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Sorbitol co-feeding reduces metabolic burden caused by the overexpression of a *Rhizopus oryzae* lipase in *Pichia pastoris*

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Abstract

To improve the specific production rate of *Rhizopus oryzae* lipase (ROL) in *Pichia pastoris*, a protein that triggers the unfolded protein response in *P. pastoris*, the effect of sorbitol/methanol mixed substrates was tested in batch and fed-batch cultures.

Remarkably, a different substrate consumption behaviour was observed depending on the host's phenotype (Mut^+ or Mut^s) in batch cultures: when the methanol assimilation capacity is genetically reduced (Mut^s phenotype), both substrates were consumed simultaneously, allowing not only a higher specific growth rate but also higher lipase levels (8.7-fold) compared to those obtained by cells growing on methanol as a sole carbon source in batch culture. This effect was not observed in Mut^+ phenotype, where the two substrates were consumed sequentially and the levels of heterologous product were only slightly higher (1.7-fold).

A mixed substrate strategy was also applied to a Mut^s fed-batch culture at a low methanol concentration set-point ($0.5\,g\,l^{-1}$). This resulted in a 2.2-fold increase in the heterologous protein level achieved, compared with the methanol-only feeding strategy. In addition, sorbitol co-feeding permitted the achievement of higher specific growth rates, and avoided the drastic decrease of the specific production rate observed after the start of the induction phase when methanol was used as sole carbon source This resulted in a significant increase in the overall bioprocess volumetric productivity (2.2-fold) and specific productivity (1.7-fold).

Moreover, whereas increased ROL gene dosage in Mut^s strains have been previously reported to be deleterious for *P. pastoris* cells growing on methanol, sorbitol co-feeding allowed for sustained cell growth and lipase production.

Keywords: *Pichia pastoris*; Mixed substrate co-feeding; Fed-batch cultivation; Sorbitol; *Rhizopus oryzae* lipase; Metabolic burden

1. Introduction

The methylotrophic yeast *Pichia pastoris* has become a well-established host system for heterologous protein production (Macauley-Patrick et al., 2005). This expression platform uses elements that include strong inducible promoters derived from genes of the methanol utilization pathway, such as the strong and tightly regulated promoter from the alcohol oxidase 1 gene P_{AOX1} (Lin Cereghino and Cregg, 2000).

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 utilisation slow (Mut^s), or both *AOX* genes, methanol utilisation minus (Mut^-). An important advantage of Mut^s strains is that the culture is not as sensitive to residual methanol in the cultivation media relative as Mut^+ strains, and hence the process of scale up can be easier (Stratton et al., 1998). However, the lower maximum specific growth rate of wild type Mut^s strains compared with wild type Mut^+ strains limits the productivity of the process.

To increase cell density and process productivity, as well as to reduce the induction time, a typical approach is the use of a multicarbon substrate in addition to methanol (Files et al., 2001). It has proved to be a straightforward strategy to increase the energy supply to recombinant cells (Katakura et al., 1998; Zhang et al., 2000a,b, 2003). This strategy has been mostly employed for fermentations using Mut^s strains because of their genetically reduced capacity to assimilate methanol, which results in long induction times (above 100 h). There are also some examples of application of such strategy for Mut^+ strains (Zhang et al., 2005).

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First attempts of fed-batch strategies using mixed substrates (glycerol/methanol) were made by Brierley et al. (1990) and Loewen et al. (1997). At limited rates, glycerol co-feeding ensured good cell growth while allowing induction of the heterologous protein expression (Thorpe et al., 1999). However, glycerol excess represses the *AOX1* promoter, which may result in lower specific productivities of recombinant protein (Xie et al., 2005).

Besides, Sreekrishna et al. (1997) successfully implemented the use of sorbitol/methanol mixtures in fed-batch fermentations for matrix metalloproteinases (MMP-2) production. Moreover, Thorpe et al. (1999) compared methanol/glycerol and methanol/sorbitol fed-batch mixed-feed strategies maintaining the residual methanol concentration between 1 and 2 g l^{-1}. Although cell yields are lower on sorbitol, this is compensated by higher specific product formation rates, which results in comparable recombinant protein levels at lower final cell concentrations. Inan and Meagher (2001) compared different carbon sources in terms of their ability to support growth and expression of an *AOX1-lac Z* fusion in shake flasks studies of a *P. pastoris* Mut^- strain confirming sorbitol as an excellent non-repressive carbon source. Recently, acid lactic has also been referred as non-repressing substrate (Xie et al., 2005).

