

Effects of different media supplements on the production of an active recombinant plant peroxidase in a *Pichia pastoris* $\Delta och1$ strain

Christoph Gmeiner[†] and Oliver Spadiut^{*}

Vienna University of Technology; Institute of Chemical Engineering; Research Area; Biochemical Engineering; Vienna, Austria

Keywords: heme precursor, media supplements, OCH1, *Pichia pastoris*, plant peroxidase, protease inhibitor

Abbreviations: HRP, horseradish peroxidase; OCH1, α -1,6-mannosyltransferase Outer Chain elongation 1; $\Delta och1$, *P. pastoris* strain with OCH1 knockout.

© Christoph Gmeiner and Oliver Spadiut

*Correspondence to: Oliver Spadiut; Email: oliver.spadiut@tuwien.ac.at

Submitted: 03/06/2015

Revised: 03/20/2015

Accepted: 03/25/2015

<http://dx.doi.org/10.1080/21655979.2015.1036208>

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.

[†]Current address: ETH Zurich; Department of Chemistry; Laboratory of Physical Chemistry; Research Area Electron-paramagnetic resonance (EPR); Zürich, Switzerland

Addendum to: Gmeiner C., Capone S., Saadati A., Krasteva S., Frank M., Herwig C. and Spadiut O. "Development of a fed-batch process for a *Pichia pastoris* OCH1 knock-out strain" *Microb Cell Fact*, 2015, **14**: 1.

Recombinant protein production in microorganisms is one of the most studied areas of research in biotechnology today. In this respect the yeast *Pichia pastoris* is an important microbial production host due to its capability of secreting the target protein and performing post-translational modifications. In a recent study, we described the development of a robust bioprocess for a glyco-engineered recombinant *P. pastoris* strain where the native α -1,6-mannosyltransferase OCH1 was knocked out ($\Delta och1$ strain). This strain produced the glycosylated enzyme horseradish peroxidase (HRP) with more homogeneous and shorter surface glycans than the respective benchmark strain. However, the recombinant $\Delta och1$ strain was physiologically impaired and thus hard to cultivate. We faced cell cluster formation, cell lysis and consequent intensive foam formation. Thus, we investigated the effects of the 3 process parameters temperature, pH and dissolved oxygen concentration on (1) cell physiology, (2) cell morphology, (3) cell lysis, (4) productivity and (5) product purity in a multivariate manner. However, not only process parameters might influence these characteristics, but also media supplements might have an impact. Here, we describe the effects of different heme-precursors as well as of a protease-inhibitor cocktail on the production of active HRP in therecombinant *P. pastoris* $\Delta och1$ strain.

Introduction

Pichia pastoris, a methylotrophic yeast, is widely used for recombinant protein production due to the possibility of

reaching high cell densities during cultivation, protein segregation and the capability of performing posttranslational modifications (e.g.¹⁻³). However, a significant disadvantage of this microorganism is its tendency to perform hyperglycosylation of proteins.⁴ This represents a significant problem for the production of biopharmaceuticals and for subsequent downstream processing.^{5,6}

Strain engineering of *P. pastoris* depicts an interesting option to conquer the problem of hyperglycosylation. In a recent study, we described the characterization of a recombinant *P. pastoris* strain where the native α -1,6-mannosyltransferase OCH1 was knocked out ($\Delta och1$ strain;⁴). This $\Delta och1$ strain, which recombinantly produced the heme-enzyme horseradish peroxidase (HRP;⁶), showed a growth impaired phenotype and considerable rearrangements of cell wall components leading to substrate dependent cell cluster formation and cell lysis. Consequent intensive foam formation made this strain hard to cultivate. In a subsequent study we developed a robust fed-batch process for this recombinant $\Delta och1$ and investigated the effects of the 3 process parameters temperature, pH and dissolved oxygen concentration on (1) cell physiology, (2) cell morphology, (3) cell lysis, (4) productivity and (5) product purity in a multivariate manner.⁷ We found out that the strain could not be cultivated at 30°C without methanol accumulation and that highest productivity and product purity were reached at 20°C, a pH of 5.0 and a dissolved oxygen concentration of 10%.⁷ However, not only process parameters can influence productivity and product purity, but also media supplements may affect these parameters. Thus we tested potential

