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Poster Presentation

Analysis of bottlenecks in *Rhizopus oryzae* lipase production in *Pichia pastoris* using the nitrogen source-regulated formaldehyde dehydrogenase promoter (PFLD1)

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Background

Methanol-free high cell density fed-batch cultivation strategies for the *P. pastoris* expression system have been recently developed by expressing a *Rhizopus oryzae* lipase (ROL) under the transcriptional control of the PFLD1 [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. Fed-batch fermentations were performed at three different specific growth rates and showed that productivities were strongly correlated with this parameter (i.e. with the cell's physiological state). 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.

In this context, several studies have pointed out the presence of an effect produced by the accumulation of misfolded proteins in the endoplasmatic reticulum, namely the unfolded protein response (UPR) of yeast, filamentous fungi and higher eukaryotes. In particular, heterologous overexpression of some proteins in *P. pastoris* has been reported to provoke an important accumulation of misfolded proteins in the endoplasmatic reticulum causing the activation of the unfolded protein response (UPR) [2]. Some approaches have been made in order to relax this bottleneck. For instance, the constitutive overexpression of the *Aspergillus niger* unfolded protein response transcription factor HacA in this host has been shown to enable an important increase of the secretion of several heterologous proteins in this host [3].

Another potential bottleneck in recombinant protein secretion is the passage of secreted proteins through the yeast cell wall [4]. Some strategies have been attempted in order to increase yeast cell wall porosity. For instance, inactivation of the Gas1p gene in *S. cerevisiae* may lead to a supersecretory phenotype yielding a considerable increase in secreted protein production [5]. The Gas1p, a glycoprotein anchored to the outer leaflet of the plasma membrane through a glycosylphosphatidylinositol, plays a key role in yeast cell wall assembly.

In this study, we report the application of flow cytometry techniques to the analysis of molecular bottlenecks during the extracellular production of the *Rhizopus oryzae* lipase (ROL) in *P. pastoris* under the transcriptional control of PFLD1. Moreover, these studies were carried out in a series of high cell density cultivation fed-batch experiments.

Results

Fed-batch fermentations were performed at two specific growth rates, namely at a low specific growth rate of about 0.005 h^{-1} (20% of μ_{max}) and a high specific growth rate of about 0.02 h^{-1} (near μ_{max}). These cultivations were performed with three different strains: i) a *P. pastoris* strain expressing ROL under the PFLD1 control, ii) a *P. pastoris* strain co-expressing the induced form of the *S. cerevisiae*'s UPR transcription factor Hac1p gene under the control of the constitutive PGAP promoter and, the ROL gene under the PFLD1 control, and iii) a *P. pastoris* strain with its Gas1p gene knocked out expressing ROL under the PFLD1 control.

Cell viability, the content of BiP, a chaperone of the HSP70 class that plays an important role in the UPR, and intracellular ROL levels were monitored throughout the fed-batch phase of the cultivations set by immunofluorescent techniques using flow cytometry.

The results obtained with the first strain demonstrated that i) cell viability is not significantly affected by ROL overexpression in cells growing on methylamine as a sole nitrogen source, ii) ROL overexpression in *P. pastoris* under the control of the PFLD1 leads to intracellular accumulation of this protein and increased BiP levels, i.e. triggering the UPR.

The use of the engineered strain constitutively expressing the activated Hac1 resulted in a 2-fold increase in the specific ROL productivity in both cells growing at the lower and higher specific growth rates. Remarkably, the maximum q_p values obtained using the Hac1p-engineered strain were 3 and 1.7-fold higher for cells growing at a μ of 0.005 h^{-1} and 0.02 h^{-1}, respectively, than those obtained in the corresponding cultivations carried out with original host strain. However, the intracellular ROL and secretion rate (q_p) profiles still followed the same pattern as in the non-engineered strain, i.e. they suffered an exponential decrease after the sharp increase during the initial stages of the induction phase.

Finally, overexpression of ROL in the Gas1p knock out strain also yielded a significant increase in extracellular lipase levels.

Conclusion

The combined use of flow cytometry, classic analytical techniques and a reduced set of engineered strains has allowed us to assess and monitor the state of the ROL secretion process under bioprocess-relevant cultivation conditions.

Overall, constitutive up-regulation of the UPR has a positive effect on ROL productivities of the process. However, results strongly suggested that ROL overexpression may be down regulated at the transcriptional level as a result of the stress response (repression under secretion stress).

In order to confirm this hypothesis, further quantitative analyses of the transcriptional levels of ROL and other key genes are under way.

Besides, the cell wall appears to pose an additional bottleneck to the excretion of ROL to the extracellular medium, i.e. that there are bottlenecks in different stages along the ROL synthesis, processing and secretion pathways. Moreover, the extent of these bottlenecks is dependent on growth conditions.

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