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Combined effect of the methanol utilization (Mut) phenotype and gene dosage on recombinant protein production in *Pichia pastoris* fed-batch cultures

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Abstract

An important number of heterologous proteins have been produced in the methylotrophic yeast *Pichia pastoris* using the alcohol oxidase promoter. Two factors that drastically influence protein production and cultivation process development in this system are gene dosage and methanol assimilation capacity of the host strain (Mut phenotype). Using a battery of four strains which secrete a *Rhizopus oryzae* lipase (ROL), the combined effects of gene dosage and Mut phenotype on recombinant protein production in *Pichia pastoris* was studied in fed-batch cultures. Regarding the effect of phenotype, the specific productivity and the $Y_{P/X}$ were 1.29- and 2.34-fold higher for Mut^s *ROL* single copy strain than for Mut$^+$ *ROL* single copy strain. On the contrary, the productivity of Mut$^+$ *ROL* single copy strain was 1.34-fold higher than Mut^s *ROL* single copy strain. An increase in *ROL* gene dosage seems to negatively affect cell's performance in bioreactor cultures, particularly in Mut^s strains. Overall, the Mut^s strain may be still advantageous to use because it allows for easier process control strategies.

Keywords: *Pichia pastoris*; Methanol utilization (Mut) phenotype; Gene dosage; Fed-batch cultivation; *Rhizopus oryzae* lipase

1. Introduction

The methylotrophic yeast *Pichia pastoris* has been widely reported as a suitable expression system for heterologous protein expression due to, among other favorable properties, the existence of a strong and tightly regulated promoter from the alcohol oxidase 1 gene, P_{AOX1} (Lin Cereghino and Cregg, 2000).

Alcohol oxidase is the first enzyme of the methanol assimilation pathway which catalyses the oxidation of methanol to formaldehyde. There are two alcohol oxidase genes in *P. pastoris* that code for the alcohol

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oxidase enzyme, the alcohol oxidase 1 gene (*AOX1*), which is responsible for greater than 90% of the enzyme in the cell, and the alcohol oxidase 2 gene (*AOX2*) for less than 10%. There are three types of *P. pastoris* host strains available that vary with regard to their ability to utilize methanol: the wild type or methanol utilization plus phenotype (Mut^+), and those resulting from deletions in the *AOX1* gene (methanol utilization slow (Mut^s)) or both *AOX* genes (methanol utilization minus (Mut^-)). Concerning the Mut^+ phenotype, the *P. pastoris* strains most widely used are GS115 (*his4*) and the wild type strain X-33, which is isogenic to GS115 except for reversion of the auxotrophy for histidine.

Heterologous protein expression under control of P_{AOX1} allows for the design of defined controlled high-cell density cultivation strategies for heterologous protein production, described in two main phases. A first phase to generate biomass utilizing carbon sources that lead to a high substrate-biomass yield, such as glycerol; and a second phase, in the presence of methanol, for induction of foreign protein expression. Optimization of cultivation processes usually involve an intermediate cultivation phase, where both carbon sources are present. Although many reports defining a successful operational scheme for cultivation of *P. pastoris* have been described, the productivity yields reached are variable, particularly with regard to the specific foreign protein expressed (Rodríguez-Jiménez et al., 1997; Katakura et al., 1998; Zhang et al., 2000; D'Anjou and Daugulis, 2001).

Besides growth conditions and cultivation process operating parameters, yields for heterologous protein production also depend on factors than influence gene expression (and secretion), and host strain physiology (Sreekrishna et al., 1997). In addition, the design of an optimal production system strongly depends on the characteristics of the foreign protein such as cell toxicity, stability, and protease sensitivity.

The presence of multiple integrated copies of a desired expression cassette has been reported to be an important factor in increasing foreign protein production in *P. pastoris* (Clare et al., 1991a,b; Werten et al., 1999; Vassileva et al., 2001). However, for Expression of extracellular proteins, maximizing gene copy number sometimes results in a decreased final productivity yield, and therefore an optimal rather than a maximal gene dosage is often best (Clare et al., 1991a; Hohenblum et al., 2004).

There are different strategies for generation of multicopy strains in *P. pastoris* (Romanos et al., 1998). The availability of small vectors in *P. pastoris* which offer drug resistance is an appropriate methodology for this purpose. Recombinant strains showing drug hyperresistance frequently correspond to strains containing multiple plasmid copies, and therefore multiple copies of the heterologous gene (for example, the vectors containing the bacterial kanamycin resistance gene, which offer resistance to the drug G418). Also, vectors containing the Zeocin resistance gene as a dominant selection marker offer the added advantage of direct screening of the transformants on Zeocin-containing plates.

Another factor that should be considered for expression optimization is the Mut phenotype. For intracellular expression, it is preferable to use Mut^s cells because of increased specific yield of heterologous protein (lower levels of alcohol oxidase protein) (Sreekrishna et al., 1997). For secretion, either one of Mut^+ or Mut^s strains can be used. Ideally, for any given product, it is better to test expression in both backgrounds (Sreekrishna and Kropp, 1996). An advantage of Mut^s strains is that the culture is not as sensitive to residual methanol in the cultivation media relative to Mut^+ strains, and hence the process of scale-up can be easier (Stratton et al., 1998). The specific growth-rate is a critical parameter in the optimization of product formation (Zhang et al., 2000; Sinha et al., 2003).

In a previous study, we have reported the optimization of production in high-cell density cultures of a lipase from *Rhizopus oryzae* lipase (ROL) (GenBank accession number AF229435) expressed in a *P. pastoris* X-33 strain (Mut^+) under control of P_{AOX1}, using selected fed-batch cultivation schemes (Minning et al., 2001). Several operational parameters strongly influenced the final yield, e.g. the methanol concentration in the growth medium. The optimization of these parameters allowed for higher ROL productivities. ROL is an enzyme with a wide range of applications in the production of enantiomeric pure fine chemicals (Suyama and Tokiwa, 1997; Demir et al., 1998; Virto et al., 1999).

A further step in the optimization of extracellular ROL protein expression in *P. pastoris* was achieved by increasing the heterologous *ROL* gene copy number in the host cell (Serrano et al., 2001). The *ROL*

multicopy Mut$^+$ strain developed in this study showed higher lipolytic activity (in low-cell density cultures), compared with the *ROL* single copy strain, and was suitable for cultivation in a defined synthetic medium. However, the multicopy strain showed a lower specific growth-rate on methanol, suggesting that these strains may constitute a useful biological model for studying foreign protein secretion bottlenecks in *P. pastoris* such as cellular stress-related effects (Hohenblum et al., 2004).

It is well known that two key parameters in the production of heterologous protein in *P. pastoris* are the phenotype and the gene dosage, however, to our knowledge, the combined effect of these parameters on productivity with the same heterologous protein has not been previously reported. The main objective of this work is to compare the effect of gene dosage on the production of extracellular ROL in *P. pastoris* fed-batch cultures under two different host Mut phenotypes (Mut$^+$ and Mut^s).

2. Materials and methods

2.1. Strains, plasmids, transformation of P. pastoris and selection of ROL multicopy transformants

The wild type *P. pastoris* X-33-derived strain (i.e. Mut$^+$) expressing a *R. oryzae* lipase extracellularly (Minning et al., 2001) was used as the *ROL* single copy strain. A *P. pastoris* strain GS115 (*his4*; Invitrogen, USA)-derived strain having multiple copies of the *ROL* gene and its histidine auxotrophy reverted has been described elsewhere (Serrano et al., 2001); therefore, this strain (named as X-33/500_3/ROL) was isogenic to X-33 (Mut$^+$ His$^+$) except for the *ROL* gene dosage. *P. pastoris* KM71 (*arg4 his4 aox1Δ::SARG4 AOX2*; Cregg and Madden, 1987), Mut^s His$^-$, was used for generation of *ROL* multicopy strains. Plasmid pPICαA-ROL (Minning et al., 1998) was linearized in the 5′AOX1 region by restriction enzyme digestion with *Sac*I and used to transform *P. pastoris* KM71 competent cells by electroporation as described by Cregg and Russell (1998). Reversion of the histidine auxotrophy in the KM71-derived strains was done by transforming them with pPIC9 vector (Invitrogen) linearized with *Sal*I.

