (Millex GS 0.22 μm, Millipore, Malsheim, France).

Fed-batch cultures were performed in a 5-L Braun Biostat ED bioreactor (Braun Biotech, Melsungen, Germany), with an initial working volume of 3.5 L. The cultivation process comprised three phases: firstly, a batch growth phase was performed using glycerol (40 g/L) as sole carbon source and ammonium sulfate at the corresponding stoichometric quantity (9.2 g/L) as sole nitrogen source. Secondly, after glycerol depletion, a batch of 10 g/L of sorbitol and 3 g/L of methylamine ($CH_3NH_2 \cdot HCl$) was added into the bioreactor to induce the P*FLD*. This transition phase lead to the third phase of the cultivation, i.e., the induction phase, which consisted in the exponential feed of sorbitol and methylamine as sole carbon and nitrogen sources, respectively. The feeding stock solution contained 300 g/L of sorbitol and 35 g/L of methylamine, and was added to the reactor by an automatic micro burette MicroBU-2031 from Crison Instruments (Alella, Barcelona, Spain). The cultivation conditions were: stirring rate 800 rpm, temperature 30°C, pH controlled at 5.5 by adding 5M KOH, dissolved oxygen controlled above 30% with an air flow rate between 1.5–20 L/min.

Glycerol, Sorbitol, Methylamine, and Ammonium Determination

Glycerol and sorbitol was determined by HPLC with a HP 1050 liquid chromatograph (Hewlett Packard, Palo Alto, CA) using an Aminex HPX-87H ion-exchange column from Bio-Rad, Hercules, CA. The mobile phase was 15 mM sulfuric acid, injection volume was 20 mL. Data was quantified by the Millenium 2.15.10 software (Waters Corporation, Mildford, MA). Methylamine was analyzed by HPLC (Hewlett Packard 1090) with UV-Vis diode array detector using a Hypersil AA-ODS column (Hewlett Packard) with a Hypersil ODS Guard pre-column. Solvent A: 20 mM sodium acetate, 0.3% (v/v) tetrahydrofurane (THF), 0.018% (v/v) triethilamine (TEA); Solvent B: 100 mM sodium acetate, 40% (v/v) methanol, 40% (v/v) acetonitrile.

The ammonium concentration was measured using a colorimetric method (LCK302 kit, Dr. Lange, Düsseldorf, Germany) with a RSD of about 8% in the concentration range used.

Lipase Activity Determination

Lipase activity determination was carried out using the Lipase colorimetric assay (LIP kit, reference no. 1821792, from Roche Diagnostics, Mannheim, Germany), as described in Resina et al. (2004).

Extracellular lipase activity was measured after removing cells from cultivation samples by centrifugation in a microcentrifuge at 12,000*g*. Intracellular lipase activity (defined as the soluble fraction of the cell bound lipase activity) was measured from cleared supernatants from cell lysates. Cells were mechanically disrupted as follows: cells were harvested by centrifugation, washed once in PBS buffer and further resuspended in lysis buffer (PBS plus Protease Inhibitor Cocktail Tablets, Roche Diagnostics). Resuspended cells were mechanically disrupted using a Constant Systems cell disrupter (Low March, Daventry, Northants, UK) following manufacturer's instructions. Lipase activity in cleared cell lysates was related to the total protein content of such lysates, as measured using the Bradford assay (Pierce, Rockford, IL).

Protease Activity Determination

Protease activity in the culture supernatant was determined using the QuantiCleave Protease Assay Kit (Pierce) according to the manufacturer's instructions.

SDS–PAGE and Western Blotting Analyses

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), 12%, was carried out in a Mini-PROTEAN II (Bio-Rad) apparatus following the standard procedures recommended by the manufacturer. Gels were stained using silver stain procedure (Bio-Rad). Western blot was performed after SDS–PAGE; proteins were transferred to a nitrocellulose membrane. ROL was detected with mouse anti-ROL antiserum (raised at the Institute of Applied Microbiology, BOKU—University of Natural Resources and Applied Life Sciences, Vienna, Austria); antimouse IgG horseradish peroxidase conjugate (Sigma, St. Louis, MO) was used as a secondary antibody. Detection was performed with the chemiluminescent substrate SuperSignal West Pico (Pierce) and photographic film.

