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Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*

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Abstract

The lipases of the *Rhizopus species* family are important and versatile enzymes that are mainly used in fat and oil modification due to their strong 1,3-regiospecificity. Inexpensive synthetic medium was used for the production of *Rhizopus oryzae* lipase in the methylotrophic yeast *Pichia pastoris*. Methanol accumulation inside the bioreactor has previously been shown to negatively influence the production level. Three different methanol fed-batch strategies for maintaining the methanol concentration within optimal limits have been assayed in high-density cultures. One methanol feeding strategy, which is based on the monitoring of the methanol concentration by gas chromatography, resulted in a 2.5-fold higher productivity compared to an initial cultivation, where the feeding rate was adjusted according to the dissolved oxygen concentration (DO) in the supernatant. Finally, productivity could be further increased by introducing a transition phase that involved the simultaneous feeding of glycerol and methanol followed by a single methanol feed. This optimized strategy resulted in the highest productivity (12 888 U l^{-1} h^{-1}), which is 13.6-fold higher than the DO-based strategy.

Keywords: Bioreactor; *Pichia pastoris*; *Rhizopus oryzae*; Lipase; Methanol; Fed-batch

1. Introduction

The methylotrophic yeast *Pichia pastoris* is an excellent host for the high level production of intracellular and extracellular proteins from different sources (Sreekrishna and Kropp, 1996; Sreekrishna et al., 1997; Cregg, 1999).

Foreign genes can be expressed at high levels by placing them under the tightly controlled *AOX1* (alcohol oxidase 1) promoter (*AOX1*p), which is inducible by methanol and repressed by other carbon sources such as glucose, glycerol or ethanol. Typically, recombinant *P. pastoris* cells are cultivated in excess of glycerol to repress foreign

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gene expression (batch phase) followed by its induction after the addition of methanol (fed-batch phase) (Cregg et al., 1993).

Methanol levels in the fermentor must be carefully controlled in order to avoid its accumulation to toxic levels. For instance, the 'Pichia Fermentation Process Guidelines' of Invitrogen Co. (San Diego, CA) indicate two different empirical methanol feeding strategies, which are commonly used by many researchers. The first is based on the dissolved oxygen (DO) spike method; the second uses preprogrammed linear feed rates that are designed to maintain very low methanol concentrations in the growth medium. These differ depending on the methanol-utilization ability phenotype of the expression host (Stratton et al., 1998). However, the selection of the production scheme and the specific parameters within each scheme (e.g. the methanol feed rate in the induction phase) must be individually optimized for each foreign protein (Cregg et al., 1993).

Several lipases have successfully been expressed in *P. pastoris*. All except one of human origin (Sahasrabudhe et al., 1998; Yang and Lowe, 1998) were from fungal species: *Candida rugosa* lipase 1 (Lip 1) (Brocca et al., 1998), lipases A and B from *Geotrichum candidum* (Catoni et al., 1997; Holmquist et al., 1997) and the *Rhizopus oryzae* lipase (ROL; Minning et al., 1998).

The first attempt to produce ROL in bioreactor cultures was successfully carried out using a rather expensive complex medium (Minning et al., 1998). This cultivation procedure involved the following fed-batch scheme: After an initial glycerol batch-phase to generate biomass, the induction phase in which methanol feeding was directly correlated with the DO of the culture was started. The lipase yield could be increased from 110 U ml^{-1} (13 mg active lipase per liter) to 500 U ml^{-1} (59 mg active lipase per liter; Minning et al., 1998). A DO-based strategy was also used for the production of dextranase (Rodríguez-Jiménez et al., 1997). With this method, the toxic effect of methanol is avoided by keeping the increment of the methanol feeding rate at a moderate level. Methanol concentration in the bioreactor will not remain constant at all times and can therefore affect the protein production and the methanol consumption rate (Katakura et al., 1998).

In this paper we describe the successful production of high amounts of ROL in *P. pastoris* with an inexpensive synthetic medium. To optimize the production levels, the evaluation of different methanol and glycerol feeding strategies and their effect in the lipase-specific productivity were investigated.

2. Material and methods

2.1. Plasmids and organisms

The ROL gene-containing plasmid pPICZαA-ROL was constructed by Minning et al. (1998). *P. pastoris* X-33 wild type strain (i.e. Methanol utilization plus, Mut^+) was transformed with *SacI*-linearized pPICZαA-ROL by electroporation following the manufacturer's instructions (Invitrogen). The integration of the ROL expression vector into the *AOX1* locus resulted in a Mut^+ phenotype (*Pichia* expression kit, Invitrogen). A *P. pastoris* X-33 control strain was constructed by transforming this strain with *SacI*-linearized pPICZαA following the manufacturer's instructions.