Selection of pPICαA-ROL-transformants was carried out in YPD agar medium (1% (w/v) yeast extract, 2% (w/v) peptone, 0.2% (w/v) glucose, 2% (w/v) agar) plates with 100 μg ml^{-1} of Zeocin (Invitrogen). Selection of *ROL* multicopy transformants was carried out using replica YPD medium agar plates containing 500, 1000 or 1500 μg ml^{-1} of Zeocin as described in Romanos et al. (1998). Selection of reverted His$^+$ strains was done on Minimal Dextrose (MD) medium agar plates containing 1.34% (w/v) YNB, 2% (w/v) dextrose and 2% (w/v) agar. Selected transformants were further checked for their Mut phenotype by growing them in Minimal Methanol (MM) medium agar plates containing 1.34% (w/v) YNB, 0.5% (w/v) methanol, and 2% (w/v) agar.

2.2. Southern-blot hybridization and quantification of ROL copy number

Genomic DNA extraction was performed according to Miles et al. (1998). DNA was digested overnight with the restriction enzymes *Eco*RI and *Not*I. The DNA probe containing the *ROL* gene was generated by PCR, using the commercial 5′AOX1 and 3′AOX1 primers from Invitrogen. Plasmid pPICαA-ROL was used as template DNA. The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen Inc., USA) and labeled with the BioPrime® DNA labeling system (Life Technologies, USA) designed to produce biotinylated (biotin-14-dCTP) DNA probes. Southern hybridization was carried out following protocols for MSI nylon membranes (http://www.msifilters.com) and the Chemiluminescent Detection System for biotin-labeled probes (Tropix Inc., MA, USA).

Vector copy number was quantified by dot-blot hybridization using purified genomic DNA as described in Romanos et al. (1998). A positively charged nylon membrane (Hybond-N+, Amersham Biosciences) was used to immobilize chromosomal DNA from *P. pastoris* strains. A PCR-amplified DNA fragment (700 bp) containing a region of the *P. pastoris URA3* gene (GenBank accession number AF321098) was used as an internal control for the amount of chromosomal DNA. Both the *ROL* and *URA3* horseradish peroxidase-labeled probes for membrane hybridization were prepared by using the ECL Direct Labeling and Detection System (Amersham Biosciences) following manufacturer's instructions.

2.3. 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 and deep frozen at −80 °C.

2.4. Inoculum preparation

Pre-inoculums for shake-flask and bioreactor cultures were grown for 24 h in baffled shake-flasks at 30 °C, 250 rpm in buffered glycerol–complex medium (BMGY) 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 $4000 \times g$ 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 $4000 \times g$. Harvested cells were resuspended in bioreactor culture medium and used to inoculate a 5-l Biostat ED bioreactor (Braun Biotech, Melsungen, Germany). Dissolved oxygen was maintained at 30% with the variation of aeration flow between 1.5 and 20 l min^{-1}. The pH was kept at 5.5 by using 2 M KOH and cultivation temperature and stirring were controlled at 30 °C and 800 rpm, respectively.

2.5. Shake-flask cultures

Shake-flask cultures were conducted in 250 ml of buffered minimal methanol ± histidine (BMM (H)) with 100 mM potassium phosphate, pH 6.0, 1.34% (w/v) YNB, 4×10^{-5}% (w/v) biotin, and 0.5% (w/v) of methanol plus 0.04% (w/v) of histidine when necessary. Cultures were incubated for 48 h at 30 °C and 250 rpm. The same amount of methanol was added every 24 h to the growing cultures.

2.6. Fed-batch cultivation set up and operational conditions

The basal salt synthetic medium for fed-batch cultivations contained per liter of distilled water: H_3PO_4 (85%) 26.7 ml, $CaSO_4$ 0.93 g, K_2SO_4 18.2 g, $MgSO_4 \cdot 7H_2O$ 14.9 g, KOH 4.13 g, glycerol 40 g, and 4.35 ml of PTM1 solution. The PTM1 solution contained per liter: $CuSO_4 \cdot 5H_2O$ 6.0 g, NaI 0.08 g, $MnSO_4 \cdot H_2O$ 3.0 g, $Na_2MoO_4 \cdot 2H_2O$ 0.2 g, H_3BO_3 0.02 g, $CoCl_2$ 0.5 g, $ZnCl_2$ 20.0 g, $FeSO_4 \cdot 7H_2O$ 65.0 g, biotin 0.3 g, and H_2SO_4 concentrated 5 ml. The antifoam agent (Mazu DF 7960—a polyoxyalkylene glycol, Mazer Chemicals, PPG Industries Inc., USA) was added when necessary at a maximal final concentration of 10 ppm.

Cells were cultured in a 5-l Braun Biostat ED bioreactor (Braun Biotech, Melsungen, Germany). The cultivation conditions were: stirring rate 800 rpm, temperature 30 °C, pH controlled at 5.5 by adding NH_4OH 30% (v/v), dissolved oxygen controlled above 30% with an air flow rate between 1.5 and 20 l min^{-1}. For fed-batch feeding, 12 ml of PTM1 were added to 1 l of glycerol (50% (v/v)) and 1 l of pure methanol, respectively.

The carbon sources, glycerol and methanol, were either added in the transition and production phases by an automatic microburette MicroBU-2031 from Crison Instruments (Alella, Barcelona, Spain). The initial working volume in the batch phase was 3.5 l. The basal salt medium was autoclaved in situ except for the PTM1 solution, which was sterile-filtered and subsequently added after the medium had cooled to room temperature.

The cultivation started with a batch phase with 40 g l^{-1} of glycerol. After that, a transition phase for 5 h followed. During the first 2 h, the glycerol feeding rate was 300 μl min^{-1}, it then decreased to 160 μl min^{-1} in the third hour to 100 μl min^{-1} in the fourth hour, and finally to 65 μl min^{-1} during the final hour. The methanol feeding was started at the third hour and was kept constant at 100 μl min^{-1} till the end of this phase. Finally, the induction phase was carried out, the methanol addition rate in the culture was manually modified attempting to maintain methanol concentration between 1 and 2 g l^{-1}.

2.7. 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 (R.S.D.) was about 5%.

2.8. Biomass elemental composition

Cells were harvested by centrifugation (CentriKon H-401 ZK401, Kontron Hermle, Zürich, Switzerland) for 5 min at 4 °C and 4500 × *g*. Pellets were collected, washed with 10 mM Tris–HCl, pH 8.0, and centrifuged twice. Washed cells were freeze-dried (Virtis Sentry 5 l, Virtis Company Gardiner, NY, USA) 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%.

2.9. Glycerol and methanol determination

Glycerol was determined by HPLC with a HP 1050 liquid chromatograph (Hewlett Packard, Palo Alto, CA, USA) using an Aminex HPX-87H ion-exchange column from Bio-Rad. The mobile phase was 15 mM sulphuric acid, injection volume was 20 ml. Data was quantified by the Millenium 2.15.10 Software (Waters Corporation, Mildford, MA, USA). The estimated R.S.D. was 3%.

