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**D I S S E R T A T I O N**

# Different approaches to make *Pichia pastoris* a more versatile expression platform – process, strain and product engineering

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Vorrei infine dedicare questa Tesi al mio amico Armando - spero tu sappia che ce l´abbiamo fatta!!

# Index

# 1.Abstract

Over the last decades, the biopharmaceutical market has become a significant segment of the global pharmaceutical market, with antibodies and antibody fragments being the leading building blocks. However, also proteins and enzymes are conquering the biopharmaceutical market now. Mainly due to the possibility of resembling human-like glycosylation, mammalian cells are still the most widely used expression system for the production of biopharmaceuticals. However, implementation of less complex microorganisms, such as yeasts or bacteria, would be highly desirable to simplify the upstream processes and improve productivity. The methylotrophic yeast *P. pastoris* is commonly used for the production of industrial and biopharmaceutical enzymes. This yeast offers many advantages such as biomass growth up to very high cell density, easiness of genetic manipulation, availability of strong and tightly regulated promoters and the possibility of producing up to gram per litre of recombinant protein, both intracellularly and extracellularly. Nevertheless, its implementation for biopharmaceuticals production is not well established yet, mainly due to the need of circumventing methanol implementation as induction substrate and due to the distance from the native human-like post-translational modifications, above all glycosylation. Methanol is a high-degree reductant with high heat of combustion and high oxygen consumption. Therefore its replacement with a different substrate is highly desirable above all for large scale fermentations. Yeast hyperglycosylation leads to the production of non-human like glycosylated proteins thus limiting their implementation as biopharmaceuticals. Additionally, the presence of non-uniform glycoforms hampers downstream processing and conjugation approaches.

Therefore the goal of this Thesis consists in solving the above mentioned issues with the aim of rendering *P. pastoris* a more suitable expression host for production of biopharmaceuticals. In compliance with the goal of this Thesis, the following methodology based on the application of three different engineering methods namely, **process, strain and product engineering** was applied. In the first chapter of this Thesis, "The biopharmaceutical market", an overview is provided about this branch of the pharmaceutical market and the recent advances with the three main microbials, namely *Saccharomyces cerevisiae*, *Pichia pastoris* and *Escherichia coli* for full length antibodies and antibody fragments production. In the second chapter of this Thesis, "Avoiding methanol requirement" I describe a process engineering approach based on the design of a mixed-feed strategy for optimizing the recombinant expression of phospholipase C enzyme (PLC) in a *P. pastoris* strain harbouring an AOX1 de-repression promoter variant. Using this strain methanol as inducer can be omitted. In the third chapter "Reducing hyperglycosylation" I describe two different engineering approaches, namely strain and product glycoengineering, which can be applied to reduce yeast hyperglycosylation. These

methods were tested for the expression of a more uniformly and less glycosylated horseradish peroxidase (HRP) isovariants. Since HRP is a heme containing enzyme, I describe and compare two different approaches based on metabolic engineering and on cofactor media supplementation for enhancing heme availability, as described in the third chapter of this Thesis "Increasing product specific cofactor availability". Over-expression of heme pathway genes versus hemin supplementation in growth media was tested for improving final yields of active HRP.

In conclusion, in compliance with the described methodology, I was able to identify the most suitable engineering approach to be applied for solving each of the above mentioned pitfalls. In particular I found out that:

- process engineering in combination with implementation of a de-repressed strain allows to circumvent methanol implementation;
- product glycoengineering proves to be a valid strategy for reducing enzyme hyperglycosylation;
- cofactor supplementation in media proves to be a valid approach to increase product specific cofactor availability.

Therefore the presented Thesis represents a basis for the combination of these engineering approaches and may work as fundamental knowledge for successful expression of recombinant biopharmaceuticals in *P. pastoris*.

# Zusammenfassung

Im Laufe der letzten Jahrzehnte etablierte sich der biopharmazeutische Markt als wichtiges Element des globalen pharmazeutischen Marktes. Zu den wichtigsten biopharmazeutischen Produkten zählen Antikörper aber auch Antikörperfragmente. Jedoch werden Protein und auch Enzyme zunehmend interessanter und wichtiger. Aufgrund der Fähigkeit menschenähnliche Glykosylierungsstrukturen auszubilden, sind Säugetierzellen die am weitest verbreitetsten Expressionssysteme für die Produktion von Biopharmazeutika. Jedoch würde die Verwendung von weniger komplexen Mikroorganismen wie Bakterien oder Hefen eine Vereinfachung des Upstream-Prozesses und außerdem eine Verbesserung der Produktivität bewirken. Ein bereits weit verbreiteter Organismus, welcher sowohl für die industrielle als auch für die biopharmazeutische Enzym-Produktion verwendet wird, ist die methylotrophe Hefe *P. pastoris*. Zu ihren Vorteilen zählen unter anderem die Fähigkeit in hohen Zelldichten zu wachsen, die Einfachheit der genetischen Manipulation, die Verfügbarkeit von starken, streng regulierten Promotoren und die Fähigkeit sowohl intrazelluläre als auch extrazelluläre rekombinante Proteine bis in Gram pro Liter zu produzieren. Nichtsdestotrotz ist die Implementierung dieses Host-Organismus in der biopharmazeutischen Produktion nicht gut etabliert. Vorwiegend wegen des Fehlens der menschenähnlichen posttranslationalen Modifikationen, allen voran der Glykosylierung. Außerdem schreckt die Verwendung von Methanol als Inducer-Substrat ab. Methanol ist ein starkes Reduktionsmittel mit hohem Sauerstoffverbrauch und starker Hitzeentwicklung. Deshalb ist die Substitution von Methanol durchein anderes Substrat sehr wünschenswert. Die Hyper-Glykosylierung, welche durch Hefen verursacht wird, führt zur Produktion von nicht human glykosylierten Proteinen. Dies ist einer der Gründe derartige Proteine nicht als Biopharmazeutika zu verwenden. Außerdem hindern die uneinheitlichen Glykosylierungsstrukturen ein effizientes Downstream Processing. Das Ziel dieser Arbeit ist es daher die genannten Probleme zu lösen und *P. pastoris* zu einem geeigneten und attraktiveren Expressionssystem für Biopharmazeutika zu machen. Um dies umzusetzen, wurden die Engineering Methoden Prozessentwicklung, Stamm-Engineering und Produkt-Engineering angewandt. Das erste Kapitel „The biopharmaceutical market“ gibt einen Überblick über den pharmazeutischen Markt und die neuesten Fortschritte im Bereich von Expressionssystemen, Stamm-Entwicklung und Produktion mit den drei wichtigsten Mikroorganismen (*Saccharomyces cerevisiae*, *P. pastoris* und *Escherichia coli*) für die Herstellung von Antikörper Fragmenten. Im zweiten Kapitel der Arbeit, „Avoiding methanol requirement“ beschreibe ich eine Prozess-Engineering Methode basierend auf einer gemischten Fütterungsstrategie zur Optimierung der rekombinanten Expression des Phospholipase C Enzyms (PLC) in einem *P. pastoris* Stamms, welcher eine AOX1 Depressionspromoter-Variante trägt. Durch Verwendung dieses

*P. pastoris* Stamms ist es möglich Methanol als Inducer zu umgehen. Das dritte Kapitel „Reducing hyperglycosylation“ beschreibt die zwei Methoden Stamm- und Produkt-Glycoengineering, welche dazu verwendet wurden um die Hyperglykosylierung der Hefe zu reduzieren. Beide Methoden wurden angewandt, um gleichmäßiger und weniger glykosylierte Isovarianten des Enzyms Horseradish Peroxidase (HRP) zu expremieren. Da HRP ein Häm-Gruppen enthaltendes Enzym ist, wurden zwei verschiedene Methoden (metabolisches Engineering und Cofaktorzusatz zum Medium) verwendet und verglichen, um die Häm-Verfügbarkeit zu steigern. Die Ergebnisse werden im vierten Kapitel “Increasing product specific cofactor availability” beschrieben. Die Methode der Überexpression von Häm-Pathway-Genen wurde mit Kultivierungen verglichen, denen Hemin in das Wachstumsmedium zugesetzt wurde, indem die endgültigen Mengen an aktiver HRP ermittelt wurden.

Zusammenfassend kann gesagt werden, dass wir die geeignetste Engineering-Methode ermitteln konnten um die zu Anfangs erwähnten Probleme bzw. Aufgaben zu lösen:

- Es war möglich einen De-repressionsstamm in einen Prozess zu implementieren um Methanol als Inducer zu vermeiden.
- Es konnte gezeigt werden, dass Glycoengineering des Produkts eine wirksame Methode ist, um die Hypergylkosylierung des Enzyms zu reduzieren.
- Die Medien-Zusammensetzung zu verändern ist eine mögliche Strategie, um die produktspezifische Cofaktor-Verfügbarkeit zu erhöhen.

Aufgrund der genannten Ergebnisse kann diese Arbeit als fundamentale Erkenntnis für eine erfolgreiche Expression von rekombinanten Biopharmazeutika in *P. pastoris* gesehen werden.

## List of abbreviations

**$\Delta_{time\ adapt}$** time for adaptation to a new substrate (methanol; h)

**$\mu$** specific growth rate ($h^{-1}$)

**$\mu_{max}$** maximum specific growth rate

**ABTS** 2,2' azino bis 3-ethylbenzthiazoline-6-sulphonic acid

**Ala** 5-aminolevulinic acid

**CER** carbon evolution rate (mol/l/h)

**C-source** carbon source

**ER** endoplasmic reticulum

**Fabs** antibody fragments

**FDA** Food and Drug Administration

**Gal** Galactose

**GlcNAc** N-acetylglucosamine

**$H_2O_2$** hydrogen peroxide

**HRP** horseradish peroxidase

**mAbs** monoclonal antibodies

**Man** Mannose

**mRNA** messanger ribonucleic acid

**pAOX** alcohol oxidase I promoter

**PLC** phospholipase C

**$q_p$** specific productivity (U/g/h)

**$q_s$** substrate specific uptake rate (g/g/h; or mmol/g/h)

**$q_{s\ adapt}$** substrate specific uptake rate

**$r_p$** volumetric production rate (U/L/h)

**Sia** Sialic acid

**UDP- GlcNAc** Uridine diphosphate N-acetylglucosamine

**(v/v)** volume per volume

# 2. Introduction

## 2.1 Biopharmaceutical market and big sellers

The biotechnological market is mainly based on the production of industrial enzymes and biopharmaceuticals, and it accounts nowadays for a multibillion dollars turnover, with steadily raising expectations. Biotechnologically produced enzymes find many different applications in production of biofuels, bioremediation, food and feed preparation, and production of bio-based chemicals. In 2010 the global market of industrial enzyme was estimated at 3.6 US billion dollars, and it is estimated to reach 6-8 US billion dollars in 2015-2016 (www.bio-economy.net) [1]. However, the biopharmaceutical market represents the real leading branch of the biotechnological market. Just to get an idea, revenues from commercialization of approved biopharmaceuticals were estimated at US 162 billion dollars in 2014, registering a constant increase of circa 15% in the latest 2012-2014 biennium (140 billion US dollar in 2013, and 120 US billion dollars of 2012) and being expected to reach US 280 billion dollar in 2020 (www.persistencemarketresearch.com) [2]. The most lucrative product class is represented by monoclonal antibodies (mAb) and antibody fragments (Fab) and with the human mAb Humira (Adulimab) being top selling product from 2010-2014 (www.statista.com) [3]. Due to the increasing demand for mAbs and, more in general, for glycosylated biopharmaceuticals mammalian cells are still the most implemented expression system. However, mammalian cell culture processes present different drawbacks such as relatively time consuming cultivations, requirement for complex media, and susceptibility to viral contaminations [4], thus leaving space for implementation of microbials as alternative expression hosts. Therefore, the microbial species, *Escherichia coli* (*E. coli*) and the two yeasts *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Pichia pastoris* (*P. pastoris*) together account for 50% of the total production of biopharmaceuticals [5].

## 2.2 *Pichia pastoris* and biopharmaceuticals production

Over the last two decades, *P. pastoris* has been frequently used as expression host in both industry and academia. Beside the large number of enzymes recombinantly expressed, this yeast has also been used for the production of biopharmaceuticals, as shown in Table1. Recently, in fact, two biopharmaceuticals Kalbitor and Jetrea, both expressed in *P. pastoris*, have been approved by the FDA [6, 7].

| Product | Citation |
|---|---|
| Human Angiogenin | [8] |
| Vascular endothelial growth factor (VEGF 165b) | [9] |
| Human interleukin-2/3/6 | [10-12] |
| Human prostaglandin H synthase-2 | [13] |
| Human adiponectin | [14] |
| Porcine Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) | [15] |
| Der p2 (major allergen from Dermatophagoides pteronyssinus) | [16] |
| Human Erythropoietin | [17] |
| Hepatitis B surface antigen | [18] |
| Human tissue plasminogen activator | [19] |
| Human neonatal Fc receptor | [20] |
| Human growth hormone | [21] |
| P32 protein of goatpox virus | [22] |
| Human Oncostatin M | [23] |
| Insulin precursor | [24] |
| Human granulocyte-macrophage colony stimulating factor (hGM-CSF) | [25] |
| Interferon-alpha mutant | [26] |
| Human apolipoprotein AI | [27] |
| Human mast cell chymase | [28] |
| Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) | [29] |
| Erythropoietin | [30] |
| Human lactoferrin | [31] |

Table1. Different biopharmaceuticals produced in *Pichia pastoris* in the last 10 years.

However, the majority of therapeutic proteins contains post-translational modifications (PTM), with glycosylation being the most common one. As previously mentioned, yeast can perform PTMs, and thus also glycosylation. However expression of glycosylated proteins in yeasts is affected by hyperglycosylation. This is why many efforts have been concentrated in yeast glycoengineering.

## 2.3 Challenges with *P. pastoris*

Among the different drawbacks associated to recombinant expression in the yeast *P. pastoris*, I focused on three main challenges to be tackled, namely methanol requirement, hyperglycosylation, and limiting product specific cofactor availability.

### 2.3.1 Methanol requirement

Recombinant expression in the methylotrophic yeast *P. pastoris* is still most commonly driven by the methanol utilisation pathway (MUT) AOX1 promoter (pAOX1) [32]. pAOX1 is strongly repressed in the presence of glucose, glycerol and ethanol, de-repressed upon depletion of these carbon sources, and strongly induced in the presence of methanol. This expression system allows uncoupling cell growth from production phase. Although this system has been and is still largely implemented, reaching high expression levels (up to 15 g/L secreted protein), methanol requirement represents a severe drawback. Its consumption during fermentations, in fact, causes high heat production and increased need for oxygen. Furthermore, on-line monitoring of methanol is desirable to avoid its accumulation during fermentation. Elevated methanol concentration in the growth media leads to production of toxic compounds and to cell growth inhibition [33]. Additionally, methanol is highly flammable and storage of great amounts is rather undesirable above all at industrial scales. Therefore, methanol is only used as induction substrate after an initial growth phase on a different substrate [34], most commonly glucose or glycerol. Specific growth rate ($\mu$) controlled fed-batch represents the most commonly implemented fermentation mode for *P. pastoris* cultivations, since it guarantees good metabolic control of cell metabolism. A normal fed-batch mode consists in an initial batch phase, which is then followed by fed-batch on a pAOX1 repressing substrate, usually at maximum specific growth rate, $\mu_{max}$, in order to achieve high cell density. Once the desired cell density is reached, the induction phase is started with methanol supplementation at $\mu$ value which allows to prevent its accumulation, thus requiring a preliminary strain characterization. This strain characterization is usually performed with continuos cultures or consecutive fed-batches cultivations thus being rather time consuming and laborious [35]. Additionally contradictory results are reported in literature concerning the existence of a direct correlation between maximum specific productivity, $q_{p\ max}$, and $\mu_{max}$ [36]. In some studies it was demonstrated that maximal specific productivity did not correlate with maximal specific growth rate [37-39], while other studies supported a growth associated productivity [40, 41]. Therefore the effect of different parameters on specific productivity has been investigated and in several studies methanol specific uptake rate, $q_s$, was identified as the most important induction parameter [42, 43]. Recently a fast approach based on dynamic batch experiment with methanol pulses was developed which allowed to determine a set of strain physiological parameters (adaptation time to methanol - $\Delta_{time\ adapt}$, the $q_s$ during adaptation - $q_{s\ adapt}$, $q_s$ maximum - $q_{s\ max}$) to be used for the development of a $q_s$ based fed batch [36, 44]. As shown in Fig.1, after a batch on a pAOX1 repressing C-source, a first methanol adaptation pulse in the final concentration of 0.5% (v/v) is applied. The time required to reach a maximum in the off-gas activity is used to define $\Delta_{time\ adapt}$, which

represents the minimum time required by the culture for complete adaptation to methanol. Another important parameter which can be extrapolated from the adaptation pulse is $q_{s\ adapt,}$ which allows to identify an optimal feeding rate to be set during the adaptation phase for preventing methanol accumulation. The methanol adaptation pulse is then followed by several 1% (v/v) methanol pulses which allow to determine $q_{s\ max}$. The $q_{s\ max}$ value represents the upper limit for the feeding rate establishment during the induction phase. Therefore dynamic batch with methanol pulses represents a fast and easy to do approach to determine a minimum set of parameters for the design of $q_s$ based fed-batch without the risk of methanol accumulation [35]. Additionally to further reduce this risk different approaches have been developed for the adjustment of $q_s$ based feeding rate according to online biomass estimation [45].

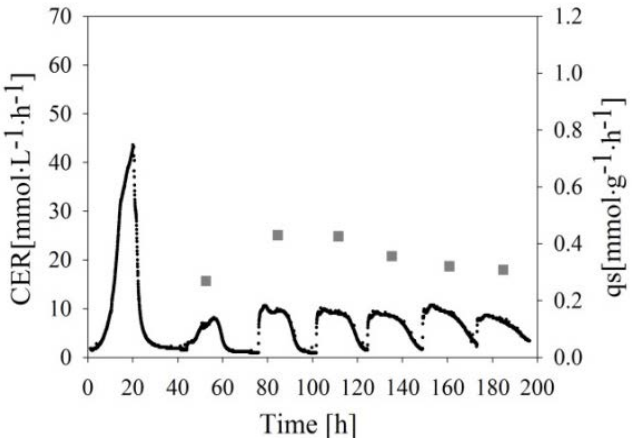


Fig1. Dynamic batch cultivation with methanol pulses of a *P. pastoris* strain. After a batch phase on glycerol, a methanol adaptation pulse is performed followed by several methanol induction pulses. Solid black line, carbon dioxide evolution rate (CER); grey squares, specific substrate uptake rate ($q_s$).

A different feeding strategy which can be implemented for reducing drawbacks associated with methanol implementation is based on the concomitant feeding of two different C-sources during the induction phase and thus it is referred as mixed-feed strategy. Most commonly, methanol/glycerol or methanol/sorbitol co-feeds are implemented [46]. This strategy offers many advantages since it is not only supposed to increase productivity, due to higher biomass yields which can be achieved during the induction phase, but it also allows lowering oxygen consumption and decreasing heat production, both drawbacks associated to methanol metabolism [33, 46]. Due to the complex interaction on cell metabolism derived by the usage of two C-sources, identification of the most appropriate feeding regime is of utmost importance [47]. Commonly, time consuming continuous cultures and fed-batches are therefore required for a preliminar characterization [33, 46, 48]

However, until when recombinant protein expression is regulated by the endogenous pAOX1 it would not be possible to completely avoid methanol implementation. This is why many efforts have been concentrated for

identifying alternative methanol-free promoters, which guarantee to achieve comparable productivity titers of the pAOX1. Numerous natural promoters have been already identified with different regulation systems [49, 50]. To clarify this concept three different promoter regulations are described here:

1. induction, the target promoter is active only in the presence of a certain component, most commonly a C-source;
2. constitutive expression, the promoter is "always active" most commonly in association to cell growth;
3. de-repression, the promoter gets activated when a certain component is available in low concentrations.

On of the most commonly implemented pAOX1 alternative promoter is the constitutive glyceraldehyde 3-phosphate dehydrogenase gene promoter (pGAP) which showed highest mRNA level on glucose [51]. Because of the constitutive expression no induction step is required thus allowing to shorten the process time. However since production is not decoupled from cell growth pGAP is not always ensuring similar production titers to pAOX1 driven expression. In addition pGAP can be used only if the recombinantly expressed protein is not toxic for cells [49]. Therefore there is a constant research for new promoters as reported in Table3.

De-repressed promoters, in particular, are highly desirable for driving recombinant protein expression. By implementing de-repressed promoters transcription is only triggered when substrate is limiting, which makes this expression profile particularly advantageous for fed-batch processes, where the feeding profile is tightly controlled [50]. However this promoter class does not guarantee high productivity titers, since substrate in limiting concentration does not allow cell growth occuring concomitantly with expression.

| **Promoters** | **Gene product** | **Regulation** | **Citations** |
|---|---|---|---|
| **ICL1** | Isocitrate lyase | De-repressed by glucose or induced by ethanol | [52] |
| **PGK1** | Phosphoglycerate kinase | Constitutive | [53] |
| **AOD** | Alternative oxidase | Expression on glucose repressed by methanol | [54] |
| **PHO89** | Sodium coupled phosphate simporter | Induce by phosphate limitation | [55] |
| **SSA4** | Heat shock protein | Constitutive | [56] |
| **TEF1** | Translation elongation factor 1 alpha | Constitutive and growth associated | [57] |
| **TPI1** | Triose phosphate isomerase | Constitutive | [56] |

Table3. *P. pastoris* endogenous methanol-free promoters.

Beside the research and characterization of natural endogenous *P. pastoris* promoters, a different input comes from synthetic biology which offers an alternative way to generate methanol-free promoters. Rational design or random mutagenesis was applied for the generation of synthetic variants of the pAOX1 promoter. The

introduction of small deletions and mutations allowed generating synthetic pAOX1 variant moderately active with glucose depletion [4].

### 2.3.2 Hyperglycosylation in *P. pastoris*

*P. pastoris*, and yeasts in general, are able to perform most of the human post-translational modifications and thus also N-linked glycosylation. However, yeast N-linked glycan structures are significantly different from those of mammalian cells and humans therefore compromising their therapeutic implementation [58] . Yeast derived high mannose N-glycans display fast serum clearance, negatively affecting pharmacokinetic properties of recombinant therapeutic glycoproteins [59]. Additionally hyperglycosylation masks the physicochemical properties of the target enzyme hampering a fast and efficient downstream processing [60]. Due to the requirement of human like glycosylation of therapeutic proteins, production of biopharmaceuticals is mainly done in mammalian cells. However, it is important to remark that mammalian cells and yeasts share the initial steps of the N-glycans biosynthetic pathway. The process starts on the cytoplasmic face of the endoplasmic reticulum (ER) with the transferring of a N-acetylglucosamine (GlcNAc) moiety from UDP-GlcNAc onto dolichol phosphate. After that, different glycosyltransferase catalyzed reactions occur leading to the addition of GlcNAc and mannose (Man) to the nascent glycan structure. When the $Man_5GlcNAc_2$-P-dolichol mature structure is assembled, it is translocated to the luminal face of the ER membrane by a flipase enzyme. In the ER this structure is further extended to $Glc_3Man_9GlcNAc_2$-Pdolichol, and transferred to the N-X-S/T motif of the target peptide. Next step consists in the removal of three glucose and one mannose residues to produce $Man_8GlcNAc_2$. The following steps for the ulterior processing of the $Man_8GlcNAc_2$ structure differ significantly between mammalians and yeasts. In mammalian cells the $Man_8GlcNAc_2$ glycan is trimmed to $Man_5$-$GlcNAc_2$ and further rearrangements lead to the production of a sialylated structure, which resembles the core of the human glycosylation pathway. Differently from mammals, yeasts rather extend the $Man_8GlcNAc_2$ glycan structure in the Golgi with additional mannose sugars, thus producing hyper-mannosylated glycans structures [61]. Therefore many efforts have concentrated on yeast glycoengineering in order to come closer to human-like glycosylation.

*Glycoengineering of P. pastoris: toward humanization*

*P. pastoris* can be used for the production of both Fabs and mAbs (e.g. [62]) For mAbs the correct human-type glycosylation is not only essential for proper folding and biological activity, but also for targeting and stability in circulation. *P. pastoris* lacks the Golgi-resident α-1,3-mannosyltransferase, but harbors four additional β-

mannosyltransferases instead [63, 64]. The absence of terminal α-1,3-mannoses on *P. pastoris* derived glycoproteins is of importance since this glycan structure causes high antigenicity in humans [65]. Thus, the humanization of the N-glycosylation pathway in *P. pastoris* has been an important goal. The Outer CHain elongation 1 gene (OCH1) coding for an α-1,6-mannosyltransferase was knocked out [66], and an α-1,2-mannosidase, β-N-acetylglucosaminyltransferase I (GnTI) and an UDP-GlcNAc transporter were introduced [67]. The *Kluyveromyces lactis* UDP-GlcNAc transporter, mouse α-1,2-mannosidase IA and *Drosophila melanogaster* mannosidase II, human GnTI and rat GnTII were introduced into an och1 knockout strain, resulting in the homogeneous formation of the complex human $GlcNAc_2Man_3GlcNAc_2$ glycan [58] . In other studies, OCH1 was inactivated via a knock-in strategy [68], an ER-targeted HDEL-tagged α-1,2-mannosidase from *Trichoderma reesei* was introduced and a chimeric human GnTI was fused to the N-terminal part of *S. cerevisiae* Kre2 for Golgi localization [69] . A further approach included the construction of a strain expressing mouse mannosidase IA, the *K. lactis* UDP-GlcNAc transporter, human GnTI and rat GnTII, in which the ALG3 gene, encoding an α-1,3-mannosyltransferase of the ER lumen, was knocked out [70], leading to the formation of $GlcNAc_2Man_3GlcNAc_2$. Additional coexpression of a fusion protein consisting of the *S. cerevisiae* Mnn2 Golgi localization domain and the activities of *Schizosaccharomyces pombe* UDP-Gal 4-epimerase and human β-1,4-galactosyl transferase allowed the production of $Gal_2GlcNAc_2Man_3GlcNAc_2$ glycans. An alternative protocol allowed production of $Gal_2GlcNAc_2Man_3GlcNAc_2$ N-glycans using the GlycoSwitch vector technology [71], where specially designed vectors are used to replace genes of the native glycosylation pathway. Further humanization was achieved by additional biosynthesis of cytidine monophosphate-linked Sia, its transport and the transfer of Sia onto the N-glycans of nascent polypeptides, leading to complex human $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$ glycans [72]. Additional glycoengineering studies included the elimination of α-1,2-mannosidase resistant high Man glycans [73] and overexpression of *Leishmania major* STT3D to increase N-glycan site occupancy [59] These steps make it possible to use glycoengineered *P. pastoris* strains for the production of full length mAbs (e.g. [74]).