P. pastoris GS115 (*his4*) and *P. pastoris* KM71 (*his4*, *aox1::ARG4*) strains were also transformed with *SacI*-linearized pPICZαA and used as Mut^+ and Mut^S (Methanol utilization slow) strain controls, respectively.

2.2. Culture maintenance

P. pastoris cells were grown on YPD 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.

2.3. Culture media

P. pastoris seed cultures were grown overnight in shake flasks at 30°C in a rich standard BMGY medium, containing 1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer pH 6.0, 4×10^{-5}% (w/v) biotin, 1% (w/v)

glycerol. Cells were centrifuged at 5000 rpm and resuspended in synthetic medium for batch and fed-batch cultivation.

To determine the Mut phenotype, MMH plates, containing 0.5% (v/v) methanol, 0.004% (w/v) histidine, 4×10^{-5}% (w/v) biotin, and MDH plates, 2% (w/v) dextrose, 0.004% (w/v) histidine, 4×10^{-5}% (w/v) biotin were used.

The basal salt synthetic medium contained the following substances 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), H_2SO_4 (concentrated, 5 ml).

2.4. Fermentation and operational conditions

The cells were cultured in a 5 l Braun Biostat E fermenter. The carbon sources were either added manually or with an automatic MicroBU-2031 burette from Crison Instruments (Alella, Barcelona, Spain). The initial working volume amounted to 3.5 l.

The basal salt medium was autoclaved in situ and sterile filtered PTM1 solution was added after the medium had cooled to room temperature. 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. Standard operating conditions were a temperature of 30°C, and a pH that was maintained at 5.5 by adding 2 M NH_4OH. To ensure a concentration of DO up to an air saturation of 20%, the stirring rate was maintained between 500 and 900 rpm and the air-flow rate between 0.5 and 10 l min^{-1}.

2.5. Feeding rate of mixed substrate

During the fermentation transition phase the culture was fed with a mixed substrate for 5 h. The methanol feeding rate was kept constant at 100 μl min^{-1} while the glycerol feeding rate decreased continuously. 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.

2.6. Biomass analysis

Biomass was determined on the basis of the protein's dry weight. Ten-milliliter samples were filtered through 0.45 μm filters. These were washed with distilled water and dried at 100°C to a constant weight.

2.7. Glycerol and methanol determination

Glycerol was determined by HPLC (Hewlett Packard 1050) analysis using an Aminex HPX-87H ion-exchange column from Bio-Rad. The mobile phase was 15 mM sulfuric acid, injection volume was 20 μl. Data was quantified by the Millenium 2.15.10 software (Waters). Methanol was analyzed by gas chromatography (GC) (Hewlett Packard 5890) using a capillary column (Tracsil TR-FFAP 25 m × 0.53 mm × 1 μm from Tracer-Teknokroma, St. Cugat del Vallès, Barcelona, Spain), an automatic injector 7376 from Hewlett Packard and a FID detector. Injector and detector temperatures were 200 and 280°C, respectively. The oven temperature was kept at 40°C. Helium was used as carrier gas at a flow rate of 9 ml min^{-1} and hydrogen was used as a fuel gas. Millenium 32 software was used for peak integration and data quantification.

2.8. Lipase activity assay

Lipolytic activity was determined by pH-stat analysis using a 718 STAT Titrino pH-stat (Metrohm) and olive oil emulsion (50%; v/v) as substrate (Sigma Diagnostics). The olive oil emulsion (0.5 ml) was mixed with 4.5 ml of a salt solution containing 0.25 M NaCl and 0.05 M $CaCl_2$. To maintain the pH at 8.1 (temperature: 30°C), 0.02 M NaOH was used as a titration agent. One unit was defined as the amount of enzyme required to release 1 μmol of fatty acid per minute under assay conditions.

2.9. Protease activity assay

Protease activity was determined using the substrate azocasein. The analysis was carried out at pH 7.6 (Scheuning and Fritz, 1976).

2.10. Digestion with endo-β-N-acetyl-glucosaminidase (Endo H)

The deglycosylation of samples was carried out with endo-β-*N*-acetylglucosaminidase H (125 U mg^{-1} enzyme, Roche Molecular Biochemicals) according to the manufacturer's instructions. The incubation buffer was 50 mM potassium acetate buffer, pH 5.5, and 0.5 mM phenylmethylsulfonyl fluoride.