Methanol was analyzed by a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) using a capillary column Tracsil TR-FFAP 25 mm × 0.53 mm × 1 μm (Tracer-Teknokroma, St. Cugat del Vallès, Barcelona, Spain) and equipped with an automatic injector (HP 7376) and a FID detector. Operating conditions were 200 and 280 °C for injector and detector, respectively, oven-temperature profile 40 °C (2 min), 20 °C min^{-1} to 200 °C, 200 °C (5 min) giving a total analysis time of about 15 min. Helium was used as carrier gas with a flow rate of 9 ml min^{-1}. The selected fuel gas was hydrogen. Internal standard was isopropanol (4 g l^{-1}) mixed at 50% (v/v) with samples. Data was quantified by Millenium 32 Software (Waters Corporation, Mildford, MA, USA). The estimated R.S.D. was about 2%.

2.10. Ammonium determination

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

2.11. Lipase activity assay

Samples were centrifuged for 10 min at 4 °C and 4500 × *g* (Mikro 12-24, Hettich, Tuttlingen, Germany). Lipolytic activity of the supernatant was determined by a modified method based on a colorimetric commercial kit assay (LIP kit from Roche Diagnostics, Mannheim, Germany), as described elsewhere (Cos et al., 2000).

This test was correlated to the lipolytic assay using pH-stat analysis and olive oil as substrate described in Minning et al. (2001). One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of fatty acid per minute under assay conditions.

2.12. Protease activity assay

Protease activity was determined using the substrate azocasein. The analysis was carried out as described elsewhere (Minning et al., 2001).

2.13. Calculation of specific rates

Estimated reaction rates were specific growth-rate (μ) (h^{-1}), specific substrate consumption rate (q_s) ($g_{methanol}\, g_{biomass}^{-1}\, h^{-1}$), and specific lipase production rate (q_p) (UA $g_{biomass}^{-1}\, h^{-1}$). The estimation procedure were performed by using suitable smoothing routines (Matlab 6.1 Curvefit Toolbox, The Mathworks Inc., Natick, USA) and mass balances that permitted to supply complete data sets with coincident off-line biomass, substrate, and lipolytic 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 sources (methanol and/or glycerol), acid and base solutions, as well as volume samples.

Finally, mass balances inside the bioreactor for the fed-batch operation were conducted to obtain the reac-

tion rates as follows:

$$\mu = \frac{1}{(XV)}\frac{\mathrm{d}(XV)}{\mathrm{d}t} \quad (1)$$

$$q_s = -\left(\frac{\mathrm{d}S}{\mathrm{d}t} + \frac{F(S-S_0)}{V}\right)\frac{1}{X} \quad (2)$$

$$q_p = \left(\frac{\mathrm{d}P}{\mathrm{d}t} + \frac{FP}{V}\right)\frac{1}{X} \quad (3)$$

where X is the biomass concentration ($g\,l^{-1}$), S the substrate concentration inside the bioreactor ($g\,l^{-1}$), S_0 the inlet substrate concentration ($g\,l^{-1}$), P the lipolytic activity ($UA\,l^{-1}$), t the time (h), F the feed-flow rate ($l\,h^{-1}$), and V is the total volume of the culture (l).

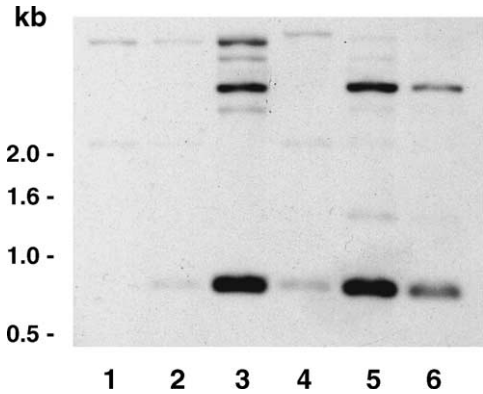

Fig. 1. Southern-blot hybridization analysis. Lane 1, control untransformed GS115 strain; lane 2, *ROL* single copy strain Mut$^+$; lane 3, *ROL* multicopy strain Mut$^+$; lane 4, *ROL* single copy strain Mut^s; and lane 5, *ROL* multicopy strain Mut^s.

3. Results and discussion

3.1. Selection of Mut^s ROL multicopy strains and shake-flask cultivations

ROL single and multicopy strains were generated by transformation of cells of the *AOX1* gene deleted (Mut^s) *P. pastoris* strain KM71 with the linearized vector pPICαA-ROL. When transforming the cells, multiple insertion events by repeated recombination at the homologous *AOX1* genomic site may occur at a low frequency. The pPICαA vector confers resistance to the antibiotic-derived Zeocin. Therefore, selection of strains containing multiple copies of the vector was achieved by dosage-dependent resistance to the drug. That is, at higher Zeocin concentrations, those transformants containing multiple copies of the vector were enriched.

Thus, after the selection of a ROL-producing KM71 transformant growing on Zeocin (100 $\mu g\,ml^{-1}$), named as KM100_0/ROL, all KM71 transformants were further screened by replica plating onto Zeocin-containing plates at concentrations of 500, 1000, and 1500 $\mu g\,ml^{-1}$ of the drug. Only two independent clones capable of growing on Zeocin at each of these concentrations were isolated (i.e. a total of six clones). Among these six clones, two growing at 1500 $mg\,ml^{-1}$ of Zeocin (named KMR1500_3 and KM1500_4, respectively) showed significant lipolytic activities when grown in shake-flask cultures.

High *ROL* gene content in KM71 transformants was verified by Southern-blot hybridization (Fig. 1). Further, the number of gene copies of Mut^s and (previously isolated, Serrano et al., 2001) Mut$^+$ multicopy strains was quantified by dot-blot analysis as described in the materials and methods section. The Mut^s multicopy clone KMR1500_3 contained about 23–24 copies of *ROL*, whereas the KM1500_4 clone contained 5–6 copies of *ROL*. Hence, clone KMR1500_3 was selected, together with a Mut$^+$ multicopy control clone for further cultivation experiments because they contained a *ROL* gene dosage of the same order.

Shake-flask cultures of strains X-33/ROL (Mut$^+$, *ROL* single copy, X-33/500_3/ROL (Mut$^+$, *ROL* multiple copy), KM100_0/ROL (Mut^s, single copy), and KMR1500_3/ROL (Mut^s, ROL multicopy) in minimal medium supplemented with histidine and methanol as a sole carbon source revealed the following observations (Fig. 2). First, specific lipolytic activities were clearly higher in the *ROL* single copy Mut^s strain than in the corresponding Mut$^+$ single copy strain. Second, although the specific lipolytic activity in the Mut$^+$ multicopy strain was clearly higher than in its corresponding single copy strain, the effect of the *ROL* gene dosage on specific lipolytic activity in Mut^s strains was not clear. Third, as previously observed in the Mut$^+$ strains (Serrano et al., 2001), despite the fact that the levels of specific lipolytic activity were higher in the multicopy strain than in the single copy strain, increased *ROL* gene dosage resulted in a clear negative effect on cell growth

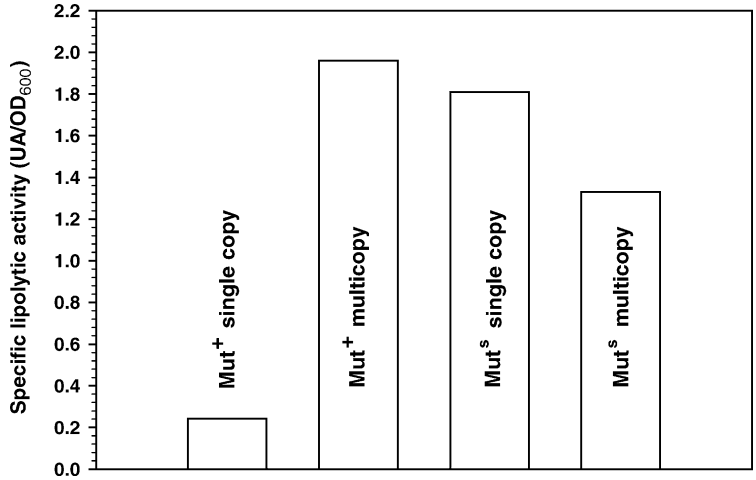

Fig. 2. Specific lipolytic activity of the ROL single and multicopy strains obtained in shake-flask cultures.