Biomass Analysis

Biomass was expressed as dry cell weight. This was measured by withdrawing 8 mL samples of the bioreactor; these were then filtered through pre-weighted glass microfibre filters (Whatman GF/F, Maidstone, UK), which were subsequently washed with two volumes of distilled water and dried at 105°C to a constant weight. Alternatively, biomass was measured by optical density at 600 nm, using a conversion factor for dry cell weight. Determinations were performed by triplicate and the relative standard deviation (RSD) was about 5%.

Biomass Elemental Composition

Cells were harvested by centrifugation (CentriKon H-401 ZK401, Kontron Hermle, Zürich, Switzerland) for 5 min at 4°C and 4,500*g*. Pellets were collected, washed with 10 mM Tris-HCl, pH 8.0, and centrifuged twice. Washed cells were freeze-dried (Virtis Sentry 5L, Virtis Company Gardiner, NY) for 24 h, further dried at 105°C to constant weight and analyzed for composition of C, H, N, O, and S in an elemental analyzer (Carlo Erba EA 1108, Carlo Erba Instruments, Milan, Italy). Ash content was quantified about 9%.

Pre-Programmed Exponential Feeding Rate

A pre-programmed exponential feeding rate strategy was designed according to D'Anjou and Daugulis (2001) with the objective to control the specific growth rate at a constant value along the fed-batch induction phase. If a quasi-steady state is assumed for the residual substrate concentration, the specific growth rate of the culture at time (t) can be obtained from fed-batch substrate balance (Eq. 1).

$$\mu(t) = \frac{Y_{X/S} F(t) S_0}{V(t) X(t)} \tag{1}$$

By integrating the fed-batch cell mass balance (Eq. 2) and combining it with Equation 1, the feeding rate $F(t)$ for a fixed specific growth rate can be expressed by Equation 3.

$$X(t)V(t) = X(t_0)V(t_0)\exp(\mu(t - t_0)) \tag{2}$$

$$F(t) = \frac{\mu[X(t_0)V(t_0)]}{Y_{X/S} S_0}\exp[\mu(t - t_0)] \tag{3}$$

This feeding rate equation can be applied if V, X, and $Y_{X/S}$ are known at t_0 and the yield can be assumed as a constant along the fermentation. At start of fed-batch phase, t_0, the biomass concentration was around 30 g/L, the culture volume was 3.6 L, and the sorbitol feed concentration was fixed at 300 g/L. The biomass/sorbitol yield was considered to be constant at 0.3 g X g/sorbitol under these conditions (Resina et al., 2004).

For programming reasons, the feeding rate (Eq. 3) was expressed as a function of the previously added feed rate in Equation 4. In this way, the feed rate (F) does not depend on the absolute time because the new addition rate can be expressed as the product of the previous feed rate and the exponential factor. The equation only requires the initial value of $F(t_o)$ when the induction phase starts and the time between two additions (Δt: 1min).

$$F(t + \Delta t) = F(t)\exp(\mu \Delta t) \tag{4}$$

For the cultivation at a constant residual sorbitol concentration, the feeding rate was manually programmed according to the consumption rate estimated from the off-line measured residual sorbitol concentration in the cultivation broth. In this way, sorbitol was maintained in excess.

Calculation of Specific Rates

Estimated reaction rates were specific growth-rate (μ) [/h], specific substrate consumption rate (q_S) [g sorbitol g/biomass · h] and specific lipase production rate (q_P) [AU g/biomass · h]. The estimation procedure were performed by using suitable smoothing routines (Matlab 6.1 Curvefit Toolbox, The Mathworks, Inc., Matlab, Natick, MA) and mass balances that permitted to supply complete data sets with coincident off-line biomass, substrate and lipase activity data.