2.11. SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) was performed in 12% polyacrylamide gels under denaturing conditions as described by Laemmli (1970). A vertical slab Mini Protein II cell (Bio-Rad) was used for electrophoresis. The proteins were visualized by silver staining according to standard procedures.

2.12. Recovery and purification of ROL from culture supernatants

The culture broth was centrifuged to remove most of the biomass. The remaining biomass was eliminated by microfiltration with 0.45 μm cut-off filters. The broth was concentrated by ultrafiltration with a Minitan® ultrafiltration system (Millipore) equipped with a membrane of 10 kDa cut-off, and subsequently dialyzed against 20 mM Tris–HCl, pH 8.1, in the same system. The concentrated liquid was lyophilized. This material was defined as crude ROL preparation.

2.13. Cation-exchange chromatography

The crude ROL preparation was resuspended (50 mg lyophilized powder ml^{-1}) in distilled water and subsequently dialyzed against 10 mM $CaCl_2$ in water, pH 6.0. The sample was centrifuged at 12 000 g for 20 min at 4°C and finally filtered through a 0.45 μm membrane prior to being loaded on the chromatographic column in a Pharmacia FPLC system.

The lipase solution was loaded on an SP sepharose XK2620 column (diameter 26 mm, 75 ml bed volume; Pharmacia) equilibrated with 10 mM $CaCl_2$ in water, pH 6.0. The flow rate was 1.5 ml min^{-1} and 5 ml fractions were collected. After the elution of two column volumes, a gradient from 0 to 1 M NaCl in 10 mM $CaCl_2$ was maintained during one additional column volume. This NaCl concentration was maintained during two more bed volumes (Fig. 6).

3. Results and discussion

3.1. Methanol feeding fed-batch under DO control

Preliminary cultivation of the cells in synthetic medium according to a methanol fed-batch scheme recommended for a Mut^+ strain by the *P. pastoris* system supplies (Fermentation guidelines, Invitrogen, San Diego, CA) led to the inhibition of cell growth. This was most likely caused by excessive methanol accumulation in the medium. In addition, experiments with minimal medium plate cultures showed that the growth-rate of the lipase-producing strain was slower than for the control Mut^+ strain (data not shown) although it still grew faster than the Mut^s control strain.

Thus, our first conservative approach followed a methanol feeding strategy suggested by Invitrogen for Mut^s strains (Fermentation guidelines; Invitrogen, San Diego, CA): A 40 g l^{-1} glycerol batch cultivation was conducted until the carbon source was completely consumed (identified by a quick increase in DO). Methanol supply was started after glycerol depletion following a pre-programmed feeding rate profile until the final feeding rate of 10 ml h^{-1} was reached. Beyond this point, the methanol feeding rate was increased in accordance with the DO of the culture. Whenever the DO increased, the methanol feeding rate was increased. The evolution of the methanol feeding rate profile and evolution of the fermentation variables are shown in Fig. 1.

After 120 h of cultivation, the lipolytic activity in the supernatant had reached 120 U ml^{-1}, the productivity was 945 U l^{-1} h^{-1} and the specific productivity was 26 U h^{-1} g biomass^{-1}. The best results previously obtained using expensive complex medium were 5435 U l^{-1} h^{-1} after 92 h of cultivation (Minning et al., 1998). However, these values are not fully comparable since assay conditions were different. It is well known that the lipolytic activity test largely depends on a variety of factors, e.g. the substrate's interfacial area that is available for the enzyme, the type of substrate, as well as the equipment used (Thomson et al., 1999). Comparative lipolytic activity measurements of the same samples were performed in parallel by both laboratories. These experiments indicated that the lipolytic activity values reported in the present work are an underestimation relative to those reported by Minning et al. (1998), i.e. they are approximately three times lower.

Comparison of the DO profiles with the methanol feed rates implemented during the culti-