(data not shown), thus suggesting a possible physiological burden (i.e. stress or toxicity) due to overexpression of *ROL* in the multicopy strain. A possible explanation for this effect could be the phospholipase activity described for mature ROL, which might be toxic for the cells when this enzyme is accumulated intracellularly (Beer et al., 1998).

Further high cell density cultivation experiments were performed in bioreactors using a synthetic medium with both single and multiple copy, Mut^+ and Mut^s strain combinations. The KM71-derived multicopy strains were histidine auxotrophic. Therefore, they were unsuitable for bioreactor-scale cultivations using defined media. In addition, supplementation of the defined medium with histidine may lead to substantial changes in the strains metabolism. Hence, the KMR1500_3/ROL strain, which had a similar *ROL* gene dosage as the X-33/500_3/ROL strain was made prototrophic using the HIS4 vector pPIC9.

3.2. Fed-batch cultivations

The selected fed-batch strategy was identical for all cultivations: after a batch phase using glycerol as single carbon source (the end of which was indicated by a sharp increase in the dissolved oxygen (DO) levels), a transition phase with simultaneous feeding of glycerol and methanol was performed according to a modified scheme from Katakura et al. (1998). After the transition phase, the induction phase was started by feeding methanol as the sole carbon source. The feeding rate was modified through the induction phase according to the residual methanol consumption rate of the culture. This was estimated in accordance with the off-line measurements of the methanol concentration. In this way, methanol concentration was kept within the range of 1–2 $g\,l^{-1}$ for both phenotypes. Invitrogen's cultivation process guidelines for Mut^s *P. pastoris* strains recommend the adjustment of the methanol feed rate to maintain an excess of methanol in the medium which does not exceed 3 $g\,l^{-1}$. Also, this strategy minimized the build up of methanol to growth-rate-inhibitory levels, which could be limiting specific growth rate of Mut^+ cells (Kobayashi et al., 2000; Zhang et al., 2000). However, methanol concentration for Mut^+ strains reached transiently maximum values of methanol concentration up to 5 $g\,l^{-1}$ due to the limitations in the control strategy.

Cultivation data from fed-batch cultivations using our battery of four strains is shown in Fig. 3. As expected, all strains behaved identically when grown on glycerol as the sole carbon source during the initial batch phase. The $Y_{X/S}$ was around 0.5 (g biomass g^{-1} glycerol) and the μ_{max} was 0.18 h^{-1} in all cases. During the transition phase, glycerol was always at limiting concentrations, i.e. below detection limits (data not shown). This allowed derepression of methanol assimilation pathway enzymes, as revealed by the observation that methanol was being consumed and low

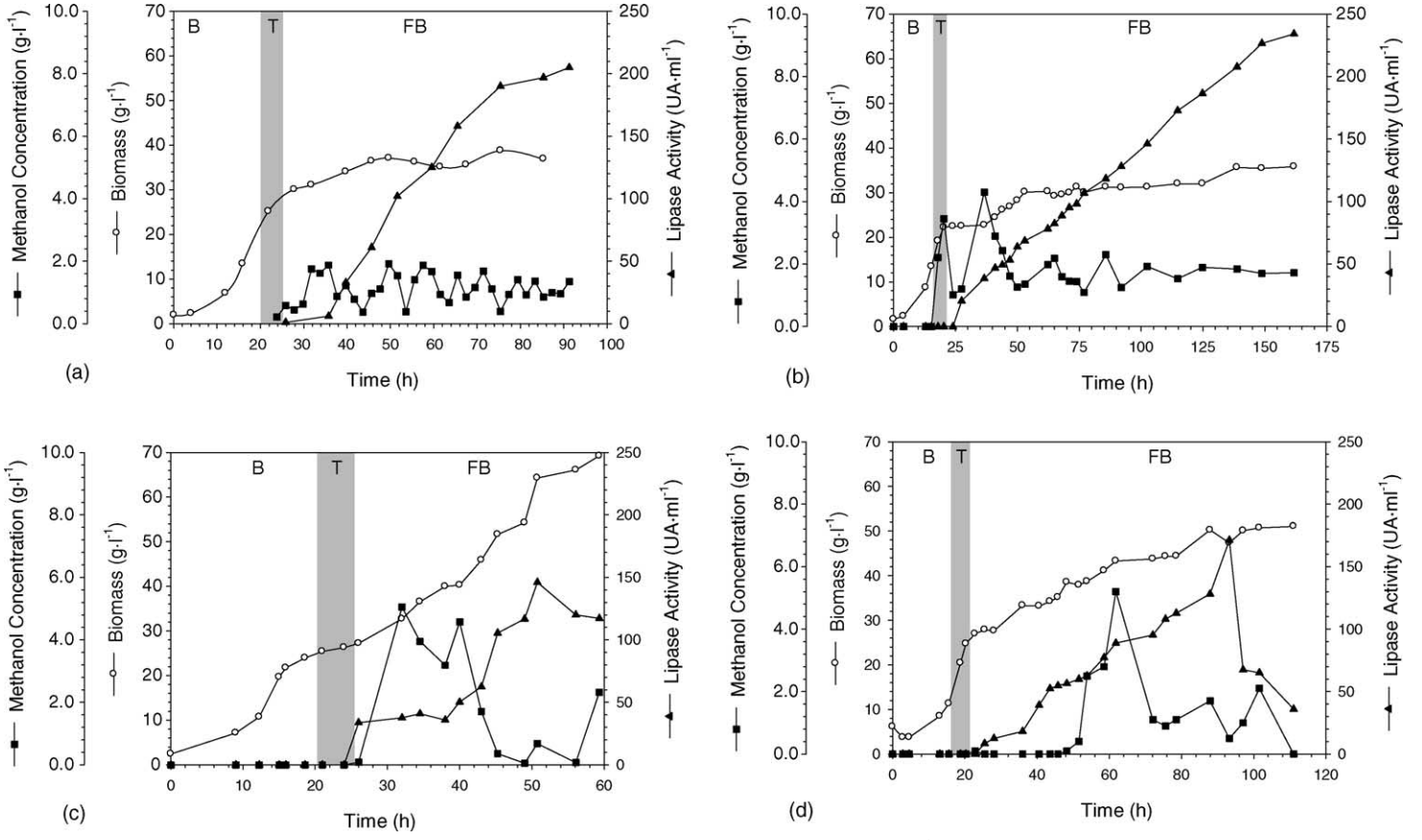

Fig. 3. Biomass, methanol concentration and lipase activity obtained in fed-batch cultivations for the Mut^{S} and Mut^{+} *Pichia pastoris* strains. Cultivation phases: B, batch culture; T, transition phase; and FB, fed-batch culture. Strain cultures: (a) Mut^{S} His^{+} single copy; (b) Mut^{S} His^{-} multicopy; (c) Mut^{+} His^{+} single copy; and (d) Mut^{+} His^{+} multicopy.

lipase activity levels were detected in the extracellular media in this phase.

3.3. Effect of gene dosage

Fed-batch cultivations with the prototrophic (His^+) Mut^s *ROL* multicopy strain did not progress for more than 24 h after starting the production phase. During this phase, oxygen demand decreased steadily, indicating a decline in the metabolic activity of cells growing on methanol. Methanol feeding rates during the initial stages of the production phase were similar to those achieved during the corresponding cultivation phase with the Mut^s single copy strain (Fig. 3); further, some extracellular lipase activity (about 5 UA ml^{-1}) was detected during this period (data not shown). However, after about 10 h, cells began to display a reduced methanol assimilation capacity, as revealed by an steady increase in the pO_2 levels.