### 2.3.3 Limitation of product specific cofactor availability

Limitation of cofactor availability can negatively affect expression of active recombinant proteins. Product specific cofactors can be defined as “those moietis, either organic or inorganic, which remain physically associated with the enzyme through a catalytic cycle” [75]. Members of the organic group are usually derivatives of vitamins, while members of the inorganic group consist in the various arrangements of the iron-sulfur cluster. In its cofactor bound state an enzyme is usually referred as holoenzyme which consists in the active form of the enzyme, whereas in an unbound state it is usually referred as apoenzyme. Therefore generation of a holoenzyme requires a polypeptide construct (apoenzyme) and a cofactor moiety which is synthesized by a metabolic pathway. A functional holoenzyme, in fact, can only be produced if the apoenzyme is correctly folded with its cofactor. Two main mechanisms have been identified for integration of the cofactor into the apo-enzyme, either in a co-translational [76] or post-translational manner [77]. Association of the cofactor to its apoenzyme can occur due to covalent linkages as described for the heme c in cytochrome c [78] or non-covalent linkages as in the case of the flavin containing enzyme acyl CoA dehydrogenase [79]. However, in order to maximize production of active holoenzyme, its apo-form and cofactor have to be both available. Two possible scenarios could impaire production of an active holoenzyme, namely complete unavailability of the desired cofactor which occurs when the implemented expression host is missing the required metabolic pathway or insufficient cofactor concentration which could be a direct consequence of the target protein (over)expression. It can happen that the amount of physiologically synthesized cofactor is not high enough for supporting recombinant expression. Two different approaches can be applied in order to overcome product specific cofactor limitation based on metabolic engineering or cofactor/precursor media supplementation [80].

In this Thesis a particular focus is dedicated to the heme pathway. This pathway has been extensively characterized in yeasts, mainly in *S. cerevisiae* [81], since heme serves as prostetic group for a large variety of proteins such as hemoglobin, myoglobin, catalases, peroxidases, cytochromes P450. These proteins are involved in important biological processes, such as respiration, photosynthesis, and the metabolism and transport of oxygen [82, 83]. Additionally as consequence of the recombinant expression of industrial and medical valuable heme containining enzymes such as peroxidases, catalases and globins in yeasts [84, 85], a lot of attention has been again addressed to this pathway, since insufficient incorporation of heme is considered a central impeding cause in the recombinant expression and stability of active heme proteins [86, 87].

## 2.4 Model proteins implemented in this Thesis

In the next two sections a brief description of the two enzymes horseradish peroxidase and phospholipase C is provided. These two enzymes were used as model proteins in the papers presented in this Thesis.

*Horseradish peroxidase*

Horseradish peroxidase is a heme containing plant enzyme which belongs to the group of Class-III peroxidases and catalyzes the oxidation of different substrates using peroxidase species (commonly $H_2O_2$) as oxidant. Horseradish peroxidase exists at least in 20 different isoenzyme forms in the plant roots [88], of which HRP C1A is the most abundant and thus the most characterized one. The catalytic mechanism of horseradish peroxidase has been characterized in great detail and it consists of three consecutive steps accompanied by the formation of three different enzyme intermediates [89]. HRP C1A contains a protoporphyrin IX prostetic group usually referred to as heme group and two calcium ions per molecule, which are essential for the stability and the functionality of the enzyme. Additionally the crystal structure allowed also the identification of nine N-glycosylation sites of the Asn-X-Ser/Thr type (X is a generic amino acid). Of these nine glycosylation sites eight are occupied when the enzyme is expressed in plant [90]. The typical glycan structure is a branched heptasaccharide, but also minor glycans have been described. The superficial N-glycosylation sites of horseradish peroxidase account for 20% of the all carbohydrate content [91].

Nowadays, horseradish peroxidase (HRP) finds application in many different branches of life science, such as diagnostic, bioremediation, biocatalysis and it has been also recently tested in combination with indol acetic acid (IAA) for target cancer treatment [92]. In comparison to other enzymes, in fact, HRP offers many different advantages like relatively small size, high catalytic conversion rates, broad variety of substrates and different methods are available for its conjugation to antibodies [92]. However and quite paradoxically enzyme preparations available on the market consist of a mixture of isoenzymes, which limits an extensive implementation in the medical field. This heterogeneity is due to the fact that HRP is still isolated from plant roots where it is produced as a mixture of isoenzymes which is seasonally variable [93]. Additionally recovery from plant requires quite cumbersome extraction and purification processes, which results in final high prices of the enzyme preparations. Therefore, different attempts have been made to recombinantly express the enzyme and different microorganisms have been used. Among the different tested microorganisms the methylotrophic yeast *P. pastoris* turned out to be the best candidate due to the possibility of performing post-translational modifications [84]. However recombinant expression in this host is negatively affected by hyperglycosylation, which leads to production of different glycoforms of the enzyme, hampering purification processes and

antibodies and lectin conjugation [60]. Therefore recombinant production of an active glycoengineered HRP isoform, characterized by a more uniform and reduced glycosylation profile for potential applications in the medical fields still represents an open challenge.

*Phospholipase C*

Phosphoinositide-specific phospholipase C (PI-PLC) is an ubiquitous enzymes class, which catalyzes the hydrolysis of phosphoinositides to soluble inositol phosphates and membrane-bound diacylglycerol (DAG). In higher eukaryotes, PLCs work as key-enzymes in most of receptor mediated signal transduction pathways [94]. These enzymes, in fact, hydrolyse phosphatidylinositol (PI) 4,5-bissphosphate into inositol 1,4,5-trisphosphate ($IP_3$) and DAG, both secondary messengers. $IP_3$ is, in fact, a universal calcium-mobilizing second messenger, and, DAG is an activator of several protein kinase C isoforms, which are involved in the regulation of a variety of biological functions such as cell motility, sensory transduction, and fertilisation [95]. It was also found that a PLC isoenzyme specific for the hydrolis of PI glycolipids was involved in the mediation of insulin effect in epatocites and different tissues [96]. Additionally aberrant regulation of PLCs was implicated in different diseases including cancer, Alzheimer's disease, and neuropathic pain [97]. Since these enzymes are involved in many signal transduction pathways in higher eukaryotes a lot of attention has been addressed for characterizing their structure and understanding their functionality. Due to the structural similarity, prokaryotic isorforms have been taken as model proteins for understanding the functioning of higher eukaryotic enzymes [98]. The crystal structures from *B. cereus* and rat PLCs revealed the presence of a high degree of structure conservation in the catalytic domain, although structural differences where found in the substrate binding pocket that result in different substrate preferences between prokaryotic and eukaryotic enzymes [99]. From a functional point of view, however, bacterial PI-PLCs share only some features of the higher eukaryotes counterpart enzymes, thus limiting their application in the medical field [100, 101]. Beside being used as model proteins, phospholipases expressed in microorganisms find also numerous industrial applications such as degumming of vegetable oils, dairy, baking or egg yolk treatment [102]. A phospholipase C from *Bacillus* will soon be introduced to the market and be used in degumming of vegetable oil [103]. Among the different bacterial PLCs the isovariant from *B. cereus* has been extensively characterized [98, 101]. Additionally PI-PLC from *B. cereus* has been already expressed in different organisms such as *E. coli* and *P. pastoris* [103-105]. In particular recombinant isoenzymes expressed in *P. pastoris* displayed higher thermal stability then the wild type enzyme from *B. cereus.* Therefore establishment of a productive process guaranteeing high productivity titers in microbials would be highly desirable.

# 3.Goal of the Thesis and applied methodology

Over the last decades, the methylotrophic yeast *P. pastoris* has attracted scientific and industrial interest emerging as a successful expression system for production of industrial enzymes as well as of biopharmaceuticals. This yeast presents many advantages such as easiness of genetic manipulation, rapid growth on inexpensive media up to high cell densities, possibility of performing many of the post-translational modifications of higher eukaryotic cell (disulphide bonds formation, glycosylation, proteolytic processing and protein folding), possibility to secrete recombinantly expressed proteins into the growth medium and availability of the strong and methanol inducible AOX promoter (pAOX1) [106]. Additionally hyperglycosylation in *P. pastoris* is less pronounced and complex than in *S. cerevisiae* [107], and the methylotrophic yeast prefers a respiratory metabolism over a fermentative growth which allows it to reach very high cell densities [108]. However, recombinant protein expression in this host is still hampered by different drawbacks. In this Thesis, I focused on two organism-related issues namely 1) avoiding methanol requirement and 2) reducing the hyperglycosylation tendency of the yeast, and on one product-related issue namely 3) increasing product specific cofactor availability, in this specific case increase heme availability for HRP. Methanol requirement constitutes a significant drawback associated to *P. pastoris* implementation. Although methanol works at the same time as carbon and energy source and as inducer for expression of recombinant proteins [48] it is a particularly undesirable substrate for high cell density cultures above all at large scale [46] (2.3.1 Introduction). Hyperglycosylation represents a huge hindrance for the expression of glycosylated proteins, above all if intended for medical purposes. Yeast glycans are immunogenic and bare low pharmacokinetic properties in humans [109]. Additionally high mannose type glycans alters physicochemical properties of the native enzyme (solubility, stability) (2.3.2 Introduction) [110], also hampering downstream processing. Limitation of product specific cofactor or precursor availability represents a common cause of suboptimal production of active proteins. Many recombinantly expressed proteins, in fact, require cofactors for the production of active biocatalysts (2.3.3 Introduction).

Therefore the main goal of this Thesis consists in solving the above mentioned pitfalls, and thus rendering *P. pastoris* a competitive host for production of biopharmaceuticals. To achieve this goal the following methodology was applied based on the separated application of the three engineering approaches process, strain and product engineering (Fig.2).

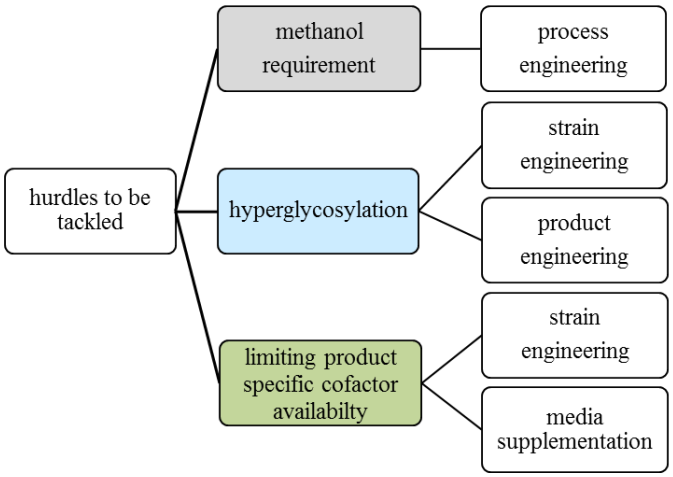


Fig2. Methanol requirement (grey), hyperglycosylation (blue) and limiting cofactor availability (green) as main hurdles to be tackled to render *P. pastoris* a more suitable expression host for the production of biopharmaceuticals. In white rectangles the different applied methods are shown.

With the aim of testing the effectiveness of the described methodology we studied the expression of the enzyme phospholipase C (PLC) under the control a glycerol de-repressed promoter and the expression of the glycosylated enzyme horseradish peroxidase (HRP).

In order to increase recombinant PLC space time yield (STY) a process engineering approach was applied for the design of a mixed-feed strategy for a glycerol de-repressed promoter which does not require methanol for production. We speculated that the design of a mixed feed strategy optimized for a glycerol de-repressed mutated AOX promoter could represent a successful process engineering approach to circumvent methanol utilization and ensuring final PLC productivity titers comparable the endogenous pAOX1 driven expression.

In order to reduce HRP hyperglycosylation two different engineering approaches were applied, namely product and strain (glyco)engineering, which resulted in two different papers. We speculated that application of these two approaches firstly separated and then in an integrated mode could lead to the production of a less hyperglycosylated HRP isovariant with a more uniform glycan content which also displays increased catalytic activity or stability.

Finally to increase heme availability as product specific cofactor for HRP we applied two different approaches, a strain (metabolic) engineering approach and cofactor/precursor media supplementation approach. We speculated that engineering of the heme pathway would allow increasing the production of active protein. Additionally we speculated that heme cofactor/precursors media supplementation could be a valid approach to increase production of active enzyme.

# 4. Summary and scientific contribution of each paper related to this Thesis

## Chapter I – Biopharmaceutical market

**"Microbials for the production of monoclonal antibodies and antibody fragments"**

mAbs and Fabs represent the most lucrative biopharmaceutical products today. Since mAbs are glycosylated mammalian cells are the most favourite expression host due to the possibility of resembling human-like glycosylation. However implementation of mammalian cells presents several drawbacks not last the requirement of cumbersome and time consuming processes. On the other hand less complex microbial species such as yeasts and bacteria are much easier to manipulate and to cultivate and they can be used for production of Fabs which are not glycosylated. Therefore in this review the most recent advances concerning the expression systems, strain engineering, and production processes are described for the three main microbial species *S. cerevisiae*, *P. pastoris* and *E.coli* implemented for the expression of mAbs and Fabs. The scientific contribution of this review consists in having provided an updated view of the ongoing studies with yeasts and *E. coli*, thus paving the way for a successful production of full length mAbs also in microbials. My contribution to this paper was related to the literature research concerning the state of the art about expression systems, strain engineering and production processes for the three main microbials namely *S. cerevisiae*, *P. pastoris* and *E. coli* used in mAbs and Fabs production. I also contributed in writing a first draft of this paper.

Spadiut, O., et al., *Microbials for the production of monoclonal antibodies and antibody fragments.* Trends in Biotechnology, 2014. **32**(1): p. 54-60.

## Chapter II – Avoiding methanol requirement

**"Development of a mixed feed strategy for a recombinant *Pichia pastoris* strain producing with a de-repression promoter"**

In order to circumvent methanol requirement, we designed a $q_s$ controlled mixed-feed fed-batch process to improve productivity titers of the PLC enzyme recombinantly expressed under the control of a glycerol de-repressed pAOX1 in *P. pastoris*. For the establishment of our mixed-feed strategy different dynamic experiments were performed. The scientific contribution of this paper consists in having proposed a process engineering approach, based on the design of a mixed feed, which allowed circumventing methanol requirement. Additionally we provided a methodological approach based on the implementation of dynamic experiments for the establishment of our mixed-feed process. My contribution to this paper consisted in planning, performing and evaluating the required fermentation experiments. Furthermore, I performed the required analytics and wrote the first draft of this paper.

Capone, S., et al., *Development of a mixed feed strategy for a recombinant Pichia pastoris strain producing with a de-repression promoter.*
Microbial Cell Factories, 2015. **14:101**.

## Chapter III – Reducing hyperglycosylation

Chapter III contains two different papers "Glyco-variant library of the versatile enzyme horseradish peroxidase" and "Combining protein and strain engineering for the production of glyco-engineered horseradish peroxidase C1A in *Pichia pastoris*". Due to the common goal which consisted in the expression of a HRP isoform with a reduced glycosylation profile these two studies have been grouped and discussed in the same chapter.

**"Glyco-variant library of the versatile enzyme horseradish peroxidase"**

In this paper application of site directed mutagenesis (glycoengineering approach) is described to mutate, and thus remove, the asparagine residue at each N-glycosylation site. After determination of the most suitable mutations we physiologically chacterized the respective *P. pastoris* strains in the bioreactor and biochemically characterized the purified HRP C1A glyco-variants. The scientific contribution of this paper consists in having demonstrated the validity of rational protein design as tool for reducing surface glycosylation. Additionally we proposed an integrated process strategy based on protein glycoengineering, physiological strain characterization, biochemical characterization of the purified isovariants, which allowed investigating the effect of single mutations in a more general way. My contribution to this paper consisted in planning and conducting the required experiments concerning the individual mutation of the eight N-glycosylation sites, the physiological characterization of *P. pastoris* strains expressing the different mutated isovariants, purification of the recombinantly expressed enzyme glycovariants and their biochemical characterization. Additionally I took care of writing a first draft of the paper.

Capone, S., et al., *Glyco-variant library of the versatile enzyme horseradish peroxidase.*
Glycobiology, 2014. 24(9): p. 852-863.

**"Combining protein and strain engineering for the production of glyco-engineered horseradish peroxidase C1A in *Pichia pastoris*"**

In this paper protein and strain engineering were integrated to obtain an active and stable HRP variant with reduced surface glycosylation. Four mutations ($^{4/8}$ HRP) were combined each being beneficial for either catalytic activity or thermal stability, and the glycoengineered enzyme variant as well as the unmutated wildtype enzyme were expressed in both a *P. pastoris* benchmark strain and a strain where the native α-1,6-mannosyltransferase was knocked out (Δoch1). The respective *P. pastoris* strains were physiologically characterized in the controlled environment of a bioreactor. After purification the recovered HRP glyco-variants were biochemically

characterized. The scientific contribution of this paper consists in having defined a methodology based on the combination of two different approaches namely product glycoengineering and strain engineering. After having tested the two methods in a separated way, we concomitantly applied both of them with the goal of reducing and rendering the glycosylation profile of our target enzyme more uniform. My contribution to this paper consisted in planning, conducting and evaluating the fermentation experiments for the physiological characterization of the benchmark and Δoch1 *P. pastoris* strains expressing the mutated $^{4/8}$ HRP variant. I also took care of the biochemical characterization of the mutated enzyme isoforms and of writing a first draft of the paper.

Capone, S., et al., *Combining Protein and Strain Engineering for the Production of Glyco-Engineered Horseradish Peroxidase C1A in Pichia pastoris*.
International Journal of Molecular Sciences, 2015. 16(10): p. 23127-42.

## Chapter IV – Increasing product specific cofactor availability

**"Optimizing cofactor availability for the production of recombinant heme peroxidase in *Pichia pastoris*"**

In this study two different approaches namely strain engineering and cofactor media supplementation were applied to increase heme availability for HRP. Co-overexpression studies allowed to identify two genes belonging to the heme biosynthetic showing potential beneficial effect for active HRP production. Additionally it was found that hemin media supplementantion produced up to 18-fold increase of HRP activity. In order to confirm these preliminary results derived from microscale cultivations, different fermentation experiments were conducted in the controlled bioreactor environment. Therefore the scientific contribution of this paper consists in having provided a methodological approach based on supplementation of the required cofactor into the growth media to maximize final yields of active target enzyme. My contribution to this paper consisted in planning and conducting the required fermentation experiments and activation studies. I also contributed in writing the first draft of the paper.

Krainer, F.W., et al., *Optimizing cofactor availability for the production of recombinant heme peroxidase in Pichia pastoris.* Microbial Cell Factories, 2015. 14: p. 4.

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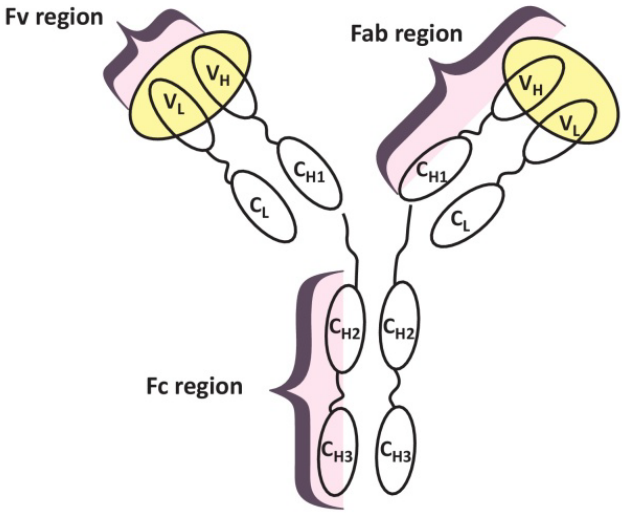
# 5. Research topics

## Chapter I – The biopharmaceutical market

**“Microbials for the production of monoclonal antibodies and antibody fragments”**

Spadiut, O., et al.Trends in Biotechnology, 2014. 32(1): p. 54-60.



Review

# Microbials for the production of monoclonal antibodies and antibody fragments

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**Monoclonal antibodies (mAbs) and antibody fragments represent the most important biopharmaceutical products today. Because full length antibodies are glycosylated, mammalian cells, which allow human-like N-glycosylation, are currently used for their production. However, mammalian cells have several drawbacks when it comes to bioprocessing and scale-up, resulting in long processing times and elevated costs. By contrast, antibody fragments, that are not glycosylated but still exhibit antigen binding properties, can be produced in microbial organisms, which are easy to manipulate and cultivate. In this review, we summarize recent advances in the expression systems, strain engineering, and production processes for the three main microbials used in antibody and antibody fragment production, namely *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Escherichia coli*.**

## Introduction

Over the past three decades, the biopharmaceutical market has become a significant component of the global pharmaceutical market accounting for around 40% of its sales. The use of organisms as biopharmaceutical production factories offers several advantages over chemical synthesis. Microorganisms can produce high molecular weight compounds such as proteins [1] and carry out highly enantio- and regio-selective reactions by their native enzymatic machinery – these reactions are hard to achieve by chemical synthesis. The use of microorganisms also enables repeated implementation of immobilized enzymes or cells resulting in the reduction of the overall production costs [2]. Finally, processes employing microorganisms do not generate organic and inorganic pollutants, such as mercury and toluene [3].

The biopharmaceutical market originated in the late 1970s with the establishment of recombinant DNA techniques. The industrial interest materialized almost immediately and in 1982 the US Food and Drug Administration (FDA) approved the commercialization of humulin, the human insulin analog, recombinantly produced in the bacterium *E. coli* [4]. For a while the FDA only allowed the transformation of bacteria and the expression of small, non-glycosylated proteins, like insulin, due to concern about introducing new toxicities such as contaminating bacterial substances, which raise immunogenic reactions in patients. However, with the development of selectable resistance markers, like antibiotic resistance markers, and the possibility of production in eukaryotic organisms, the FDA began showing increasing flexibility towards biotechnological innovation, leading to a continually increasing number of approved new biological entities (NBEs). In 2012, the biopharmaceutical market turnover was estimated at around 100–120 billion US dollars per year [5], with more than 200 biopharmaceutical proteins already on the market [6], and is expected to reach 170 billion US dollars in 2014. This exceptionally high market turnover is largely derived from the marketing of mAbs and antibody fragments that currently represent the fastest growing class of approved biopharmaceutical products. In fact, production of full length mAbs (Figure 1) is the most important biopharmaceutical venture to date, with several therapeutic products reaching blockbuster status (e.g., Avastin, Herceptin, Remicade, Rituxan, Humira, and Erbitux).

More recently, interest has grown in the production of antibody fragments that can be used not only in therapeutic applications but also in immunodetection, purification, and bioseparation applications [7]. Antibody fragments still exhibit antigen binding properties and can be produced in microbials, which are easy to manipulate and cultivate. In this review, we summarize recent advances in the expression system, strain engineering, and production process for the three main microbials for antibody fragment production, namely *S. cerevisiae*, *P. pastoris*, and *E. coli*, and highlight ongoing research that may allow full

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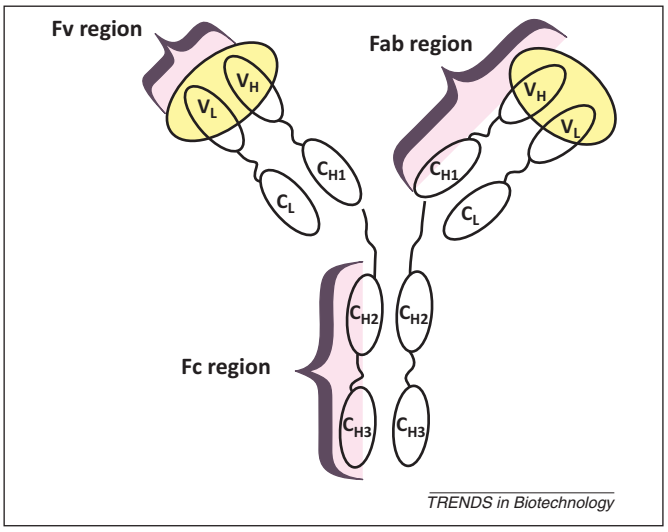


**Figure 1**. Schematic view of a full length antibody (the antigen binding sites are highlighted in yellow).

length antibody production in these organisms in the future.

## mAbs and antibody fragments: an overview

A full length mAb consists of the constant Fc (crystallizable fragment) domain and an antigen binding domain, comprising the Fv (variable fragment) and the Fab region (antibody binding fragment; Figure 1). Native full length mAbs are glycosylated during their synthesis. Although the glycosylated Fc domain does not directly interact with antigens, it stabilizes the antibody and is important for antibody-dependent, cell-mediated cytotoxicity. Moreover, glycosylation strongly impacts the clearance rate of the recombinant mAb from the body, and incompatible glycoforms can cause severe immunogenic effects in patients. Thus, much current work is focused on optimizing and controlling glycosylation events in mammalian cells [8], which at this time are the most often used cell type for the production of mAbs (Box 1).

Nevertheless, a full length antibody with a glycosylated Fc domain is not necessary for antigen recognition. In fact, both the Fv and the Fab region alone (Figure 1) exhibit antigen binding properties. Furthermore, antibody fragments show increased tissue penetration and a lower retention time in non-target tissues compared to mAbs [9]. Although the lack of the stabilizing Fc domain causes reduced stability [10], the absence of glycosylation on both the Fv and the Fab regions allows their production to be less complex and enables easier engineering and cultivation of microbial host organisms such as bacteria and yeasts.

## Microbial expression hosts for mAbs and antibody fragments

### *The yeast S. cerevisiae*

*S. cerevisiae* was the first yeast employed in the production of recombinant proteins, and several biopharmaceuticals produced in this yeast have since been successfully marketed [11]. There are several intrinsic characteristics, like the stability of the expression system and the ease of cultivation, as well as advances in host engineering, that make *S. cerevisiae* an attractive host for the production of mAbs and antibody fragments. In fact, the production of Llama heavy chain antibody fragments (Hvv) in *S. cerevisiae* already represents a well-established industrial process, ensuring production titers up to hundreds of mg/l [12].

*Expression system.* *S. cerevisiae* is easy to transform either chemically or by electroporation. There are three main types of shuttle vectors in use: (i) yeast episomal plasmids (Yep), which contain the 2 μ origin of replication, allowing gene expression without genomic integration at high copy numbers; (ii) yeast centromeric plasmids (Ycp), which contain an autonomously replicating sequence and replicate with single or very low gene copy number; and (iii) yeast integrative plasmids (Yip), which lack the yeast origin of replication and are integrated into the host genome [13]. Although genomic integration of the target gene leads to a reduced expression level, it is highly desirable in terms of process quality and stability [14]. To overcome the disadvantage of low expression, targeted integration of the heterologous gene at the highly transcribed ribosomal DNA locus was developed recently [15]. In addition, commonly used promoters derived from the native glycolytic pathway, such as the promoters for glyceraldehyde-3-phosphate dehydrogenase (GAP), alcohol dehydrogenase1 (ADH1), phosphoglycerate kinase (PGK), and phosphoglycerate kinase (PGK1), allow high transcription levels [16]. Finally, new cloning strategies introduced recently allow the concomitant expression of two or more genes located on specially designed self-replicating plasmids [17], which also addresses the issue of low expression levels of heterologous genes caused by genomic integration.