The production of the heterologous *Rhizopus oryzae* lipase (ROL) has been shown to have a negative effect on *P. pastoris* growth (Minning et al., 2001). This effect was even more drastic when ROL gene dosage was increased, particularly when using a Mut^s host strain growing on methanol as a sole carbon source in fed-batch cultures (Cos et al., 2005b). Also, this study revealed that ROL gene dosage had a clear effect in terms of specific productivity and $Y_{P/X}$, and that the extent of this effect was dependant on the Mut phenotype, i.e. on the capacity of substrate assimilation.

High-level protein expression influences cell physiology of *P. pastoris* and a common effect is a reduction of specific growth rate (Zhang et al., 2000a,b; Curvers et al., 2002; Zhou and Zhang, 2002). This effect has also been described in the heterologous production of ROL in fed-batch cultures (Minning et al., 2001). Recently, a μ_{max} of 0.06 h^{-1} has been determined for a Mut^+ strain expressing the ROL under the P_{AOX1} (Cos et al., 2005a), i.e. far from μ_{max} of 0.14 h^{-1} reported for the wild strain (Brierley et al., 1990). Also, despite the fact that the levels of specific lipolytic activity were higher in the ROL multi-copy Mut^+ strain than in the corresponding single copy strain in shake-flasks cultures, increased ROL gene dosage resulted in a clear negative effect on cell growth. Moreover, the negative impact of ROL expression on cell growth was also clearly observed in fed-batch cultures. For instance, the mean specific growth rate achieved with the Mut^+ ROL single copy strain in fed-batch cultures where the residual methanol concentration was controlled between 1 and 2 g l^{-1} was 0.036 h^{-1}, i.e. significantly lower than the μ_{max} achieved by the same strain growing on methanol as a sole carbon source in batch cultures (Cos et al., 2005b).

This effect was more pronounced in fed-batch cultures of a ROL-producing Mut^s strain, as the observed specific growth rate of a Mut^s ROL single copy strain was extremely low (mean μ of 0.005 h^{-1}). The ROL gene dosage effect was even more striking when attempting to grow the prototrophic Mut^s ROL multi-copy strain in fed-batch cultures, as growth on methanol during the fed-batch induction phase did not progress for more than 24 h (Cos et al., 2005b).

Overall, these studies suggested that ROL overexpression using the PAOX-based expression system posed a serious physiologic burden in *P. pastoris* cells. Interestingly, recent studies strongly suggest that ROL overexpression triggers the unfolded protein response (UPR) in *P. pastoris* (Marx et al., 2006). Moreover, such response is dependant on the specific growth rate.

The objective of the present comparative study is to investigate the effect of sorbitol/methanol mixed carbon source (versus methanol as a sole carbon source) used for growing both Mut^+ and Mut^s *P. pastoris* cells in batch and fed-batch cultures in terms of production, productivities, specific growth and heterologous product production rates. In addition, the study aims at revealing potential operational strategies for alleviating the effects of cellular stress responses of ROL overexpression in single and multi-copy strains.

2. Materials and methods

2.1. Strains

The wild type *P. pastoris* X-33-derived strain expressing a *R. oryzae* lipase (ROL) extracellularly was used as the *ROL* single copy Mut^+ strain (Minning et al., 2001). A *P. pastoris* GS115 (*his4*; Invitrogen, USA)-derived strain having multiple copies of the *ROL* gene and its histidine auxotrophy reverted has been described elsewhere (Cos et al., 2005b). *P. pastoris* X-33 and GS115 are isogenic strains except for GS115's histidine auxotrophy; in particular, the X-33 strain is a His^+ derivative of strain GS115 generated by transformation of the latter strain with a DNA fragment containing the *P. pastoris his4* gene (Higgins et al., 1998). Therefore, the ROL multi-copy strain (named as X-33/500_3/ROL) was essentially isogenic to X-33/ROL (Mut^+ His^+) except for the *ROL* gene dosage. The *P. pastoris* KM71 (*arg4 his4 aox1Δ::SARG4 AOX2*; (Cregg et al., 1987))-derived Mut^s strains having single and multiple copies of the ROL gene and its histidine auxotrophy reverted have been described in Cos et al. (2005b).