These results were in clear contrast to the expression levels observed in shake-flask cultivations of both Mut^s strains, thus indicating that expression becomes limited at higher gene dosage under the more efficient induction conditions found in controlled cultivations. Similar observations have been previously reported (Clare et al., 1991b). Sinha et al. (2003) found that if the specific growth-rate drops to values as close as the maintenance metabolism, production may not be sustained. This may become critical at higher gene dosage under the more efficient induction conditions of the bioreactor culture.

In fact, cultivation of the KM71-derived (Mut^s) *ROL* multicopy strain without its histidine auxotrophy being reverted in a fed-batch mode in the presence of an excess of histidine allowed sustained cell growth and lipase production during the methanol feeding phase (Fig. 3b). The use of the auxotrophic strain provided a means of ensuring/assessing that histidine was always in excess at all times during cultivation. Nevertheless, the maximal lipase activity levels achieved after 90 h of cultivation (140 UA ml^{-1}) were substantially lower than the corresponding values obtained with the *ROL* single copy strain (205 UA ml^{-1}) after the same period of time. Biomass levels at that stage were of the same order in both cultivations (about 38 g dry cell weight per liter). Further, the yield of lipolytic activity per biomass unit, $Y_{P/X}$, was 1.3-fold higher in the Mut^s *ROL* multicopy strain compared to its single copy counterpart, thus showing that the cell's specific productivity increased at high *ROL* gene dosages in Mut^s strains (Table 1). In Fig. 4, the evolution of the specific growth-rate (μ), the methanol feeding rate, and the residual methanol concentration in the medium during the fed-batch cultivations with Mut^s strains are presented. The corresponding data for specific methanol consumption rate (q_s) and lipase production rate (q_p) evolution throughout the course of the fed-batch cultivations are presented in Fig. 5.

In both Mut^s strains, the specific growth-rate decreased to values below 0.02 h^{-1} after the transition phase was completed. These values are somewhat lower than those reported for the untransformed KM71 strain (0.04 h^{-1}, Brierley et al., 1990). Since the biomass generation throughout the production phase was virtually nil, the possible impact of gene dosage on the microorganism's growth cannot be assessed. In both Mut^s cultivations (Fig. 5a and b), a maximum for q_s was reached at early stages of the production phase, being followed by an exponential decrease along this phase; a similar behavior was observed for the q_p. That is, as the fed-batch phase progressed, the methanol consumption capacity, and therefore the ROL production capacity decreased. Further, at later stages of these cultivations, the apparent μ was virtually zero. However,

Table 1
Maximal lipase activity, lipase yield, and productivities obtained in fed-batch operation for the different *Pichia pastoris* strains

	Mut^s His^+ single copy	Mut^s His^- multicopy[b]	Mut^+ His^+ single copy[a]	Mut^+ His^+ multicopy[a]
Maximal lipase activity (UA ml^{-1})	205	270	150	175
$Y_{P/X}$ (UA $g^{-1}_{biomass}$)	5775	7500	2470	3578
Productivity (UA l^{-1} h^{-1})	2246	1500	3000	1894
Specific productivity (UA $g^{-1}_{biomass}$ h^{-1})	63	46	49	40

[a] Calculated for the maximal lipase activity in Mut^+ strain.
[b] Cultivation supplemented with histidine.

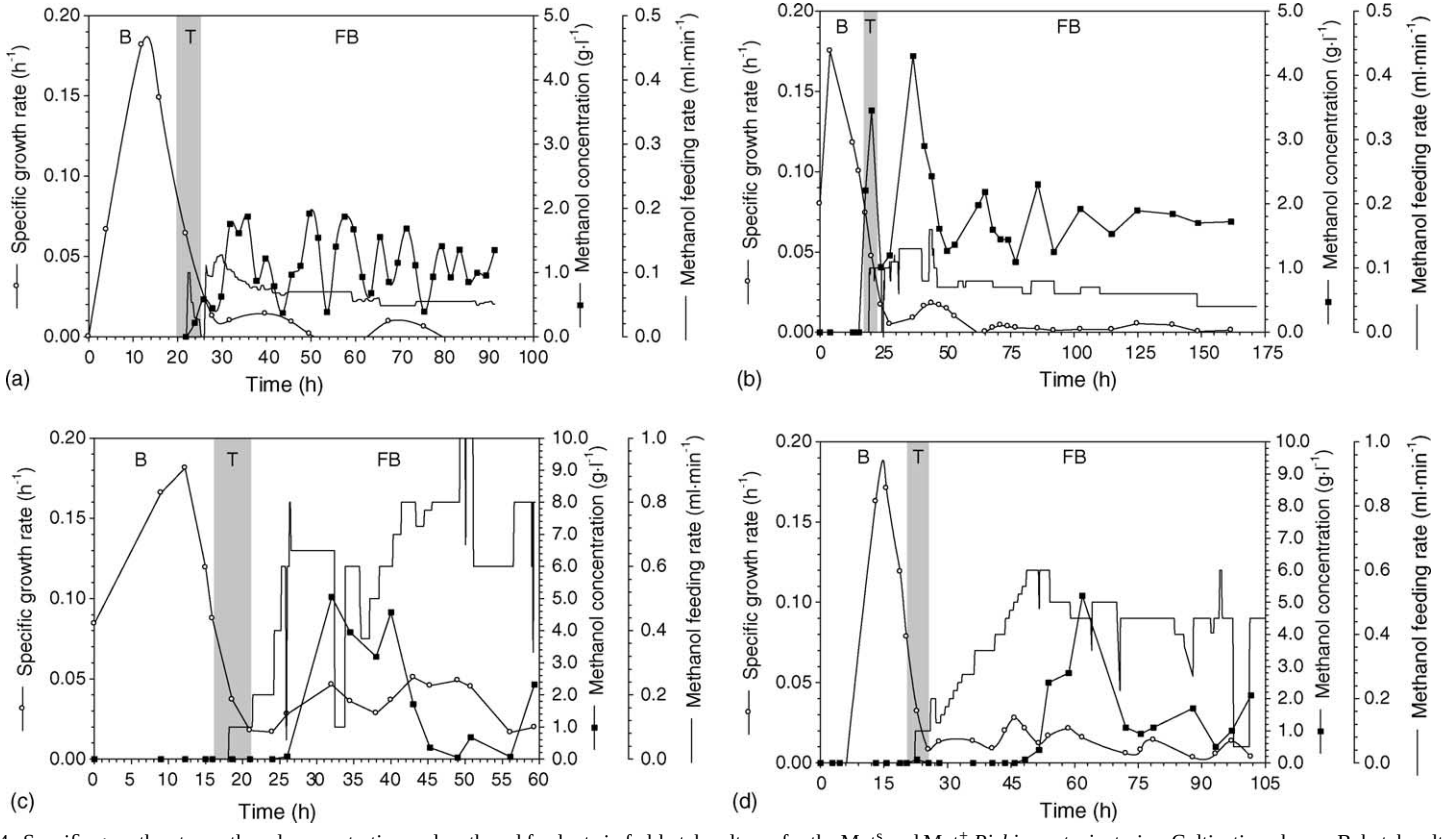

Fig. 4. Specific growth-rate, methanol concentration and methanol feed rate in fed-batch cultures for the Mut^{s} and Mut^{+} *Pichia pastoris* strains. Cultivation phases: B, batch culture; T, transition phase; and FB, fed-batch culture. Strain cultures: (a) Mut^{s} His^{+} single copy; (b) Mut^{s} His^{-} multicopy; (c) Mut^{+} His^{+} single copy; and (d) Mut^{+} His^{+} multicopy.