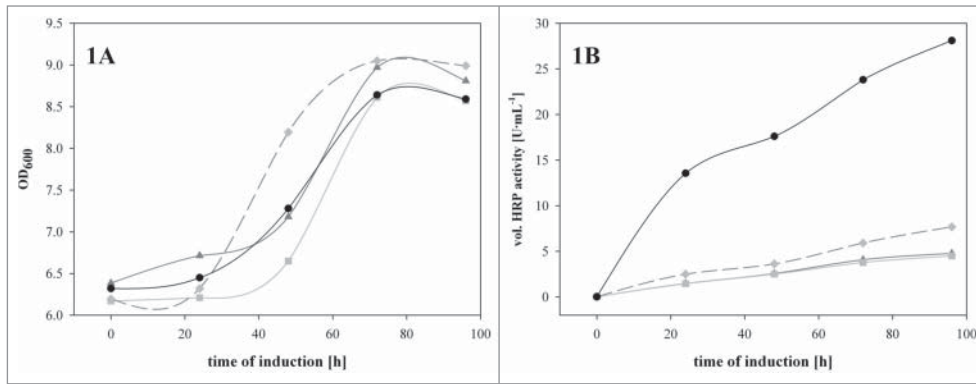


Figure 1. The recombinant $\Delta och1$ strain was cultivated under inducing conditions in the presence of different heme precursors in shake flasks at 25°C for 96 hours. (A) OD₆₀₀ values over induction time; (B) volumetric enzyme activity in U·mL⁻¹ over induction time. Dark gray solid line with triangles, no heme precursor; light gray solid line with squares, ALA; dark dashed line with diamonds, FeSO₄; back solid line with dots, hemin.

effects of (1) different heme precursors and (2) a protease inhibitor cocktail on productivity and product purity by adding these compounds to shake flask cultivations of the recombinant $\Delta och1$ strain.

Effect of heme precursors

The enzyme HRP comprises 4 disulfide-bridges, 2 Ca²⁺-ions as prosthetic group and an iron-protoporphyrin-ring (a heme group) as cofactor in the active site.^{8,9} It is known that the addition of a heme-precursor supports the production of active heme enzymes.¹⁰ In a recent study, we investigated the effect of adding either the heme-precursors Δ -aminolevulinic acid (ALA and ferric sulfate (FeSO₄) or hemin on the production of active HRP using a recombinant *P. pastoris* benchmark strain with active OCH1.¹¹ We found out that medium supplementation with the traditionally used and pricy heme precursor ALA did not increase the yield of active product. FeSO₄ and hemin on the other hand turned out to be useful medium supplements to increase the yield of active heme protein.¹¹ Thus, we tested these

precursors also for the production of HRP with the recombinant $\Delta och1$ strain.⁷

After overnight cultivation in 5 mL BMGY/Zeocin (1% yeast extract; 2% peptone; 100 mM potassium phosphate buffer, pH 6.0; 1,34% YNB; 4·10⁻⁵% biotin; 1% glycerol; 50 μg·mL⁻¹ ZeocineTM) in 100 mL baffled shake flasks at 30°C and 230 rpm, the cell suspension was transferred into 20 mL BMMY/Zeocin (1% yeast extract; 2% peptone; 100 mM potassium phosphate buffer, pH 6.0; 1,34% YNB; 4·10⁻⁵% biotin; 0.5% methanol; 50 μg·mL⁻¹ ZeocineTM) in 250 mL baffled shake flasks and cultivated at 20°C and 230 rpm. We reduced the cultivation temperature from 30°C to 20°C upon induction since we had seen highest productivity and purity for the recombinant $\Delta och1$ strain at the lower temperature previously.⁷ The BMMY/Zeocin contained either no heme-precursor or ALA [1 mM], FeSO₄ [1 mM] or hemin [30 μM], respectively. Cells were cultivated for 96 hours under these inducing conditions. Every day, samples were taken and analyzed for OD₆₀₀,

extracellular protein content and enzymatic HRP activity and 0.5% (v/v) pure methanol was pulsed. In Figure 1 the time courses of biomass growth (followed by OD₆₀₀ values; Fig. 1A) and the extracellular HRP activity (Fig. 1B) are shown.

