Microbial Cell Factories

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Transcriptional analysis of protein production and induction of unfolded protein response in *Pichia pastoris* expressing a *Rhizopus oryzae* lipase under the FLD1 promoter

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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):P49 doi:10.1186/1475-2859-5-S1-P49

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Background

Methanol-free high cell density fed-batch cultivation strategies for the *Pichia pastoris* expression system have been recently developed by expressing a *Rhizopus oryzae* lipase (ROL) under the transcriptional control of FLD1 promoter (P *FLD1*)[1]. These cultivation strategies were based on the use of sorbitol and methylamine as carbon and nitrogen source, respectively, during the induction phase of the cultivation process. The specific growth rate proved to be an important parameter in the productivity of secreted ROL. Moreover, intracellular active product accumulation and a decrease in the specific product secretion rate were observed along the induction phase of the fermentation process. These results suggested the presence of a bottleneck(s) throughout the synthesis and secretion process of the heterologous lipase. Recently [2], flow cytometry analyses of intracellular ROL levels confirmed that a fraction of the product was retained within the cell. Further, this intracellular product accumulation was concomitant with an increase on the BiP protein, a chaperone of the HSP70 class that plays an important role in the unfolded protein response (UPR). Notably, the increase of BiP and ROL content in the cell was detected soon after the beginning of the induction phase. Interestingly, the intracellular BiP and ROL profiles were different depending on the specific growth rate of the cells.

In this study, we report the application of a sandwich hybridization assay-based technique [3] for quantification of specific mRNAs levels during the extracellular production of ROL in *P. pastoris* under the transcriptional control of P*FLD1*. These studies have been carried out in fed-batch cultures at two different specific growth rates.

Results

Fed-batch fermentations were performed at two different controlled specific growth rates, namely at a limited specific growth rate of about 0.005 h^{-1} (20% of μ_{max}) and under carbon excess (i.e. achieving a near- μ_{max} specific growth rate of about 0.02 h^{-1}). These cultivations were performed with two different strains: i) a *P. pastoris* X33-derived strain expressing ROL under the transcriptional control of the P *FLD1* and, 2) a *P. pastoris* GS115H-derived strain co-expressing the induced form of the *S. cerevisiae*'s UPR transcription factor Hac1p gene under the control of the constitutive GAP promoter and, the ROL gene under the P*FLD1* control [2].

The transcriptional levels of key genes involved in *P. pastoris*' C1 carbon compounds and amines metabolism (formaldehyde dehydrogenase, *FLD1*, and alcohol oxidase, *AOX1*), protein folding (the BiP-encoding gene, Kar2, and protein disulfide isomerase gene, PDI), as well as the ROL

gene were monitored throughout the fed-batch phase of cultivations.

As expected, the quantitative RNA analyses demonstrated that transcriptional levels of AOX and FLD are growth-rate dependant, remaining relatively low and constant at growth-limiting rates, but clearly induced by 2~3-fold during the induction phase (growth on sorbitol and methylamine as sole carbon and nitrogen sources, respectively). Notably, induction levels were higher in the X33-derived strain than in GS115H/HAC1.

PDI mRNA levels and profiles were also μ-dependant. PDI mRNA levels remained low and constant at low growth rate, whereas they were clearly induced (about 2-fold) under carbon excess growth conditions, reaching a maximum after 70 h of induction in the X33-derived strain; after that point, PDI mRNA levels decreased to the initial basal level towards the end of the induction phase. Such induction was more moderate in the GS115H/HAC1 strain. Interestingly, PDI mRNA profiles followed a similar pattern as ROL mRNA levels, which clearly increased during the induction phase under carbon excess growth conditions, reaching a maximum after 60–70 h of induction. In contrast, Kar2 profiles were strikingly different, particularly in the X33-derived strains: at growth-limiting conditions Kar2 mRNA levels were sharply increased by 2~3 fold, reaching a maximum soon after the onset of the induction phase. After reaching this maximum, Kar2 mRNA levels decreased exponentially along the induction phase. This profile was similar but less pronounced under carbon excess conditions. In contrast, Kar2 mRNA levels in the GS115H/HAC1 strain remained rather constant along the induction phase of the cultivation.

Conclusion

Overall, the bead-based mRNA sandwich hybridization assay is a useful instrument to follow transcriptional evolution of key genes in protein production processes. In this study, a clear cellular (stress) response to ROL production is shown at the transcriptional level, consistent with previous data on intracellular stress protein markers levels [2]. Also, this study provides new insights on the interactions between physiological state (growth rate) and protein expression in *P. pastoris*. These preliminary results strongly suggest that the decrease in ROL secretion rates observed along the induction phase of fed-batch cultivations [2] may be partially related with a downregulation of ROL transcription levels at later stages of the induction phase.

Acknowledgements

The present work has been supported by the Spanish Programme on Chemical Processes Technologies (Project CTQ2004-00300) and the grant No. 2005SGR 00698 from the DURSI (Generalitat de Catalunya) as well as by projects from Academy of Finland (project no. 203839) and the Finnish Funding Agency for Technology and Innovation (TEKES, CORF project of the Neobio program). The Department of Chemical Engineering of the Universitat Autònoma de Barcelona is the Unit of Biochemical Engineering of the Centre de Referència en Biotecnologia de la Generalitat de Catalunya. D.R. acknowledges the Spanish MEC for a predoctoral grant. We thank Dr. Bernard de la Cruz (KGI, U.S.A.) for providing the putative nucleotide sequence from the Kar2 gene from *P. pastoris*.

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