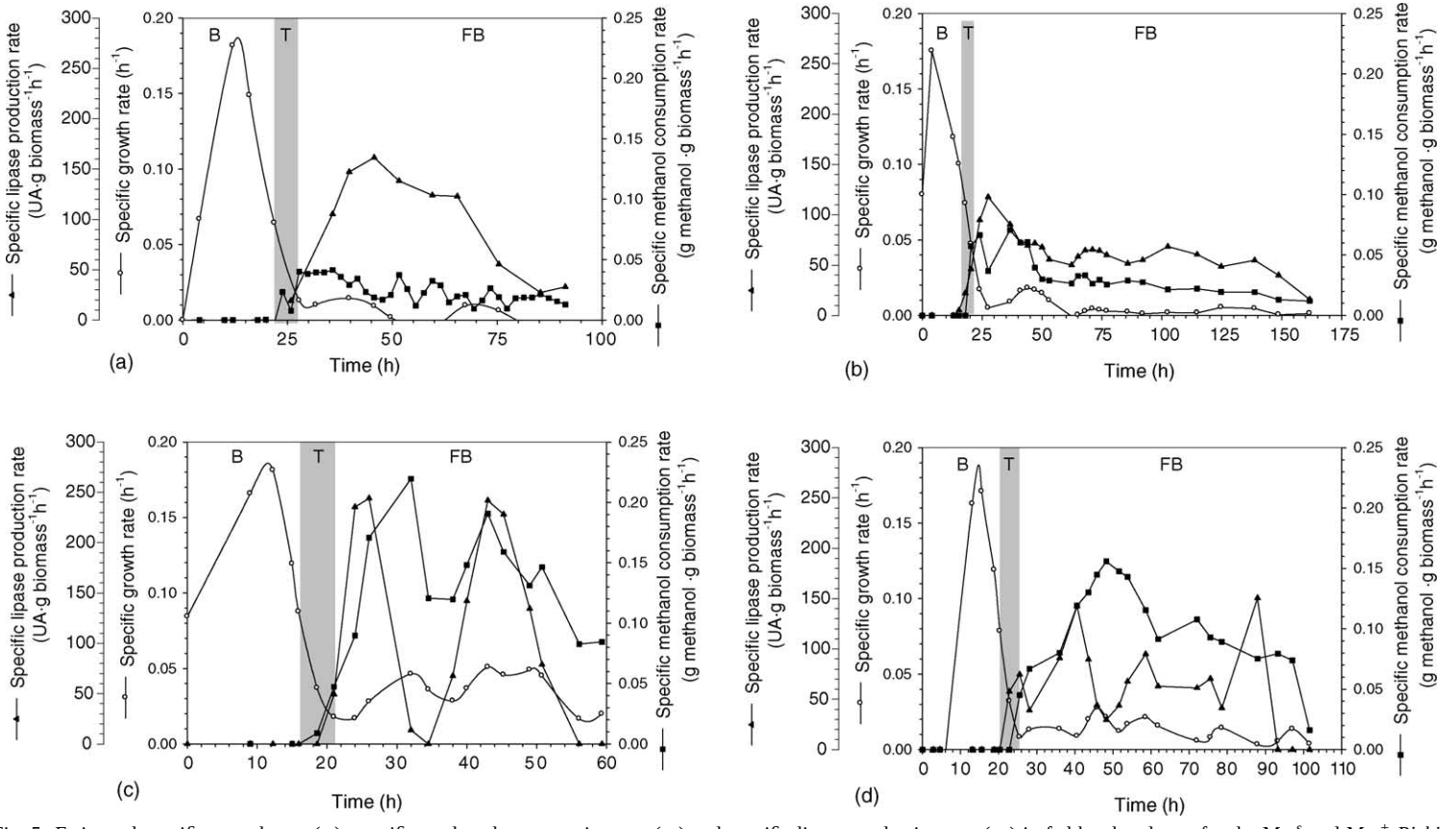

Fig. 5. Estimated specific growth-rate (μ), specific methanol consumption rate (q_s) and specific lipase production rate (q_p) in fed-batch cultures for the Mut^s and Mut$^+$ *Pichia pastoris* strains. Cultivation phases: B, batch culture; T, transition phase; and FB, fed-batch culture. Strain cultures: (a) Mut^s His$^+$ single copy; (b) Mut^s His$^-$ multicopy; (c) Mut$^+$ His$^+$ single copy; and (d) Mut$^+$ His$^+$ multicopy.

the $q_{p,max}$ and $q_{p,mean}$ were about 1.45- and 1.4-fold higher for the Mut^s single copy strain cultivation, respectively. Lower q_p values in multicopy strains suggest a negative effect of gene dosage on the specific production rate.

In fed-batch cultivations of Mut$^+$ strains, it was not possible to maintain the residual methanol concentration within the same limits as in Mut^s fed-batch cultivations (1–2 g l^{-1}). Also, this parameter had bigger oscillations than in the Mut^s fed-batch cultivations due to the limitations of the control strategy and the higher methanol assimilation capacity of Mut$^+$ strains in relation to Mut^s strains (Fig. 3c and d).

In the Mut$^+$ *ROL* multicopy strain fed-batch cultivation, the extracellular lypolytic activity reached a maximum of 175 UA ml^{-1} after approximately 70 h of induction phase, with a biomass of about 50 g l^{-1}, whereas in the cultivation with the Mut$^+$ *ROL* single copy strain, a maximum of 150 UA ml^{-1} was reached after only 24 h of induction phase and a biomass of about 55 g l^{-1}. The $Y_{P/X}$ is 1.4-fold higher for the Mut$^+$ *ROL* multicopy strain in relation to its single copy counterpart (Table 1), a value very similar to that found for Mut^s strains.

Hence, gene dosage in fed-batch cultivations with both phenotypes is not correlated with production levels and $Y_{P/X}$ values. This is reflected in the volumetric productivity (units of lipase per liter of reactor per hour) levels calculated for the single copy strains, which are significantly higher than for the multicopy strains, regardless their Mut phenotype (about 2250–3000 UA l^{-1} h^{-1} for *ROL* single copy strains versus 1500–1900 UA l^{-1} h^{-1} for *ROL* multicopy strains). Notably, the ratio of volumetric productivities between single copy:multiple copy strains is very similar for Mut^s and Mut$^+$ strains (1.5 and 1.6, respectively).

The effect of gene dosage in Mut$^+$ strains on the specific growth-rate is more clear than in Mut^s cultivations due to the higher specific growth-rates. Thus, in the cultivation with the *ROL* single copy strain (Fig. 4c), the maximum specific growth-rate reached is about 0.04 h^{-1}, whereas in the *ROL* multicopy strain only a maximum of 0.02 h^{-1} (Fig. 4d). In both cases, the observed maximum growth-rate is clearly below the maximum reported for Mut$^+$ strains in fed-batch cultivations under similar conditions (for instance, 0.071 h^{-1}, Zhang et al., 2002). Since concentrations higher than 2 g l^{-1} were only reached transiently in a fraction of about 20% of the total fed-batch phase time in the Mut$^+$ *ROL* multicopy cultivation, the lower maximum specific growth-rate observed for this strain can not be related to methanol accumulation in the culture medium, but to the *ROL* gene dosage, i.e. to *ROL* overexpression.

The behavior of μ, q_s, and q_p values in cultivations with Mut$^+$ strains (Fig. 5c and d) are more difficult to analyze because the off-line strategy used to control the residual methanol concentration in the reactors resulted in oscillations of this parameter, from 0 to 5 g l^{-1}, with important consequences: In the early stage of the production phase, sudden decreases in q_p were correlated with sudden increases of the residual methanol concentrations in the medium (in the case of the Mut$^+$ *ROL* single copy cultivation), or with prolonged periods under methanol-limiting conditions (in the case of the Mut$^+$ *ROL* multicopy strain cultivation). Further, the transient accumulation of methanol at other stages of the fed-batch phase of cultivations with Mut$^+$ strains corresponded with an important decrease in q_p values (Fig. 5c and d). These observations are consistent with the fact that transcription levels from P_{AOX1} are reported to be three to five times higher at methanol-limiting concentrations (Lin Cereghino and Cregg, 2000). Overall, one can still observe that q_s and q_p values tended to decrease during the fed-batch phase of both single and multicopy Mut$^+$ strains. However, such a decrease was not so pronounced as that observed in cultivations with Mut^s strains (particularly for q_s values).

q_s Values in the initial stages of the production phase were close to those observed for the corresponding untransformed strains in all cases and after reaching a maximum, these values decreased. Notably, average methanol feeding rates were clearly lower in both Mut$^+$ and Mut^s *ROL* multicopy strain cultivations than in the cultivations with their corresponding *ROL* single copy strains. It is possible that accumulation of intracellular *ROL* along cultivation time gradually inhibits methanol assimilation capacity of the host organism, becoming critical when methanol assimilation is genetically controlled and *ROL* transcription levels are higher (i.e. Mut^s *ROL* multicopy strain).