2.2. Inoculum preparation

Pre-inocula for bioreactor culture 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 200 ml of final volume in a 1 l. The culture was centrifuged at 4000 × *g*, the harvested cells were resuspended in a sterilized 0.9% NaCl isotonic solution and used to inoculate a 2-l Biostat B batch bioreactor or 5-l Biostat ED fed-batch bioreactor (Braun Biotech, Melsungen, Germany).

2.3. Batch fermentation culture medium

The medium has the following composition per litre: $(NH4)_2SO_4$ 5 g, YNB (yeast nitrogen base without aminoacids and ammonium sulphate, Difco Laboratories, Detroit, MI, USA) 3.4 g, methanol 10 g and/or sorbitol 10 g, 0.1 ml of antifoam Mazu DF 7960 (Mazer Chemicals, PPG Industries Inc., USA) and 4.35 ml of PTM1. The PTM1 solution contained per litre: $CuSO_4 \cdot 5H_2O$ 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). The pH was controlled using KOH 1 M. The ammonium sulphate and sorbitol were autoclaved in situ in the bioreactor. YNB, methanol and PTM1 solution were sterile filtered and added to the bioreactor at room temperature.

2.4. Fed-batch cultivation set up and operational conditions

The basal salt synthetic medium for fed-batch cultivations contained per litre 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. Also 0.1 ml l^{-1} of antifoam agent (Mazu DF 7960) – a polyoxyalkylene glycol – was added.

Cells were cultured in a 5 l Braun Biostat ED bioreactor (Braun Biotech, Melsungen, Germany). The cultivation conditions were: stirring rate 900 rpm, temperature 30 °C, pH controlled at 5.5 by adding NH_4OH 30% (v/v), dissolved oxygen above 30% with a constant air flow rate of 1.5 l min^{-1}.

The cultivation started with a 40 g l^{-1} of glycerol batch phase. After that, a pulse of sorbitol and methanol was added to achieve a concentration of 10 g l^{-1} of sorbitol and 5 g l^{-1} of methanol into the bioreactor as a transition phase.

For the induction phase a feeding medium containing per litre 300 g of sorbitol and 7.5 ml of trace salts solution was used in a pre-programmed exponential feeding rate strategy at a specific growth rate lower than maximum specific growth rate determined in batch fermentation (0.02 h^{-1}).

Methanol addition rate was programmed by a control algorithm developed (Cos et al., 2006a) attempting to maintain a 0.5 g l^{-1} constant methanol concentration adding a solution of pure methanol with 12 ml l^{-1} of PTM1. Methanol was analyzed on-line by the commercial equipment MC-168 (PTI Instruments, Kathleen, USA) (Ramon et al., 2004). Both carbon sources were also added in the production phase by an automatic microburette MicroBU-2031 from Crison Instruments (Alella, Barcelona, Spain).

2.5. Biomass analysis

Biomass was expressed as dry cell weight as reported elsewhere (Resina et al., 2005).

2.6. Glycerol, sorbitol and methanol determination

Off-line glycerol, sorbitol and methanol determination was made as reported elsewhere (Cos et al., 2005a).

2.7. Lipolytic activity assay

Lipolytic 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 lipolytic activity was measured after removing cells from cultivation samples by centrifugation in a microcentrifuge at 12,000 × g. Intracellular lipolytic activity (defined as the soluble fraction of the cell bound lipolytic activity) was measured from cleared supernatants from cell lysates as described in Resina et al. (2005). Lipolytic activity in cleared cell lysates was related to the total protein content of such lysates, as measured using the Bradford assay (Pierce, Rockford, IL), and expressed as lipolytic activity UA μg total protein^{-1}.

2.8. Calculation of specific rates

Estimated specific growth-rate (μ) (h^{-1}) and specific lipase production rate (q_p) [UA gX^{-1} h^{-1}] were determined applying the methodology developed for this estimation (Cos et al., 2005b).