*Strain engineering.* Despite continuing advances in genetic manipulation, efficient production of mAbs and antibody fragments in *S. cerevisiae* can still be impaired by endoplasmic reticulum (ER) misfolding and inefficient trafficking. Although Hvv can be produced successfully in sufficient amounts [12], the expression of the significantly smaller single chain Fv (scFv) region (Figure 1) leads to intracellular accumulation of misfolded proteins in the ER or in vacuolar-like organelles. A possible explanation for this is the higher hydrophobicity of the variable light and heavy chains of scFv compared to Hvv [18]. However, additional overexpression of chaperones and foldases can correct protein folding and allow subsequent scFv secretion [19].

Several strategies have been developed to increase the overall secretory capacity and productivity of *S. cerevisiae*. These approaches include engineering intracellular protein trafficking by over-expression of soluble N-ethylmaleimide-sensitive factor (NFS) attachment protein receptor proteins (SNAREs) [20], reduction of proteolytic degradation by multiple protease gene deletions [21], and engineering of the heat shock response (HSR) pathway by overexpressing the heat shock transcription factor (Hsf) [22]. Although these engineered strains have not yet been used for the production of mAbs and antibody fragments, they demonstrate the ongoing, intensive strain engineering work that is being done with *S. cerevisiae*.

**Box 1. Production of mAbs in mammalian cells: advantages and drawbacks**

Mammalian cells are used most often for production of mAbs due to their ability to perform post-translational modifications (PTM), especially human-like N-glycosylation. Their use simplifies subsequent medical applications by eliminating the risk of an immunogenic response in patients due to incompatible N-glycans on the protein. Chinese Hamster Ovary (CHO) cell lines are used most frequently to generate full length mAbs with human-like Fc N-glycosylation and production titers of around 10 g/l [8]. However, the use of mammalian cells for heterologous protein expression holds several drawbacks such as low product yield and growth rate, risk of viral contamination, and requirement for serum. Despite the introduction of serum-free (SF) chemically defined media (CDM) encountering regulatory requirements [56], the addition of chemically undefined hydrolysates is still necessary to support cell growth. This, however, highly contradicts QbD guidelines demanding defined growth media [57]. Furthermore, the current standard production process is cumbersome and time-consuming. Cell transfection leads to high clone heterogeneity, necessitating repeated screening procedures at increasing drug concentrations for the isolation of a positive, highly productive clone [8]. Clone evaluation and culture condition optimization is then performed in shake flasks and lab-scale bioreactors before production processes can be set up. However, scale-up is also very challenging. The catabolism of the main carbon sources, glucose and glutamine, leads to formation of the inhibiting metabolites lactate and ammonium, respectively; hence batch and fed-batch operation modes, both representing closed cultivation systems, are only possible for a restricted timeframe. Because the metabolism of mammalian cells is highly sensitive and responsive to changing culture conditions, bioprocesses are hard to model – in fact only unstructured models are possible – and to control, which again contradicts QbD guidelines [57]. Consequently, chemostat cultivations, which describe open cultivation systems where substrate is constantly fed and cultivation broth is continuously removed, are generally employed to avoid metabolite inhibition. To avoid a critical wash out of mammalian cells, perfusion systems that provide cell retention by employing membranes are mainly used. However, operating a continuous culture with a perfusion system requires more devices and control systems than a batch or fed-batch system and also bears the elevated risk of contamination. Another drawback associated with scaling-up mammalian cell cultures is their sensitivity to shear stress, creating further challenges to efficient aeration in large vessels. Thus, although mammalian cells can produce mAbs with compatible PTMs, several drawbacks in bioprocessing are yet to be overcome.

*Production process.* Production of antibody fragments in *S. cerevisiae* is generally done in glucose-limited fed-batch cultivations [12]. Yeast shows a mixed oxidative/fermentative metabolism, which can result in the undesired production of toxic metabolites. Fermentative mode shift is triggered by oxygen depletion or by elevated carbon source concentration. Limiting glucose is therefore a valid strategy for preventing fermentation during cultivation processes with this yeast. Recently, a fully aerobically engineered strain, in which glucose uptake was reduced, was developed, allowing a full aerobic respiration even at elevated glucose concentrations [23].

As this discussion indicates, there are ongoing efforts to optimize the yeast *S. cerevisiae* for the production of mAbs and antibody fragments. Because antibody fragments are not glycosylated, they can be produced successfully in this yeast and are not affected by hypermannosylation, which characterizes *S. cerevisiae* [24]. Furthermore, current studies are investigating the possibility of humanizing the glycosylation machinery in *S. cerevisiae* [25], in an attempt to engineer this yeast for the production of full length mAbs.

### *The yeast P. pastoris*

As an alternative to *S. cerevisiae*, the methylotrophic yeast *P. pastoris*, which is closely related to *S. cerevisiae*, can be used for the production of mAbs and antibody fragments as it also holds a generally recognized as safe (GRAS) status [26].

*Expression system.* Similar to the process in *S. cerevisiae*, the target gene is integrated into the genome of *P. pastoris* to guarantee reproducibility and stability of the expression system. However, a major obstacle in *P. pastoris* is the substantial degree of non-homologous recombination. One solution to this challenge is the use of a recently developed *P. pastoris* strain with an inactivated non-homologous end joining pathway [27].

*P. pastoris* can use methanol as a sole carbon source, as it is a crucial part of its metabolism (e.g., [28]). However, instead of the traditional hard-to-control alcohol oxidase promoter system typically used for *P. pastoris*, alternative adjustable promoters are currently under investigation [29]. Furthermore, the generation of artificial and semi-artificial, tunable promoter variants are the subject of recent synthetic biology approaches [30].

*Strain engineering.* The genome sequences of the wild type strains NRRL Y-1603 (identical to DSMZ 70382 or CBS704) [7], NRRL Y-11430 (identical to ATCC 7673 or CBS7435), and GS115 are available online [31,32] and a genome-scale metabolic model of *P. pastoris* was published recently [33], allowing straight-forward strain engineering approaches. For example, co-overexpression of helper proteins, such as the protein disulfide isomerase or the transcription factor of the unfolded protein response Hac1 [34], as well as inactivation of endogenous proteases (e.g., [35]) enhances the production and secretion of recombinant proteins. Engineering the protein trafficking pathway represents another successful approach to improve secretion [36]. In addition, intensive glycoengineering work is ongoing to humanize the glycosylation events in *P. pastoris* and allow production of full length mAbs in this yeast (Box 2).

*Production process.* In contrast to *S. cerevisiae*, *P. pastoris* prefers respiratory over fermentative growth, allowing cultivations to very high cell densities, for example, 160 g/l cell dry weight [37], on inexpensive, defined media without the risk of accumulating ethanol. The very well-studied production processes in *P. pastoris* are most commonly performed as fed-batch processes. The possibility of performing mixed-feed fed-batch cultivations, where two substrates are concomitantly fed facilitating biomass growth due to higher biomass yields on the second substrate and leading to lower oxygen consumption and lower heat production, is a significant advantage of yeasts over mammalian cells and has already been applied successfully for the production of scFvs with *P. pastoris* [38]. In addition, a recent study presented a dynamic approach for determining strain-specific parameters in simple batch

**Box 2. Glycoengineering of *Pichia pastoris* allows mAb production**

*P. pastoris* can be used for the production of both antibody fragments and mAbs (e.g., [58]). For mAbs, the correct human-type glycosylation is not only essential for proper folding and biological activity, but also for targeting and stability in circulation. *P. pastoris* lacks the Golgi-resident α-1,3-mannosyltransferase, but harbors four additional β-mannosyltransferases instead [59,60]. The absence of terminal α-1,3-mannoses on *P. pastoris*-derived glycoproteins is of importance because this glycan structure causes high antigenicity in humans [61]. Thus, the humanization of the N-glycosylation pathway in *P. pastoris* has been an important goal. The Outer CHain elongation 1 gene (*OCH1*) coding for an α-1,6-mannosyltransferase was knocked out [62], and an α-1,2-mannosidase, β-N-acetylglucosaminyltransferase I (GnTI) and an UDP-GlcNAc transporter were introduced [63]. The *Kluyveromyces lactis* UDP-GlcNAc transporter, mouse α-1,2-mannosidase IA, *Drosophila melanogaster* mannosidase II, human GnTI, and rat GnTII were introduced into an *och1* knockout strain, resulting in the homogeneous formation of the complex human $GlcNAc_2Man_3GlcNAc_2$ glycan [64]. In other studies, *OCH1* was inactivated via a knock-in strategy [65], an ER-targeted HEDL (His-Asp-Glu-Leu; C-terminal tetrapeptide involved in the lumen sorting of soluble proteins)-tagged α-1,2-mannosidase from *Trichoderma reesei* was introduced, and a chimeric human GnTI was fused to the N-terminal part of *Saccharomyces cerevisiae* Kre2 for Golgi localization [66]. A further approach included the construction of a strain expressing mouse mannosidase IA, the *K. lactis* UDP-GlcNAc transporter, human GnTI, and rat GnTII, in which the *ALG3* gene, encoding an α-1,3-mannosyltransferase of the ER lumen, was knocked out [67], leading to the formation of $GlcNAc_2Man_3GlcNAc_2$. Additional coexpression of a fusion protein consisting of the *S. cerevisiae* Mnn2 Golgi localization domain and the activities of *Schizosaccharomyces pombe* UDP-Gal 4-epimerase and human β-1,4-galactosyl transferase allowed the production of $Ga_{l2}GlcNAc_2Man_3GlcNAc_2$ glycans. An alternative protocol allowed production of $Gal_2GlcNAc_2Man_3GlcNAc_2$ N-glycans using the GlycoSwitch vector technology [68], where specially designed vectors are used to replace genes of the native glycosylation pathway. Further humanization was achieved by additional biosynthesis of cytidine monophosphate-linked Sia, its transport and the transfer of Sia onto the N-glycans of nascent polypeptides, leading to complex human $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$ glycans [69]. Additional glycoengineering studies included the elimination of α-1,2-mannosidase-resistant high Man glycans [70] and overexpression of *Leishmania major* STT3D to increase N-glycan site occupancy [71]. These steps make it possible to use glycoengineered *P. pastoris* strains for the production of full length mAbs (e.g., [72]).

cultivations. This approach enables the design of efficient mixed-feed strategies for this yeast [39].

In conclusion, *P. pastoris* is a well-established host system for the production of antibody fragments. In fact, two recombinant therapeutic antibody fragments are already on the market: Nanobody ALX0061, which is a recombinant anti-IL6 receptor single domain antibody fragment used for rheumatoid arthritis treatment, and Nanobody® ALX00171, a recombinant anti-RSV single domain antibody fragment used for respiratory syncytial virus (RSV) infection treatment. Given recent and ongoing advances in glycoengineering, *P. pastoris* is of increasing interest for the production of glycosylated full length mAbs (Box 2).

### *The bacterium E. coli*

Due to rapid growth on inexpensive substrates, the ability to reach high cell densities, well-understood genetics, and easy genetic manipulation, prokaryotic expression systems are widely used for the production of recombinant proteins. The gram-negative bacterium *E. coli* was the first microbial organism employed for the production of recombinant biopharmaceuticals and still accounts for nearly 40% of all the marketed biopharmaceutical compounds produced today. After the approval of humulin in 1982, several different therapeutic proteins, such as antibody fragments [e.g., the antitumor necrosis factor (TNF)-α Fab], have been successfully produced in this prokaryotic organism [11].

*Expression system.* Recombinant protein expression in bacterial hosts is generally driven by self-replicating multicopy plasmids carrying a strong promoter, like the bacteriophage T7, the *E. coli* lactose operon *(lac)* or the synthetic tryptophan operon (*trp*) promoter, and a ribosome binding site allowing high gene dosages [40]. Although greater volumetric productivity can be reached by implementing self-replicating multicopy plasmids, these cause a severe metabolic burden for *E. coli*, including cell growth inhibition and cell death. Thus, new plasmid-free expression systems, based on site-directed chromosomal integration of the heterologous DNA, have been developed [41]. In order to eliminate the metabolic burden associated with the selection marker, a novel marker-free plasmid selection system using a genomically modified *E. coli* strain was also engineered [42].

*Strain engineering.* Although cytoplasmic production in *E. coli* allows high intracellular product yields, it is often associated with inclusion body formation (e.g., [43]). This *E. coli* characteristic phenomenon arises from unbalanced expression of folding helper elements and the fact that disulfide bridges cannot be formed correctly in the reductive environment of the cytoplasm. This problem can be overcome by the co-expression of chaperones [44] or by the transport of the target protein to the periplasmic space by fusion to a leader peptide at the N terminus [45]. Secretion into the periplasm has already been successfully performed for antibody fragments [46]. However, if efficient refolding is possible, recombinant protein production in inclusion bodies also describes a valuable production strategy, as already described for Fc-fusion proteins [47].

*Production process.* Due to the intrinsic high growth rate of *E. coli*, high cell density cultures are currently used for the production of antibody fragments [48]. Production processes with *E. coli* are commonly conducted in stirred tank reactors (STR) as limited glucose fed-batch processes because glucose excess induces overflow metabolism and causes the production of the inhibiting metabolite acetate. As an alternative to a carbon source-limited feeding strategy, different metabolic engineering approaches have been designed to prevent or at least reduce acetate formation. These approaches include manipulating the native acetate formation pathway [49] and engineering the endogenous glucose uptake system [50]. Another recent advance is to improve the bioprocess via the identification and characterization of key strain-specific physiological parameters instead of excessive strain engineering. The knowledge of the strain characteristic parameters specific substrate uptake rate ($q_s$) and maximum specific substrate uptake rate ($q_{s\ max}$), for example, allows the design of tailored

**Table 1. Recent advances in the production of full length mAbs and antibody fragments with different host organisms**

| Production milestone | Recent advances | | | | | |
|---|---|---|---|---|---|---|
| | Mammalian cells | Refs | Yeasts | Refs | *Escherichia coli* | Refs |
| Stable and efficient expression system | Site-specific homologous recombination<br>Vector engineering and marker attenuation<br>Expression of anti-apoptotic genes | [73]<br>[74,75]<br>[76] | Targeted gene integration<br>Concomitant expression of several genes<br>Co-expression of chaperones<br>Reduction of proteolysis<br>Over-expression of Hsf | [15]<br>[17]<br>[34]<br>[21,35]<br>[22] | Plasmid-free expression system<br>Marker-free selection system<br>Co-expression of chaperones | [41]<br>[42]<br>[44] |
| Clone selection | Robotics and fluorescence-activated cell sorting | [75] | Targeted gene integration<br>Optimization of codons, gene copy number, and promoters | [15]<br>[77] | Not an issue | |
| Disulfide bridges | Intrinsic feature of the ER | | Intrinsic feature of the ER | | Transport to the periplasm | [46] |
| Product secretion | Intrinsic feature | | Over-expression of SNAREs<br>Mutation studies on MFα1<br>System biological analysis | [20]<br>[78]<br>[77] | Transport to the periplasm | [46] |
| Chemically defined medium (CFD) | Serum-free CFD | [56] | Already applied | | Already applied | |
| Efficient bioprocess | Concentrated fed-batch strategy | [79] | Fully aerobic strain<br>Dynamic processes | [23]<br>[80] | Manipulating the native acetate formation pathway<br>Engineering the glucose uptake system | [49]<br>[50] |

bioprocesses avoiding overflow metabolism. Soft-sensor tools, which are virtual sensors processing different signals measured online that give real-time information on a non-measurable process parameter, are powerful tools for that purpose [51]. Besides, strain-specific physiological parameters can easily be determined by applying dynamic changes of process parameters during cultivation [52]. The availability of detailed physiological data enables design that follows the Quality by Design (QbD) guidelines [53].

In summary, antibody fragments, which are not glycosylated, can be produced in *E. coli* and the required tools are already in place (e.g., [11]). Remarkably, successful production of full length mAbs in *E. coli* was achieved recently, although the mAbs were not glycosylated [47]. The identification of the N-glycosylation pathway in *Campylobacter jejuni* and the possibility of introducing it into *E. coli* [54] may pave the way for the production of the glycosylated Fc domain [55] and the successful expression of full length mAbs in *E. coli*. For this reason, pharmaceutical companies are now investing effort and capital in re-introducing *E. coli* to their production facilities.

## Concluding remarks and future perspectives

Full length mAbs as well as antibody fragments represent the most important and valuable class of biopharmaceuticals today. Due to the requirement for surface glycosylation, mAbs are still predominantly produced in mammalian cells, which possess several drawbacks relating to bioprocessing and scale-up. By contrast, antibody fragments, which are not glycosylated but retain antigen binding properties, can also be produced in microbial organisms. Recent advances in the production of full length mAbs and antibody fragments with both mammalian cells and microbials are summarized in Table 1.

As shown in Table 1, current efforts are directed towards optimizing the production of mAbs and antibody fragments in microbial organisms, because they outpace mammalian cells in several aspects, such as the ease of genetic manipulation, greater productivity, and high cell density cultivation processes on inexpensive and defined substrates. Although mAbs are still most frequently produced in mammalian cells, ongoing glycoengineering studies with yeasts (Box 2) and *E. coli* [54,55] are paving the way for the successful production of glycosylated full length mAbs in microbial host organisms.

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## Chapter II – Avoiding methanol requirement

**"Development of a mixed feed strategy for a recombinant *Pichia pastoris* strain producing with a de-repression promoter"**

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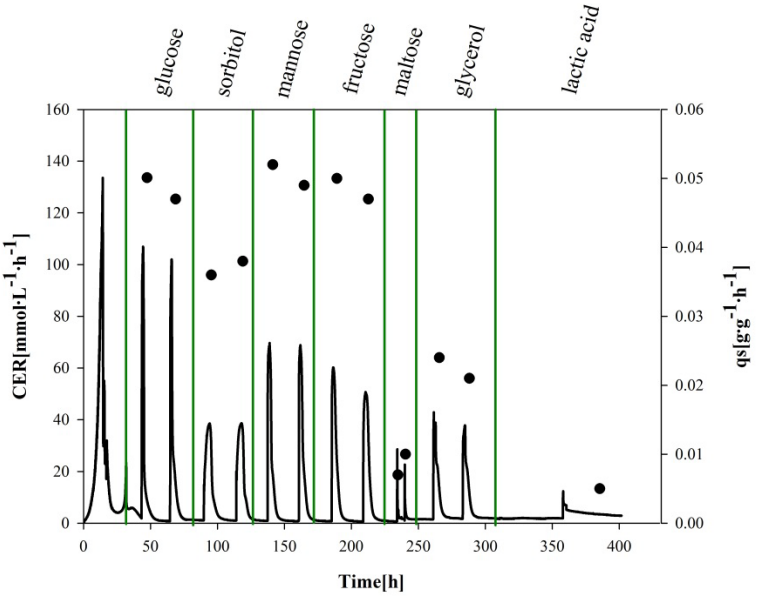

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MICROBIAL CELL FACTORIES

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# Development of a mixed feed strategy for a recombinant *Pichia pastoris* strain producing with a de-repression promoter

Simona Capone, Jernej Horvat, Christoph Herwig and Oliver Spadiut*

## Abstract

**Background:** Recombinant protein production in the yeast *Pichia pastoris* is usually based on the alcohol oxidase promoters pAOX1 and pAOX2, which are regulated by methanol and strongly repressed by other C-sources, like glycerol and glucose. However, the use of methanol brings several disadvantages, which is why current trends in bioprocess development with *P. pastoris* are focussing on minimizing the required amount of methanol or even avoid its employment. In this respect novel promoter systems which do not rely on methanol have been investigated and promoter variants were designed to fine-tune gene expression. Amongst these novel promoter systems, mutated AOX promoters, which are regulated by available carbon source concentration (so-called de-repressed promoters), are currently raising attention. However, the main disadvantage of such a production system is that expression and growth usually cannot happen concomitantly resulting in low space–time-yields.

**Results:** Here we show the development of a mixed-feed strategy for an industrial recombinant *P. pastoris* de-repression strain aiming at increased productivity and maximum space–time-yield. By doing dynamic experiments we determined a ratio between the specific substrate uptake rates of glycerol and sorbitol allowing a more than 2-fold increased productivity compared to the conventional single substrate de-repression strategy.

**Conclusion:** Based on our results we recommend adjusting $q_{s\ glycerol} = 0.04\ g\ g^{-1}\ h^{-1}$ and $q_{s\ sorbitol} = 0.055\ g\ g^{-1}\ h^{-1}$ to obtain highest productivity with a *P. pastoris* de-repression strain. Our methodological approach of designing mixed-feed strategies based on physiological strain characterization using dynamic experiments proved to be beneficial.

**Keywords:** *Pichia pastoris*, Mutated AOX1 promoter, De-repression, Phospholipase C, Glycerol, Sorbitol, Mixed feed, Productivity

## Background

The methylotrophic yeast *Pichia pastoris* is widely used for recombinant protein production in industrial biotechnology. Recombinant protein production in this yeast is usually based on the transcriptional activity of the alcohol oxidase promoters pAOX1 and pAOX2, which are regulated by methanol and strongly repressed by other C-sources, like glycerol and glucose [1]. However, the use of methanol brings several disadvantages as methanol metabolism causes heat production and increased need of oxygen, on-line methanol monitoring and especially feedback control is difficult and methanol accumulation leads to the production of toxic compounds [2]. Consequently, current trends in bioprocess development with *P. pastoris* are focussing on minimizing the required amount of methanol.

Bioprocesses employing *P. pastoris* can on the one hand be improved by developing mixed feed strategies, where a primary C-source (e.g. glycerol) is used for biomass growth in non-repressing concentrations, whereas methanol is used for induction [2–4]. In this respect, positive effects of mixed-feed strategies on productivity have been

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demonstrated for both *P. pastoris* Mut$^S$ and Mut$^+$ strains [5]. To analyze the benefit of a mixed-feed strategy on productivity in more detail, recent studies are aiming at a better understanding of its impact on cell physiology by performing metabolomics and metabolic flux analysis [2, 6]. In a recent study, we were able to show considerable improvement by determining the specific substrate uptake rates ($q_s$) and employing dynamic experiments to examine physiological conditions allowing high productivity in a methanol-glycerol mixed feed environment [7]. However, even in these mixed feed systems methanol is still required for induction making it less attractive for industrial large-scale production processes.

Besides bioprocess engineering approaches, also strain engineering can be applied to minimize or even avoid the use of methanol as inducer. Novel promoter systems which do not rely on methanol have been investigated and promoter variants were designed to fine-tune gene expression [8–11]. Amongst these novel promoter systems, mutated AOX promoters, which are regulated by carbon source depletion (so-called de-repressed promoters), are currently raising attention [8, 12]. These promoter systems are especially interesting for bioreactor cultivations, where substrate concentrations can be tightly controlled at levels allowing full promoter de-repression. However, the main disadvantage of such a production system is that expression and growth usually cannot happen concomitantly. Thus, a typical bioprocess with these systems usually comprises three steps: (1) batch, (2) repressed fed-batch at a high growth rate, and (3) de-repressed fed-batch at limited carbon source concentration for production. Thus, space–time-yields (STY) with these systems are typically rather low.

In the present study we physiologically characterized an industrial *P. pastoris* strain harbouring the recombinant product phospholipase C (PLC) from *Bacillus cereus* under the control of a mutated AOX1 promoter, which gets de-repressed at limiting concentrations of glycerol. Due to high structural and catalytic similarity of PLC from *B. cereus* with mammalian PLCs, it is currently used as a good model system and is thus a highly important subject in medical research [13]. Since this enzyme is hard to produce, the current price for an enzyme preparation from *B. cereus* lies at 320 Euros for 5 Units, which corresponds to only 5 μg of protein (P5542-5UN; Sigma Aldrich).

Based on physiological parameters, we cultivated the recombinant *P. pastoris* strain under optimized conditions following the typical feeding regime for such de-repressed strains comprising of the three phases (1) batch, (2) repressed fed-batch at high $q_{s\ glycerol}$ and (3) de-repressed fed-batch at low $q_{s\ glycerol}$. However, our main goal was the development of a mixed feed strategy for this strain aiming at increased productivity and a higher STY. Thus, we also propose a novel methodology to develop a mixed-feed strategy for industrial recombinant *P. pastoris* de-repression strains using dynamic experiments.

## Methods

### Microorganism

A *P. pastoris* CBS7435 Mut$^S$ strain carrying the recombinant gene for phospholipase C from *Bacillus cereus* (PLC; EC 3.1.4.3) under the control of a mutated AOX1 promoter, conferring high expression upon de-repressing conditions (*i.e.* at limiting glycerol concentrations), was kindly provided by VTU Technology GmbH (Grambach, Austria). This strain will be referred to as "recombinant *P. pastoris* de-repression strain" in this article.

### Culture media

Precultures were done in yeast nitrogen base medium (YNB; 0.1 M potassium phosphate buffer pH 6.0, 3.4 g $L^{-1}$ YNB w/o amino acids and ammonia sulfate, 10 g $L^{-1}$ $(NH_4)_2SO_4$, 400 mg $L^{-1}$ biotin, 20 g $L^{-1}$ glucose). Zeocine was added to a concentration of 100 μg $L^{-1}$.

Batch and fed-batch cultivations were performed in 2-fold concentrated basal salt medium (BSM; 21.6 mL $L^{-1}$ 85% phosphoric acid, 0.36 g $L^{-1}$ $CaSO_4{\cdot}2H_2O$, 27.24 g $L^{-1}$ $K_2SO_4$, 4.48 g $L^{-1}$ $MgSO_4{\cdot}7H_2O$, 8.26 g $L^{-1}$ KOH, 0.3 mL $L^{-1}$ Antifoam Struktol J650, 4.35 mL $L^{-1}$ PTM1, $NH_4OH$ as N-source). Trace element solution (PTM1) was made of 6.0 g $L^{-1}$ $CuSO_4{\cdot}5H_2O$, 0.08 g $L^{-1}$ NaI, 3.0 g $L^{-1}$ $MnSO_4{\cdot}H_2O$, 0.2 g $L^{-1}$ $Na_2MoO_4{\cdot}2H_2O$, 0.02 g $L^{-1}$ $H_3BO_3$, 0.5 g $L^{-1}$ $CoCl_2$, 20.0 g $L^{-1}$ $ZnCl_2$, 65.0 g $L^{-1}$ $FeSO_4{\cdot}7H_2O$, 0.2 g $L^{-1}$ biotin, 5 mL $L^{-1}$ $H_2SO_4$. The concentration of the base $NH_4OH$ was determined by titration with 0.25 M potassium hydrogen phthalate.

### Bioreactor cultivations

#### *Preculture*

Frozen stocks (−80°C) were cultivated in 100 mL YNB-Zeocine in 1,000 mL shake flasks at 30°C and 230 rpm for 24 h. Then, the preculture was transferred aseptically to the culture vessel. The inoculum volume was 10% (v/v) of the final starting volume.

#### *Batch and fed-batch cultivations*

Batch and fed-batch cultivations were carried out in a 5 L working volume glass bioreactor (Infors, Switzerland). Dissolved oxygen ($dO_2$) was measured with a sterilizable fluorescence dissolved oxygen electrode (Visiferm DO425, Hamilton, Germany). The pH was measured with a sterilizable electrode (Easyferm™, Hamilton, Switzerland) and maintained constant at pH 5.0 with a PID controller using $NH_4OH$ (2–3 M). Base consumption was determined gravimetrically. Cultivation temperature was

set to 30°C and agitation was fixed to 1,000 rpm. The culture was aerated with 2.0 vvm dried air to keep dissolved oxygen level above 30%. In case of $dO_2$ limitation pure oxygen was added. Off-gas was measured by an infrared cell for $CO_2$ and a zirconium dioxide sensor for $O_2$ concentration (DasGip, Germany). Temperature, pH, $dO_2$, agitation as well as $CO_2$ and $O_2$ in the off-gas were measured online and logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland).