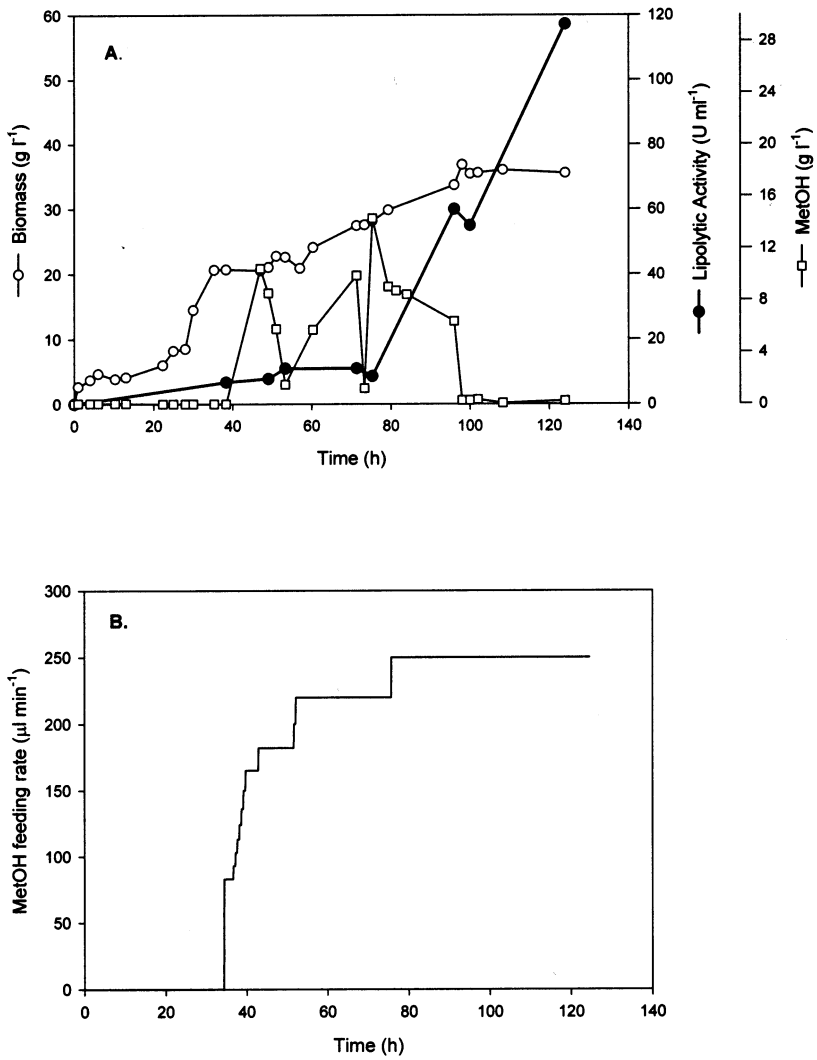

Fig. 1. (a) Cell growth, ROL production, and methanol concentration in the culture broth of a fed-batch cultivation based on the DO strategy, and (b) the corresponding profile of the methanol feeding rates.

vation period to maintain a relatively constant methanol concentration in the bioreactor, clearly indicated that the DO value was not suitable for monitoring methanol concentration. During 40–80 h of cultivation, the methanol concentration reached toxic levels of approximately 12 g l^{-1} (the recommended methanol concentration lies below 5 g l^{-1}, Stratton et al., 1998; Fig. 1).

At growth-limiting rates, the transcription levels, initiated by the *AOX1* promoter, can be 3–5-times higher than transcription in cells grown in conditions with excess methanol. Therefore, periods characterized by excess methanol during the induction phase may be one of the reasons why the values for the lipolytic activity and productivity obtained in this cultivation were significantly lower than those from previous cultivations involving complex medium (Minning et al., 1998).

Overall, it appeared that the indirect measurement of DO to control the methanol feeding rate did not guarantee maintenance of methanol levels below inhibiting concentrations. It also produced fluctuations in methanol concentration during cultivation. Remarkably, a very small number of biochemical engineering studies has so far been reported on the production of foreign proteins in the presence of constant methanol concentration (Katakura et al., 1998).

3.2. Methanol feeding fed-batch under off-line GC control

Low productivity in the DO controlled experiment demonstrated that a more rigorous control of methanol concentration was necessary. In the subsequent experiment, the methanol concentration was therefore analyzed by off-line GC. Due to the easy sample preparation and the fast analysis time, this procedure might prove suitable for controlling the methanol feeding rate in quasi real time.

In this cultivation, the methanol feeding-rate strategy was retained but was altered slightly in that methanol feeding was now based on the data obtained from off-line GC. The evolution of the main parameters and the profile of methanol addition are depicted in Fig. 2.

With this new strategy, the methanol concentration never exceeded 2.5 g l^{-1}, thus avoiding the inhibitory effects of high methanol levels. In two different periods (45 and 90 h of fermentation), however, the methanol consumption rate was higher than the methanol feeding rate and could have negatively affected the protein expression levels. The final lipolytic activity (320 U ml^{-1}) and productivity (2318 U l^{-1} h^{-1}) obtained were around 2.5-fold higher than in the previous strategy, whereas the specific productivity (40.6 U h^{-1} g biomass^{-1}) was 1.6-fold higher. Although increased lipase productivity was observed, lipolytic activity could not be detected until 40 h after methanol induction.