As shown in Fig. 1A, the growth of the recombinant $\Delta och1$ strain was not affected by the presence of either heme precursor. For all the cultivations the strain grew in the first 70 hours of induction, before limitations caused reduced growth. However, in terms of the amount of active extracellular HRP, we observed drastic differences (Fig. 1B). The

traditionally used and pricy heme precursor ALA did not have any impact on the amount of active HRP but we observed a slight positive effect of FeSO₄ (1.6-times more active enzyme). However, we obtained 7-times more active HRP when we induced the recombinant $\Delta och1$ strain in the presence of 30 μM hemin. In a previous study we observed a boost in the amount of active HRP of up to fold18-when we added 10 μM hemin to minimal media in microscale cultivations of a recombinant *P. pastoris* benchmark strain.¹¹ In comparison, in the present study performed with the recombinant $\Delta och1$ strain we obtained only a 7-fold boost. This discrepancy might be explained by the different media used in the 2 studies. In our previous study we used minimal media, whereas here we used complex BM(G/M)Y medium, which already contained a certain amount of heme-precursors. Thus the observed boost was not that pronounced. However, we can conclude that the boosting effect by hemin is product related and not strain dependent.

Table 1 Values for OD₆₀₀, HRP activity, total protein content and specific activity for the recombinant $\Delta och1$ strain induced in the presence of different heme precursors for 96 hours

Heme precursor	OD ₆₀₀	HRP activity [U·mL ⁻¹]	Protein content [mg·mL ⁻¹]	Specific activity [U·mg ⁻¹]
No precursor	8.81	4.79	0.056	84.8
ALA [1 mM]	8.57	4.51	0.053	85.5
FeSO ₄ [1 mM]	8.99	7.70	0.065	117.9
hemin [30 μM]	8.59	28.1	0.101	278.1

Interestingly, FeSO_4 and hemin did not only affect the enzymatic activity, but also the total amount of extracellular protein. In Table 1 the values for OD_{600} , HRP activity, extracellular protein content and specific activity at the end of cultivation are shown. Clearly we obtained the highest amount of active HRP in the presence of hemin, however we also reached the highest extracellular protein concentration under these conditions. In terms of specific activity, we observed a more than 3-fold higher value for HRP produced in the presence of hemin compared to the traditionally used ALA.

Furthermore, we investigated if the different heme precursors also influenced cell morphology.¹² We analyzed all the samples under the microscope and with a Malvern Mastersizer but could not determine any effect on cell morphology (data not shown). Thus, we conclude that addition of hemin is an effective strategy to obtain more active recombinant peroxidase not only for the benchmark strain, but also for the growth impaired $\Delta och1$ strain.

Effect of protease inhibitor cocktail

In our previous studies we had shown that the recombinant $\Delta och1$ strain was physiologically impaired and strongly affected by cell lysis.^{4,7} It is well known that cell lysis also means the release of intracellular proteases, which could potentially degrade the target product.¹³ Thus, we tested the effect of the presence of a protease inhibitor cocktail (cOmplete Mini EDTA-freeTM; Roche, Switzerland) on the total amount of extracellular protein and active HRP in shake flask experiments. We conducted this study with the *P. pastoris* benchmark strain with intact OCH1 and with the recombinant $\Delta och1$ strain. The cultivations were again performed in shake flasks, as described above. Both strains showed growth in the first 70 hours of induction, before limitations caused reduced growth at the later phases of cultivation. However, when we analyzed the growth of these 2 strains during