Before fed-batch experiments, a single dynamic batch cultivation with substrate pulses was performed, as we described previously [14–16], to determine the 2nd C-source for the development of a mixed feed strategy. After the complete consumption of glucose at a concentration of 40 g $L^{-1}$ in the batch, the C-sources glucose, sorbitol, mannose, fructose, maltose, glycerol and lactic acid were sequentially pulsed twice to the culture each in a final concentration of 45 mM. For each pulse, at least two samples were taken for offline sample analysis to calculate specific rates and yields.

Fed-batch experiments were conducted as follows: after a batch on a C-source at a final concentration of 40 g $L^{-1}$, either a dynamic or an exponential fed-batch was performed, where the feeding rate based on $q_s$ was constantly adjusted according to the total amount of biomass in the bioreactor and controlled by the PIMS. Real-time measurement of total biomass was done by a soft-sensor tool as we described previously [17]. Off-line measurements of biomass were done every 2 h to correct for potential soft sensor errors. Dynamic feeding was controlled by a built-in online calculator [18] according to Eq. 1:

$$F = \frac{q_s \text{ theoretical } \times X \times \rho \text{ feed } \times \text{reactor weight}}{S \times \rho \text{broth}} \quad (1)$$

$q_s$ theoretical = $q_s$ set point (g $g^{-1}$ $h^{-1}$); X = biomass estimated by soft sensor (g $L^{-1}$); ρ feed = density of feed (g $L^{-1}$); reactor weight (g); S = feed concentration (g $L^{-1}$); ρ broth = density of culture broth (g $L^{-1}$).

The different fed-batch experiments, the respective feeding rates as well as the respective goals of each experiment are summarized in Table 1.

## Offline sample analysis

### *Analysis of growth and expression parameters*

Dry cell weight (DCW) was determined by centrifugation of 5 mL culture broth (5,000 rpm, 4°C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11,156), washing the pellet with 5 mL deionized water and subsequent drying at 105°C to a constant weight in an oven. Optical density at 600 nm ($OD_{600}$) was measured in a photometer (U-1100 Hitachi, Japan). A linear correlation between DCW and $OD_{600}$ was experimentally determined (Eq. 2).

$$\text{DCW}\left(\text{g L}^{-1}\right) = (0.506 \times \text{OD}_{600} + 0.0006) \times \text{dilution factor} \quad (2)$$

**Table 1 Overview of dynamic fed-batch experiments**

| Experiment | Substrate | Feeding strategy based on $q_s$ (g $g^{-1}$ $h^{-1}$) | Goals |
|---|---|---|---|
| FB1 | Glycerol | Batch on glycerol–step-wise decrease of $q_{s\ glycerol}$: 0.338–0.063–0.054–0.029–0.014–0.005 g $g^{-1}$ $h^{-1}$ | Determination of strain physiological parameters<br>Characterization of recombinant expression profile |
| FB2 | Glycerol | Batch on glycerol–repression phase on glycerol ($q_s$ = 0.29 g $g^{-1}$ $h^{-1}$)–de-repression phase on glycerol ($q_s$ = 0.035 g $g^{-1}$ $h^{-1}$) | Mimic optimized industrial process comprising of three phases |
| FB3 | Sorbitol | Batch on sorbitol–step-wise increase of $q_{s\ sorbitol}$: 0.033–0.060–0.127–0.176–0.197 g $g^{-1}$ $h^{-1}$ | Determination of strain physiological parameters<br>Characterization of recombinant expression profile |
| | | Glycerol pulse at a final concentration of 10 g $L^{-1}$ at highest $q_{s\ sorbitol}$ | $q_s$ in presence of both substrates |
| FB4 | Mixed feed | Batch on glycerol–repression phase on glycerol ($q_s$ = 0.33 g $g^{-1}$ $h^{-1}$)–de-repression phase on glycerol ($q_s$ = 0.054 g $g^{-1}$ $h^{-1}$)–mixed-feed: glycerol ($q_s$ = 0.054 g $g^{-1}$ $h^{-1}$) and sorbitol ($q_s$ = 0.070 g $g^{-1}$ $h^{-1}$)–mixed-feed: glycerol ($q_s$ = 0.026 g $g^{-1}$ $h^{-1}$) and sorbitol ($q_s$ = 0.015 g $g^{-1}$ $h^{-1}$) | Analyze physiology and productivity in mixed feed environment |
| FB5 | Mixed feed | batch on glycerol– repression phase on glycerol ($q_s$ = 0.33 $g^{-1}$ $h^{-1}$)–mixed-feed: glycerol ($q_s$ = 0.040 $g^{-1}$ $h^{-1}$) and sorbitol ($q_s$ = 0.055 g $g^{-1}$ $h^{-1}$) | Verify increased STY in mixed feed environment |
| FB6 | Mixed feed | batch on glycerol–repression phase on glycerol ($q_s$ = 0.23 g $g^{-1}$ $h^{-1}$)–mixed-feed: glycerol ($q_s$ = 0.026 g $g^{-1}$ $h^{-1}$) and sorbitol ($q_s$ = 0.027 g $g^{-1}$ $h^{-1}$)–mixed-feed: glycerol ($q_s$ = 0.026 g $g^{-1}$ $h^{-1}$) and sorbitol ($q_s$ = 0.061 g $g^{-1}$ $h^{-1}$) | Verify increased STY in mixed feed environment<br>Identification of $q_{s\ glycerol}/q_{s\ sorbitol}$ ratio allowing highest productivity and STY |

Since the regression coefficient $R^2$ was 0.996, Eq. 2 was used for regular $q_s$ adjustments of the feed based on $OD_{600}$ measurements.

#### *Substrates and metabolites*

Concentrations of carbon sources and metabolites were determined in cell free cultivation broth using HPLC (Agilent Technologies, USA), equipped with a Supelcoguard column, a Supelcogel C-610 H ion exchange column (Sigma-Aldrich, USA) and a refractive index detector (Agilent Technologies, USA). The mobile phase was 0.1% $H_3PO_4$ with a constant flow rate of 0.5 mL $min^{-1}$ and the system was run isocratically at 30°C. All measurements were executed in duplicates.

#### *PLC activity assay*

Recombinant PLC activity in cell-free cultivation broth was determined with a colorimetric method based on the hydrolysis of *p*-nitrophenylphosphorylcholine (*p*-NPPC) [19]. Quantification was done with a photometer (U-1100 Hitachi, Japan). The standard curve was prepared with commercial PLC from *B. cereus* (Sigma-Aldrich, P6621-250UN) dissolved in HEPES buffer (50 mM, pH 7.0) and *p*-NPPC (Melford, 21064-69-7) substrate solution (100 mM in 250 mM HEPES buffer, 0.1 mM $ZnCl_2$, pH 7.0, 30% sorbitol). 540 µL of cell-free cultivation broth were mixed with 60 µL of *p*-NPPC substrate solution. The mixture was incubated at 37°C for 60 min [20] and enzymatic activity was followed at 410 nm [21]. Total extracellular protein content was determined by the Bradford Reagent (Sigma-Aldrich, B6919) at 595 nm.

## Results and discussion

### Dynamic batch cultivation with substrate pulses

To identify a second C-source for developing the mixed-feed strategy for the recombinant *P. pastoris* de-repression strain, we performed a dynamic batch cultivation with substrate pulses. After a batch on glucose, the C-sources glucose, sorbitol, mannose, fructose, maltose, glycerol and lactic acid were sequentially pulsed twice to the culture each in a final concentration of 45 mM. In Figure 1 the carbon dioxide evolution rate (CER), depicting metabolic activity, and the calculated specific substrate uptake rates ($q_s$) are shown.

Based on offline analysis, specific rates and yields were calculated. We also analyzed productivity, formation of metabolites as well as economic aspects. As shown in Table 2, all substrates were taken up; however, maltose and lactic acid were only metabolized at low rates. Metabolism of glucose and mannose led to the formation of ethanol, which could be problematic in mixed feed experiments. We analyzed the volumetric productivity ($r_p$) for each substrate pulse and found promoter de-repression in each phase. However, fructose and lactic

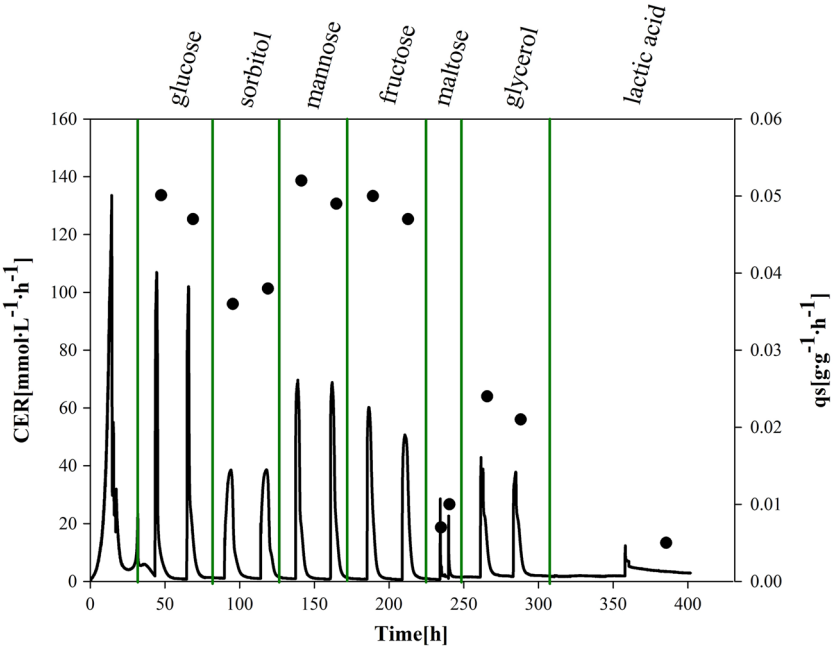


**Figure 1** Dynamic batch cultivation with substrate pulses. Glucose, sorbitol, mannose, fructose, maltose, glycerol and lactic acid were sequentially pulsed after a batch on glucose. The CER signal (*continuous black line*) was used to follow metabolic activity. The specific substrate uptake rate ($q_s$, *black dot*) was determined for the different substrates.

**Table 2 Summary of strain specific parameters, metabolites and STY during dynamic batch experiment with substrate pulses as well as economic aspects**

| C-source | $q_s$ (g $g^{-1}$ $h^{-1}$) | $Y_{CO_2/S}$ (mol $Cmol^{-1}$) | $Y_{X/S}$ (Cmol $Cmol^{-1}$) | C-balance | Metabolites | $r_p$ (U $L^{-1}$ $h^{-1}$) per pulse | Price (€ $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| Glucose (batch) | 0.059 | 0.53 | 0.498 | 1.03 | Acetate (3.0 g $L^{-1}$) Ethanol (0.6 g $L^{-1}$) | 6.2 | |
| Glucose | 0.048 | 0.93 | 0.004 | 0.94 | Ethanol (0.3 g $L^{-1}$) | 4.2 | 40 |
| Sorbitol | 0.037 | 0.94 | 0.002 | 0.94 | nd | 4.8 | 20 |
| Mannose | 0.051 | 0.93 | 0.008 | 0.94 | ethanol (1.0 g $L^{-1}$) | 4.7 | 2,000 |
| Fructose | 0.048 | 0.90 | 0.036 | 0.94 | nd | 2.2 | 62 |
| Maltose | 0.006 | 0.93 | 0.014 | 0.94 | nd | 5.4 | 252 |
| Glycerol | 0.023 | 1.04 | 0.011 | 1.05 | nd | 4.3 | 26 |
| Lactic acid | 0.005 | 1.04 | 0.000 | 1.04 | nd | 0.5 | 24 |

$q_s$ specific substrate uptake rate, $Y_{CO_2/S}$ *and* $Y_{X/S}$ yields of $CO_2$ and biomass, *C-balance* sum of $Y_{CO_2/S}$and $Y_{X/S}$ which should ideally give 1.0, $r_p$ volumetric productivity per pulse and *nd* none determined.

acid gave lower $r_p$ compared to the other substrates. Closing C-balances confirmed data validity (Table 2). Taken together, based on physiology, productivity and economic aspects we chose sorbitol as 2nd C-source for the development of a mixed feed strategy.

## Fed-batch cultivations

### *Dynamic fed-batch on glycerol as sole carbon-source (FB1)*

In order to physiologically characterize the recombinant *P. pastoris* de-repression strain and identify the glycerol concentration allowing for full promoter de-repression, we performed FB1 where we stepwise adapted the feeding rate to correspond to lower $q_{s\ glycerol}$ (Table 1; Additional file 1: Figure S1).

As expected, decreasing $q_{s\cdot glycerol}$ resulted in a decreased specific growth rate (μ) and a decreased $Y_{X/S}$ (Table 3). In particular, by adjusting the feeding rate to correspond to a $q_{s\ glycerol}$ between 0.029 g $g^{-1}$ $h^{-1}$ and 0.054 g $g^{-1}$ $h^{-1}$ we could nicely determine the maintenance level of this yeast strain (Additional file 2: Figure S2). At $q_{s\ glycerol}$ lower than 0.05 g $g^{-1}$ $h^{-1}$, the cells use the C-source for maintenance metabolism and not for growth, which is shown in increasing $Y_{CO_2/S}$ values. Only at $q_{s\ glycerol}$ higher than 0.05 g $g^{-1}$ $h^{-1}$, the cells can efficiently produce biomass and product.

When we analyzed $r_p$ and $q_p$ during the single $q_{s\ glycerol}$ steps, we determined apparent full promoter de-repression at a $q_{s\ glycerol} = 0.054$ g $g^{-1}$ $h^{-1}$ (Table 3). Interestingly, by further lowering $q_{s\ glycerol}$, also productivity decreased. Apparently, maximum productivity for the recombinant *P. pastoris* de-repression strain is directly linked to an optimum $q_{s\ glycerol}$. As soon as the cells come close to their maintenance level, $r_p$ and $q_p$ decrease dramatically, leaving only a narrow operating window for the design of an efficient de-repression fed-batch (Additional file 3: Figure S3, Additional file 4: Figure S4).

### *Production fed-batch on glycerol as sole carbon-source (FB2)*

Based on data from FB1, we performed FB2 to mimic an optimized industrial process comprising of the three phases (1) batch on glycerol, (2) repressed fed-batch at high $q_{s\ glycerol}$ and (3) de-repressed fed-batch at low $q_{s\ glycerol}$ (Table 1). After the batch, we cultivated the cells at a repressing $q_{s\ glycerol} = 0.29$ g $g^{-1}$ $h^{-1}$ to a biomass concentration of 60 g $L^{-1}$. Then we adjusted the feeding rate to correspond to a de-repressing $q_{s\ glycerol} = 0.035$ g $g^{-1}$ $h^{-1}$ and continued the cultivation for another 24 h. The final biomass concentration was 65 g $L^{-1}$. In Table 4 the physiological strain characteristic parameters are summarized. In

**Table 3 Strain characteristic parameters during the dynamic fed-batch on glycerol (FB1)**

| $q_{s\ glycerol}$ (g $g^{-1}$ $h^{-1}$) | μ ($h^{-1}$) | $Y_{CO_2/S}$ (mol $Cmol^{-1}$) | $Y_{X/S}$ (Cmol $Cmol^{-1}$) | C-balance | $r_p$ (U $L^{-1}$ $h^{-1}$) | $q_p$ (U $g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|
| 0.338 | 0.199 | 0.29 | 0.70 | 0.99 | 2.45 | 0.03 |
| 0.063 | 0.026 | 0.52 | 0.47 | 0.99 | 5.38 | 0.06 |
| 0.054 | 0.024 | 0.43 | 0.53 | 0.96 | 54.8 | 0.57 |
| 0.029 | 0.008 | 0.61 | 0.34 | 0.95 | 13.8 | 0.14 |
| 0.014 | 0.000 | 0.91 | 0.03 | 0.94 | 13.5 | 0.13 |
| 0.005 | 0.000 | 1.01 | 0.01 | 1.02 | 6.08 | 0.06 |

**Table 4 Strain characteristic parameters during the production fed-batch on glycerol (FB2)**

| $q_{s\ glycerol}$ (g $g^{-1}$ $h^{-1}$) | μ ($h^{-1}$) | $Y_{CO_2/S}$ (mol $Cmol^{-1}$) | $Y_{X/S}$ (Cmol $Cmol^{-1}$) | C-balance | $r_p$ (U $L^{-1}$ $h^{-1}$) | $q_p$ (U $g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|
| 0.29 | 0.104 | 0.40 | 0.58 | 0.98 | 0 | 0 |
| 0.035 | 0.012 | 0.73 | 0.30 | 1.03 | 23.6 | 0.36 |

the de-repressed phase we determined $r_p = 23.6$ U $L^{-1}$ $h^{-1}$ and $q_p = 0.36$ U $g^{-1}$ $h^{-1}$.

#### *Dynamic fed-batch on sorbitol as sole carbon-source (FB3)*

To characterize the recombinant *P. pastoris* de-repression strain on sorbitol, we performed a batch on sorbitol which was followed by a fed-batch where we stepwise increased $q_{s\ sorbitol}$ (Table 1; Additional file 5: Figure S5).

We observed an extremely long lag phase during the batch at a concentration of 40 g $L^{-1}$ sorbitol (Additional file 5: Figure S5). We speculate that an osmotic shock caused by the high sorbitol concentration could have caused this long lag phase [22, 23]. However, when we analyzed the different $q_{s\ sorbitol}$ steps, we observed a nice correlation between $q_{s\ sorbitol}$, μ and $Y_{X/S}$ (Table 5) and thus were again able to determine the maintenance level of the strain between $q_{s\ sorbitol} = 0.060$ and 0.127 g $g^{-1}$ $h^{-1}$ (Additional file 6: Figure S6).

When we raised $q_{s\ sorbitol}$ >0.197 g $g^{-1}$ $h^{-1}$, we observed sorbitol accumulation. Interestingly, productivity increased concomitantly with $q_{s\ sorbitol}$ and the substrate did not repress the promoter at any concentration (Table 5; Additional file 7: Figure S7, Additional file 8: Figure S8). At the end of cultivation we determined a biomass concentration of around 40 g $L^{-1}$.

To analyze if sorbitol and glycerol can be taken up concomitantly and identify the respective $q_s$ values, we pulsed glycerol at a final concentration of 10 g $L^{-1}$ to the culture while sorbitol was fed at a constant $q_{s\ sorbitol} = 0.197$ g $g^{-1}$ $h^{-1}$. The cells immediately reacted to glycerol, as indicated by a sudden increase in the CER (Figure 2).

Sampling before and after the glycerol pulse and offline analysis allowed the determination of physiological parameters. As shown in Table 6, glycerol was specifically taken up at a rather high rate of 0.193 g $g^{-1}$ $h^{-1}$, whereas $q_{s\ sorbitol}$ decreased from 0.197 to 0.071 g $g^{-1}$ $h^{-1}$. Although the recombinant *P. pastoris* de-repression strain preferred glycerol as substrate, both C-sources were taken up concomitantly. This was a crucial observation, as the concomitant uptake was an essential requirement for the development of a mixed feed strategy. As expected, we did not determine an increase of the total amount of PLC in the cultivation broth after pulsing glycerol in a repressing concentration. However, we even determined a lower total amount of active PLC in the cultivation broth after the pulse, indicating PLC to be a very unstable product, which constantly degraded and/or lost activity in the bioreactor.

#### *Dynamic mixed feed fed-batch (FB4)*

In order to verify $q_s$ for both glycerol and sorbitol, we performed a dynamic mixed feed fed-batch. After a fed-batch phase on glycerol at a repressing concentration of $q_{s\ glycerol} = 0.33$ g $g^{-1}$ $h^{-1}$ to a biomass concentration of around 40 g $L^{-1}$, we de-repressed the promoter at a $q_{s\ glycerol} = 0.054$ g $g^{-1}$ $h^{-1}$ for 24 h to get a biomass concentration of around 60 g $L^{-1}$. Then we added the 2nd C-source sorbitol and performed two different mixed feed phases: first we concomitantly fed glycerol at $q_{s\ glycerol} = 0.054$ g $g^{-1}$ $h^{-1}$ and sorbitol at $q_{s\ sorbitol} = 0.070$ g $g^{-1}$ $h^{-1}$ for 8 h giving a biomass concentration of 78 g $L^{-1}$, before we adapted the feeding rates to correspond to a $q_{s\ glycerol} = 0.026$ g $g^{-1}$ $h^{-1}$ and $q_{s\ sorbitol} = 0.015$ g $g^{-1}$ $h^{-1}$ for another 52 h resulting in a biomass concentration of around 66 g $L^{-1}$. The results of this dynamic experiment are summarized in Table 7.

As shown in Table 7, the concomitant presence of sorbitol boosted μ more than 4-fold from 0.018 to 0.082 $h^{-1}$. We could also follow the positive effect of sorbitol on cell growth by shifts in both yields. When we reduced $q_s$ for both substrates in the later phase of the mixed feed

**Table 5 Strain characteristic parameters during the dynamic fed-batch on sorbitol (FB3)**

| $q_{s\ sorbitol}$ (g $g^{-1}$ $h^{-1}$) | μ ($h^{-1}$) | $Y_{CO_2/S}$ (mol $Cmol^{-1}$) | $Y_{X/S}$ (Cmol $Cmol^{-1}$) | C-balance | $r_p$ (U $L^{-1}$ $h^{-1}$) | $q_p$ (U $g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|
| 0.033 | 0.005 | 0.76 | 0.16 | 0.92 | 0.63 | 0.028 |
| 0.060 | 0.018 | 0.57 | 0.36 | 0.93 | 1.37 | 0.032 |
| 0.127 | 0.059 | 0.47 | 0.55 | 1.02 | 4.74 | 0.051 |
| 0.176 | 0.084 | 0.45 | 0.56 | 1.01 | 10.4 | 0.170 |
| 0.197 | 0.088 | 0.48 | 0.53 | 1.01 | 33.0 | 0.951 |

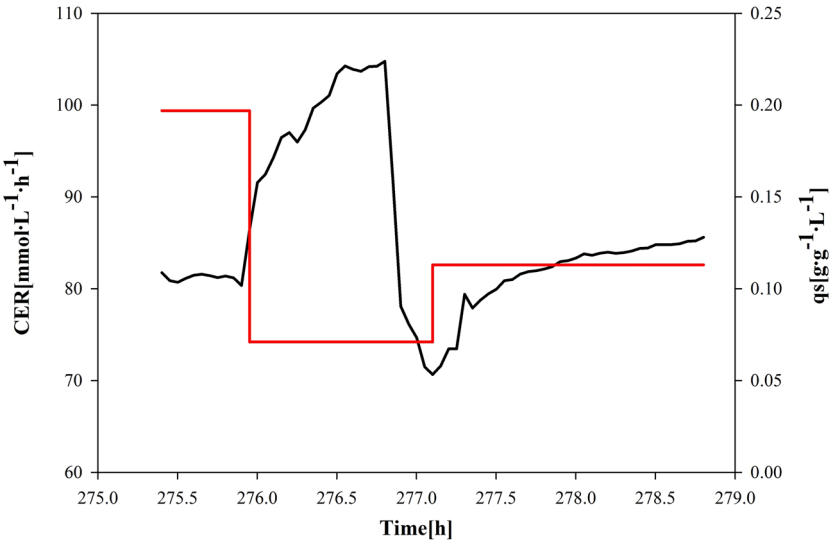


**Figure 2** Glycerol pulse during sorbitol fed-batch. Carbon dioxide evolution rate (CER, *continuous black line*) and specific sorbitol uptake rate ($q_{s\ sorbitol}$, *continuous red line*). After glycerol pulse a sudden increase in the CER signal and a concomitant decrease in $q_{s\ sorbitol}$ were observed.

**Table 6 Strain characteristic parameters during a glycerol pulse in sorbitol fed-batch FB3 (glycerol was pulsed at a final concentration of 10 g $L^{-1}$ to the culture while sorbitol was fed at a constant $q_{s\ sorbitol} = 0.197$ g $g^{-1}$ $h^{-1}$)**

| $q_{s\ glycerol}$ (g $g^{-1}$ $h^{-1}$) | $q_{s\ sorbitol}$ (g $g^{-1}$ $h^{-1}$) | μ ($h^{-1}$) | $Y_{CO_2/S}$ (mol Cmol$^{-1}$) | $Y_{X/S}$ (Cmol Cmol$^{-1}$) | C-balance | $r_p$ (U $L^{-1}$ $h^{-1}$) | $q_p$ (U $g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|---|
| 0.193 | 0.071 | 0.186 | 0.26 | 0.71 | 0.97 | 0* | 0* |

* We even measured less active PLC after the glycerol pulse than before, indicating product instability.

**Table 7 Strain characteristic parameters during dynamic mixed feed fed-batch FB4**

| $q_{s\ glycerol}$ (g $g^{-1}$ $h^{-1}$) | $q_{s\ sorbitol}$ (g $g^{-1}$ $h^{-1}$) | μ ($h^{-1}$) | $Y_{CO_2/S}$ (mol Cmol$^{-1}$) | $Y_{X/S}$ (Cmol Cmol$^{-1}$) | C-balance | $r_p$ (U $L^{-1}$ $h^{-1}$) | $q_p$ (U $g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|---|
| 0.33 | – | 0.174 | 0.38 | 0.63 | 1.01 | 0 | 0 |
| 0.054 | – | 0.018 | 0.63 | 0.42 | 1.05 | 5.24 | 0.080 |
| 0.054 | 0.070 | 0.082 | 0.44 | 0.57 | 1.01 | 13.25 | 0.143 |
| 0.026 | 0.015 | 0.011 | 0.80 | 0.20 | 1.00 | 5.57 | 0.099 |

fed-batch, also μ dramatically decreased. In terms of productivity, we obtained a 2-fold increase in the first mixed feed phase compared to the glycerol de-repression phase. When we decreased both $q_s$ values, also productivities decreased. Interestingly, for the de-repression phase at $q_{s\ glycerol} = 0.054$ g $g^{-1}$ $h^{-1}$ we only obtained $r_p = 5.24$ U $L^{-1}$ $h^{-1}$ and $q_p = 0.08$ U $g^{-1}$ $h^{-1}$ which was 4-fold lower compared to the results obtained in FB2 at $q_{s\ glycerol} = 0.035$ g $g^{-1}$ $h^{-1}$. Based on our observations in FB1, we actually expected an even higher productivity at $q_{s\ glycerol} = 0.054$ g $g^{-1}$ $h^{-1}$. We currently have no explanation for this mismatch, however we speculate that a different batch of p-NPPC substrate might have caused this aberration. Thus, for the following experiments we always used the same batch of substrate. However, for FB4 a direct comparison between the different phases was still possible and legitimate, showing a beneficial effect of a mixed feed environment on productivity compared to a single substrate de-repression strategy.