Also, a clear negative effect of the *ROL* gene dosage was observed on q_p values in both Mut phenotypes. The estimated q_p mean values were about 1.4 and 1.8 times higher for Mut^s and Mut$^+$ *ROL* single copy strains, re-

spectively, compared with their *ROL* multicopy counterparts.

3.4. Effect of the Mut phenotype

The effect of Mut phenotype on ROL production can be assessed when comparing the major parameters calculated for fed-batch cultivations with Mut^+ and Mut^s *ROL* single copy strains (Fig. 3a and c, Table 1). In the Mut^+ strain cultivation, a maximum extracellular lipase activity of 150 UA ml^{-1} was achieved after 50 h of cultivation at a biomass of about 55 g l^{-1}. After this point, lipase activity decreased. In the Mut^s strain cultivation, equivalent lipase activity levels were also reached after 75 h of cultivation but at a much lower biomass (ca. 35 g l^{-1}). Further, extracellular lipase levels steadily increased up to about 205 UA ml^{-1} after 90 h of cultivation, with biomass levels around 38 g l^{-1}.

Overall, the maximum lipase activity achieved, the specific productivity (UA $g^{-1}_{biomass}$ h^{-1}), and the $Y_{P/X}$ obtained with the Mut^s were 1.4, 1.3, and 2.3 times higher, respectively, than with the Mut^+ strain, indicating that the Mut^s strain was more efficient for ROL production. However, in terms of productivity (units of lipase per liter of reactor per hour), the value for the Mut^+ was 1.3-fold higher than for the Mut^s strain, i.e. compensating the lower production levels achieved with the Mut^+ strain with its shorter cultivation time and similar biomass levels.

As expected, the methanol feeding rates achieved for the Mut^+ *ROL* single copy strain of about 800 μl min^{-1} were clearly higher than those for the Mut^s *ROL* single copy strain (Fig. 4), approximately 100 μl min^{-1}. In turn, the mean μ reached throughout the production phase with methanol as sole carbon source was 7.2-fold higher for the Mut^+ strain culture. These differences are also observed from the analysis of the maximum and mean specific methanol consumption rates (q_s) (Table 2). The evolution of q_s during the production phase of Mut^+ and Mut^s cultivations showed quite different profiles in qualitative terms. Although in both cultivations, a maximum q_s value was obtained in the initial stage of the production phase, in the Mut^s cultivation an exponential decay of this parameter was observed after reaching its maximum value. Conversely, for the Mut^+ cultivation, the q_s value did not experience an exponential decay after the maximum was reached. Rather, it seemed to decrease and stabilize to values around 0.15 $g_{methanol}$ $g^{-1}_{biomass}$ h^{-1}.

The maximum q_p for the Mut^+ cultivation was 244 UA $g^{-1}_{biomass}$ h^{-1}, higher (1.4-fold) than that estimated for the Mut^s cultivation, 170 UA $g^{-1}_{biomass}$ h^{-1}. This relation was maintained within similar terms when the mean q_p values were compared: 130 and 83 UA $g^{-1}_{biomass}$ h^{-1} for the Mut^+ and Mut^s, respectively, as the higher specific lipase productivity of the Mut^s strain compensated its longer cultivation time.

A problem observed in the cultivations with both Mut^+ *ROL* single and multicopy strains is the product degradation (revealed by the sharp decrease in extracellular lipase activity, as well as in SDS-PAGE of cultivation samples, data not shown) and concomitant cell lysis when biomass levels of 50 g l^{-1} (dry cell weight) or higher were reached, as observed in Fig. 3c and d. Product proteolytic degradation has also been detected in *P. pastoris* expressing human serum albumin at the same biomass levels, using the same growth medium (Kobayashi et al., 2000). These authors detected an increase of proteolytic activity when the ammonium levels in the cultivation medium decreased below 0.3 mg l^{-1}, thus suggesting that the protease activity may be induced by nitrogen starvation. Indeed,

Table 2
Mean and maximal specific growth-rate (μ), specific substrate consumption rate (q_s), and specific lipase production rate (q_p) for the different *Pichia pastoris* strains. Mean specific growth-rates were estimated as time-averaged

	Mut^s His^+ single copy	Mut^s His^- multicopy	[a]Mut^+ His^+ single copy	[a]Mut^+ His^+ multicopy
μ_{max} (h^{-1})	0.014	0.018	0.051	0.028
μ_{mean} (h^{-1})	0.005	0.004	0.036	0.012
$q_{s,max}$ ($g_{methanol}$ $g^{-1}_{biomass}$ h^{-1})	0.05	0.071	0.22	0.16
$q_{s,mean}$ ($g_{methanol}$ $g^{-1}_{biomass}$ h^{-1})	0.024	0.028	0.14	0.10
$q_{p,max}$ (UA $g^{-1}_{biomass}$ h^{-1})	170	118	244	150
$q_{p,mean}$ (UA $g^{-1}_{biomass}$ h^{-1})	83	59	130	71

[a] Calculated for the maximal lipase activity in Mut^+ strain.

analysis of the ammonium levels throughout the production phase of our fed-batch cultivations confirmed that ammonium became limiting above biomass levels of about 50 g l^{-1}. Furthermore, based on the elemental composition obtained from *P. pastoris* Mut^+ cells growing on a methanol batch culture ($C\ H_{1.78\pm0.01}\ O_{0.62\pm0.05}\ N_{0.18\pm0.001}\ S_{0.006\pm0.001}$), and considering a biomass/substrate yield of 1.00 (C-molar basis), the maximal ratio ammonium sulfate/methanol was calculated as 2.71 g Methanol g^{-1} ammonium sulfate. On the basis of this calculation, it was determined that the cultivation would become nitrogen-limited at biomass concentrations above 50–60 g l^{-1} dry cell weight of biomass.

4. Conclusions

The methanol feeding strategy, which also dictates the specific growth-rate, is a key parameter for maximizing recombinant protein production (Shioya, 1992). However, strategies to control cell growth may increase instrumentation and expertise requirements. In this context, the use of a Mut^s strain allowed us to readily maintain residual methanol concentrations close to a constant level, as well as smoothing the fluctuations of μ, q_s, and q_p during the fed-batch phase. For Mut^+ strains, the need for an effective control system to avoid abrupt fluctuations in the residual methanol concentration in the medium is clearly shown in this study.

Notably, a clear difference in terms of specific productivity and $Y_{P/X}$ were observed depending on the phenotype. Mut^s strain showed higher values than Mut^+ strains of these two parameters. However, although Mut^+ *ROL* single copy strain showed a 34% higher process productivity than Mut^s single copy strain, the Mut^s strain may still be advantageous to use because it allows for easier process control strategies.