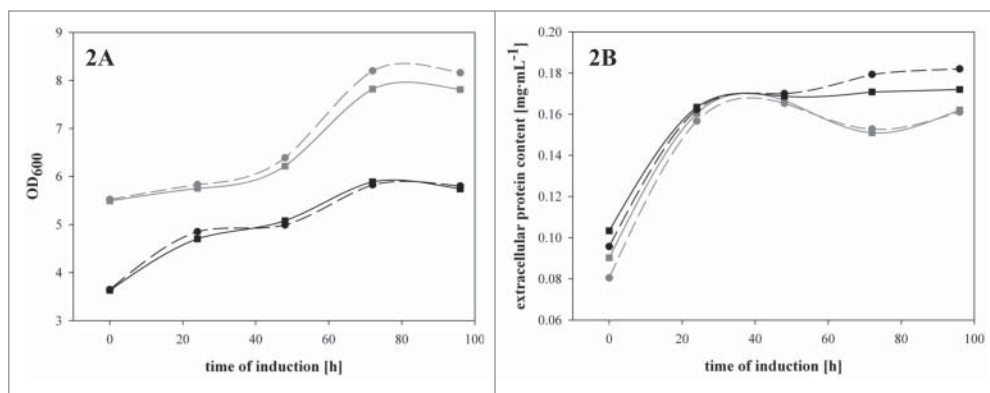


Figure 2. A recombinant benchmark strain and the recombinant $\Delta och1$ strain were cultivated under inducing conditions in the presence or absence of a protease inhibitor cocktail in shake flasks at 25°C for 96 hours. (A) OD_{600} values over induction time; (B) total extracellular protein concentration in $\text{mg}\cdot\text{mL}^{-1}$ over induction time. Dark gray solid line with squares, benchmark strain without protease inhibitor; dark gray dashed line with dots, benchmark strain with protease inhibitor; black solid line with squares, $\Delta och1$ strain without protease inhibitor; black dashed line with squares, $\Delta och1$ strain with protease inhibitor.

the induction phase in more detail, we clearly confirmed the impaired growth of the recombinant $\Delta och1$ strain (Fig. 2A). Interestingly, when we analyzed the total extracellular protein content (Fig. 2B) and the enzymatic HRP activity in the cultivation broths of the 2 strains, cultivated in the presence or absence of the protease inhibitor cocktail, we could not determine any differences. Thus, we concluded that the presence of protease inhibitors does not affect the amount of product for the recombinant benchmark strain nor for the recombinant $\Delta och1$ strain, respectively. As shown in our previous study, cell cluster formation and consequent cell lysis for the recombinant $\Delta och1$ strain are C-source dependent and mainly happen in cultivation phases on glycerol – once the cells switch to methanol cell cluster formation as well as lysis are diminished.⁷ As we can see from the present data, it is not necessary to add protease inhibitors to the cultivation broth of the recombinant $\Delta och1$ strain to protect the product from the proteases released before.

In conclusion we showed that heme precursors can also be used successfully for the recombinant $\Delta och1$ strain to produce more active HRP. In agreement with our previous study performed with a recombinant benchmark strain,¹¹ we found out that addition of hemin gave the highest amount of active product. Furthermore, we showed that endogenous proteases released by cell lysis events during glycerol

cultivation phases of the recombinant $\Delta och1$ strain do not degrade the product during subsequent induction phases, thus making the addition of protease inhibitors unnecessary.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

The authors thank the Austrian Science Fund FWF (project P24861-B19) for funding.