Firstly, biomass, substrate, and lipase activity data were smoothed. After that, the first derivatives of the smoothed curves were obtained. Feed-flow rate was time-averaged between consecutive off-line biomass samples. Total volume was estimated taking into account feed-flow rate including carbon source (sorbitol), acid and base solutions, as well as volume samples.

Finally, mass balances inside the bioreactor for fed-batch operation were conducted to obtain the reaction rates as follows:

$$\mu = \frac{1}{(X \cdot V)}\frac{\mathrm{d}(X \cdot V)}{\mathrm{d}t} \tag{5}$$

$$q_s = -\left(\frac{\mathrm{d}S}{\mathrm{d}t} + \frac{F \cdot (S - S_0)}{V}\right) \cdot \frac{1}{X} \tag{6}$$

$$q_p = \left(\frac{\mathrm{d}P}{\mathrm{d}t} + \frac{F \cdot P}{V}\right) \cdot \frac{1}{X} \tag{7}$$

RESULTS

Previous studies in batch cultures have demonstrated that the combined use of sorbitol and methylamine as carbon and nitrogen sources, respectively, allowed for heterologous ROL expression in *P. pastoris* under the control of P*FLD*, which turned out to be as least as efficient as the conventional P*AOX*-based ROL expression in batch cultivations with methanol and ammonium as substrates. Although the substitution of methanol-ammonium for sorbitol-methylamine provoked a clear decrease in the specific growth rate (μ_{max} ~0.04 vs. ~0.025/h), the productivity (AU/h) values were quite similar, i.e., providing a basis for the design of methanol-free heterologous protein production processes using P*FLD*-based *P. pastoris* systems (Resina et al., 2004).

In order to perform high cell density fed-batch cultivations of *P. pastoris* for heterologous protein production using the P*FLD*, the following strategy was designed: first, a fast-growing batch phase was implemented similar to that used for P*AOX*-based systems, with an initial amount of 40 g/L of glycerol, but with only the stoichiometric amount of ammonium sulfate to achieve the exhaustion of both substrates at the end of the batch phase. Such amount was calculated on the basis of the elementary composition of *P. pastoris* cells growing in batch cultivations on glycerol and ammonium as sole carbon and nitrogen sources, respectively, C $H_{1.87}$ $O_{0.56}$ $N_{0.18}$ $S_{0.008}$, and the biomass/substrate yield, $Y_{X/S}$, 0.5 g biomass/g glycerol. The calculated stoichioemetric ratio was 0.23 g $(NH_4)_2SO_4$/g glycerol. Ammonium analysis at the end of the batch phase confirmed this value, i.e., at the time of glycerol exhaustion the ammonium concentration was virtually zero. Second, after this batch phase (the end of which was indicated by a sharp increase in the dissolved oxygen [DO] levels), a transition phase involving the addition of a sorbitol plus methylamine pulse (10 g/L sorbitol and 3 g/L methylamine) was performed. This allowed pre-adapting *P. pastoris* cells

metabolism to the carbon and nitrogen sources used in the induction phase. Third, such induction phase was initiated when sorbitol levels were near exhaustion. During this phase, a pre-programmed exponential feeding rate strategy ensured a constant specific growth rate along the fed-batch induction phase. In the induction phase, the sorbitol:methylamine ratio in the feeding stock solution reflected the stoichioemetric ratio between these substrates, 0.118 g methylamine/g sorbitol, as calculated on the basis of the elementary composition of *P. pastoris* cells growing in batch cultivations on sorbitol and methylamine, $CH_{1.74}O_{0.51}N_{0.14}S_{0.008}$, and a yield biomass/substrate, $Y_{X/S}$, of about 0.3 g X/g sorbitol (Resina et al., 2004).