3.3. Mixed feeding methanol–glycerol fed-batch under off-line GC control

In the final strategy, a transition phase was introduced prior to the methanol induction phase. This involves the simultaneous feeding of glycerol and methanol and was previously reported to result in higher growth rates and higher rates of methanol uptake (Brierley et al., 1989). With mixed feeding, cultures can be primed for methanol induction, which leads to an essential reduction in the length of this phase.

Hence, we implemented a fed-batch cultivation scheme introducing a mixed glycerol–methanol feed. This was based on the general mixed-substrate feeding scheme described by Katakura et al. (1998) for the human β_2-glycoprotein domain *V*. To keep the overall amount of glycerol comparable to previous cultivation, the initial batch cultivation was performed only with 20 g l^{-1} of glycerol. The transient phase was initiated during the exponential growth phase with a reduced glycerol feeding rate (see materials and methods) until a final glycerol concentration of 40 g l^{-1} was reached. Simultaneously, methanol was continuously fed at 6 ml h^{-1}. Once this transition phase had ended, methanol feeding was adjusted according to the data obtained from GC.

During the transient phase, methanol was not consumed until glycerol was completely exhausted (Fig. 3). Therefore, methanol accumulated in the culture broth, reaching a maximum concentration

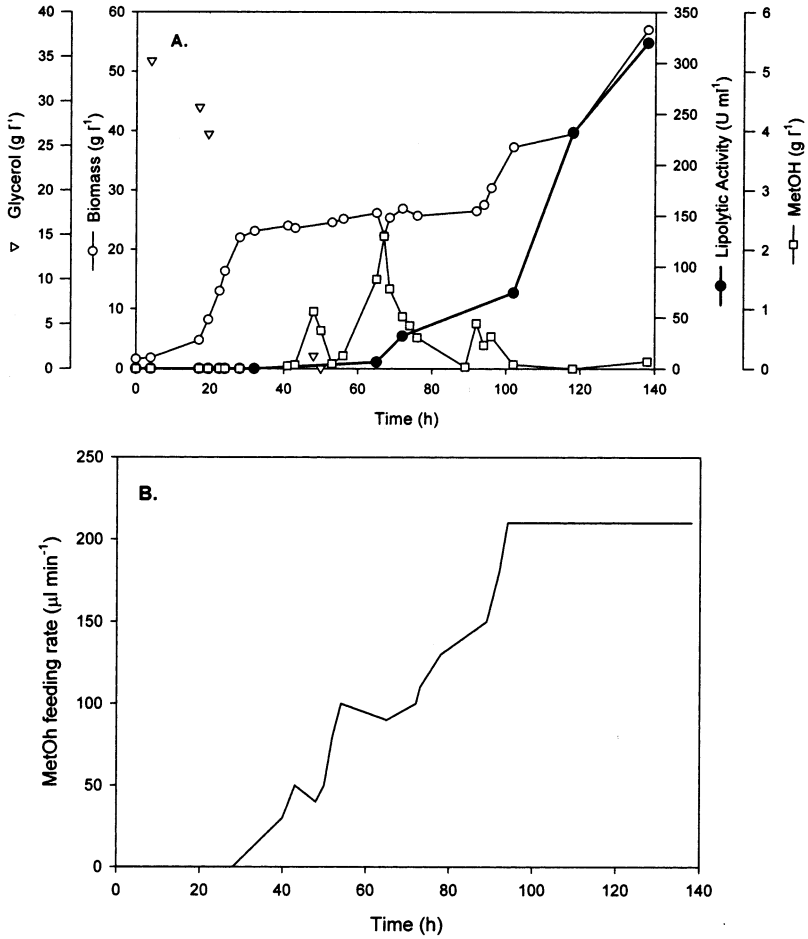

Fig. 2. (A) Cell growth, ROL production, and methanol concentration in the culture broth of a fed-batch cultivation based on off-line methanol monitoring, and (B) the corresponding profile of the methanol feeding rates.

of 5 g l^{-1}. Nevertheless, as soon as the glycerol was consumed, the microorganisms started to assimilate methanol in rates that were higher than in previously applied strategies. Furthermore, no accumulation ethanol – a strong inhibitor of the *AOX1*p was observed throughout the glycerol–methanol co-feed phase (when measured by off-line GC, data not shown). After 103.5 h of cultivation, the lipolytic activity was 1334 U ml^{-1}. This led to a final productivity of as much as 12 888 U l^{-1} h^{-1} of ROL, due to the reduction in the operation time, and also to a greater specific productivity (268 U h^{-1} g biomass^{-1}).