References

1. Cregg JM, Cereghino JL, Shi JY, Higgins DR. Recombinant protein expression in *Pichia pastoris*. *Mol Biotechnol* 2000; 16:23-52; PMID:11098467; <http://dx.doi.org/10.1385/MB:16:1:23>
2. Cregg JM, Tschopp JF, Stillman C, Siegel R, Akong M, Craig WS, Buckholz RG, Madden KR, Kellaris PA, Davis GR, et al. High-Level Expression and Efficient Assembly of Hepatitis-B Surface-Antigen in the Methylophilic Yeast, *Pichia-Pastoris*. *Nat Biotechnol* 1987; 5:479-85; <http://dx.doi.org/10.1038/nbt0587-479>
3. Zalai D, Dietzsch C, Herwig C, Spadiut O. A dynamic fed batch strategy for a *Pichia pastoris* mixed feed system to increase process understanding. *Biotechnol Prog* 2012; 28:878-86; PMID:22505140; <http://dx.doi.org/10.1002/btpr.1551>
4. Krainer FW, Gmeiner C, Neutsch L, Windwarder M, Pletzenauer R, Herwig C, Altmann F, Glieder A, Spadiut O. Knockout of an endogenous mannosyltransferase increases the homogeneity of glycoproteins produced in *Pichia pastoris*. *Scientific reports* 2013; 3:3279; PMID:24252857; <http://dx.doi.org/10.1038/srep03279>
5. Spadiut O, Rossetti L, Dietzsch C, Herwig C. Purification of a recombinant plant peroxidase produced in *Pichia pastoris* by a simple 2-step strategy. *Protein Expr*

- Purif 2012; 86:89-97; PMID:23026679; <http://dx.doi.org/10.1016/j.jpep.2012.09.008>
6. Spadiut O, Herwig C. Production and purification of the multifunctional enzyme horseradish peroxidase: a review. *Pharm Bioproc* 2013; 1:283-95; <http://dx.doi.org/10.4155/pbp.13.23>
 7. Gmeiner C, Saadati A, Maresch D, Krasteva S, Frank M, Altmann F, Herwig C, Spadiut O. Development of a fed-batch process for a recombinant *Pichia pastoris* inverted question mark och1 strain expressing a plant peroxidase. *Microb Cell Fact* 2015; 14:1; PMID:25567661; <http://dx.doi.org/10.1186/s12934-014-0183-3>
 8. Smith AT, Santama N, Dacey S, Edwards M, Bray RC, Thorneley RNF, Burke JF. Expression of a Synthetic Gene for Horseradish Peroxidase-C in Escherichia-Coli and Folding and Activation of the Recombinant Enzyme with Ca-2+ and Heme. *J Biol Chem* 1990; 265:13335-43; PMID:2198290
 9. Veitch NC, Smith AT. Horseradish peroxidase. *Adv Inorg Chem* 2001; 51:107-62; [http://dx.doi.org/10.1016/S0898-8838\(00\)51002-2](http://dx.doi.org/10.1016/S0898-8838(00)51002-2)
 10. Jiang H, Morgan JA. Optimization of an in vivo plant P450 monooxygenase system in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 2004; 85:130-7; PMID:14704995; <http://dx.doi.org/10.1002/bit.10867>
 11. Krainer FW, Capone S, Jager M, Vogl T, Gerstmann M, Glieder A, Herwig C, Spadiut O. Optimizing cofactor availability for the production of recombinant heme peroxidase in *Pichia pastoris*. *Microb Cell Fact* 2015; 14:4; PMID:25586641; <http://dx.doi.org/10.1186/s12934-014-0187-z>
 12. Mellitzer A, Ruth C, Gustafsson C, Welch M, Birner-Grunberger R, Weis R, Purkarthofer T, Glieder A. Synergistic modular promoter and gene optimization to push cellulase secretion by *Pichia pastoris* beyond existing benchmarks. *J Biotechnol* 2014; 191:187-95; PMID:25193713; <http://dx.doi.org/10.1016/j.jbiotec.2014.08.035>
 13. Sinha J, Plantz BA, Inan M, Meagher MM. Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-tau. *Biotechnol Bioeng* 2005; 89:102-12; PMID:15580575; <http://dx.doi.org/10.1002/bit.20318>