3. Results and discussion

3.1. Batch cultures

P_{AOX} non-repressing carbon substrates such as sorbitol have been used as methanol co-substrates to minimize the effects derived from its use as sole carbon and energy source, particularly in Mut^s cells, as they have genetically-limited methanol assimilation capacity (Cos et al., 2006b).

Nevertheless, to our knowledge, there is no information about the regulation of methanol/sorbitol assimilation in *P. pastoris* cells growing in batch cultures, as well as of the potential effect of the host's Mut phenotype on such phenomena. With this objective, three batch fermentations with methanol and sorbitol as co-substrates were performed for both ROL single copy Mut$^+$ and Mut^s strains and, a ROL multi-copy Mut^s strain.

In the culture with Mut$^+$ phenotype cells, the consumption of mixed substrates was sequential: *P. pastoris* cells first metabolized methanol, and once it was practically exhausted, cells started consuming sorbitol (Fig. 1). The extracellular lipolytic activity levels achieved at the end of the methanol growth phase (46 h) were similar (about 6 UA ml^{-1}) to those obtained in a batch culture using methanol as sole carbon source at the same initial concentration (10 g l^{-1}) (Cos et al., 2005a). However, extracellular lipolytic activity continued increasing in the sorbitol consumption growth phase, thereby achieving a maximum value of 10.4 UA ml^{-1} at the end of the cultivation (Table 1).

In contrast, during growth of the ROL-producing Mut^s both carbon substrates were consumed simultaneously (Fig. 2). Interestingly, the use of sorbitol and methanol as mixed substrates not only allowed faster cell growth but also higher secreted heterologous protein levels. Extracellular lipolytic activity levels were detected in the culture supernatant from the early phase

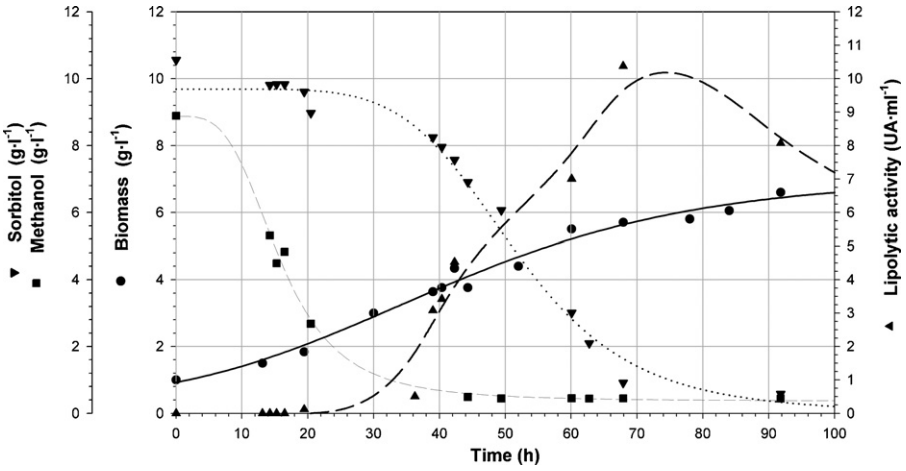

Fig. 1. Time evolution of biomass, methanol, sorbitol and lipolytic activity in batch cultures for the P_{AOX1} single copy (Mut$^+$ strain) using methanol and sorbitol.

Table 1
Comparison of fermentation parameters of different phenotypes *P. pastoris* expressing ROL under control of P_{AOX1} in bioreactor batch cultures with and without sorbitol

	Mut$^+$, methanol + sorbitol single copy	Mut^s, methanol + sorbitol single copy	Mut^s, methanol + sorbitol multi-copy	Mut$^+$, methanol (Cos et al., 2005a)
Lipolytic activity[a] (UA ml^{-1})	10.4	53	29.5	6.1
Y_{lipase}[a] (UA gX^{-1})	1825	10,600	7284	1413
Volumetric productivity[a] (UA l^{-1} h^{-1})	153	757	258	201
Specific productivity[a] (UA gX^{-1} h^{-1})	27	151	64	47

[a] Calculated for the maximal lipolytic activity.

of the cultivation and increased steadily along the cultivation time, reaching a maximum value at the end of the culture of 53 UA ml^{-1}, i.e. 5.1-fold higher than in the corresponding culture using the Mut$^+$ strain.