The specific growth rate has been proven to have an important effect on secreted recombinant protein productivities in *P. pastoris* (Sinha et al., 2003; Zhang et al., 2000). Hence, two fed-batch cultivations were initially performed using such strategy, with their fed-batch phases pre-set at a 0.005/h (i.e., about 20% of the μ_{max}) and 0.01/h (i.e., about 40% of the μ_{max}), respectively. The evolution of the key parameters during the transition and induction phases of these cultivations is shown in Figure 1A and B.

Adaptation of metabolism to sorbitol and methylamine and P*FLD* induction was quite fast, as both intracellular and extracellular lipase activities could be detected soon after the sorbitol + methylamine pulse. Moreover, extracellular lipase activity levels detected at the end of the transition phase were similar in both cultivations. This observation is indicative of the reproducibility of the cultivation strategy for product induction.

The experimentally estimated μ values during the fed-batch phase (Fig. 2A) closely matched the pre-set values within a $\pm 0.001 \sim 0.0015$ variation/range. Only in later stages of the lower growth rate cultivation there is a slight decrease in the experimental μ values. As expected, during this phase, residual sorbitol levels were below 0.5 and 1.0 g/L in the cultivations at a μ of 0.005 and 0.01/h, respectively, and only in the later stages of the cultivations these values showed a slight increase. Consistently, methylamine residual levels in the lower growth rate cultivation (0.005/h) were very low ($0 \sim 0.5$ g/L), and somewhat higher ($0.5 \sim 2$ g/L) in the cultivation at μ of 0.01/h. Also, the evolution of the q_s values are well in agreement with the pre-programmed exponential feeding parameters and, consistently, q_s values stayed constant along the fed-batch phase in both cultivations (data not shown).

Remarkably, the extracellular lipase activity profiles in these two cultivations are very different between the lower and the higher growth rate fed-batch phases. In the 0.005/h fed-batch cultivation, extracellular lipase exponential accumulation ceased soon after the transition phase, reaching only a maximum of 42 AU/mL. In contrast, in the fed-batch at 0.01/h extracellular lipase levels increased steadily up to 60 h of cultivation. These extracellular lipase accumulation profiles are reflected in the evolution of q_p values throughout the cultivation (Fig. 2B). In both cases, q_p values in the early stages of the transition phase are similar (about $70 \sim 110$ AU/g X · h). However, in the lower growth rate cultivation, a maximum q_p of 88 AU/g X · h is obtained at the end of the transition phase, whereas in the 0.01/h specific growth rate a maximum q_p of 206 AU/g X · h is reached soon after the start of the induction. After these maxima, an exponential decrease in q_p was observed in both cases, except for a transient increase in the 0.01/h fed-batch cultivation at $30 \sim 45$ h after the start of the induction phase.