Overall, this strategy led to a 20–30 h reduction in the production time, a 11-fold higher final lipolytic activity, a 13.6-fold higher productivity, and a 10.3-fold higher specific productivity compared to the DO-based strategy.

The comparative analysis of these data showed that the observed lipolytic activity in the medium is slightly delayed in cultivations using synthetic media instead of complex media. This can probably be attributed to the fact that in *P. pastoris* ROL production in synthetic medium requires de novo synthesis of lipase precursors (e.g. aminoacids). These precursors are already present in the complex medium.

3.4. Effect of ROL expression on cell growth and rate of methanol consumption

The effect of the expression of the recombinant protein (ROL) in yeast cells was examined. A *P. pastoris* strain, transformed with the pPICZαA vector lacking the *ROL* gene, was used to perform a fed-batch cultivation following the same scheme in feeding methanol as used in the last fermentation. Thus, the effect of *ROL* expression on *P. pastoris* growth could be assessed. The maximal methanol consumption rate is quite similar in both ROL-producing cultures and this control experiment. However, a dramatic delay was observed for the respective strains before they reached the maximum methanol assimilation rate. The highest rate of methanol consumption (8.5 g l^{-1} h^{-1}) was achieved after 45 h of cultivation; this value was only reached after 85 h when the ROL gene was present (Fig. 4). Nevertheless, in both experiments the maximum specific growth rate was quite similar (≈ 0.03 h^{-1}).

This experiment showed that ROL expression clearly affected the methanol assimilation rate during the initial stages of the induction phase. This is in agreement with the reduced growth of the ROL-producing strain observed in methanol agar plates in relation to a control Mut^+ strain (data not shown).

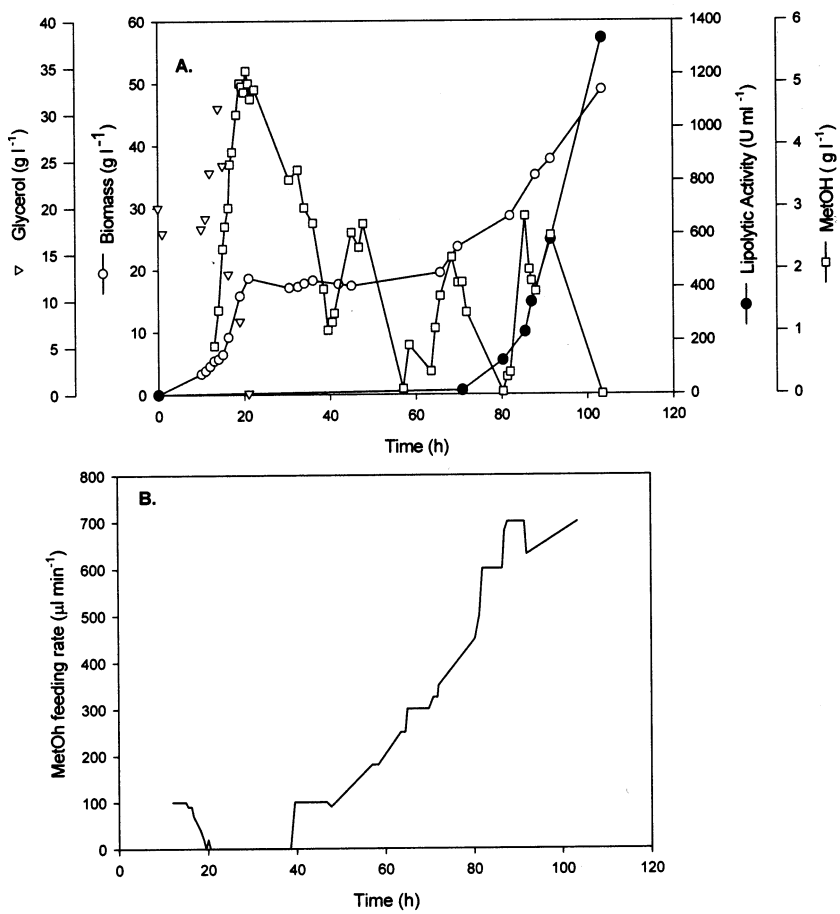

Fig. 3. (A) Cell growth, ROL production, methanol and glycerol concentrations in the culture broth of a fed-batch cultivation with a glycerol/methanol transition feeding phase, followed by a methanol fed-batch phase based on off-line methanol monitoring, and (B) Corresponding profile of the methanol feeding rates.