A decrease in the extracellular lipolytic activity levels was observed in Mut$^+$ phenotype towards the end of the cultivation, probably due to the effect of proteases, as it was previously observed in fed-batch cultures with the same heterologous protein (Cos et al., 2005b). Interestingly, this effect was not observed in Mut^s strain.

Growth on methanol as sole carbon source of the prototrophic Mut^s ROL multi-copy strain in fed-batch cultures leads to cell death (Cos et al., 2005b). In order to evaluate whether the use of a mixed-substrate strategy could sustain cell growth and heterologous production of this strain, a batch fermentation was also made using sorbitol and methanol as carbon sources. As observed in Fig. 3, the microorganism grew but the specific growth rate and specific consumption substrate rate were lower than those observed with the single copy strain (Fig. 2). The final level of extracellular lipolytic activity and $Y_{P/X}$ was 1.8- and

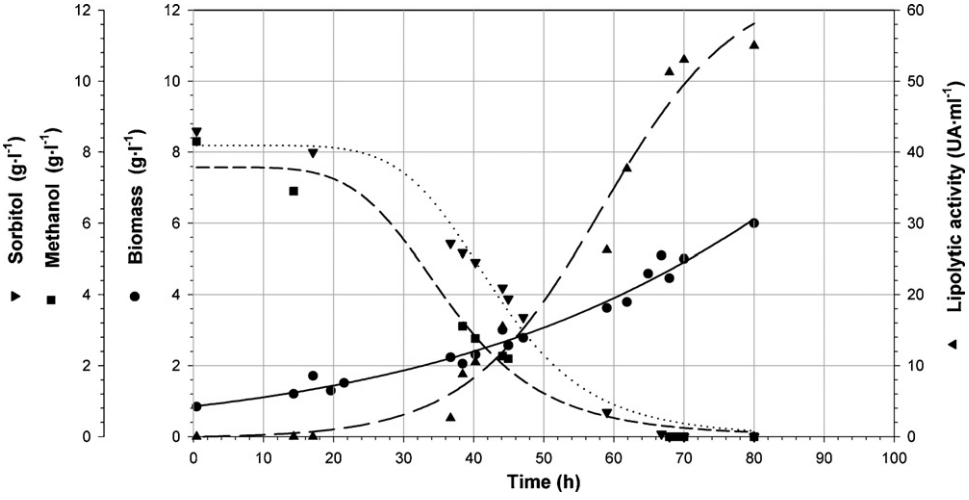

Fig. 2. Time evolution of biomass, methanol, sorbitol and lipolytic activity in batch cultures for the P_{AOX1} single copy (Mut^s strain) using methanol and sorbitol.

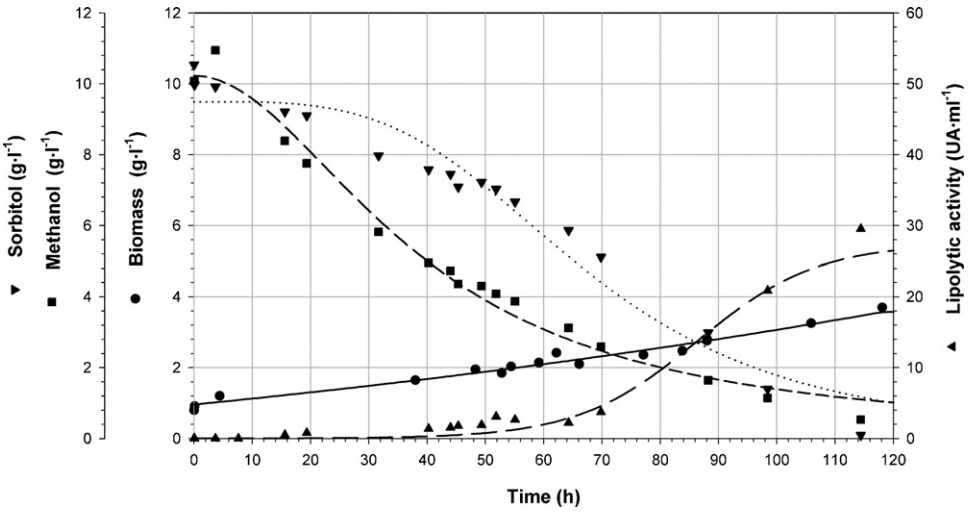

Fig. 3. Time evolution of biomass, methanol, sorbitol and lipolytic activity in batch cultures for the P_{AOX1} multi-copy (Mut^s strain) using methanol and sorbitol.