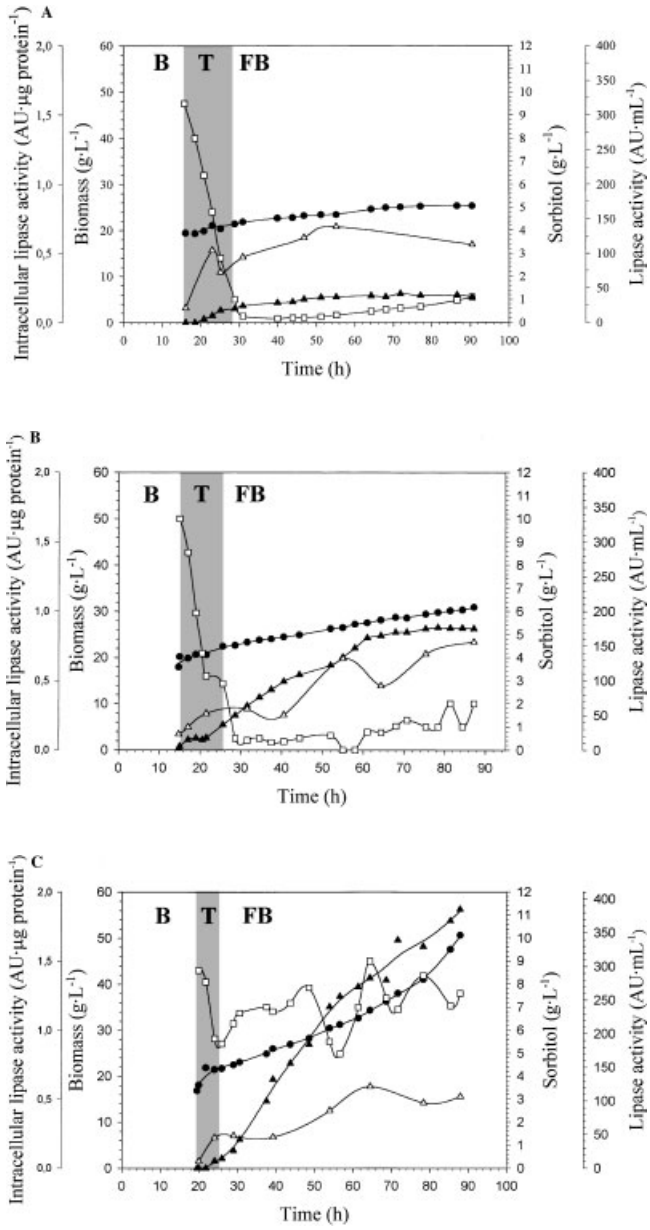

Figure 1. **A**: Fed-batch cultivation of *P. pastoris* expressing ROL at a controlled specific growth rate of 0.005/h. Biomass (dry cell weight) (●), sorbitol concentration (□), extracellular lipase activity (▲), and intracellular lipase activity (△) are indicated. **B**: Fed-batch cultivation of *P. pastoris* expressing ROL at a controlled specific growth rate of 0.01/h. Biomass (dry cell weight) (●), sorbitol concentration (□), extracellular lipase activity (▲), and intracellular lipase activity (△) are indicated. **C**: Fed-batch cultivation of *P. pastoris* expressing ROL at constant residual sorbitol concentration of 8 g/L (near μ_{max}). Biomass (dry cell weight) (●), sorbitol concentration (□), extracellular lipase activity (▲), and intracellular lipase activity (△) are indicated.