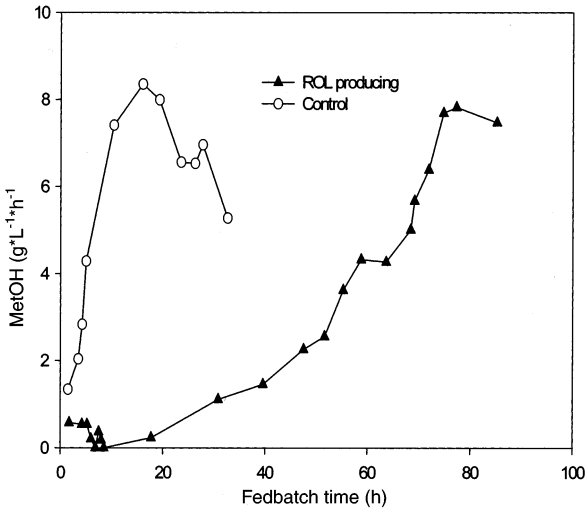

Fig. 4. Comparison of the methanol consumption rates and biomass between ROL-producing and control-strain fed-batch cultivations with a glycerol/methanol transition phase and a methanol fed-batch phase based on off-line methanol monitoring.

3.5. Recovery and partial purification of the recombinant ROL secreted from P. pastoris

After cultivation, the cells were centrifuged; the supernatant was concentrated by ultrafiltration, and subsequently lyophilized with minimized loss (over 95% recovery) compared to the previously described procedure (75% recovery; Minning et al., 1998).

Unexpectedly, silver-stained SDS-PAGE gels revealed that the supernatants derived from synthetic medium cultivations had a larger number of protein contaminants than the supernatants of complex medium (Fig. 5). Protease activity was not detected in any of the supernatants. However, since the host was a wild-type strain and neither casaminoacids nor peptones were present in the culture media, we could not exclude the presence of basal levels of endogenous proteases, which could have caused partial degradation of the protein. Limited cell lysis due to transient substrate limitations might be another plausible explanation.

In complex media, *N*-glycosylation of the 30 kDa ROL did not occur despite three potential *N*-glycosylation sites (Minning et al., 1998). SDS-PAGE analysis of the crude preparations obtained from the bioreactor cultures in synthetic medium revealed a minor fraction of approximately 32 kDa. Further investigation of this band with endo-β-*N*-acetyglycosamide H identified this fraction as an *N*-glycosylated 30 kDa protein. However, the N-terminal sequence of the major protein component of the 32 kDa band did not correspond to the mature ROL or unprocessed PrePro-α-ROL (ROL with the unprocessed α-factor from *S. cerevisiae*). There are no glycosylation studies of the native ROL. However, Hiol et al. (2000) have reported very low or no glycosylation for a 32 kDa lipase isoenzyme from the same microorganism.

The purification procedure for the recombinant ROL expressed by *P. pastoris* in bioreactor cultivations with synthetic medium was essentially the same as that employing complex medium (Minning et al., 1998). It consisted of a single cation-exchange chromatography (CEC) purification step (Fig. 6). However, it was essential to dialyze the concentrated suspensions of crude ROL preparations against water or 10 mM $CaCl_2$ ($pH \leqslant 6.0$) prior to their application to the chromatographic column. Otherwise, the relatively high amounts of Tris–HCl (pH 8.1) present in the

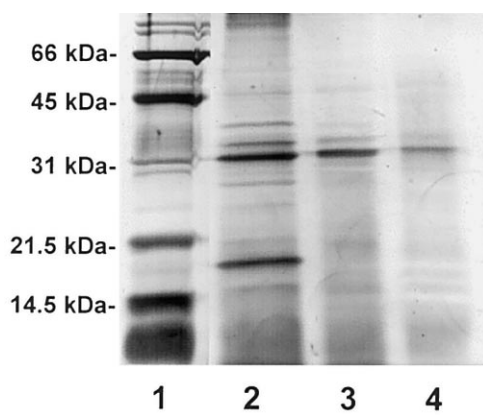

Fig. 5. SDS-PAGE of crude ROL preparations obtained from fed-batch cultivations in synthetic medium (lane 2) and complex medium (lane 3), and of fractions with ROL activity collected from CEC (peak Lip in Fig. 6), (Lane 4); Lane 1: molecular weight standards indicated in kDa; 12% acrylamide gel, silver-stained.