1.5-fold lower than that of the single copy strain, respectively (Table 1). A higher negative effect were observed when volumetric and specific productivity were compared. The respective values were 2.9- and 2.4-fold higher for the Mut^s ROL single copy strain. Similarly to fed-batch experiments using methanol as sole carbon source (Cos et al., 2005b), a clear negative effect of the ROL gene dosage in heterologous protein production was observed. However, the use of sorbitol as co-substrate allowed cell growth and heterologous production when using the Mut^s multi-copy strain.

The analysis of intracellular lipolytic activity for both ROL single copy strains batch fermentations (Fig. 4) revealed important features: in both cultures, an accumulation of intracellular activity levels was observed at the beginning of the methanol consumption phase, suggesting that secretion, but not synthesis, is the limiting step of the process at the start of the expression phase. Notably, the intracellular activity levels were two-fold higher in Mut^s cells compared to Mut$^+$ cells, although the observed extracellular lipolytic activity was 5.1-fold higher in the Mut^s strain culture. Its lower intra/extracellular lipase activity ratio observed in Mut^s phenotype suggest that sorbitol co-assimilation alleviates the bottleneck in ROL secretion.

The increase in ROL production during sorbitol consumption phase of Mut$^+$ cells could be the result of a double effect: the secretion to the extracellular medium of an significant amount of intracellular product produced under methanol induction phase, and the production of low levels of heterologous product (around 1 UA ml^{-1}) because of partial P_{AOX1} derepression occurring when sorbitol is used as sole carbon source (data not shown). The analysis of the specific production rates (q_p) profiles for all batch cultures (Fig. 5) is coherent with this hypothesis: in cultures using both Mut^s ROL single and multi-copy strains, a single q_p maximum was observed. This maximum was reached at the end of substrates consumption phase. However in cultures using Mut$^+$ cells, in addition to a maximum q_p value observed when methanol was exhausted, a second local maximum was detected when sorbitol was practically depleted, i.e. low specific production rates were also observed during the sorbitol consumption phase.

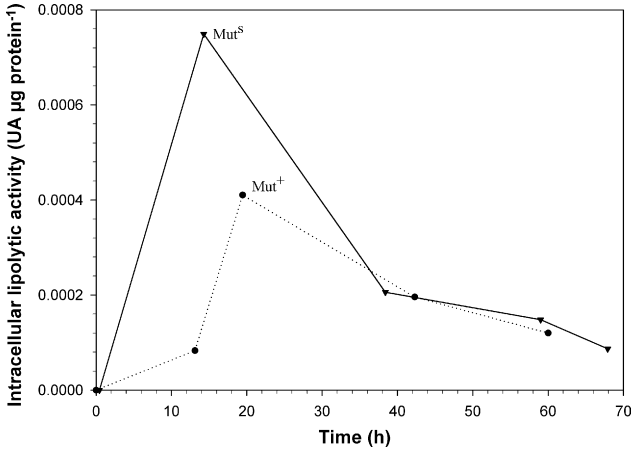

Fig. 4. Intracellular lipolytic activity comparison between single copy Mut$^+$ (●) and Mut^s (▼) phenotypes batch fermentation using sorbitol as methanol as carbon sources.

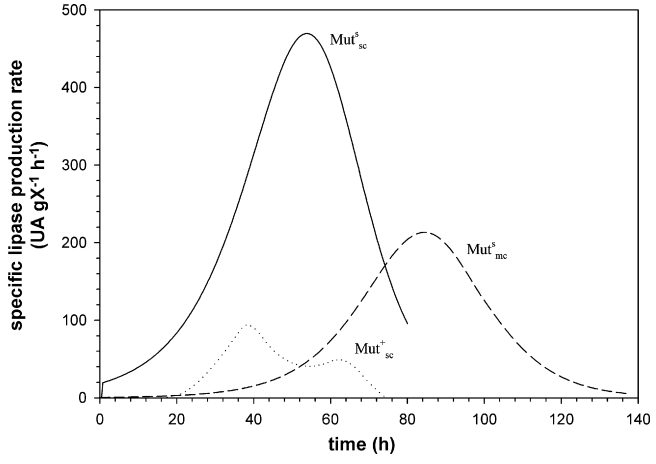

Fig. 5. Time evolution of specific protein production rate in mixed substrates batch cultures for single copy Mut$^+$ (Mut$^+$sc) and Mut^s (Mut^{s}sc) phenotypes and multi-copy Mut^s (Mut^{s}mc) strains.