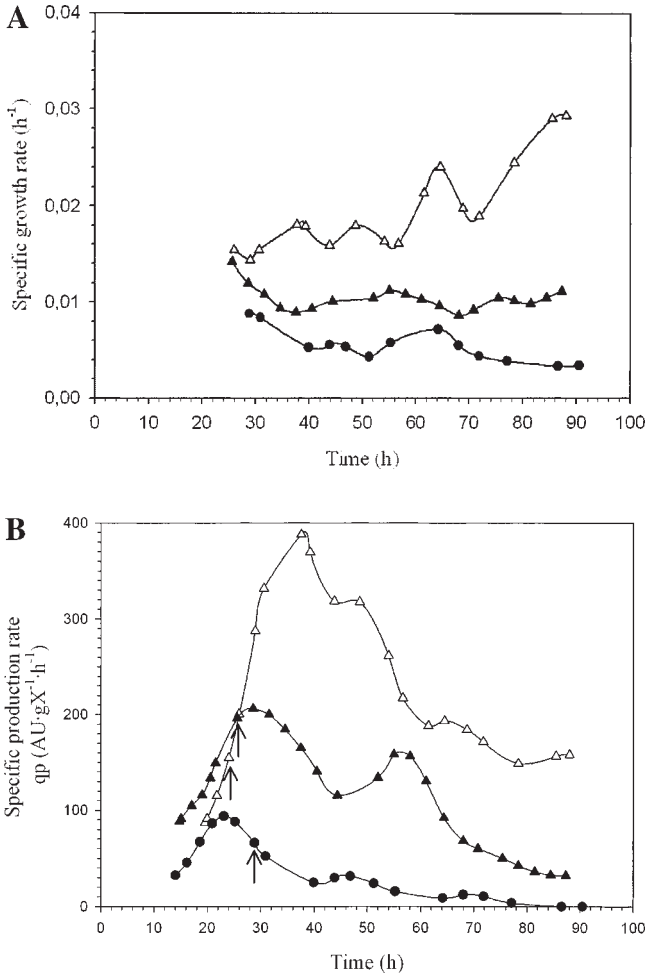

Figure 2. **A**: Specific growth rate profiles of three different fed-batch cultures of *P. pastoris* expressing ROL. Mean specific growth rate of 0.005 (/h) (●), mean specific growth rate of 0.01 (/h) (▲) and at constant residual sorbitol concentration of 8 g/L (near μ_{max}) (△). The first point plotted for each culture corresponds to the start time of its induction phase. **B**: Specific product production rate (q_p) profiles of three different fed-batch cultures of *Pichia pastoris* expressing ROL at different specific growth rate. Mean specific growth rate of 0.005 (/h) (●), mean specific growth rate of 0.01 (/h) (▲) and constant residual sorbitol concentration of 8 g/L (near μ_{max}) (△). The first point plotted for each culture corresponds to the start time of its transition phase. The vertical arrows point to the start time of the induction phase in each culture.

Hence, it appears that ROL secretion can not be sustained after induction under growth limiting conditions. Further, the decrease in q_p (i.e., in the ROL secretion rate) does not appear to be concomitant with an increasing intracellular product accumulation: After an initial exponential increase of intracellular lipase levels upon ROL expression induction, these values stayed rather constant along the induction phase.

The exponential decrease in q_p could also be attributed to the presence of endogenous proteases released to the extracellular medium, e.g., as a result of cell lysis phenomena. Nevertheless, extracellular protease levels in the lower growth rate fed-batch cultivation were very low/basal, i.e., indicating low cell lysis and product degradation levels. Western blotting analyses of cultivation broth samples using

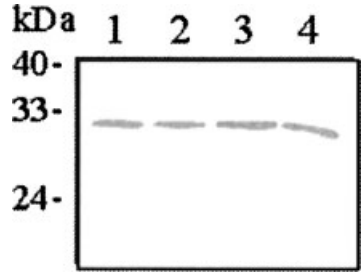

Figure 3. Western blot analysis of culture supernatants from fed-batch cultivations of *P. pastoris* expressing ROL. **Lane 1**: Culture supernatant at 86 h of fed-batch cultivation performed at a low specific growth rate (0.005/h). **Lane 2**: Culture supernatant at 88 h of fed-batch cultivation performed at a medium specific growth rate (0.01/h). **Lanes 3** and **4**: Culture supernatants at 75 and 88 h of fed-batch cultivation, respectively, performed at constant residual sorbitol concentration of 8 g/L (near μ_{max}).

anti-ROL antibodies confirmed that ROL degradation was minimal (Fig. 3). In addition, cell viability values were >90% (data not shown).

On the other hand, these results were suggesting that extracellular ROL production seemed to be favored when the microorganism was growing at higher growth rates and, therefore, in a situation of carbon excess. Thus, a third fermentation was performed where the feeding rate was manually programmed to maintain sorbitol in excess of 8 g/L. This defined concentration value allowed to maintain the cultivation under carbon and nitrogen excess conditions, as well as keeping sorbitol residual concentrations well below growth-inhibitory levels (>40 g/L, Thorpe et al., 1999).

The results of this cultivation are shown in Figure 1C. As observed, the simple off-line control strategy allowed maintaining sorbitol residual levels within ±2 g/L range of the set point, i.e., allowing cells to grow at a near-μ_{max} growth rate. Under these conditions, extracellular lipase levels steadily increased until the end of the cultivation time (90 h), reaching a maximum of 385 AU/mL, i.e., 2.2-fold higher than in cultivation at μ 0.01/h and 9.2-fold higher than in cultivation at μ 0.005/h (Table I).

Table I. Maximal lipase activity, lipase yield, mean specific growth rate and productivities obtained in fed-batch cultivations of P*FLD* and P*AOX*-based *P. pastoris* strains expressing ROL.