### *Production mixed feed fed-batch (FB5)*

In order to verify the higher productivity observed for a mixed feed environment compared to a de-repression strategy on glycerol as sole C-source, we performed FB5. First we performed a de-repressed fed-batch phase at a $q_{s\ glycerol} = 0.33$ g $g^{-1}$ $h^{-1}$ to a biomass concentration of

**Table 8 Strain characteristic parameters during production mixed feed fed-batch FB5**

| $q_{s\ glycerol}$ (g $g^{-1}$ $h^{-1}$) | $q_{s\ sorbitol}$ (g $g^{-1}$ $h^{-1}$) | μ ($h^{-1}$) | $Y_{CO_2/S}$ (mol $Cmol^{-1}$) | $Y_{X/S}$ (Cmol $Cmol^{-1}$) | C-balance | $r_p$ (U $L^{-1}$ $h^{-1}$) | $q_p$ (U $g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|---|
| 0.33 | – | 0.189 | 0.32 | 0.69 | 1.01 | 0 | 0 |
| 0.040 | 0.055 | 0.062 | 0.41 | 0.63 | 1.04 | 64.7 | 0.86 |

65 g $L^{-1}$, before we started the mixed feed where we kept $q_s$ values for both substrates constant (Table 8).

Compared to the production fed-batch on glycerol as sole carbon-source (FB2), where we determined $\mu = 0.012\ h^{-1}$, $r_p = 23.6$ U $L^{-1}$ $h^{-1}$ and $q_p = 0.36$ U $g^{-1}$ $h^{-1}$ at a $q_{s\ glycerol} = 0.035$ g $g^{-1}$ $h^{-1}$ (Table 3), we increased μ 5-fold to $\mu = 0.062\ h^{-1}$, $r_p$ 2.8-fold to $r_p = 65.7$ U $L^{-1}$ $h^{-1}$ and $q_p$ 2.4-fold to $q_p = 0.86$ U $g^{-1}$ $h^{-1}$ at a comparable $q_{s\ glycerol}$ in a mixed feed environment with concomitant uptake of sorbitol. This clearly shows the beneficial effect of the mixed feed system on productivity and STY compared to the single substrate strategy.

### *Production mixed feed fed-batch (FB6)*

To determine a potential optimal ratio between $q_{s\ glycerol}$ and $q_{s\ sorbitol}$, we performed FB6, where, after a repression phase on glycerol to a biomass concentration of 60 g $L^{-1}$, we tested 2 different mixed feed environments (Table 9). The productivity values determined in the first mixed feed phase were comparable to the values determined in the previous fed-batch experiments. Interestingly, by keeping $q_{s\ glycerol}$ constant and increasing $q_{s\ sorbitol}$ around 2-fold we increased μ, but significantly decreased the productivity (Table 9). This was rather surprising, since we expected to further increase productivity by increasing $q_{s\ sorbitol}$ and thus μ. However, the results suggest, that the boost in productivity does not result from a higher μ, but rather from a certain ratio of $q_{s\ glycerol}$ to $q_{s\ sorbitol}$. Thus, we plotted the ratio $\frac{q_{s\ glycerol}}{q_{s\ sorbitol}}$ of FB3, FB5 and FB6 against productivity values (Figures 3, 4). Due to the different substrate batches for activity measurements and thus possibly non-comparable productivity values, we did not consider FB4 for this mechanistic

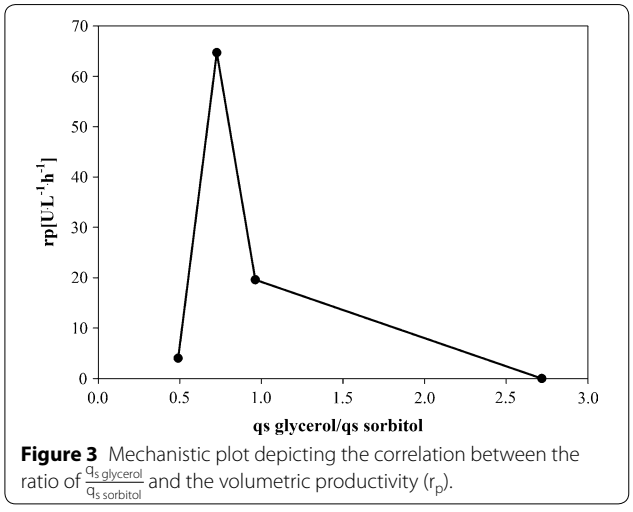


**Figure 3** Mechanistic plot depicting the correlation between the ratio of $\frac{q_{s\ glycerol}}{q_{s\ sorbitol}}$ and the volumetric productivity ($r_p$).

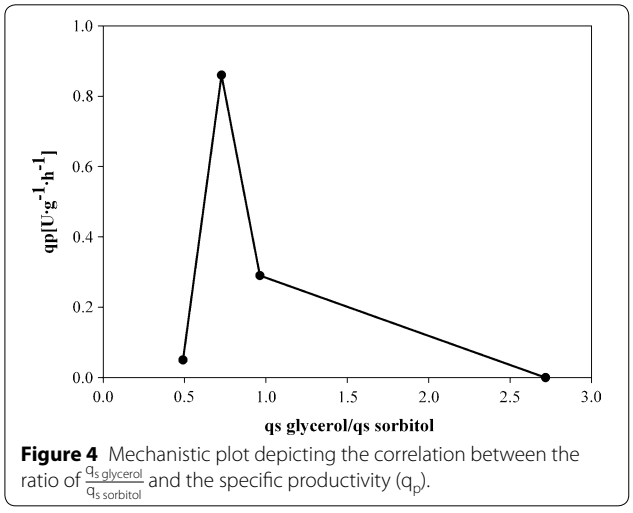


**Figure 4** Mechanistic plot depicting the correlation between the ratio of $\frac{q_{s\ glycerol}}{q_{s\ sorbitol}}$ and the specific productivity ($q_p$).

**Table 9 Strain characteristic parameters during production mixed feed fed-batch FB6**

| $q_{s\ glycerol}$ (g $g^{-1}$ $h^{-1}$) | $q_{s\ sorbitol}$ (g $g^{-1}$ $h^{-1}$) | μ ($h^{-1}$) | $Y_{CO_2/S}$ (mol $Cmol^{-1}$) | $Y_{X/S}$ (Cmol $Cmol^{-1}$) | C-balance | $r_p$ (U $L^{-1}$ $h^{-1}$) | $q_p$ (U $g^{-1}$ $h^{-1}$) |
|---|---|---|---|---|---|---|---|
| 0.23 | – | 0.127 | 0.35 | 0.67 | 1.02 | 0 | 0 |
| 0.026 | 0.027 | 0.022 | 0.52 | 0.50 | 1.02 | 19.6 | 0.29 |
| 0.026 | 0.061 | 0.053 | 0.30 | 0.72 | 1.02 | 4.03 | 0.05 |

plot. As shown in Figures 3 and 4, a ratio $\frac{q_{s\ glycerol}}{q_{s\ sorbitol}}$ of around 0.7 is most beneficial for productivity.

## Conclusions

In this study we physiologically characterized a recombinant *P. pastoris* strain, where the gene coding for the recombinant product phospholipase C (PLC) was under the control of a mutated AOX1 promoter, which gets de-repressed at limiting concentrations of glycerol. Based on physiological data we developed a mixed feed strategy for this novel de-repression strain and compared productivity data to the commonly used single substrate strategy. Our findings can be summarized as:

- A dynamic batch with substrate pulses revealed sorbitol as second C-source for the development of a mixed feed strategy.
- The specific substrate uptake rate for glycerol allowing full promoter de-repression was determined with $q_{s\ glycerol} = 0.054\ g\ g^{-1}\ h^{-1}$. However, maximum productivity could only be achieved in a rather small operating window of $q_{s\ glycerol}$, which underlines the importance of precise and robust process control.
- Sorbitol did not repress the mutated AOX1 de-repression promoter. The maximum specific substrate uptake rate of this strain for sorbitol was determined with $q_{s\ sorbitol} = 0.197\ g\ g^{-1}\ h^{-1}$, where also highest productivity was reached.
- An easy-to-perform pulse experiment showed that the yeast strain was able to take up both glycerol and sorbitol concomitantly and revealed maximum $q_s$ values for both substrates.
- The highest productivity was reached at a ratio of $q_{s\ glycerol}$ to $q_{s\ sorbitol}$ of 0.7. In this study, we were able to boost the productivity more than 2-fold in the mixed feed environment compared to the commonly used single substrate strategy, where we fed glycerol in de-repressing conditions.

Summarizing, we have employed a methodological approach based on dynamic experiments to establish a mixed-feed strategy for a recombinant *P. pastoris* de-repression strain comprising three phases (1) batch, (2) repressed fed-batch at high $q_{s\ glycerol}$ and (3) de-repressed mixed feed fed-batch at a ratio $q_{s\ glycerol}$ to $q_{s\ sorbitol}$ of 0.7. Based on our results we recommend adjusting $q_{s\ glycerol} = 0.04\ g\ g^{-1}\ h^{-1}$ and $q_{s\ sorbitol} = 0.055\ g\ g^{-1}\ h^{-1}$ to obtain highest productivity. Our methodological approach of designing mixed-feed strategies based on physiological strain characterization using dynamic experiments proved to be beneficial.

## Additional files

**Additional file 1: Figure S1.** Dynamic fed-batch on glycerol as sole carbon source (FB1). The carbon dioxide evolution rate signal (CER, continuous black line) was used to follow metabolic activity. The specific glycerol uptake rate ($q_{s\ glycerol}$) is depicted as continuous red line.

**Additional file 2: Figure S2.** Carbon dioxide yield ($Y_{CO_2/S}$, black dots) and biomass yield ($Y_{X/S}$, white squares) at different specific glycerol uptake rates ($q_{s\ glycerol}$).

**Additional file 3: Figure S3.** Volumetric productivity ($r_p$) at different specific glycerol uptake rates ($q_{s\ glycerol}$).

**Additional file 4: Figure S4.** Specific productivity ($q_p$) at different specific glycerol uptake rates ($q_{s\ glycerol}$).

**Additional file 5: Figure S5.** Dynamic fed-batch on sorbitol as sole carbon source (FB3). The carbon dioxide evolution rate signal (CER, continuous black line) was used to follow metabolic activity. The specific sorbitol uptake rate ($q_{s\ sorbitol}$) is depicted as continuous red line.

**Additional file 6: Figure S6.** Carbon dioxide yield ($Y_{CO_2/S}$, black dots) and biomass yield ($Y_{X/S}$, white squares) at different specific sorbitol uptake rates ($q_{s\ sorbitol}$).

**Additional file 7: Figure S7.** Volumetric productivity ($r_p$) at different specific sorbitol uptake rates ($q_{s\ sorbitol}$).

**Additional file 8: Figure S8.** Specific productivity ($q_p$) at different specific sorbitol uptake rates ($q_{s\ sorbitol}$).

#### Authors' contributions

OS panned the study, SC and JH conducted experiments. SC, JH and OS analyzed the data and wrote the manuscript. CH and OS supervised research and conceived of the study. All authors read and approved the final manuscript.

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#### Compliance with ethical guidelines

#### Competing interest

The authors declare that they have no competing interests.

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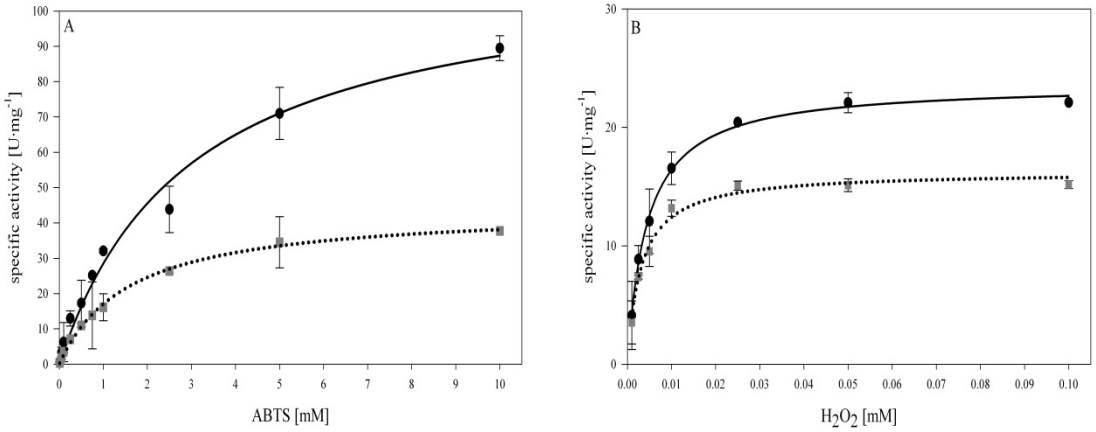
## Chapter III – Reducing hyperglycosylation

**"Glyco-variant library of the versatile enzyme horseradish peroxidase"**

Capone, S., et al., Glycobiology, 2014. 24(9): p. 852-863.

**"Combining protein and strain engineering for the production of glyco-engineered horseradish peroxidase C1A in *Pichia pastoris*"**

Capone, S., et al., International Journal of Molecular Sciences, 2015. 16(10): p. 23127-42.



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# Glyco-variant library of the versatile enzyme horseradish peroxidase

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**When the glycosylated plant enzyme horseradish peroxidase (HRP) is conjugated to specific antibodies, it presents a powerful tool for medical applications. The isolation and purification of this enzyme from plant is difficult and only gives low yields. However, HRP recombinantly produced in the yeast *Pichia pastoris* experiences hyperglycosylation, which impedes the use of this enzyme in medicine. Enzymatic and chemical deglycosylation are cost intensive and cumbersome and hitherto existing *P. pastoris* strain engineering approaches with the goal to avoid hyperglycosylation only resulted in physiologically impaired yeast strains not useful for protein production processes. Thus, the last resort to obtain less glycosylated recombinant HRP from *P. pastoris* is to engineer the enzyme itself. In the present study, we mutated all the eight N-glycosylation sites of HRP C1A. After determination of the most suitable mutation at each N-glycosylation site, we physiologically characterized the respective *P. pastoris* strains in the bioreactor and purified the produced HRP C1A glyco-variants. The biochemical characterization of the enzyme variants revealed great differences in catalytic activity and stability and allowed the combination of the most promising mutations to potentially give an unglycosylated, active HRP C1A variant useful for medical applications. Interestingly, site-directed mutagenesis proved to be a valuable strategy not only to reduce the overall glycan content of the recombinant enzyme but also to improve catalytic activity and stability. In the present study, we performed an integrated bioprocess covering strain generation, bioreactor cultivations, downstream processing and product characterization and present the biochemical data of the HRP glyco-library.**

*Keywords:* bioprocess technology / glyco-engineering / glycosylation / horseradish peroxidase / *Pichia pastoris*

## Introduction

The heme-containing plant enzyme horseradish peroxidase (HRP; EC 1.11.1.7) is a Class III peroxidase catalyzing the oxidation of various substrates (e.g., amines, aromatic phenols, indoles, phenolic acids and sulfonates) using hydrogen peroxide ($H_2O_2$) as oxidant. Horseradish peroxidase exists in at least 19 different isoenzyme forms in the horseradish root (*Armoracia rusticana*), of which isoenzyme C1A is the most abundant and thus the most studied one (e.g., Dunford 1999; Veitch and Smith 2001; Veitch 2004; Carlsson et al. 2005; Krainer et al. 2013; Spadiut and Herwig 2013). It is a 34 kDa monomeric oxidoreductase containing a heme-group as well as two $Ca^{2+}$-ions as prosthetic groups. The crystal structure of HRP C1A led to the identification of nine N-glycosylation sites of the Asn-X-Ser/Thr type, where X can be any amino acid but proline, of which eight are occupied when the enzyme is expressed in plant (Smith et al. 1990), which is why the molecular mass of HRP C1A increases from 34 to ~44 kDa (Veitch 2004; Spadiut and Herwig 2013). Due to glycosylation and the presence of both, the heme-group and disulfide bridges, the recombinant production and subsequent preparative purification of HRP has proven to be very difficult (Smith et al. 1990; Gajhede et al. 1997; Lavery et al. 2010; Spadiut et al. 2012), which is why HRP is still mainly isolated from plant (Lavery et al. 2010). However, HRP preparations from plant describe a mixture of isoenzymes, which seasonally varies in composition and concentration, and yields are extremely low (Jermyn 1952; Jermyn and Thomas 1954; Shannon et al. 1966). Since HRP is a versatile enzyme used in numerous, quite diverse industrial and medical applications, such as waste water treatment, fine chemical synthesis, immunoassays, biosensors and coupled enzyme assays (e.g., Krieg and Halbhuber 2003), the controllable recombinant production and subsequent efficient purification of single HRP isoenzymes is highly desired. Thus, we have not only investigated and improved the recombinant production of the isoenzyme HRP C1A with *Pichia pastoris* in the past few years (Dietzsch et al. 2011a,b; Krainer et al. 2012; Zalai et al. 2012; Spadiut et al. 2013), but also developed an efficient downstream process for the hypermannosylated enzyme recombinantly derived from this yeast (Spadiut et al. 2012; Krainer, Pletzenauer, et al. 2013). However, for medical applications where HRP is conjugated to antibodies, like antibody-directed enzyme-prodrug therapy (Folkes and Wardman 2001; Wardman 2002) and medical

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diagnostics (Romero et al. 1999; Huang 2001; Palmgren et al. 2011; Dotsikas and Loukas 2012), the degree of glycosylation of HRP is of utmost importance, since not only the stability of the enzyme but also the conjugation with antibodies is expected to change with varying glycosylation. Besides, the untrimmed yeast-derived high-mannose containing glycosylation can trigger immune responses in humans (personal communication with Dr. Lisa Folkes; Gray Institute for Radiation Oncology and Biology, Department of Oncology, University of Oxford).

Obviously, the availability of an enzyme without or at least with reduced surface glycosylation would solve abovementioned problems. However, the biological role and importance of glycans for plant peroxidases is still not completely understood and is topic of numerous studies in glycobiology. So far, some studies report stabilizing effects of the glycans (Narhi et al. 1991; Wang et al. 1996), whereas other studies do not show such effects (Ehlers et al. 1992; Powell and Pain 1992). In 1990, Smith et al. (1990) were able to produce active and correctly folded HRP without any glycans in *Escherichia coli*. In a following study, Tams and Welinder analyzed the importance of the glycosylation for HRP in more detail (Tams and Welinder 1998). They showed that the removal of most of the glycans, except the *N*-acetylglucosamine residues, by a mild chemical deglycosylation with trifluoromethanesulfonic acid resulted in a fully active, but less stable enzyme (Tams and Welinder 1995, 1998). Both studies showed that glycans on the surface of HRP affect the physicochemical properties of the enzyme but are not required for catalytic activity. However, chemical deglycosylation only left 60% of HRP active and also describes a quite cumbersome procedure (Tams and Welinder 1995, 1998), which is why it is not a useful method to obtain unglycosylated HRP. Although recombinant proteins from *P. pastoris* can also be deglycosylated enzymatically (Sainz-Pastor et al. 2006; Kang et al. 2012), also this option has to be reconsidered, since (1) enzymatic deglycosylation is only quantitative when the target protein is denatured and (2) the additional endoglycosidases have to be removed again to obtain pure product. Another way to control and reduce the complexity of native yeast-like glycosylation on glycoproteins secreted from *P. pastoris* is through glyco-engineering (Choi et al. 2003; Hamilton et al. 2003; Vervecken et al. 2004; Hamilton and Gerngross 2007). A key event in such engineering is the knockout of the OCH1 gene, which initiates outer-chain elongation, leading to hypermannosylation. However, in a previous study, where we knocked out this gene, we observed that HRP with reduced glycan complexity possessed hampered downstream processing and that the glyco-engineered *P. pastoris* strains generated were physiologically impaired, impeding efficient production processes (Krainer et al. 2013).

Consequently, the last resort to efficiently produce HRP with a reduced amount of surface glycosylation is to glyco-engineer the enzyme itself. In a recent study, two selected Asn residues of HRP were mutated to Asp to analyze effects on the stability of the enzyme and to produce more properly folded HRP in *E. coli* (Asad, Khajeh, et al. 2011). Asad, Khajeh, et al. (2011) showed that introducing the mutations Asn13Asp and Asn268Asp did not just affect the production of HRP in *E. coli*, but also increased the catalytic constants as well as the thermal stability. These results did not only underline the possibility of obtaining active and correctly folded HRP with reduced glycosylation but also showed that mutating the glycosylation sites may even have beneficial effects on catalytic activity and stability.

In the present study, we generated a glyco-variant library of HRP C1A exchanging all the eight Asn serving as glycosylation sites by the structurally similar amino acids Asp, Gln or Ser. We did not only investigate the effects of the single mutations on enzyme activity and stability but also on protein purification following an integrated bioprocess technology approach. After determining the most suitable mutation at the single N-glycosylation sites, we physiologically characterized the respective *P. pastoris* strains in the controlled environment of a bioreactor. A two-step purification procedure, where both chromatography steps were performed in the flow-through mode, enabled us to recover purified HRP glyco-variants for subsequent biochemical characterization. Based thereon, we combined the most suitable mutations to potentially obtain an unglycosylated, active HRP variant suitable for medical applications. Summarizing, we conducted an integrated bioprocess study and present the bioprocess technological and biochemical results for the HRP C1A glyco-variant library.

## Results and discussion

### *Screening procedure*

Every transformation into *P. pastoris* CBS7435 Mut$^S$ yielded several dozens of transformants. We randomly picked five transformants per mutation and screened them for cell growth and production of active HRP in shake flasks. With only few exceptions, all the picked transformants produced active HRP; however, on average only three of five showed comparable growth and productivity. PCR analysis confirmed the presence of the target gene in the genome of *P. pastoris*. Although we did not analyze the exact number of gene integration events by real-time polymerase chain reaction, we assumed the integration of a comparable amount of gene copies into the yeast genome due to the fact that we always transformed the same amount of linearized vector DNA into the *P. pastoris* cells (i.e., 2 μg DNA). We had observed such a correlation in a previous study (Krainer et al. 2012). Although we did not check for the exact integration site of the target gene in the host genome, we ascribe the observed differences in protein production during the screening procedure to a fair amount of non-homologous recombination of the transformed HRP C1A gene into the genome of *P. pastoris* (Naatsaari et al. 2012), most likely at different loci in the chromosome, which consequently influences the accessibility of the transcription machinery to the transformed gene (Krainer et al. 2012). In Table I, we compared the growth of the best transformant per mutation as well as the specific activity and thermal stability of the produced HRP C1A glyco-variants. Although diagnostic applications are normally not performed at 60°C, we determined the stability of HRP at this temperature since we observed nice differences for the glyco-variants at this temperature and the assay could be easily performed in the laboratory. Furthermore, we used the thermal stability as a measure for kinetic stability, as also discussed elsewhere (Polizzi et al. 2007; Spadiut et al. 2009). We always included a *P. pastoris* strain expressing the unmutated wild-type (wt) HRP C1A in the screening experiments as control. Although we obtained slightly different results for the wt depending on the screening round, we included the average values for

**Table I.** Results of screening experiments to identify the most suitable mutation at the single N-glycosylation sites of HRP C1A

| Mutation | Results after 96 h of induction | | | | | Chosen mutation |
|---|---|---|---|---|---|---|
| | $OD_{600}$ | Cat. activity (U $mL^{-1}$) | Protein content (mg $mL^{-1}$) | Spec. activity (U $mg^{-1}$) | Residual activity after 4 h at 60°C (%) | |
| wt | 25.2 | 7.5 | 0.13 | 48.4 | 75.0 | |
| N13D | 31.8 | 0.69 | 0.09 | 7.29 | 27.0 | →N13D |
| N13Q | 42.3 | 0.74 | 0.08 | 9.10 | 10.5 | |
| N13S | 35.1 | – | – | – | – | |
| N57D | 31.4 | 0.77 | 0.11 | 7.00 | 53 | →N57S |
| N57Q | 23.4 | 2.48 | 0.13 | 19.1 | 43 | |
| N57S | 30.3 | 7.20 | 0.13 | 55.4 | 74 | |
| N158D | 32.7 | 6.11 | 0.23 | 26.6 | 100 | →N158D |
| N158Q | 34.1 | 0.49 | 0.37 | 1.32 | 81.8 | |
| N158S | 30.0 | 0.87 | 0.13 | 6.23 | 90.8 | |
| N186D | 36.8 | 0.10 | 0.09 | 1.06 | 43.1 | →N186D |
| N186Q | 47.6 | 0.07 | 0.10 | 0.70 | 0 | |
| N186S | 46.2 | 0.14 | 0.09 | 1.60 | 36.2 | |
| N198D | 21.3 | 12.1 | 0.23 | 53.1 | 68.1 | →N198D |
| N198Q | 19.7 | 4.31 | 0.22 | 20.1 | 34.1 | |
| N198S | 25.1 | 1.25 | 0.22 | 5.84 | 20.5 | |
| N214D | 17.9 | 5.03 | 0.11 | 46.6 | 46.1 | →N214S |
| N214Q | 18.1 | 4.92 | 0.13 | 39.0 | 41.5 | |
| N214S | 14.2 | 3.17 | 0.12 | 25.6 | 96.0 | |
| N255D | 13.6 | 7.24 | 0.13 | 53.9 | 66.5 | →N255D |
| N255Q | 14.2 | 8.59 | 0.18 | 48.3 | 78.8 | |
| N255S | 11.9 | 5.49 | 0.15 | 35.9 | 74.9 | |
| N268D | 13.6 | 0.56 | 0.27 | 2.07 | 70.6 | →N268D |
| N268Q | 12.9 | 0.40 | 0.26 | 1.54 | 71.0 | |
| N268S | 12.6 | 0.43 | 0.32 | 1.34 | 54.0 | |

growth, protein production, enzyme activity and thermal stability in Table I for comparison. Based on the determined specific activity and thermal stability, we chose the most suitable mutation at the single N-glycosylation sites. Except for N13S all the produced HRP glyco-variants showed catalytic activity; however, replacement of Asn by Gln never turned out to be the most suitable mutation at any of the eight N-glycosylation sites. Since we did not measure any detectable extracellular protein content for N13S either, we speculate that this mutation caused problems in protein folding and/or secretion, a phenomenon described before (Zhu et al. 1998; Ito, Ishimaru, et al. 2007; Ito, Seri, et al. 2007; Zou et al. 2013). Interestingly, we observed significant differences in enzyme activity and stability depending on the introduced mutation (Table I), and identified three mutations which had been described before, namely at positions N13 and N268 (Asad, Khajeh, et al. 2011) and N255 (Lin et al. 1999), respectively.

*Physiological strain characterization in the bioreactor*

The different *P. pastoris* strains carrying the respective mutated HRP gene were physiologically characterized in single dynamic batch cultivations in the controlled environment of a bioreactor. After exhaustion of glycerol which was indicated by an increase in the off-gas signal, a 0.5% (v/v) methanol adaptation pulse was applied which was followed by several, subsequent 1.0% (v/v) methanol pulses (an example for this procedure is illustrated for the *P. pastoris* strains expressing the wt enzyme and variant N57S in Figure 1, while illustrations for the other strains are shown in Supplementary data, Figure S1). This dynamic strategy has repeatedly proven to be a very efficient method to physiologically characterize *P. pastoris* strains in a fast and simple manner (Dietzsch et al. 2011a,b; Krainer et al. 2012; Zalai et al. 2012).