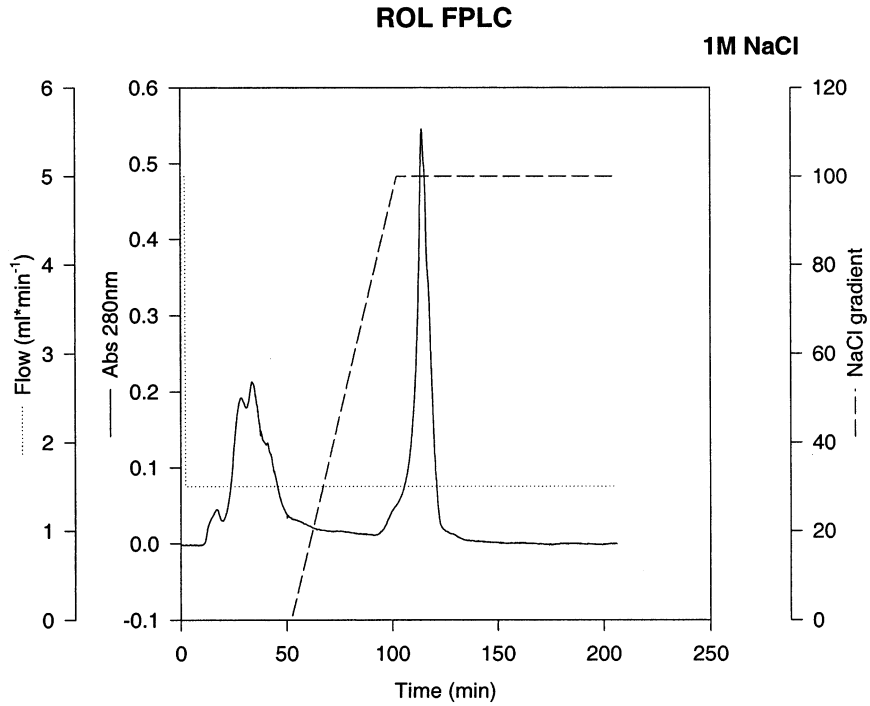

Fig. 6. CEC on SP sepharose of the crude ROL preparation obtained from fed-batch cultivations in synthetic medium.

lyophilized material, prevented the binding of the lipase (pI $\geqslant$ 9.3, Minning et al., 1998) to the cation-exchange resin (SP sepharose).

After chromatographic purification, the lipolytic activity was close to 90%. However, the lipase still showed a significantly reduced specific activity compared to the lipase obtained from cultures with complex medium. This can probably be attributed to the protein contaminants that were still present in the ROL preparation (Fig. 5). Nevertheless, the degree of purity obtained was sufficient to perform target reactions in fine chemical processes (Ferrer et al., 2000). Minning et al. (1998) reported a specific activity of 8571 U mg^{-1} (using triolein as substrate, at 30°C, and pH 8.1) for the recombinant ROL produced in *P. pastoris*, comparable with the native enzyme (10 000 U mg^{-1}, olive oil, pH 8.5, 37°C) (Ben Salah et al., 1994). Also, Hiol et al. (2000), have recently purified a 32 kDa-lipase isoenzyme from *R. oryzae* with a specific activity of 8800 U mg^{-1} (trioctanoylglycerol, 30°C, pH 7.0).

4. Conclusions

We demonstrated that the careful off-line monitoring of the methanol concentration by means of GC and the consequent control of the methanol feeding rates are the key parameters in obtaining a significant increase in the final protein yields in the ROL – *P. pastoris* system. This methodology offers an increased robustness and reliability over DO control-based methanol fed-batch strategies, especially when maintenance of relatively constant (i.e., limiting) and non-toxic methanol concentrations is desired, which is essential for the optimization of product yield.

In addition, the introduction of a transition phase with a mixed glycerol–methanol fed-batch before the methanol induction phase resulted in an increase in methanol consumption rates during the induction phase and, therefore, in productivity and also specific productivity. This allowed the further optimization of the production system. Higher active lipase yields could be achieved than in fed-batch cultivations in expensive complex medium.

Nevertheless, there are some observations that need further investigation. First, the appearance of lipolytic activity in the medium was slightly delayed in synthetic medium with respect to complex medium cultivations. Second, as deduced from the comparison between ROL-producing and control cultivations, ROL expression clearly affected the methanol assimilation rate in a negative way.

Also, glycosylation of a minor fraction of ROL can not be totally excluded. Additional characterization studies might be important. Glycosylation has been shown to have an effect on synthesis/secretion rate and functional properties of the Lip1 lipase from the fungus *Candida rugosa* (Brocca et al., 2000).

Finally, cultivation conditions (medium composition) affected the lipase downstream process. Additional chromatographic steps are needed to improve lipase purity, if required. In particular, HIC steps have proven effective for the selective purification of fungal lipases (Sabuquillo et al., 1998; Sánchez et al., 1999) and can be scaled-up to accommodate larger volumes.

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