	AU_{max} (AU/mL)	$Y_{P/X}$ (AU/g X)	μ_{mean} (/h)	Productivity (AU/L · h)	Specific productivity (AU/g X · h)
P*AOX*[a] Mut^s	205	5,775	0.005	2,246	63
P*AOX*[a] Mut$^+$	150	2,470	0.036	3,000	49
P*FLD* $\mu = 0.005$/h	42	1,658	0.005	579	23
P*FLD* $\mu = 0.01$/h	177	5,932	0.010	2,254	76
P*FLD* $\mu = 0.02$/h	385	7,634	0.020	4,379	87

[a]Data taken from Cos et al. (2005).

The average specific growth rate during the fed-batch phase was 0.02 ± 0.004/h, i.e., about 80% of the μ_{max} observed in batch cultivations (0.025/h, Resina et al., 2004). As expected, q_s values were higher than in fed-batch cultivations at lower specific growth rates (data not shown). Most interestingly, q_p profiles during the fed-batch phase at near-μ_{max} were remarkably different from those observed in growth-limiting conditions (Fig. 2B): The maximum q_p value (388 AU/g X · h) was obtained after about 20 h of the induction phase start, that is, 1.9-fold and 4.4-fold higher than in cultivations at μ 0.01 and 0.005/h, respectively. Moreover, this maximum was reached in a later stage compared with the cultivations under growth-limiting conditions. After reaching this maximum, q_p values decreased exponentially down to a value of about 150 AU/g X · h. After this point, reached 50 h after the start of the induction phase, q_p values remained stable. Interestingly, the intracellular lipase activity levels followed similar profiles and maximum values to those observed in the cultivations at lower growth rates, except for the case of the cultivation at the highest growth rate, where maximum activity levels were slightly lower. This could be the result of differences in the relation between synthesis and secretion rates. Notably, the fact that intracellular lipase activity is detected in all three cultivations reflects that some active (i.e., properly folded and processed) ROL is retained within the cell's periplasm, and/or in the secretion vacuoles.

A comparative analysis of the fed-batch cultivations in terms of $Y_{P/X}$, productivity and specific productivity (Table I) revealed that the overall efficiency of the system increased together with the specific growth rate of the corresponding fed-batch cultivation. That is, at the highest specific growth rate cultivations, $Y_{P/X}$ values were 1.3-fold and 4.6-fold higher, the productivity was 1.9-fold and 7.6-fold higher, and the specific productivity was 1.1-fold and 3.8-fold higher than in cultivations at μ 0.01 and 0.005/h, respectively (Table I). These results clearly indicate that the best strategy for ROL extracellular production using the P*FLD*-based system is a fed-batch induction phase with a constant sorbitol excess residual concentration in the cultivation broth.

DISCUSSION

Recently, the performance of the classical P*AOX*-based system for ROL expression has been evaluated in both Mut^s and Mut$^+$ in high cell density cultures (Cos et al., 2005). Here, we compare (Tables I and II) this earlier study with the new data for *P. pastoris* expressing *ROL* under the control of the P*FLD* in high cell density fed-batch cultivations at equivalent constant growth rates. Specifically, the cultivation of the *P. pastoris* Mut^s strain expressing *ROL* was carried out at about the same growth rate than for the present *P. pastoris* cultivation at the lower growth rate (about 0.005/h). Remarkably, we find that, *P. pastoris* growing on methanol and ammonium sulfate as sole carbon and nitrogen sources, respectively, allow for sustained ROL production, in clear contrast to the cultivation with the combination sorbitol plus methylamine, clearly showing the impact of cells' metabolism on process

Table II. Mean and maximal specific growth rate (μ), and specific lipase production rate (q_p) obtained in fed-batch cultivations of P*FLD* and P*AOX*-based *P. pastoris* strains expressing ROL.