In Table II, the determined strain characteristic parameters of all the strains are summarized. Apparently, the introduction of the respective recombinant HRP C1A gene had an impact on the physiology of the *P. pastoris* strains. Although the majority of the strains showed similar maximum specific growth rates on glycerol ($\mu_{max\ gly}$) between 0.24 and 0.28 $h^{-1}$, the strain carrying the gene HRP C1A N13D showed a nearly 1.3-fold higher $\mu_{max\ gly}$. This effect was even more pronounced with respect to the specific methanol uptake rate during the adaptation pulse ($q_{s\ adapt}$). Surprisingly, when we calculated the average-specific methanol uptake rate during the consecutive 1% (v/v) methanol pulses ($q_{s\ average\ MeOH}$), we observed striking differences between all the strains. One can speculate that the reason for the altered strain physiology lies in the produced HRP glyco-variant itself, since it is known that N-glycosylation can influence protein folding and protein production and thus might affect cell physiology (Zhu et al. 1998; Ito, Ishimaru, et al. 2007; Ito, Seri, et al. 2007; Zou et al. 2013). However, as shown in Table II, the amount of total extracellular protein for each strain at the end of the dynamic batch cultivation was basically the same, indicating that the single mutations did not cause significant problems in protein folding or secretion. We also analyzed the cell-free cultivation broths on SDS gels and obtained the same pattern of protein bands at comparable intensity (graphs not shown). Thus, apparently not the mutated product but rather the locus of the respective introduced gene in the yeast genome had a significant influence on the methanol metabolism of the cells. This influence is also obvious in both yield coefficients (biomass yield,

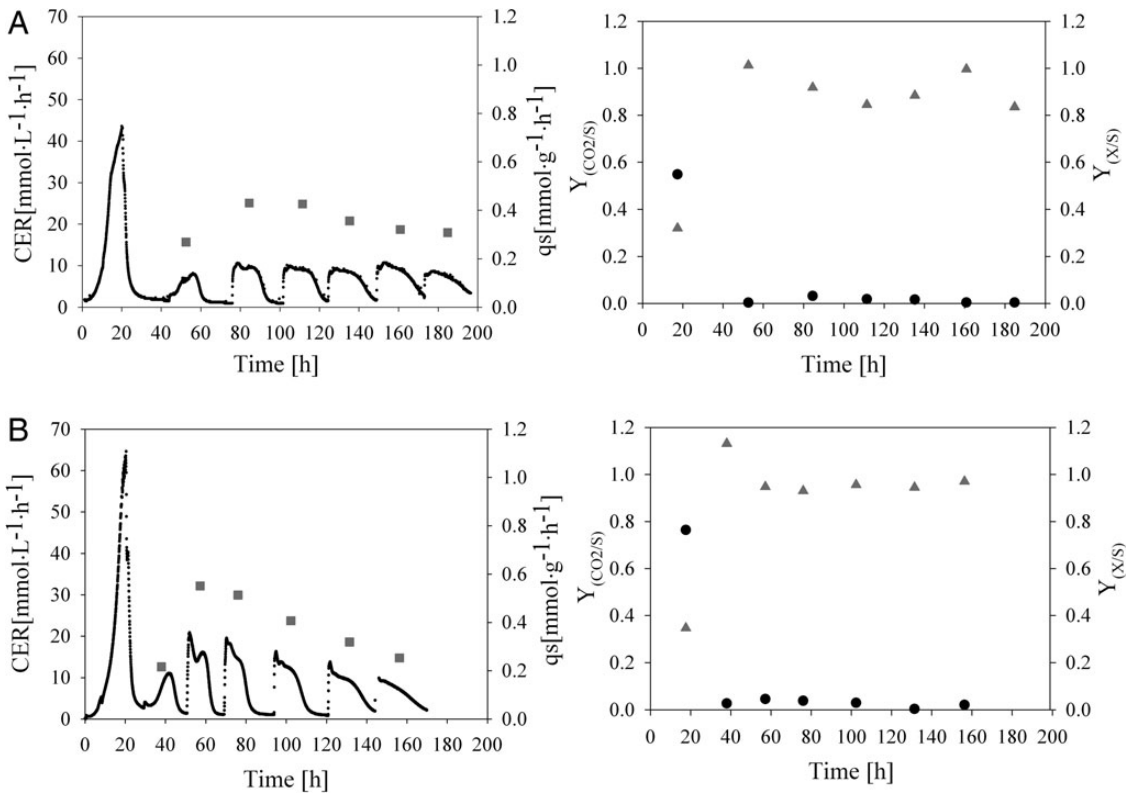



**Fig. 1.** Batch cultivation of a *P. pastoris* CBS7435 Mut$^S$ strain carrying either the unmutated HRP C1A gene (designated as "wt") or the glyco-variant HRP C1A N57S. A1, batch cultivation with methanol pulses of wt; B1, batch cultivation with methanol pulses of N57S. Solid black line, carbon dioxide evolution rate (CER); gray square, specific substrate uptake rate ($q_s$). A2, calculated yields of wt; B2, calculated yields of N57S. Gray triangle, carbon dioxide yield ($Y_{CO2/S}$); black dot, biomass yield ($Y_{X/S}$).

**Table II.** Strain characteristic parameters determined for recombinant *P. pastoris* strains harboring either the wt HRP C1A gene or a glyco-variant thereof and the amount of total extracellular protein at the end of cultivation

| Strain | $\mu_{max\ gly}$ ($h^{-1}$) | $\Delta_{time\ adapt}$ (h) | $q_{s\ adapt}$ (mmol $g^{-1}$ $h^{-1}$) | $q_{s\ average\ MeOH}$ (mmol $g^{-1}$ $h^{-1}$) | $Y_{X/S}$ ($C_{mol}$ $C_{mol}^{-1}$) | $Y_{CO2/S}$ ($C_{mol}$ $C_{mol}^{-1}$) | C-balance | Protein (mg $mL^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| wt | 0.277 | 11.1 | 0.269 | 0.370 | 0.013 | 0.92 | 0.93 | 0.08 |
| N13D | 0.330 | 8.7 | 0.317 | 0.592 | 0.063 | 0.88 | 0.95 | 0.08 |
| N57S | 0.245 | 12.8 | 0.216 | 0.409 | 0.027 | 0.95 | 1.02 | 0.09 |
| N158D | 0.251 | 13.5 | 0.211 | 0.304 | 0.065 | 1.00 | 1.07 | 0.09 |
| N186D | 0.268 | 13.1 | 0.211 | 0.273 | 0.019 | 1.00 | 1.02 | 0.10 |
| N198D | 0.244 | 8.3 | 0.292 | 0.372 | 0.022 | 0.90 | 0.96 | 0.08 |
| N214S | 0.267 | 14.4 | 0.219 | 0.213 | 0.012 | 0.95 | 0.95 | 0.08 |
| N255D | 0.253 | 8.4 | 0.291 | 0.537 | 0.006 | 0.96 | 1.00 | 0.11 |
| N268D | 0.258 | 14.4 | 0.253 | 0.256 | 0.038 | 1.00 | 1.04 | 0.08 |

$Y_{X/S}$; carbon dioxide yield, $Y_{CO2/S}$). However, we did not investigate the exact locus of gene integration for the single strains in more detail. Closing C-balances for all cultivations confirm the accuracy of the calculated strain specific parameters. It is remarkable that although we used the same *P. pastoris* strain CBS7435 Mut$^S$, the same vector and basically the same gene except for single point mutations, we obtained physiologically diverging strains. This actually underlines the importance of a detailed physiological strain characterization using the dynamic method applying methanol pulses, especially if subsequent fed-batch cultivations for protein production are envisioned.

*Protein purification*

After cultivation, the respective HRP glyco-variant was purified from the cell-free cultivation broth using a previously reported two-step flow-through strategy (Spadiut et al. 2012; Krainer, Pletzenauer, et al. 2013). Total protein content and enzymatic activity were determined in the flow-through and the eluates and the respective recovery yield of HRP activity in percentage ($R$%) and the purification factor (PF) were calculated for each single purification step (Table III).

After hydrophobic charge induction chromatography (HCIC), we recovered >80% of wt HRP C1A and of most

**Table III.** Results of the two-step purification approach for HRP C1A applying HCIC and AEC both operated in flow-through mode

| Variant | HCIC | | | AEC | | | Combined | | Spec. activity (U mg$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| | *R*% total | *R*% FT | PF | *R*% total | *R*% FT | PF | *R*% | PF | |
| wt | 83.6 | 83.6 | 1.95 | 85.9 | 77.1 | 3.46 | 64.5 | 6.7 | 248 |
| N13D | 91.5 | 91.5 | 2.94 | 85.6 | 24.5 | 7.92 | 22.4 | 23.3 | 689 |
| N57S | 80.7 | 80.4 | 2.70 | 86.2 | 70.9 | 3.32 | 56.3 | 9.0 | 461 |
| N158D | 53.8 | 53.6 | 1.85 | 90.1 | 75.0 | 5.02 | 40.2 | 9.3 | 167 |
| N186D | 86.8 | 86.8 | 1.48 | 91.4 | 57.1 | 10.2 | 49.6 | 15.1 | 198 |
| N198D | 51.3 | 51.1 | 1.30 | 84.0 | 50.4 | 1.89 | 25.8 | 2.5 | 114 |
| N214S | 82.4 | 82.3 | 1.66 | 94.4 | 45.3 | 4.53 | 37.3 | 7.5 | 113 |
| N255D | 94.9 | 94.9 | 2.95 | 96.4 | 75.4 | 6.59 | 71.6 | 19.4 | 236 |
| N268D | 94.7 | 94.7 | 3.43 | 82.0 | 74.9 | 4.21 | 70.9 | 14.4 | 274 |

enzyme variants except for N158D and N198D. In agreement with our previous observations (Spadiut et al. 2012; Krainer et al. 2013), the whole activity was found in the flow-through. The remaining 5–20% of the enzymes did not elute from the column under the conditions applied, which actually proofs the existence of a variety of enzyme species in the cultivation broth differing in glycosylation and thus a varying degree of interaction with the resin. Interestingly, for glyco-variant N158D and N198D, we only recovered 50% of the enzyme in total, which we also found in the flow-through. Apparently, by mutating these two glycosylation sites and thus reducing the overall amount of surface N-glycosylation, the masking effect thereof was reduced leading to a different HCIC performance for these two enzyme variants. However, compared with the other glycosylation sites we could not identify a particular location of these two sites which could potentially explain this phenomenon (Supplementary data, Figure S2). By HCIC, the wt enzyme was purified 2-fold, whereas the success of purification varied between 1.3- and 3.4-fold for the different glyco-variants highlighting the importance of an integrated bioprocess aspect—already little changes of protein properties, as the degree of surface N-glycosylation, might have a significant impact on following unit operations. The difference in the PF for wt HRP C1A compared with previous studies where we achieved a PF of 7.0 (Krainer, Pletzenauer, et al. 2013) might be explained by the different cultivation strategies. In our previous study, we cultivated *P. pastoris* in shake flasks, where conditions were not controlled and limitations in nutrients and oxygen occurred, which is why cells were more sensitive to cell lysis. Consequently, more contaminating proteins were found in the cell-free cultivation broth. In the present study, we cultivated the different strains in a bioreactor where parameters, such as pH and temperature, were controlled and thus undesired cell lysis was reduced. Consequently, the cell-free cultivation broth contained less contaminating proteins. This is also obvious when looking at the cell-free cultivation broth before purification, which showed a specific activity of only 20 U mg$^{-1}$ from shake flasks (Krainer, Pletzenauer, et al. 2013) but ~40 U mg$^{-1}$ in the present study.

In the subsequent anion exchange chromatography (AEC) step, we recovered >80% of the initially applied HRP for all enzyme variants (Table III). However, the amount of HRP we found in the flow-through vastly differed between the glyco-variants. For the wt enzyme and variants N57S, N158D, N155D and N268D, we found a comparable amount of ~75% of HRP in the flow-through, whereas for variants N13D, N186D, N198D and N214S, the recovery in the flow-through was only 50% or less. For N13D, we even only found 25% of the enzyme in the flow-through, whereas the rest was found in the eluate together with contaminating proteins. However, when we looked at the location of N13 in comparison to the other glycosylation sites we could not identify a particularity which could explain this phenomenon (Supplementary data, Figure S2). This again highlights the importance of the single N-glycosylation sites and the respective surface N-glycosylation for the physicochemical properties of HRP and the applicability of the flow-through chromatography approach. With regard to AEC purification success, we obtained a PF of >3 for the wt enzyme and similar values for N57S, N214S and N268D. Although we only recovered 25% of the initial amount of N13D in the flow-through, this glyco-variant was purified nearly 8-fold. Also N158D, N186D and N255D were purified with great success (5-, 10- and more than 6-fold, respectively), whereas for variant N198D, a PF of only two was determined in the flow-through.

With respect to the overall purification efficiency for the glyco-variant library of HRP C1A using a two-step flow-through approach, we observed vastly different results (Table III). The overall recovery of the initial amount of HRP after the two purification steps varied from only 20% to >70%. Also the obtained total PF varied immensely between 2.5 for variant N198D and >23 for N13D. Remarkably, these vast differences only originated from single point mutations of HRP C1A and consequent changes in surface N-glycosylation. Hence, for some of these variants, such as for N198D, we recommend to adapt the downstream strategy to obtain an enzyme preparation with higher purity.

### *Biochemical enzyme characterization*

After protein purification, the enzyme variants were biochemically characterized. In Table IV, the kinetic constants for the substrates 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and $H_2O_2$ are shown. The here presented apparent $K_m$ of 1.60 mM of the wt HRP C1A preparation for ABTS was higher than the previously published $K_m$ values of 0.27 and 0.18 mM for C1A preparations from plant and *E. coli*, respectively (Gilfoyle et al. 1996). However, in previous studies on recombinant HRP C1A from *P. pastoris*, $K_m$ values of 0.68 mM (Morawski et al. 2000) and 1.01 (Krainer, Pletzenauer, et al. 2013) were reported. Apparently, yeast-derived HRP C1A preparations generally have a

**Table IV.** Kinetic constants of wt HRP C1A and the glyco-variants for the substrates ABTS and $H_2O_2$ as well as thermal stability

| Variant | ABTS | | | $H_2O_2$ | | | $\tau_{1/2}$ (min) |
|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ (U mg$^{-1}$) | $V_{max}/K_m$ (U mg$^{-1}$ mM$^{-1}$) | $K_m$ (mM) | $V_{max}$ (U mg$^{-1}$) | $V_{max}/K_m$ (U mg$^{-1}$ mM$^{-1}$) | |
| wt | 1.60 | 44.2 | 27.7 | 0.003 | 16.3 | 5433 | 20.6 |
| N13D | 2.90 | 47.2 | 16.3 | 0.005 | 14.7 | 3066 | 28.9 |
| N57S | 2.98 | 113 | 38.1 | 0.004 | 23.7 | 5378 | 38.5 |
| N158D | 3.08 | 16.3 | 5.30 | 0.005 | 51.7 | 10,342 | 3.2 |
| N186D | 4.24 | 77.4 | 18.2 | 0.004 | 7.63 | 2179 | 18.8 |
| N198D | 1.21 | 14.9 | 12.3 | 0.003 | 19.1 | 5795 | 18.5 |
| N214S | 3.48 | 41.1 | 11.8 | 0.004 | 9.36 | 2531 | 6.3 |
| N255D | 1.72 | 51.5 | 29.9 | 0.005 | 21.6 | 4506 | 11.6 |
| N268D | 1.89 | 32.5 | 17.2 | 0.003 | 10.6 | 3642 | 61.9 |

tendency for a lowered affinity to ABTS compared with preparations from plant and *E. coli*, indicating a crucial role of glycosylation for enzyme activity.

As shown in Table IV, the $K_m$ values of the glyco-variants for ABTS were higher than for the wt, except for variant N198D. Mutating the N-glycosylation sites on the surface of enzyme HRP C1A also affected the reaction rate (Table IV). Summarizing, in terms of catalytic efficiency with ABTS only variants N57S and N255D showed slightly higher or similar values compared with the wt. A similar effect for glyco-variant N255D had already been described elsewhere (Lin et al. 1999). The other glyco-variants were characterized by an up to 5-fold reduced catalytic efficiency. Interestingly, in the work of Asad, Khajeh, et al. (2011) HRP variants N13D and N268D also showed higher catalytic activity compared with the wt. However, Asad et al. used a different reducing substrate as well as different assay conditions, which is why a direct comparison with the present study is not feasible.

We observed similar trends of $K_m$ and $V_{max}$ for the substrate $H_2O_2$, as the majority of HRP glyco-variants showed a reduced catalytic efficiency compared with the wt enzyme (Table IV). In fact, N57S was the only glyco-variant showing similar or even higher catalytic efficiency with both substrates compared with the wt. The Michaelis–Menten kinetics for the wt enzyme and for variant N57S for both substrates is exemplarily shown in Figure 2, whereas illustrations for the other enzyme variants are shown in the Supplementary data, Figure S3.

To potentially explain the observed effects of the mutations on the surface of the enzyme on the catalytic activity, we determined the distance of the respective N-glycosylation site to the amino acid His170, which is linked to the heme group in the active site (Figure 3). However, we were not able to identify a direct correlation between the distance of the N-glycosylation site to the active site and observed changes in catalytic activity. Only resolving the crystal structures of the single HRP C1A glyco-variants and the subsequent analysis of structure–function relationships could potentially explain the observed effects of the respective mutation on the catalytic behavior.

Finally, we also tested the enzymes for thermal stability, since it is known that mutating N-glycosylation sites on the surface of proteins might affect stability (Asad, Khajeh, et al. 2011). In order to investigate if the protein concentration affects thermal stability, as described elsewhere (Asad, Khajeh, et al. 2011), two different concentrations, i.e., 0.01 and 0.02 mg mL$^{-1}$, of the wt HRP were tested. In fact, we observed a huge

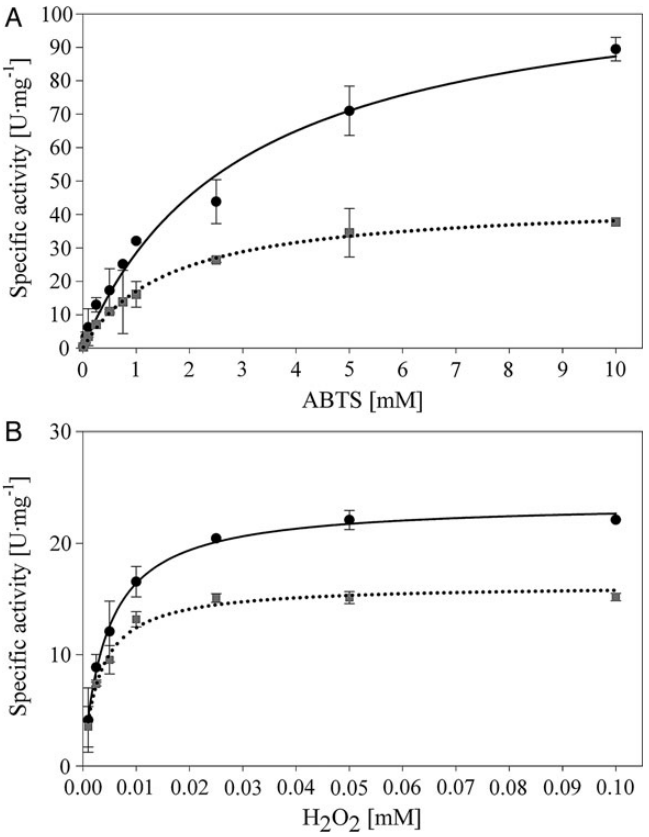
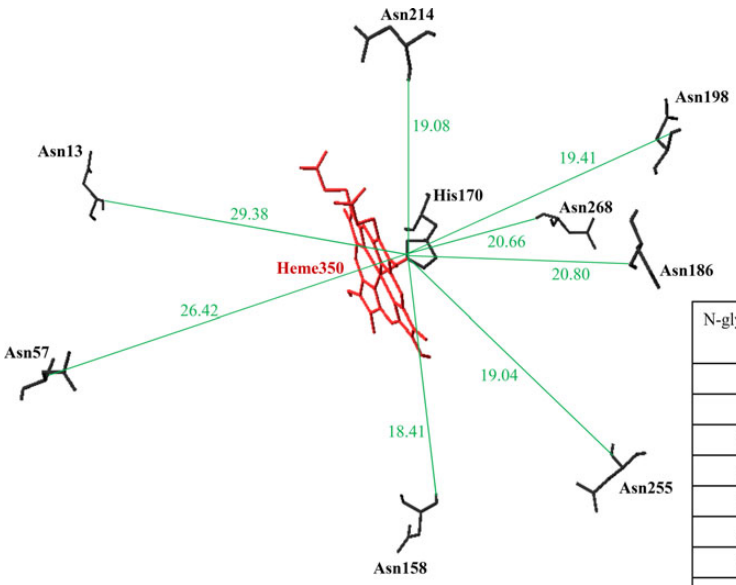



**Fig. 2.** Michaelis–Menten kinetics of the unmutated wt HRP C1A and the glyco-variant N57S for ABTS and $H_2O_2$. (**A**) Kinetics for ABTS, (**B**) kinetics for $H_2O_2$. Black dots, N57S; gray squares, wt.

difference in the half-life time at 60°C, which we determined with 20.6 min for the less concentrated protein solution and with 121 min for the more concentrated one. Consequently, we normalized all the different HRP glyco-variant solutions to a concentration of 0.01 mg mL$^{-1}$ before heat treatment. Interestingly, we found striking differences in the thermal stability of the enzyme glyco-variants (Table IV). For N158D, N214S and N255D stability was significantly reduced, whereas N186D and N198D showed half-life times comparable to the wt. Interestingly, N13D and N57S showed a higher thermal stability and N268D had an even 3-fold higher half-life time ($\tau_{1/2}$)




| N-glycosylation site | distance to His170 [Å] |
|---|---|
| N13 | 29.38 |
| N57 | 26.42 |
| N158 | 18.41 |
| N186 | 20.80 |
| N198 | 19.41 |
| N214 | 19.08 |
| N255 | 19.04 |
| N268 | 20.66 |

**Fig. 3.** Distances of the N-glycosylation sites on the surface of HRP C1A to the heme-binding site His170 in the active site.

than the fully glycosylated enzyme. Comparable observations for variants N13D and N268D were also made by Asad, Khajeh, et al. (2011). The differences between the determined half-life times in this study compared with the study of Asad et al., who determined the stability at 50°C, can be explained by differences in the assay conditions. Whereas Asad, Torabi, et al. (2011) used a 200 mM phosphate buffer, we only used a 50 mM phosphate buffer, which is known to positively affect HRP stability. Summarizing, it is remarkable that mutating glycosylation sites of a protein and thus reducing its overall glycosylation pattern does not only reduce protein stability but might also increase it.

*Glycosylation analysis*. To prove the absence of surface glycosylation on the respective mutated N-glycosylation site, we exemplarily performed glycosylation analysis for the glyco-variant N57S by digesting the protein with either chymotrypsin or trypsin and subsequently analyzing the peptides by liquid-chromatography mass spectrometry. Furthermore, aliquots of the chymotryptic digest were incubated with PNGase A and released glycans were analyzed by mass spectrometry (Figure 4). The success of mutating Asn57 to a non-glycosylated Ser was confirmed by mass spectrometric analysis of chymotryptic peptides. We clearly see the absence of surface N-glycosylation on N57S (Figure 4).

*Combination of mutations*

To potentially obtain an enzyme variant without any N-glycosylation, we combined all the eight mutations described in chapter Screening procedure (hereafter called "mutant"). The resulting *P. pastoris* strain was again cultivated in a batch with consecutive methanol pulses for physiological strain characterization (Table V). Again, we observed very different strain characteristic parameters, although we used the same *P. pastoris* strain CBS7435 Mut$^S$, the same vector and basically the same gene except for eight point mutations. Closing C-balances for both cultivations confirm the accuracy of the calculated strain specific parameters. In contrast to the strain carrying the wt HRP C1A gene, we were not able to detect any HRP activity in the cell-free cultivation broth for the strain carrying the mutated gene. Only after ultrafiltration and 20-fold concentration of the cultivation broth, we were able to measure activity for the mutated HRP C1A glyco-variant. We concentrated the enzyme further and diafiltrated it before we determined the catalytic constants with ABTS and $H_2O_2$ as well as thermal stability (Table VI). As shown in Table VI, the combination of all eight mutations to obtain a HRP C1A variant without any *N*-glycans resulted in an enzyme variant with extremely reduced catalytic efficiency and thermal stability. Although the affinity towards ABTS basically remained the same, the catalytic activity was reduced nearly 300-fold. The effects for $H_2O_2$ were even more severe, as $K_m$ was increased >8-fold and $V_{max}$ decreased >100-fold resulting in a nearly 1000-fold reduced catalytic efficiency. As judged by SDS–PAGE analysis, the size of this variant was significantly reduced compared with the glycosylated wt enzyme (figure not shown). However, since catalytic activity and stability were that low, making this variant not useful for medical applications, we did not analyze the surface N-glycosylation of this glyco-variant in more detail.

Summarizing, enzyme engineering describes a valid approach to obtain active HRP C1A variants with a reduced amount of surface N-glycosylation. Although an enzyme variant where all the eight N-glycosylation sites were mutated hardly showed catalytic activity and thus does not describe a meaningful tool for medical applications, the here described glyco-library of HRP C1A describes a very useful basis for further enzyme engineering approaches. Studies, where we only combine up to four mutations, namely N13D, N57S, N255D and N268D, and then express these variants in a *P. pastoris OCH*1 knockout strain (Krainer, Gmeiner, et al. 2013) to obtain a HRP C1A variant useful for targeted cancer treatment are currently ongoing.

