Microbial Cell Factories

BioMed Central

Oral Presentation

Open Access

Substrate feeding strategies in *Pichia pastoris* fed-batch cultivation processes: Analysis of key parameters influencing recombinant protein production

Ramon Ramon, Oriol Cos†, Pau Ferrer†, José Luis Montesinos and Francisco Valero*

Address: Departament d'Enginyeria Química, E.T.S.E., Universitat Autònoma de Barcelona, 08193-Bellaterra (Cerdanyola del Vallès), Spain

* Corresponding author †Equal contributors

from The 4th Recombinant Protein Production Meeting: a comparative view on host physiology
Barcelona, Spain. 21–23 September 2006

Published: 10 October 2006

Microbial Cell Factories 2006, **5**(Suppl 1):S13 doi:10.1186/1475-2859-5-S1-S13

© 2006 Ramon et al; licensee BioMed Central Ltd.

Background

An important number of heterologous proteins have been produced in the methylotrophic yeast *Pichia pastoris* using the methanol-inducible alcohol oxidase promoter [1]. Cultivation conditions and host physiology have an important impact on the final yields and productivities for heterologous protein production [2]. Recently, the effect of the Mut phenotype and gene dosage on the heterologous production of a *Rhizopus oryzae* lipase (ROL) in *P. pastoris* has been studied in fed-batch bioprocesses with a manual (off-line) methanol concentration control [4]. These studies demonstrated that variations of the residual methanol concentration influence drastically in the specific consumption and production rates. To avoid this problem a predictive control algorithm coupled with a PI feedback controller has been satisfactorily implemented [5].

This set-up has allowed for further analysis of several key parameters influencing heterologous protein production in *P. pastoris* fed-batch cultivation processes. In particular, the impact of i) the residual methanol concentration present in the culture broth and ii) co-feeding of a multi-carbon substrate and methanol on process performance will be illustrated in a *P. pastoris* Mut^s phenotype strain secreting a *Rhizopus oryzae* lipase (ROL) as a reporter protein.

Results

The effect of methanol concentration on heterologous ROL production during the fed-batch phase was analysed by performing cultivations at different methanol set points, ranging from 0.5 to 1.75 $g \cdot L^{-1}$. The maximal lipase activity (490 $UA \cdot mL^{-1}$), specific yield (11236 $UA \cdot g^{-1}_{biomass}$), productivity (4901 $UA \cdot L^{-1} \cdot h^{-1}$) and specific productivity (112 $UA \cdot g^{-1}_{biomass} \cdot h^{-1}$) were reached for a residual methanol concentration set point of 1 $g \cdot L^{-1}$. Notably, these parameters are almost 2-fold higher than those obtained with a manual control at a similar methanol set-point. The study of the consumption (q_s) and production rates (q_p) showed very different patterns for these rates depending on the methanol concentration set-point:

In all cultivations maximal q_s values were obtained at the beginning of the induction phase; shortly after this point, q_s started to exponentially decrease.

The evolution of the extracellular lipolytic activity was completely different depending on the residual methanol concentration at the fed-batch phase. In particular, when the methanol set point was set at 0.5 $g \cdot L^{-1}$, the q_p reached a maximum of 340 $UA \cdot gX^{-1} \cdot h^{-1}$ at the beginning of the induction phase, followed by a sharp decrease to almost zero values after 20 h of induction. Since no proteolytic degradation of the product was observed, the exponential decrease in the product secretion rate was probably indicative that ROL synthesis had also stopped. In the fed-

batch cultivation carried out at a set point of 1 g· L^{-1}, the lipolytic activity values remained very low during a considerable period (20 h of induction phase); after this lag phase, a maximum q_p value of 440 UA· gX^{-1}· h^{-1} is reached after 40 h of induction phase, which was followed by an exponential decrease to values below 100 UA· gX^{-1}· h^{-1} after 75 h of fed-batch phase. Again, neither extracellular protease activity nor important levels of cellular lysis were detected. The q_p values during the fed-batch cultivation at a set point of 1.75 g· L^{-1} were kept rather constant throughout the bioprocess with significantly lower maximal (60 UA· gX^{-1}· h^{-1}) and mean q_p values. In this cultivation, extracellular lipolytic activity increased steadily until 110 h. After this point, lipolytic activity slightly decreased by the effect of cellular lysis. However, obtained levels (150 UA· mL^{-1}) were similar to those obtained at the beginning of the induction phase when a methanol set-point of 0.5 g· L^{-1} was used (137 UA· mL^{-1}) in only 4 hours.