	μ_{mean} (/h)	μ_{max} (/h)	q_p mean (AU/g X · h)	q_p max (AU/g X · h)
P*AOX*[a] Mut^s	0.005	0.014	83	170
P*AOX*[a] Mut$^+$	0.036	0.051	130	244
P*FLD* $\mu = 0.005$/h	0.005	0.009	29	88
P*FLD* $\mu = 0.01$/h	0.010	0.014	132	206
P*FLD* $\mu = 0.02$/h	0.020	0.029	244	388

[a]Data taken from Cos et al. (2005).

productivity. Also, whereas in the P*AOX*-based system using the Mut^s strain, q_p maximum values are reached after 20 h of methanol induction, in the P*FLD*-based system these were achieved only 4∼5 h after methylamine induction. This indicates that q_p profiles over cultivation time (i.e., the kinetics of product secretion) are significantly different. Overall, ROL expression and secretion levels over the cultivation time seem to be substrate (C-source and/or N-source) dependent. This has also been observed for other proteins expressed in *P. pastoris* (Hohenblum et al., 2004).

In terms of process productivity, the comparison of the results obtained with the P*FLD*-based system cultivation at near μ_{max}, 0.025/h with those obtained with both Mut^s and Mut$^+$ P*AOX*-based strains clearly indicate that the performance of the P*FLD* system is better than the classic P*AOX* system (1.9- and 1.5-fold higher productivity than the Mut^s and Mut$^+$ system, respectively).

The q_p profiles observed in all cultivations could reflect possible effects of protein turnover by proteolysis, which have also been reported to be a potential bottleneck for protein production in *P. pastoris* (Cuvers et al., 2001; Jahic et al., 2003; Kobayashi et al., 2000). Further, ammonium limitation has been shown to trigger proteolytic degradation of ROL in high cell density cultivations using the classic P*AOX*-based systems with Mut$^+$ strains (Cos et al., 2005). Notably, we could not detect significant product degradation nor extracellular protease levels in our cultivations, even in the case where methylamine levels were very low or below detection limits (cultivation at 0.005/h).

Thus, the q_p and intracellular lipase profiles could reflect an adaptation of cell's physiological state to ROL overexpression along the cultivation time leading to a down regulation of the *ROL* transcription levels. Remarkably, maximum intracellular lipase levels in growth-limited cultivations were very similar, i.e., suggesting that a threshold level of intracellular ROL may exist. Interestingly, down regulation of secreted recombinant protein expression levels in stressed cells has recently been shown to be related to the triggering of the unfolded protein response (UPR) in the fungus *Trichoderma reesei* (Pakula et al., 2003). Further, the

UPR has been also described in yeast such as *S. cerevisiae* (Valkonen et al., 2003) and *P. pastoris* (Hohenblum et al., 2004).

CONCLUSIONS

We have developed a simple and reliable fed-batch strategy for recombinant protein production in *P. pastoris* high cell density cultivations using the P*FLD*. Overall, in this study we have shown that the P*FLD* system can be successfully used for protein production in *P. pastoris* using methanol-free high cell density cultivation strategies, being comparable to the P*AOX*-based system in terms of process productivity. However, current limitations in the knowledge of the regulation of protein processing processes and methylamine metabolism regulation in *P. pastoris* limit the rational design of fermentation strategies. Notably, the strong effect of the specific growth rate (i.e., of cell's physiological state) on product (ROL) synthesis and secretion needs further characterization. Physiological studies are in progress in order to characterize the cell's responses to ROL overexpression and secretion.

NOMENCLATURE

X	biomass concentration [g/L]
S	substrate concentration inside the bioreactor [g/L]
S_0	substrate feed concentration [g/L]
P	lipase activity [AU/L]
T	time[h]
F	feed flow-rate [L/h]
V	total volume of the culture [L]
μ	specific growth rate (/h).
q_s	specific substrate consumption rate [g sorbitol/g biomass · h]
q_p	specific lipase production rate [UA/g biomass · h].
$Y_{X/S}$	biomass/substrate yield [g biomass g/substrate].

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