3.2. Fed-batch cultures

In previous Mut^S fed-batch experiments using methanol as sole carbon source it was observed that the highest secretion levels were detected at the lowest methanol set-point tested (0.5 g l^{-1}). However, the specific production rate profile under those conditions showed a maximum value soon after the start of the induction phase, followed by an abrupt decrease to almost zero values after 20 h of induction (Cos et al., 2006a). Under these conditions, the negative effect of ROL production on cell growth is combined with the reduced methanol assimilation capacity of Mut^S cells. This effect became more striking when attempting to grow the prototrophic Mut^S ROL multicopy strain in fed-batch cultures, as growth on methanol during the fed-batch induction phase did not progress for more than 24 h.

Based on the ROL expression results obtained in mixed sorbitol/methanol batch cultures using Mut^S strains, a fed-batch culture was performed to evaluate the effect of mixed substrates in ROL production under such conditions at a methanol set-point of 0.5 g l^{-1}. The evolution of biomass, lipolytic activity, sorbitol and methanol concentration, as well as the specific growth and production rates profiles are shown in Fig. 6A and B. The maximal lipolytic activity obtained using this strategy was 2.8-fold higher compared with those from a fed-batch culture using only methanol during the induction phase. No accumulation of sorbitol, except at the beginning of the induction phase, was detected along the fed-batch, assuring that this co-substrate was also added in limiting conditions according with the pre-programmed exponential feeding rate strategy designed. Importantly, the specific production rate, after reaching a maximum at the beginning of the induction phase, did not fall down abruptly as observed when growing cells on methanol as sole carbon source at the same set-point (0.5 g l^{-1}) (Cos et al., 2006a). The specific production rate was stabilized at intermediate values around 125 UA gX^{-1} h^{-1} between 50 and 70 h. Besides, the use of the mixed substrate strategy allowed to increase the lipase/biomass yield 2.1-fold and the mean specific growth rate

Table 2
Comparison of mean and maximal specific growth rate (μ) and specific lipase production rate (q_p) in fed-batch bioprocess at a methanol concentration set-point of 0.5 g l^{-1} with and without sorbitol as co-substrate

	MeOH 0.5 g l^{-1} (Cos et al., 2006a)	MeOH 0.5 g l^{-1} + sorbitol
Max. lipolytic activity (UA ml^{-1})	137	380
$Y_{P/X}$ (UA gX^{-1})	3917	8260
Volumetric productivity (UA l^{-1} h^{-1})	1996	4285
Specific productivity (UA gX^{-1} h^{-1})	57	99
μ_{mean}[a] (h^{-1})	0.003	0.012
$q_{p,max}$[a] (UA gX^{-1} h^{-1})	340	350
$q_{p,mean}$[a] (UA gX^{-1} h^{-1})	58	165

Mean specific growth rates were estimated as time-averaged.
[a] Values calculated in the induction phase.

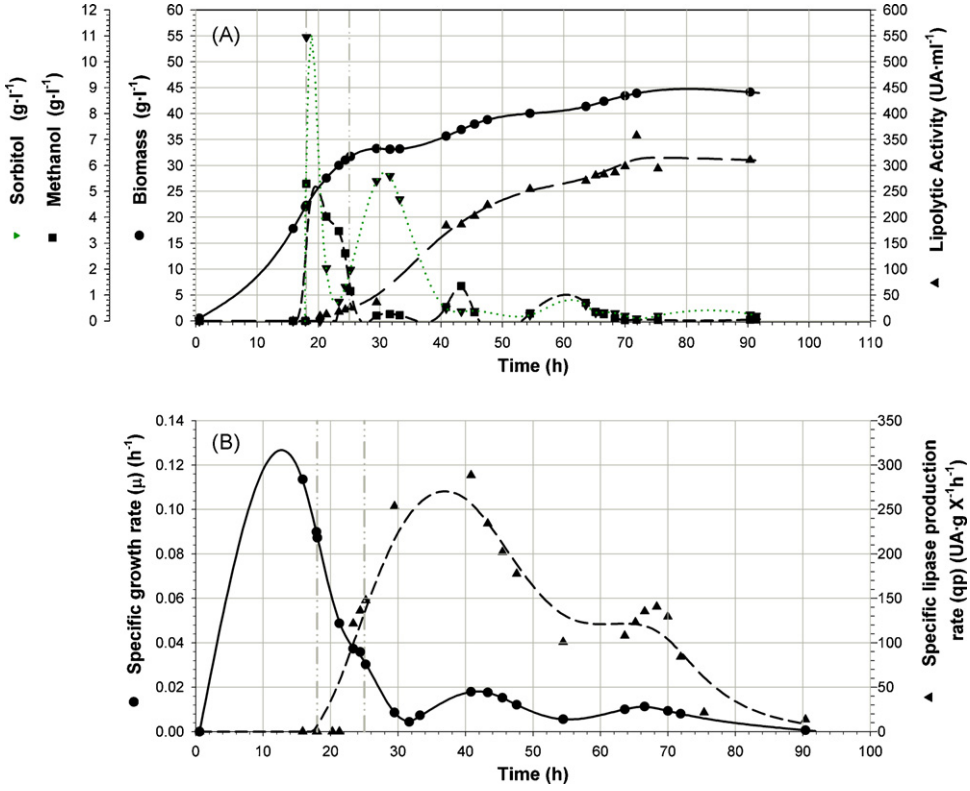

Fig. 6. (A) Time evolution of biomass, methanol, sorbitol and lipolytic activity in mixed substrates fed-batch culture for the P_{AOX1} single copy (Mut^S strain). (B) Time evolution of specific growth and protein production rates in mixed substrates fed-batch culture for the P_{AOX1} single copy (Mut^S strain).

four-fold, with a significant increase in both volumetric productivity and specific productivity (Table 2).

An indirect approach on the level of expression could be reached by the analysis of the slope of the specific production rate before the maximum is reached (as described in Cos et al., 2006a). When methanol was used as sole carbon source at the same set-point of 0.5 g l^{-1} this value was 106 UA gX^{-1} h^{-2} (Cos et al., 2006a). The slope when sorbitol was used as co-substrate was 40 UA gX^{-1} h^{-2}. Thus, the use of sorbitol at growth limiting rates decrease the level of expression. However, this value was two-fold higher compared to fed-batch cultivation using methanol as sole carbon source at a 1 g l^{-1} set-point.

Nevertheless, the modulation of transcription level and the use of a co-substrate such as sorbitol minimizes the negative impact of high-level protein expression on cell physiology and growth rate reduction.

4. Conclusions

The sorbitol assimilation profile of *P. pastoris* cells growing on sorbitol/methanol mixtures in batch cultures presents a different behaviour depending on the Mut phenotype (Mut^+ or Mut^s) used. When the capacity of methanol assimilation is restricted (Mut^s phenotype), both substrates were consumed simultaneously, allowing not only a higher specific growth rate but also higher secretion levels of heterologous product compared to cells growing on methanol as a sole carbon. This effect was not observed in the Mut^+ phenotype where the two substrates were consumed sequentially and the levels of heterologous product were slightly higher than the values obtained for cells growing on methanol as a sole carbon source.

The mixed substrate strategy applied to a Mut^s ROL single copy fed-batch culture at a low residual methanol concentration set-point (0.5 g l^{-1}) has allowed to increase the production of heterologous protein in Mut^s phenotype, avoiding the drastic reduction of the specific production rate observed after the start of the induction phase when methanol is used as a sole carbon source. This fact, together with the higher specific growth rate achieved resulted in a significant increase of the bioprocess volumetric and specific productivities.

Remarkably, sorbitol co-feeding in Mut^s ROL multi-copy strain batch cultivations allowed sustained cell growth and lipase productivity, strongly suggesting that cells growing on mixed substrates can – at least partially – overcome the stress caused by ROL overexpression in Mut^s cells. Furthermore, this observation reveals that the (stress) cellular response to heterologous protein overexpression and secretion is interconnected with the energetic state of the cells.

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