As shown in previous studies, an increase in expression cassette copy number can be an effective means to increase product synthesis up to a certain limit of gene dosage (McGrew et al., 1997; Vassileva et al., 2001). This limit is product-dependent and a further increase of gene dosage can result in a decrease of expression in several cases (Parekh et al., 1995; Hohenblum et al., 2004). Further, heterologous expression of multiple copies of a given gene may be influenced by the promoter used and growth conditions (Kobayashi et al., 2000; Hohenblum et al., 2004). This is further supported by this study showing that the methanol assimilation capacity of the host strain has an important impact on ROL expression levels. Although Kobayashi et al. (2000) suggested that limitations in the translation machinery or limitation of precursors may become critical at high gene dosages, the moderate specific productivities may also indicate limitations in folding and secretion. Current studies are in progress to identify possible bottlenecks in expression and secretion processes.

A biological system has been developed to study the effects of gene dosage on the production of a heterologous model protein ROL. Also, the strains constructed and characterized in this study seem to be appropriated as a model system for studying possible effects on the cells physiology brought on by the overproduction of extracellular heterologous proteins in *P. pastoris*.

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References

Beer, H.D., McCarthy, J.E., Bornscheuer, U.T., Schmid, R.D., 1998. Cloning, expression, characterization and role of the leader sequence of a lipase from *Rhizopus oryzae*. Biochim. Biophys. Acta 1399, 173–180.

Brierley, R.A., Bussineau, C., Kosson, R., Melton, A., Siegel, R.S., 1990. Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme. Ann. N Y Acad. Sci. 589, 350–362.

Clare, J.J., Romanos, M.A., Rayment, F.B., Rowedder, J.E., Smith, M.A., Payne, M.M., Sreekrishna, K., Henwood, C.A., 1991a. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. Gene 105, 205–212.

Clare, J.J., Rayment, F.B., Rowedder, J.E., Ballantine, S.P., Sreekrishna, K., Romanos, M.A., 1991b. High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. Biotechnology 9, 455–460.

Cregg, J.M., Madden, K.R., 1987. Development of yeast transformation systems and construction of methanol-utilization-defective mutants of *Pichia pastoris* by gene disruption. In: Stewart, G.G., Russell, I., Klein, R.D., Hiebsch, R.R. (Eds.), Biological Research on Industrial Yeasts, vol. II. CRC Press, Boca Raton, FL, USA, pp. 1–18.

Cregg, J.M., Russell, K.A., 1998. Transformation. Methods Mol. Biol. 103, 27–39.

Cos, O., Montesinos, J.L., Lafuente, J., Solà, C., Valero, F., 2000. On-line monitoring of lipolytic activity by sequential injection analysis. Biotechnol. Lett. 22, 1783–1788.

D'Anjou, M.C., Daugulis, A.J., 2001. A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. Biotechnol. Bioeng. 72, 1–11.

Demir, A.S., Hamamci, H., Tanyeli, C., Akhmedov, I.M., Doganel, F., 1998. Synthesis and *Rhizopus oryzae* mediated enantioselective hydrolysis of α-acetoxy aryl alkyl ketones. Tetrah. Asym. 9, 1673–1677.

Hohenblum, H., Gasser, B., Maurer, M., Borth, N., Mattanovich, D., 2004. Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. Biotechnol. Bioeng. 85, 367–375.

Katakura, Y., Zhang, W., Zhuang, G., Omasa, T., Kishimoto, M., Goto, Y., Suha, K., 1998. Effect of methanol concentration on the production of human β_2-glycoprotein I Domain V by recombinant *Pichia pastoris*: a simple system for the control of methanol concentration using a semiconductor gas sensor. J. Ferm. Bioeng. 86, 482–487.

Kobayashi, K., Kuwae, S., Ohya, T., Ohda, T., Ohyama, M., Ohi, H., Tomomitsu, K., Ohmura, T., 2000. High-level expression of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. J. Biosci. Bioeng. 89, 55–61.

Lin Cereghino, J., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 45–66.

McGrew, J.T., Leiske, D., Dell, B., Klinke, R., Krasts, D., Wee, S., Abbot, N., Armitage, R., Harrington, K., 1997. Expression of trimeric CD40 ligand in *Pichia pastoris*: use of a rapid method to detect high-level expressing transformants. Gene 187, 193–200.

Miles, D.J., Busser, K., Stalder, C., Higgins, D.R., 1998. Isolation of nucleic acids. Methods Mol. Biol. 103, 73–80.

Minning, S., Schmidt-Dannert, C., Schmid, R.D., 1998. Functional expression of *Rhizopus oryzae* lipase in *Pichia pastoris*: high-level production and some properties. J. Biotechnol. 66, 147–156.

Minning, S., Serrano, A., Ferrer, P., Solà, C., Schmid, R.D., Valero, F., 2001. Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. J. Biotechnol. 86, 59–70.

Parekh, R., Forrester, K., Wittrup, D., 1995. Multicopy overexpression of bovine pancreatic trypsin inhibitor saturates the protein folding and secretory capacity of *Saccharomyces cerevisiae*. Protein Expr. Purif. 6, 537–545.

Rodríguez-Jiménez, E., Sánchez, K., Roca, H., Delgado, J.M., 1997. Different methanol, feeding strategies to recombinant *Pichia pastoris* cultures producing high level of dextranase. Biotechnol. Techniq. 11, 461–466.

Romanos, M., Scorer, C., Sreekrishna, K., Clare, J., 1998. The generation of multicopy recombinant strains. Methods. Mol. Biol. 103, 55–72.

Serrano, A., Cereghino, G.L., Ferrer, P., Cregg, J.M., Valero, F., 2001. Overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris* strains containing multiple copies of the target gene. In: Merten, O.-W., et al. (Eds.), Recombinant Protein Production with Prokaryotic and Eucaryotic Cell. A Comparative View on Host Physiology. Kluwer Academic Publishers, Dordrecht, pp. 259–267.

Shioya, S., 1992. Optimization and control in fed-batch bioreactors. In: Fiechter, A. (Ed.), Advances in Biochemical Engineering & Biotechnology, vol. 46. Springer, Berlin, pp. 111–142.

Sinha, J., Plantz, B.A., Zhang, W., Gouthro, M., Schlegel, V., Liu, C.-P., Meagher, M.M., 2003. Improved production of recombinant ovine interfereon-τ by Mut^+ strain of *Pichia pastoris* using an optimized methanol feed profile. Biotechnol. Prog. 19, 794–802.

Sreekrishna, K., Kropp, K., 1996. *Pichia pastoris*. In: Wolf, K. (Ed.), Non-Conventional Yeasts in Biotechnology. Springer, Berlin, pp. 203–252.

Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J., Smith, P.L., Wierschke, J.D., Subramaniam, A., Birkenberger, L.A., 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. Gene 190, 55–62.

Stratton, J., Chiruvolu, V., Meagher, M., 1998. High cell-density fermentation. Methods Mol. Biol. 103, 107–120.

Suyama, T., Tokiwa, Y., 1997. Enzymatic degradation of an aliphatic polycarbonate, poly(tetramethylene carbonate). Enzyme Microb. Technol. 20, 122–126.

Vassileva, A., Chugh, D.A., Swaminathan, S., Khanna, N., 2001. Effect of copy number on the expression levels of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris*. Protein Expr. Purif. 21, 71–80.

Virto, C., Svensson, I., Adlercreutz, P., 1999. Enzymatic synthesis of lysophosphatidic acid and phosphatidic acid. Enzyme Microb. Technol. 24, 651–658.

Werten, M.W.T., Van den Bosch, T.J., Wind, R.D., Mooibroek, H., de Wolf, F.A., 1999. High-yield secretion of recombinant gelatins by *Pichia pastoris*. Yeast 15, 1087–1096.

Zhang, W., Bevins, M.A., Plantz, B.A., Smith, L.A., Meagher, M.M., 2000. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnol. Bioeng. 70, 1–8.

Zhang, W., Smith, L.A., Plantz, B.A., Schlegel, V.L., Meagher, M.M., 2002. Design of methanol feed control in *Pichia pastoris* fermentations based upon a growth model. Biotechnol. Prog. 18, 1392–1399.