Overall, these results indicated that, although cell growth is genetically limited (the host strain is Mut^s and, therefore, has a very limited methanol assimilation capacity), the synthesis and secretion rates are still greatly influenced by the residual methanol concentration (as expected from the observation that transcription levels from the AOX1 promoter are highest at methanol limiting concentrations [1]).

Since ROL expression has been shown to trigger the unfolded protein response [6], cells growing under carbon/energy limitation may not be able to sustain highest ROL production rates. To overcome this problem, we tested the effect of an additional, non-repressing, carbon source (sorbitol) on cell performance and heterologous protein expression and secretion during the methanol-fed induction phase. Remarkably, growth of Mut^s cells in a batch cultivation using sorbitol and methanol as carbon sources showed that both substrates were co-assimilated simultaneously along the growth phase. Conversely, sorbitol and methanol were assimilated sequentially by wild type cells.

Hence, we performed replica fed-batch cultivations at a methanol residual concentration of 0.5 g L^{-1} and sorbitol below 2 g L^{-1} simultaneously fed. The maximum q_p values reached at the beginning of the induction phase were similar (about 340 UA· gX^{-1}· h^{-1}). Although the q_p decreased after this point, production rate stabilized at around 200 UA· gX^{-1}· h^{-1} until the end of the bioprocess. By using this strategy, the maximal lipolytic activity was 3.5 fold higher than in the fed-batch at 0.5 g L^{-1} of methanol as a single carbon source. The specific yield, productivity and specific productivity were also improved by 2.5, 2.6 and 2-fold, respectively.

Conclusion

The combined use of a *P. pastoris* Mut^s strain and the control of the residual methanol concentration during the fed-batch phase allow for the modulation of the ROL production rates. Since ROL expression triggers the UPR, cells growing under carbon and energy source limitation appear particularly sensitive to ROL production rates (highest process productivities are not achieved under methanol-limiting conditions i.e. when AOX1 promoter transcription levels are probably highest).

Notably, mixed carbon source co-assimilation seems to support cell's adaptation to the stress caused by ROL secretion, i.e. allowing for sustained specific secretion rates and boosting process productivities and yields.

Acknowledgements

This work was supported by a grant from the Spanish Program on Chemical Processes Technologies (CTQ2004-00300).

References

1. Lin-Cereghino J, Cregg JM: **Heterologous protein expression in the methylotrophic yeast *Pichia pastoris* .** *FEMS Microbiol Rev* 2000, **24:**45-66.
2. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM: **Heterologous protein production using the *Pichia pastoris* expression system.** *Yeast* 2005, **22:**249-270.
3. Zhang W, Inan M, Meagher MM: **Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*.** *Biotechnol Bioprocess Eng* 2000, **5:**275-287.
4. Cos O, Serrano A, Montesinos JL, Ferrer P, Cregg JM, Valero F: **Combined effect of methanol utilization (Mut) phenotype and gene dosage on recombinant protein production in *Pichia pastoris* fed-batch cultures.** *J Biotechnol* 2005, **116:**321-335.
5. Cos O, Ramon R, Montesinos JL, Valero F: **A simple model-based control for *Pichia pastoris* allows a more efficient heterologous protein production bioprocess.** in press.
6. Resina D, Cos O, Gasser B, Mauer M, Valero F, Mattanovich D, Ferrer P: **Analysis and engineering of bottlenecks in *Rhizopus oryzae* lipase production in *Pichia pastoris* using the mitrogen source-regulated *FLD1* promoter.** in press.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp