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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.
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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 multicopy 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).

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