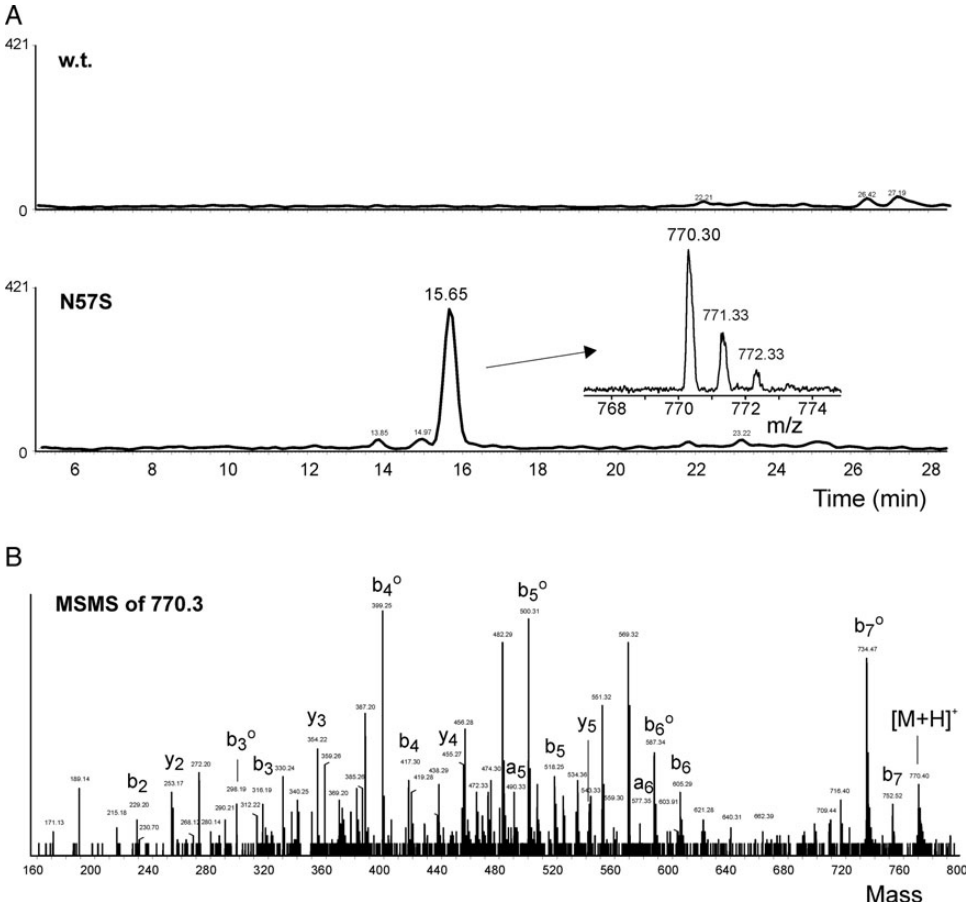



**Fig. 4.** Verification of the mutated peptide LD$^{57}$STTSF by MS. (A) The extracted ion chromatograms for the mass of LDSTTSF ([M+H]$^+$ 770.36 Da) in N57S HRP. The mutant exhibited the relevant peak, whose identity was confirmed by CID fragmentation as shown in (B). B-fragments with loss of $H_2O$ are designated as b$^o$ fragments.

**Table V.** Strain characteristic parameters determined for recombinant *P. pastoris* strains harboring either the wt HRP C1A gene or a variant where all the eight N-glycosylation sites were mutated (mutant)

| Strain | $\mu_{max\ gly}$ ($h^{-1}$) | $\Delta_{time\ adapt}$ [h] | $q_{s\ adapt}$ (mmol $g^{-1}$ $h^{-1}$) | $q_{s\ average\ MeOH}$ (mmol $g^{-1}$ $h^{-1}$) | $Y_{X/S}$ ($C_{mol}$ $C_{mol}^{-1}$) | $Y_{CO2/S}$ ($C_{mol}$ $C_{mol}^{-1}$) | C-balance | Protein (mg $mL^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Wt | 0.277 | 11.1 | 0.269 | 0.370 | 0.013 | 0.92 | 0.93 | 0.08 |
| Mutant | 0.222 | 4.7 | 0.660 | 0.882 | 0.153 | 0.81 | 0.97 | 0.10 |

**Table VI.** Kinetic constants of wt HRP C1A and the variant where all the eight N-glycosylation sites were mutated (mutant) for the substrates ABTS and $H_2O_2$ as well as thermal stability

| Variant | ABTS | | | $H_2O_2$ | | | $\tau_{½}$ (min) |
|---|---|---|---|---|---|---|---|
| | $K_m$ (mM) | $V_{max}$ (U $mg^{-1}$) | $V_{max}/K_m$ (U $mg^{-1}$ $mM^{-1}$) | $K_m$ (mM) | $V_{max}$ (U $mg^{-1}$) | $V_{max}/K_m$ (U $mg^{-1}$ $mM^{-1}$) | |
| Wt | 1.60 | 44.2 | 27.7 | 0.003 | 16.3 | 5433 | 20.6 |
| Mutant | 1.44 | 0.15 | 0.10 | 0.026 | 0.14 | 5.38 | 3.2 |

## Material and methods

### *Chemicals*

Enzymes were purchased from Fermentas GmbH (Vienna, Austria). ABTS diammonium salt was obtained from Sigma–Aldrich Handels GmbH (Vienna, Austria). Difco™ yeast nitrogen base w/o amino acids (YNB), Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson and Company (Schwechat, Austria). Zeocin™ was obtained from Invitrogen (Vienna, Austria). D-Biotin was obtained from Fluka Chemia AG (St. Gallen, Switzerland). All other chemicals were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

*Strain and gene*

*P. pastoris* CBS7435 Mut$^S$ (Dietzsch et al. 2011a,b; Krainer et al. 2012; Zalai et al. 2012; Krainer, Gmeiner, et al. 2013; Spadiut et al. 2013) and vector pPpT4_S harboring the HRP isoenzyme C1A, which was codon-optimized for high-level expression in *P. pastoris* (Krainer et al. 2012), were used in this study. The codon table described in Abad et al. (2010) was applied for codon optimization. Secretion of HRP C1A to the cultivation broth was facilitated by an N-terminally fused prepro-signal sequence of the *S. cerevisiae* alpha-factor.

*Site-directed mutagenesis*

The eight Asn, representing the glycosylation sites of HRP C1A, were mutated to either Asp, Gln or Ser, which are amino acids providing a certain structural similarity to Asn, by site-directed mutagenesis and subsequent digestion with DpnI (Li and Wilkinson 1997). The mutagenic PCR was performed as: 98°C for 30 s; then 10 cycles of 98°C for 10 s, 57°C for 20 s, 72°C for 1 min—10 cycles of 98°C for 10 s, 60°C for 20 s, 72°C for 1 min—10 cycles of 98°C for 10 s, 63°C for 20 s, 72°C for 1 min; with a final incubation at 72°C for 10 min. Each reaction contained 1× HF buffer (Fermentas), 0.1 µg of plasmid DNA, 2.5 U *Phusion* DNA polymerase (Fermentas), 10 µM of each dNTP and 5 pmol of each primer in a total volume of 50 µL. All primers are listed in Table VII and were purchased from Microsynth (Vienna, Austria).

After PCR, the methylated template DNA was degraded by digestion with 10 U of DpnI at 37°C for at least 3 h. The remaining PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Vienna, Austria) and 5 µL of each purified PCR product were transformed into electro-competent *E. coli* TOP10 F′ cells. The successful introduction of the desired mutation and the absence of further mutations were confirmed by DNA sequencing (Microsynth). Transformation of ~2 µg SwaI-linearized pPpT4_S plasmid DNA harboring the respective mutated HRP C1A gene (Supplementary data, Figure S4) into *P. pastoris* was done as described by Lin-Cereghino et al. (2005). Stable transformants were generated via homologous recombination between the linearized plasmid DNA and genomic yeast DNA. Selection of successfully transformed clones was performed on yeast extract peptone dextrose medium (YPD; 10 g L$^{-1}$ yeast extract, 20 g L$^{-1}$ peptone, 20 g L$^{-1}$ glucose, 20 g L$^{-1}$ agar) supplemented with 100 mg L$^{-1}$ Zeocin.

*Screening procedure*

Screening of five randomly picked *P. pastoris* transformants per mutation was done in 1000 mL shaking flasks. We also included a *P. pastoris* CBS7435 Mut$^S$ strain carrying the unmutated HRP C1A gene (henceforth designated as "wt") as well as an untransformed *P. pastoris* CBS7435 Mut$^S$ strain as negative control, resulting in a total of 17 shaking flasks per screening experiment. First the clones were cultivated in 10 mL buffered glycerol complex medium supplemented with 100 mg L$^{-1}$ Zeocin (BMGY_Zeo; 10 g L$^{-1}$ yeast extract; 20 g L$^{-1}$ peptone; 3.4 g L$^{-1}$ YNB w/o amino acids and ammonia sulfate, 10 g L$^{-1}$ $(NH_4)_2SO_4$, 400 mg L$^{-1}$ biotin; 1 g L$^{-1}$ glycerol; 0.1 M potassium phosphate buffer, pH 6.0) in 100 mL shaking flasks at 30°C and 230 rpm overnight. The next day the $OD_{600}$ was measured, an appropriate aliquot of the culture was taken, and after centrifugation the cells were resuspended in selective buffered methanol complex medium supplemented with 100 mg L$^{-1}$ Zeocin (BMMY_Zeo; 10 g L$^{-1}$ yeast extract; 20 g L$^{-1}$ peptone; 3.4 g L$^{-1}$ YNB w/o amino acids and ammonia sulfate, 10 g L$^{-1}$ $(NH_4)_2SO_4$, 400 mg L$^{-1}$ biotin; 0.5% methanol; 0.1 M potassium phosphate buffer, pH 6.0) to an $OD_{600}$ of 1.0. The cells were again cultivated at 30°C and 230 rpm. Every day, 1% (v/v) methanol was pulsed to the culture and a 1 mL sample was taken, analyzed for optical density ($OD_{600}$), catalytic activity and protein content. The catalytic activity of HRP was measured using an ABTS assay in a CuBiAn XC enzymatic robot (Bielefeld, Germany). Ten microliters of sample were mixed with 140 µL 1 mM ABTS solution (50 mM $KH_2PO_4$, pH 6.5). The reaction mixture was incubated at 37°C for 5 min before the reaction was started by the addition of 20 µL 0.078% $H_2O_2$ (v/v). Changes in absorbance at 415 nm were measured for 80 s and rates were calculated. The standard curve was prepared using a commercially available HRP preparation (Type VI-A; Sigma–Aldrich) in the range from 0.02 to 2.0 U mL$^{-1}$. Protein concentrations were determined at 595 nm by the Bradford assay using the Sigma–Aldrich Protein Assay Kit with bovine serum albumin as standard in the range of 0.2–1.2 mg mL$^{-1}$.

After 96 h of induction, the HRP glyco-variants in the cell-free supernatants were tested for thermal stability. Therefore, aliquots of 1 mL were incubated in a waterbath at 60°C for up to 4 h, before the samples were centrifuged (14,000 rpm; 10 min)

**Table VII.** Oligonucleotide primers to mutate the eight Asn residues of the enzyme HRP C1A, which act as N-glycosylation sites, to either Asp, Gln or Ser

| N-site | Name | Sequence (5′→3′) |
|---|---|---|
| N13 | N13D_fwd | AAC TCT TGT CCT *GAT* GTG TCC AAC ATC |
| | N13Q_fwd | AAC TCT TGT CCT *CAG* GTG TCC AAC ATC |
| | N13S_fwd | AAC TCT TGT CCT *AGT* GTG TCC AAC ATC |
| | N13_rev | AGG ACA AGA GTT ATC GTA GAA GGT TGG AGT |
| N57 | N57D_fwd | TCC ATC TTG CTG GAC *GAC* ACT ACC TC |
| | N57Q_fwd | TCC ATC TTG CTG GAC *CAG* ACT ACC TC |
| | N57S_fwd | TCC ATC TTG CTG GAC *AGC* ACT ACC TC |
| | N57_rev | GTC CAG CAA GAT GGA AGC ATC ACA ACC |
| N158 | N158D_fwd | C AGA AAC GTT GGT CTT *GAC* AGA TCA TCC |
| | N158Q_fwd | C AGA AAC GTT GGT CTT *CAG* AGA TCA TCC |
| | N158S_fwd | C AGA AAC GTT GGT CTT *AGC* AGA TCA TCC |
| | N158_rev | AAG ACC AAC GTT TCT GAA AGA GTC TTT CAA TTG |
| N186 | N186D_fwd | ATG GAT CGT CTG TAC *GAC* TTC TCT AAC AC |
| | N186Q_fwd | ATG GAT CGT CTG TAC *CAG* TTC TCT AAC AC |
| | N186S_fwd | ATG GAT CGT CTG TAC *AGC* TTC TCT AAC AC |
| | N186_rev | GTA CAG ACG ATC CAT GAT GAA TCT ACA TTG GTT |
| N198 | N198D_fwd | CCA GAT CCT ACT CTG *GAC* ACC ACT TAC |
| | N198Q_fwd | CCA GAT CCT ACT CTG *CAG* ACC ACT TAC |
| | N198S_fwd | CCA GAT CCT ACT CTG *AGC* ACC ACT TAC |
| | N198_rev | CAG AGT AGG ATC TGG CAA ACC GG |
| N214 | N214D_fwd | CCA CTT AAC GGA *GAC* CTG TCT GC |
| | N214Q_fwd | CCA CTT AAC GGA *CAG* CTG TCT GC |
| | N214S_fwd | CCA CTT AAC GGA *AGC* CTG TCT GC |
| | N214_rev | TCC GTT AAG TGG GCA CAA ACC TC |
| N255 | N255D_fwd | TTG TTC TCC TCT CCT *GAC* GCT ACT GAT |
| | N255Q_fwd | TTG TTC TCC TCT CCT *CAG* GCT ACT GAT |
| | N255S_fwd | TTG TTC TCC TCT CCT *AGC* GCT ACT GAT |
| | N255_rev | AGG AGA GGA GAA CAA CTC CTG GTC |
| N268 | N268D_fwd | G AGA TCC TTC GCA *GAC* TCC ACT CAA |
| | N268Q_fwd | G AGA TCC TTC GCA *CAG* TCC ACT CAA |
| | N268S_fwd | G AGA TCC TTC GCA *AGC* TCC ACT CAA |
| | N268_rev | TGC GAA GGA TCT CAC CAA TGG AAT G |

The mutation sites are depicted in italics.

and the supernatants were analyzed for remaining HRP activity. These values were then compared with the initial activity before heat treatment. Based on activity and stability measurements, the most suitable mutation at a respective N-glycosylation site was chosen and the corresponding *P. pastoris* strain was physiologically characterized in the bioreactor. Before bioreactor cultivation, the presence of the correctly mutated HRP gene in the respective *P. pastoris* transformant was verified by colony PCR using the primers AOX_fwd (5′-ACTCCAACCTTCTACGATAACTC-3′) and AOX_rev (5′-ACTGTGTCATGTGCTGACC-3′) and subsequent sequencing (Microsynth).

*Strain characterization in the bioreactor*

*Culture media*. Precultures were done in yeast nitrogen base medium with 100 mg $L^{-1}$ Zeocin (YNBM_Zeo; 3.4 g $L^{-1}$ YNB w/o amino acids and ammonia sulfate, 10 g $L^{-1}$ $(NH_4)_2SO_4$, 400 mg $L^{-1}$ biotin, 20 g $L^{-1}$ glucose, 0.1 M potassium phosphate buffer, pH 6.0). Batch cultivations were performed in basal salt medium (26.7 mL $L^{-1}$ 85% phosphoric acid, 1.17 g $L^{-1}$ $CaSO_4{\cdot}2H_2O$, 18.2 g $L^{-1}$ $K_2SO_4$, 14.9 g $L^{-1}$ $MgSO_4{\cdot}7H_2O$, 4.13 g $L^{-1}$ KOH, 40 g $L^{-1}$ glycerol, 0.2 mL $L^{-1}$ Antifoam Struktol J650, 4.35 mL $L^{-1}$ PTM1, $NH_4OH$ as N-source). Trace element solution (PTM1) was made of 6.0 g $L^{-1}$ $CuSO_4{\cdot}5H_2O$, 0.08 g $L^{-1}$ NaI, 3.0 g $L^{-1}$ $MnSO_4{\cdot}H_2O$, 0.2 g $L^{-1}$ $Na_2MoO_4{\cdot}2H_2O$, 0.02 g $L^{-1}$ $H_3BO_3$, 0.5 g $L^{-1}$ $CoCl_2$, 20.0 g $L^{-1}$ $ZnCl_2$, 65.0 g $L^{-1}$ $FeSO_4{\cdot}7H_2O$, 0.2 g $L^{-1}$ biotin, 5 mL $L^{-1}$ $H_2SO_4$. Induction was carried out in presence of 1 mM Δ-aminolevulinic acid. The concentration of the base $NH_4OH$ was determined by titration with 0.25 M potassium hydrogen phthalate.

*Experimental procedure*

*Preculture*. Frozen stocks (−80°C) were cultivated in 100 mL YNBM in 1000 mL shake flasks at 30°C and 230 rpm. The grown preculture was transferred aseptically to the respective culture vessel. The inoculation volume was 10% of the final starting volume.

*Batch cultivation*. Batch cultivations were carried out in either a 3 L or a 5 L working volume glass bioreactor (Infors, Bottmingen, Switzerland). Basal salt medium was sterilized in the bioreactor and pH was adjusted to pH 5.0 by using concentrated $NH_4OH$ solution after autoclaving. Sterile filtered trace elements were transferred to the reactor aseptically. Dissolved oxygen ($dO_2$) was measured with a sterilizable $dO_2$ electrode (Visiferm™, Hamilton, Bonaduz, Switzerland). The pH was measured with a sterilizable electrode (Easyferm™, Hamilton, Bonaduz, Switzerland) and maintained constant with a PID controller using $NH_4OH$ solution (1–2 M). Base consumption was determined gravimetrically. Cultivation temperature was set to 30°C and agitation was fixed to 1200 rpm. The culture was aerated with 1.0 vvm dried air and off-gas of the culture was measured by using an infrared cell for $CO_2$ and a paramagnetic cell for $O_2$ concentration (Servomax, Hyderabad, India). Temperature, pH, $dO_2$, agitation as well as $CO_2$ and $O_2$ in the off-gas were measured online and logged in a process information management system (Lucullus, Biospectra, Schlieren, Switzerland). After the complete consumption of the substrate glycerol, indicated by an increase of $dO_2$ and a drop in off-gas activity, the first methanol pulse of a final concentration of 0.5% (v/v) was conducted with methanol supplemented with 12 mL $L^{-1}$ PTM1. Following pulses were performed with 1% methanol/PTM1 (v/v) (Figure 2). For each pulse, two samples were taken to determine the concentrations of substrate and product, as well as dry cell weight to calculate specific rates and yields.

*Analysis of growth and expression parameters*

Dry cell weight was determined by centrifugation of 5 mL culture broth (5000 rpm, 4°C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156), washing the pellet twice with 5 mL deionized water and subsequent drying at 105°C to a constant weight.

*Substrate concentrations*

Concentrations of methanol were determined in cell-free samples by HPLC (Vienna, Austria) equipped with an ion-exchange column (Supelcogel C-610H Sigma–Aldrich) and a refractive index detector (Agilent Technologies). The mobile phase was 0.1% $H_3PO_4$ with a constant flow rate of 0.5 mL $min^{-1}$ and the system was run isocratic at 30°C. Calibration was done by measuring standard points in the range from 0.1 to 10 g $L^{-1}$ methanol. Measurements of biomass, product and substrate concentration were executed in duplicates.

*Protein purification*

After bioreactor cultivation, the cell-free cultivation broth was diafiltrated for subsequent HCIC using a Centramate 500S TFF system (PALL, Vienna, Austria) with a 10 kDa MWCO membrane. The buffer was HCIC-A (500 mM NaCl, 20 mM NaOAc, pH 6.0) and the protein solution was concentrated to a final volume of 40–50 mL. All further steps of concentration and buffer change were performed using Amicon Ultra-15 Centrifugal Filter Units with 10 kDa MWCO (Merck Millipore; Vienna, Austria). The HCIC resin MEP HyperCel™ was obtained from PALL and HCIC was performed in flow-through mode: a column containing 25 mL of MEP HyperCel™ resin was equilibrated with at least 4 column volumes (CV) of buffer HCIC-A. Forty to 50 mL concentrated HRP solution in HCIC-A were loaded onto the column which was then washed with at least 5 CV of HCIC-A at a flow rate of 55 cm $h^{-1}$. Then a step elution to 100% buffer HCIC-B (1.0 M NaCl, 20 mM NaOAc, pH 8.0) was performed. After elution, the column was washed with 5 CV 0.8 M NaOH before it was stored in EtOH 20%, 1.0 M NaCl. During all the different steps, fractions of 10 mL were collected and analyzed for protein content and catalytic activity.

HCIC flow-through fractions showing HRP activity were pooled, concentrated and rebuffered in AEC-A (50 mM Tris–HCl, pH 8.0) for subsequent AEC using an 8 mL CIM®-DEAE monolithic column (Krainer, Pletzenauer, et al. 2013) (BIAseparations, Ajdovščina, Slovenia). The column was equilibrated with 5 CV of AEC-A at a flow rate of 16.8 cm $h^{-1}$. Diafiltrated post-HCIC pools were subsequently loaded onto the AEC column at an average linear flow rate of 16.8 cm $h^{-1}$ before a post-load wash with 5 CV of AEC-A was performed. Elution was performed in a single step from 0 to 100% AEC-B (50 mM Tris–HCl, 1.0 M NaCl, pH 8.0). The column was washed with 5 CV of a 1 M NaOH/1 M NaCl solution at an average linear flow rate of 33.6 cm $h^{-1}$ for column recovery, before the column was stored in 20% EtOH.

The efficiency of each purification step was evaluated by determining the PF and the recovery yield of HRP activity in percentage ($R$%). PF and $R$% were calculated by Eqs (1) and (2) (Krainer, Pletzenauer, et al. 2013). The suffixes "pre" and "post" indicate the respective values before and after a purification step.

$$\mathrm{PF} = \frac{\text{specific activity}_{\text{post}}}{\text{specific activity}_{\text{pre}}}, \quad (1)$$

$$R\% = 100 \times \frac{\text{volumetric activity}_{\text{post}} \times \text{volume}_{\text{post}}}{\text{volumetric activity}_{\text{pre}} \times \text{volume}_{\text{pre}}}. \quad (2)$$

Finally, the pooled active fractions after AEC were diafiltrated in 50 mM potassium phosphate buffer, pH 6.5, and concentrated to a volume of ~1.5 mL for the subsequent biochemical characterization.

*Biochemical enzyme characterization*

Biochemical characterization of the purified HRP glyco-variants included the determination of the basic kinetic parameters $K_m$ and $V_{max}$ for the two substrates $H_2O_2$ and ABTS in a spectrophotometer UV-1601 from Shimadzu (Korneuburg, Austria). The reaction mixture with a final volume of 1.0 mL contained 20 µL of HRP glyco-variant, 50 mM potassium phosphate buffer, pH 6.5, and either varying concentrations of ABTS (0.01–10 mM) and a saturating concentration of $H_2O_2$ of 1.0 mM or varying concentrations of $H_2O_2$ (0.001–1.0 mM) and a saturating concentration of ABTS of 10.0 mM, respectively. The increase in absorption was followed at 420 nm at 30°C for 180 s. Absorption curves were recorded with a software program (UVPC Optional Kinetics software, Shimadzu). The maximum reaction rate ($V_{max}$) and the Michaelis constant ($K_m$) were calculated with the Sigma Plot software (Version 11.0; Systat Software Inc.).

The thermal stability of individual HRP glyco-variants was tested at 60°C. The residual activity towards ABTS was measured after 1, 5, 10, 15, 30, 45, 60, 90 and 120 min of incubation at 60°C in a water bath. Protein concentrations were normalized to 0.01 mg mL to limit possible effects of the different protein concentrations on thermal stability (Asad, Khajeh, et al. 2011) and to obtain comparability. Residual activities were plotted versus the incubation time and the half-life times of thermal inactivation at 60°C ($\tau_{½}$) were calculated using Eq. (3):

$$\tau_{1/2} = \frac{\ln 2}{k_{in}}, \quad (3)$$

$k_{in}$ rate of inactivation (slope of the logarithmic residual activity).

*Glycosylation analysis*. Purified HRP sample was buffered in 0.1 M $NH_4HCO_3$ and reduced with dithiothreitol (5 mM) for 45 min at 56°C and alkylated using iodoacetamide (25 mM) at room temperature for 30 min. The protein was precipitated with 4 volumes of acetone for 45 min at −20°C, dried in a vacuum centrifuge and resuspended in 0.1 M $NH_4HCO_3$ buffer to yield a protein concentration of ~1 µg µL$^{-1}$. Digests were performed overnight with either chymotrypsin or trypsin (Promega, Mannheim, Germany) at 37°C at an enzyme-to-substrate ratio of 1:50 (w/w). The digested peptides were analyzed by liquid-chromatography mass spectrometry as follows: 1 µg of sample was loaded on a BioBasic-18 column (150 × 0.32 mm; 5 µm; Thermo Scientific, Vienna, Austria) and eluted with a gradient from 1 to 60% acetonitrile in 0.3% formic acid buffered to pH 3.0 at flow rate of 6 µL min. Eluted peptides were analyzed on an Ultima Global Q-TOF mass spectrometer (Waters, Manchester, UK) operated in positive-ion mode, which was previously calibrated with a cesium iodide standard in the range of 400–1800 *m/z*. Additionally, the peptide harboring the site N57S within the mutated HRP variant was subjected to collision-induced dissociation MS-MS with Argon as collision gas. Data were manually evaluated and deconvoluted using the Software MassLynx V4.00.00 (Waters).

An aliquot of the chymotryptic digest was heat inactivated and then incubated with 0.03 mU PNGase A (Proglycan, Vienna, Austria) in 50 mM citrate buffer, pH 5.5. Glycans were purified using porous graphitic carbon cartridges (Thermo Scientific) as described (Pabst et al. 2012). Glycans were analyzed by mass spectrometry as described in chapter Glycosylation analysis for peptides with the sole divergence of using a 100 × 0.32 mm hypercarb column (Thermo Scientific) and 1 h gradient from 1 to 50% acetonitrile.

## Supplementary Data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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## Conflict of interest statement

None declared.

## Abbreviations

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); AEC, anion exchange chromatography; CV, column volumes; HCIC, hydrophobic charge induction chromatography; HRP, horseradish peroxidase; $H_2O_2$, hydrogen peroxide; mutant, HRP C1A, where all eight N-glycosylation sites were mutated; PF, purification factor; $R$%, recovery yield of HRP activity in percentage; $\tau_{½}$, thermal half-life time; wt, wild type; X, any amino acid but proline; $Y_{X/S}$, biomass yield (C-mol·C-mol$^{-1}$); $Y_{CO2/S}$, carbon dioxide yield (C-mol·C-mol$^{-1}$).

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*Article*

# Combining Protein and Strain Engineering for the Production of Glyco-Engineered Horseradish Peroxidase C1A in *Pichia pastoris*

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**Abstract:** Horseradish peroxidase (HRP), conjugated to antibodies and lectins, is widely used in medical diagnostics. Since recombinant production of the enzyme is difficult, HRP isolated from plant is used for these applications. Production in the yeast *Pichia pastoris* (*P. pastoris*), the most promising recombinant production platform to date, causes hyperglycosylation of HRP, which in turn complicates conjugation to antibodies and lectins. In this study we combined protein and strain engineering to obtain an active and stable HRP variant with reduced surface glycosylation. We combined four mutations, each being beneficial for either catalytic activity or thermal stability, and expressed this enzyme variant as well as the unmutated wildtype enzyme in both a *P. pastoris* benchmark strain and a strain where the native α-1,6-mannosyltransferase (OCH1) was knocked out. Considering productivity in the bioreactor as well as enzyme activity and thermal stability, the mutated HRP variant produced in the *P. pastoris* benchmark strain turned out to be interesting for medical diagnostics. This variant shows considerable catalytic activity and thermal stability and is less glycosylated, which might allow more controlled and efficient conjugation to antibodies and lectins.

**Keywords:** horseradish peroxidase; glyco-engineering; strain engineering; bioreactor cultivation; OCH1; site-directed mutagenesis

## 1. Introduction

The methylotrophic yeast *Pichia pastoris* is a widely used host organism for recombinant protein production. It can grow on cheap and defined media to high cell densities, is robust against stressful conditions and able to perform post-translational modifications, like glycosylation [1–4]. However, glycosylation in *Pichia pastoris* (*P. pastoris*) is characterized by a severe drawback: native glycosyltransferases recognize the amino acid motif Asn-X-Ser/Thr and link many glycan moieties to the asparagine residues of recombinant proteins yielding a hyperglycosylated product (e.g., [5–7]). Hyperglycosylation causes many problems as it alters the physicochemical properties of the product, hampers downstream processing [5,8] and prevents medical applications. One of the main disadvantages of yeast-derived glycosylation lies in its heterogeneous nature making subsequent conjugation to antibodies and lectins, a prerequisite for applications in medical diagnostics, extremely difficult. Two different approaches can be applied to reduce yeast-derived hyperglycosylation, namely (1) protein engineering and (2) strain engineering. The yeast *P. pastoris* is a prominent example for such efforts. In several studies detailed strain engineering approaches describing humanization of this yeast were reported (Table 1; e.g., [7–14]).

**Table 1.** Selected studies focusing on the humanization of *N*-glycosylation in *P. pastoris*.

| Goal of the Study | Citation |
|---|---|
| Introduction of α-1,2-Mns and GntI, *OCH1* inactivation via a knock-in plasmid | [9] |
| Introduction of an UDP-GlcNAc transporter, α-1,2-MnsIA, MnsII, GntI, GntII in a *Δoch1*::*URA3* strain | [10] |
| Introduction of sialic acid biosynthesis pathway to produce sialylated glycoproteins | [11] |
| *OCH1* knock-out and introduction of glycosidases and glycosyltransferases to produce terminally galactosylated glycoproteins | [14] |

Mns, mannosidase; Gnt, β-*N*-acetylglucosaminyltransferase; UDP-GlcNAc, uridine diphosphate-*N*-acetylglucosamine; *OCH1*, outer chain elongation gene.

We used *P. pastoris* for the recombinant production of the heme-containing plant enzyme horseradish peroxidase (HRP), an enzyme widely used in medical diagnostics (e.g., [5,15]). We chose this yeast as expression host because Morawski *et al.* [16,17] had shown *P. pastoris* to produce recombinant proteins with significant shorter surface glycans than *Saccharomyces cerevisiae* (*S. cerevisiae*). We investigated various aspects of the expression of HRP in *P. pastoris*: different HRP isoenzymes were produced [18,19], production strategies were developed and optimized [20–23], media supplementation and strain engineering for increased heme-incorporation were analyzed [24] and the methanol utilization pathway of *P. pastoris* was manipulated for higher productivity [25].

In more recent studies we applied both protein and strain engineering with the goal of producing more homogenously glycosylated HRP variants in *P. pastoris*. However, we did not use fully humanized yeast strains, which are proprietary, but produced HRP in a *P. pastoris* strain with a deleted

*och1* gene (Δoch1 strain; [26]). This gene codes for an α-1,6-mannosyltransferase responsible for triggering the uncontrolled addition of mannose residues to the recombinant protein. Although HRP produced in the Δoch1 strain was more homogeneously glycosylated with more than 70% of the enzyme being of the $Man_8$ glycosylation type, the Δoch1 strain showed a growth impaired phenotype and was hard to cultivate [26]. In a subsequent study, we analyzed the effect of the process parameters temperature, pH and dissolved oxygen concentration on strain physiology and productivity [6]. We found that the space-time-yield (STY) of the glyco-engineered strain was eight-fold lower compared to an unmodified *P. pastoris* benchmark strain, making HRP production in the Δoch1 strain unattractive.

Thus, we glyco-engineered the enzyme HRP by mutating each of the eight *N*-glycosylation sites to produce in a benchmark strain but still get HRP with reduced surface glycosylation [27]. We found different effects of the single mutations on enzyme activity and stability and demonstrated that mutation of the respective *N*-glycosylation site in fact resulted in the absence of glycans there [27]. Although the combination of all eight mutations gave a non-glycosylated enzyme, both catalytic activity and thermal stability were dramatically reduced leading to the hypothesis that some of the *N*-glycosylation sites must not be mutated to obtain active and stable HRP.

In the present study we combined both (1) protein engineering and (2) strain engineering to obtain a stable and active HRP variant with reduced and more homogeneous surface glycosylation. We combined the four mutations we had identified as being beneficial for either catalytic activity or stability in a previous study (Table 2; [27]).

**Table 2.** Biochemical characteristics of the unmodified horseradish peroxidase (HRP) wildtype enzyme (wt) and four different HRP variants [27].

| Enzyme | ABTS | | | $H_2O_2$ | | | Thermal Half-Life Time |
|---|---|---|---|---|---|---|---|
| Variant | $K_m$ (mM) | $v_{max}$ (U/mg) | $v_{max}/K_m$ (U/mg/mM) | $K_m$ (mM) | $v_{max}$ (U/mg) | $v_{max}/K_m$ (U/mg/mM) | $\tau_{½}$ (min) |
| wt | 1.60 | 44.2 | 27.7 | 0.003 | 16.3 | 5433 | 20.6 |
| N13D | 2.90 | 47.2 | 16.3 | 0.005 | 14.7 | 3066 | 28.9 |
| N57S | 2.98 | 113 | 38.1 | 0.004 | 23.7 | 5378 | 38.5 |
| N255D | 1.72 | 51.5 | 29.9 | 0.005 | 21.6 | 4506 | 11.6 |
| N268D | 1.89 | 32.5 | 17.2 | 0.003 | 10.6 | 3642 | 61.9 |

ABTS, 2-2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); $H_2O_2$, hydrogen peroxide.

We produced the enzyme variant, called 4/8 HRP, in both an unmodified *P. pastoris* benchmark strain and a Δoch1 strain. Summarizing, enzymes produced in the Δoch1 strain were characterized by lower catalytic activity and stability. However, 4/8 HRP produced in the *P. pastoris* benchmark strain (enzyme $wt^{4/8\ HRP}$) showed considerable catalytic activity and increased thermal stability. In combination with its significantly reduced surface glycosylation, which might allow more controlled conjugation to antibodies and lectins, this variant might be useful for applications in medical diagnostics in the future.

## 2. Results and Discussion

### *2.1. Strain Physiology*

We cultivated the four recombinant *P. pastoris* strains in dynamic batch cultivations with methanol pulses at different temperatures and analyzed the effect of temperature on strain physiology. A typical cultivation is exemplarily shown for strain $wt^{4/8\ HRP}$ in Figure 1.

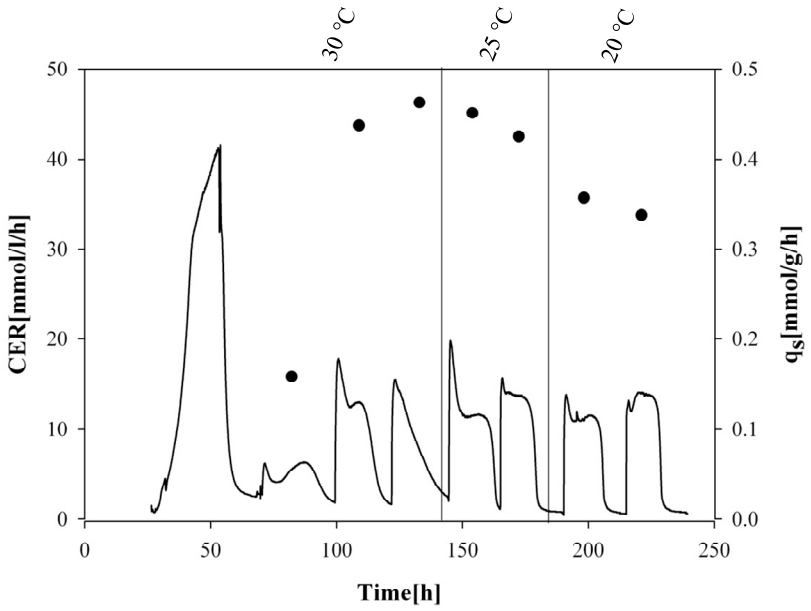


**Figure 1.** Schematic overview of the dynamic batch cultivation of strain $wt^{4/8\ HRP}$ with methanol pulses at different temperatures. Black continuous line, carbon dioxide evolution rate (CER); black dots, specific methanol uptake rate ($q_{s\ MeOH}$).

The most important strain physiological parameters are summarized in Table 3. Closing C-balances for the benchmark strains underline data validity. Similar to our previous studies, we observed that the Δoch1 strains lost metabolic activity over time and were affected by cell lysis [26]. Thus, C-balances did not close.

When comparing the recombinant benchmark strains, great differences in specific methanol uptake rates ($q_{s\ MeOH}$) were identified. While for both strains $q_{s\ MeOH}$ increased with increasing temperature, a correlation we also had observed before [6], the strain producing the 4/8 HRP variant showed a three-fold lower $q_{s\ MeOH}$ compared to the strain producing the unmutated enzyme at the respective temperature. Apparently, production of the glyco-engineered 4/8 HRP caused a physiological burden for the yeast, decelerating metabolism and thus methanol uptake.

When comparing the recombinant Δoch1 strains, we observed that $q_{s\ MeOH}$ for both strains decreased with increasing temperature, a phenomenon we also had described before [6]. However, $q_{s\ MeOH}$ of both Δoch1 strains were comparable at each temperature indicating that the mutated product did not cause any physiological burden. We speculate that the glyco-engineered 4/8 HRP variant might cause problems in the *P. pastoris* benchmark strain during secretion as it might get stuck in the cell wall. On the contrary, the same enzyme variant can be secreted without any problems in the Δoch1 strain, which has a completely altered cell wall structure [24]. However, this remains to be elucidated in detail.

**Table 3.** Physiological parameters of strains $wt^{wt\ HRP}$, benchmark strain expressing the unmutated HRP enzyme; $wt^{4/8\ HRP}$, benchmark strain expressing the mutated 4/8 HRP variant; $OCH1^{wt\ HRP}$, deleted OCH1 gene strain expressing the unmutated HRP enzyme and $OCH1^{4/8\ HRP}$ delete OCH1 gene strain expressing the mutated 4/8 HRP variant were determined in dynamic batch cultivations.

| Strain | $\mu_{max\ gly}$ ($h^{-1}$) | $\Delta_{time\ adapt}$ (h) | $q_{s\ adapt}$ (mmol/g/h) | $q_{s\ MeOH\ 20\ °C}$ (mmol/g/h) | $q_{s\ MeOH\ 25\ °C}$ (mmol/g/h) | $q_{s\ MeOH\ 30\ °C}$ (mmol/g/h) | C-Balance |
|---|---|---|---|---|---|---|---|
| $wt^{wt\ HRP}$ | 0.271 | 11.1 | 0.272 | 0.931 | 1.190 | 1.32 | 0.96 |
| $wt^{4/8\ HRP}$ | 0.200 | 16.0 | 0.158 | 0.354 | 0.438 | 0.450 | 0.97 |
| $OCH1^{wt\ HRP}$ | 0.199 | 4.5 | 0.370 | 0.891 | 0.780 | 0.632 | const. Decreasing |
| $OCH1^{4/8\ HRP}$ | 0.182 | 3.8 | 0.400 | 1.02 | 0.955 | 0.800 | const. Decreasing |

### *2.2. Protein Purification*

After harvest, we purified the different enzyme variants by a 1-step hydrophobic charge interaction chromatography (HCIC) purification strategy [8,19]. As shown in Table 4, the majority of each HRP variant was found in the respective flow-through fraction, where purification factors (PF) between 1.34 and 1.53 were achieved. However, enzyme $OCH1^{4/8\ HRP}$ showed a different result, as nearly 20% of the enzyme was retained on the resin. The eluate fraction showed a higher PF compared to the flow-through fraction (Table 4). We ascribe this phenomenon to the fact that this HRP variant was the least glycosylated one, as the four remaining glycosylation sites mainly carried $Man_8$ instead of longer Man chains (respective detailed analyses had been performed in our previous study [26]). Apparently, the reduced glycosylation of enzyme $OCH1^{4/8\ HRP}$ allows physico-chemical interactions of the enzyme variant and the resin. One might speculate that the amount of enzyme variant able to interact with the resin should be even higher. We think that the rather stressful manner of cultivation with pulses and temperature shifts might have caused very heterogeneous glycosylation on the four remaining *N*-glycosylation sites and thus resulted in the still rather low fraction of only around 20% enzyme interacting with the resin. Detailed analysis of surface glycosylation by mass spectrometry, as we have done previously [24], could shed light on this speculation. However, we did not perform this analysis in this study, since variant $OCH1^{4/8\ HRP}$ did not turn out to be interesting for further applications due to low catalytic activity (see below).

After HCIC, flow-through fractions showing highest PFs were pooled and concentrated to around 1.5 mL. To assess purity of the different enzyme preparations Reinheitszahl values (RZ; $A_{404}/A_{280}$) were determined (Table 4). Highly pure HRP preparations are known to have RZ values of more than 3.0 [16]. Although we did not get pure enzyme preparations by the one-step HCIC purification in this study, the RZ values of the different enzyme variants were in the same range. We also analyzed the different enzyme variants on SDS-PAGE gels, to identify potential differences in apparent size (Figure 2). Furthermore, interesting protein bands were excised and analyzed by mass spectrometry (respective bands are indicated in black boxes in Figure 2).

**Table 4.** Results of the hydrophobic charged interaction chromatography (HCIC) purification and Reinheitszahl (RZ) measurements for the four different HRP enzyme variants.

| | HCIC | | | | Concentrated Fraction |
|---|---|---|---|---|---|
| **Enzyme Variant** | **R% Total** | **R% FT** | **PF FT** | **Specific Activity (U/mg)** | **RZ ($A_{404}/A_{280}$)** |
| $wt^{wt\ HRP}$ | 87.2 | 87.2 | 1.49 | 273.3 | 0.50 |
| $wt^{4/8\ HRP}$ | 97.4 | 95.6 | 1.34 | 36.4 | 0.51 |
| $OCH1^{wt\ HRP}$ | 99.8 | 98.7 | 1.53 | 59.8 | 0.63 |
| $OCH1^{4/8\ HRP}$ | 93.1 | 76.1 | 1.47 | 11.5 | 0.19 |
| **Variant** | **R% Total** | **R% Eluate** | **PF Eluate** | **Specific Activity (U/mg)** | **RZ ($A_{404}/A_{280}$)** |
| $OCH1^{4/8\ HRP}$ | 93.1 | 17.5 | 2.43 | 19.1 | 0.31 |

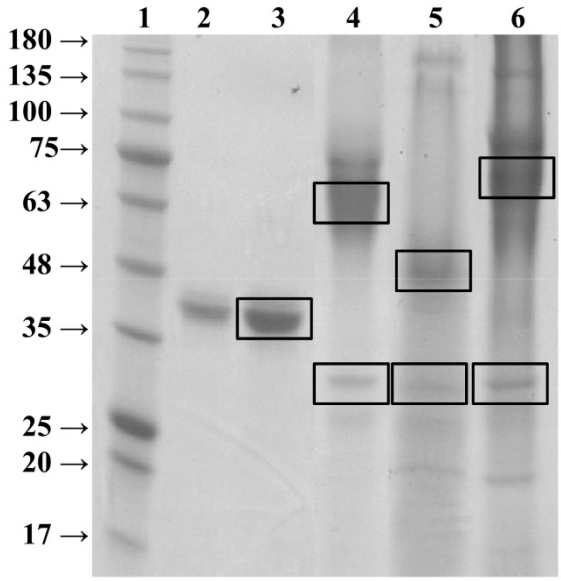


**Figure 2.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of different horseradish peroxidase (HRP) variants. Black boxes indicate protein bands analyzed by mass spectrometry. Lane 1, BLUeye Prestained Protein Ladder; lanes 2 and 3, HRP from plant in two different concentrations (4 µg protein and 8 µg protein, respectively); lane 4, $wt^{wt\ HRP}$, unmutated HRP enzyme expressed in the benchmark strain; lane 5, $wt^{4/8\ HRP}$, mutated 4/8 HRP variant expressed in the benchmark strain; lane 6, $OCH1^{4/8\ HRP}$, mutated 4/8 HRP variant expressed in the deleted OCH1 gene strain.

The enzyme preparation from plant showed a rather distinct band at an apparent size of around 40 kDa on the SDS gel (lanes 2 and 3 in Figure 2), whereas the recombinant enzyme $wt^{wt\ HRP}$ showed a smear at an apparent size of around 65 kDa (lane 4 in Figure 2). Both proteins were identified as HRP by mass spectrometry (Table 5). The apparent size difference of around 20 kDa and the smeary appearance of $wt^{wt\ HRP}$ result from the heterogeneous yeast-derived glycosylation [17,20,21]. The second prominent protein band in lane 4 at an apparent size of around 30 kDa was identified as a glucosidase from *P. pastoris* (Table 5).

As shown in Figure 2, the preparation of enzyme $wt^{4/8\ HRP}$ showed a different protein pattern on the SDS gel, as the band at an apparent size of 65 kDa disappeared, whereas a prominent band at an apparent size of around 50 kDa appeared. In fact, this protein was identified as HRP (Table 5). Apparently, the mutation of four of the eight *N*-glycosylation sites resulted in the absence of glycans

there and thus a size reduction of around 15 kDa. This nicely underlines the feasibility of reducing the vast and heterogeneous glycosylation of recombinant proteins from yeast by protein design. The second prominent band at an apparent size of around 30 kDa was again identified as a glucosidase (Table 5).

Lane 6 in Figure 2 shows the protein bands of enzyme preparation OCH1$^{4/8\ HRP}$. Again, we observed a different protein pattern. The prominent band at an apparent size of around 70 kDa was identified as an oxidase and the band at an apparent size of around 30 kDa again as a glucosidase (Table 5). We expected to see a protein band at an apparent size of around 45 kDa representing enzyme OCH1$^{4/8\ HRP}$. However, no respective band was visible on the SDS gel. We ascribe this absence to the extremely low protein production in the Δoch1 strain [6,26]. Furthermore, cell lysis during bioreactor cultivation resulted in a rather high impurity pattern, which is also demonstrated by the low RZ value for this enzyme variant (Table 4). However, we still measured enzymatic activity for OCH1$^{4/8\ HRP}$ and thus included this enzyme variant in the comparative biochemical characterization.

**Table 5.** Identification of prominent protein bands by mass spectrometry.

| Lane | Apparent Size [kDa] | Rank | Peptides | Scores | Protein | Accession |
|---|---|---|---|---|---|---|
| 3 | 45 | 1 | 11 | 608.9 | Peroxidase C1A Organism species (OS)=Armoracia rusticana | PER1A_ARMRU |
| 4 | 65 | 1 | 6 | 386.9 | 1,3-β-glucanosyltransferase OS=Komagataella pastoris | Q0QCW1_PICPA |
| | | 2 | 9 | 368.0 | Peroxidase C1A OS=Armoracia rusticana | PER1A_ARMRU |
| | 30 | 1 | 9 | 411.5 | Glucan 1,3-β-glucosidase OS=Komagataella pastoris | F2QPL8_PICP7 |
| 5 | 50 | 1 | 9 | 454.5 | Alpha-1-antichymotrypsin 2 OS=Sus scrofa | Q9GMA6_PIG |
| | | 2 | 9 | 386.6 | Keratin, type II cytoskeletal 1 OS=Homo sapiens | K2C1_HUMAN |
| | | 3 | 9 | 317.4 | Peroxidase C1A OS=Armoracia rusticana | PER1A_ARMRU |
| | 30 | 1 | 9 | 411.5 | Glucan 1,3-β-glucosidase OS=Komagataella pastoris | F2QPL8_PICP7 |
| 6 | 70 | 1 | 20 | 1136.6 | Primary-amine oxidase OS=Komagataella pastoris | F2QTE6_PICP7 |
| | | 2 | 10 | 685.6 | 1,3-β-glucanosyltransferase OS=Komagataella pastoris | Q0QCW1_PICPA |
| | | 3 | 9 | 548.8 | 1,3-β-glucanosyltransferase OS=Komagataella pastoris | F2QQJ2_PICP7 |
| | 30 | 1 | 9 | 411.5 | Glucan 1,3-β-glucosidase OS=Komagataella pastoris | F2QPL8_PICP7 |

### *2.3. Biochemical Enzyme Characterisation*

After purification, we biochemically characterized the different HRP variants. The kinetic parameters for ABTS and $H_2O_2$ are summarized in Table 6. We also included plant HRP for comparison. Introducing the four mutations N13D, N57S, N255D and N268D into HRP did not affect the affinity towards ABTS and $H_2O_2$ as $K_m$ values of enzymes wt$^{wt\ HRP}$ and wt$^{4/8\ HRP}$ were comparable.

However, the catalytic efficiency was reduced seven-fold for ABTS and six-fold for $H_2O_2$, respectively. Apparently, the introduced mutations did not only reduce surface glycosylation (Figure 2) but also affected the active site and reduced catalytic activity.

When we produced the HRP variants in the Δoch1 strain, we found that reduced surface glycosylation, caused by the absence of the native enzyme OCH1 [26], did not alter substrate affinity either. In fact, $K_m$ values of all four HRP variants for both ABTS and $H_2O_2$ were comparable. However, reducing surface glycosylation affected catalytic efficiency. Enzyme $OCH1^{wt\ HRP}$ showed a nearly six-fold reduced $v_{max}/K_m$ compared to enzyme $wt^{wt\ HRP}$. Apparently, the benefit of having a more homogeneously glycosylated enzyme produced in the Δoch1 strain comes at cost of catalytic activity. Finally, we characterized the mutated 4/8 HRP produced in the Δoch1 strain (enzyme $OCH1^{4/8\ HRP}$). This enzyme is the least glycosylated one in this study, only providing four out of eight *N*-glycosylation sites, which are mainly occupied by $Man_8$-glycan structures [26]. However, compared to enzyme $wt^{wt\ HRP}$ the catalytic efficiency for ABTS was reduced 119-fold and for $H_2O_2$ 76-fold, respectively. Although, enzyme $OCH1^{4/8\ HRP}$ is basically fit for medical applications, as it misses the heterogeneous yeast-derived glycan structures, the highly reduced catalytic activity as well as low productivity in the bioreactor will most likely prevent future applications.

**Table 6.** Kinetic constants of four different HRP enzyme variants and plant HRP.

| Enzyme | ABTS | | | $H_2O_2$ | | |
|---|---|---|---|---|---|---|
| Variant | $K_m$ (mM) | $v_{max}$ (U/mg) | $v_{max}/K_m$ (U/mg/mM) | $K_m$ (mM) | $v_{max}$ (U/mg) | $v_{max}/K_m$ (U/mg/mM) |
| $wt^{wt\ HRP}$ | 1.50 | 152.9 | 101.9 | 0.009 | 55.2 | 6133 |
| $wt^{4/8\ HRP}$ | 0.99 | 13.6 | 13.7 | 0.015 | 15.5 | 1030 |
| $OCH1^{wt\ HRP}$ | 1.56 | 26.8 | 17.2 | 0.008 | 10.4 | 1300 |
| $OCH1^{4/8\ HRP}$ | 1.34 | 1.28 | 0.96 | 0.016 | 1.29 | 80.7 |
| plant HRP | 1.75 | 567.2 | 324.8 | 0.033 | 377.8 | 11,589 |

ABTS, 2-2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); $H_2O_2$, hydrogen peroxide.

Compared to the commercially available HRP preparation from plant, all recombinant HRP variants showed comparable $K_m$ values but reduced catalytic activity. This might be a result from incomplete heme incorporation or different surface glycosylation. However, the plant preparation contains a mixture of different HRP isoenzymes with varying surface glycosylation, which has to be isolated from its natural source in a rather cumbersome way. Furthermore, seasonal variation in the isoenzyme content and thus the variable production scenario describe only some of the disadvantages of plant HRP. Thus, even though recombinant HRP variants show lower catalytic activity, they are still interesting for industry, as they only describe a single isoenzyme that can be produced in a predictable manner in the controlled environment of a bioreactor. Furthermore, less glycosylated enzymes might allow more controlled and efficient conjugation to antibodies and lectins, which outweighs reduced catalytic activity.

### *2.4. Thermal Stability*

Since we had determined mutations N13D, N57S and N268D to positively affect thermal stability of HRP before (Table 2; [27]), we also analyzed thermal half-life times of the four HRP variants at

60 °C. We again included plant HRP as standard and normalized all enzyme preparations to a protein concentration of 0.1 mg/mL before incubation to guarantee comparability. In Table 7 the respective results are summarized.

**Table 7.** Thermal half-life times of four different HRP variants and the HRP plant preparation at 60 °C. All enzymes were normalized to a protein concentration of 0.1 mg/mL before incubation.

| Enzyme Variant | Protein Concentration (mg/mL) | $\tau_{1/2\ 60\ °C}$ (min) |
|---|---|---|
| $wt^{wt\ HRP}$ | 0.1 | 31.5 |
| $wt^{4/8\ HRP}$ | | 173.2 |
| $OCH1^{wt\ HRP}$ | | 3.3 |
| $OCH1^{4/8\ HRP}$ | | 19.3 |
| plant HRP | | 53.3 |

As shown in Table 7, the introduction of the four mutations into HRP caused a significant increase in thermal stability. Enzyme $wt^{4/8\ HRP}$ had a 5.5-fold higher $\tau_{1/2\ 60\ °C}$ than enzyme $wt^{wt\ HRP}$ and was even 3.3-fold more stable than the enzyme preparation from plant. The same HRP variants produced in the Δoch1 strain showed a 10-fold reduced thermal stability compared to their respective counterparts from the benchmark strain. Reducing glycosylation to $Man_8$ structures apparently affected stability to a greater extent than completely removing four *N*-glycosylation sites. In fact, mutating the four *N*-glycosylation sites significantly increased stability instead of decreasing it. It is remarkable that enzyme $OCH1^{4/8\ HRP}$ actually showed a similar thermal half-life time as enzyme $wt^{wt\ HRP}$ (Table 7).

Considering both, kinetic parameters and thermal stability, enzyme $wt^{4/8\ HRP}$ might be interesting for future applications in medical diagnostics. This enzyme variant can be efficiently produced in bioreactor cultivations, is less glycosylated, which might allow more controlled and efficient conjugation to antibodies and lectins, still shows considerable catalytic activity and a 5.5-fold higher thermal stability compared to enzyme $wt^{wt\ HRP}$. In fact, higher stability and reduced glycosylation could compensate for reduced catalytic activity.

## 3. Experimental Section

### *3.1. Chemicals*

2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), D(+)-biotin and hemin were purchased from Sigma-Aldrich. Difco™ yeast nitrogen base w/o amino acids (YNB), Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Zeocin™ was obtained from InvivoGen (San Diego, CA, USA). Other chemicals were obtained from Carl Roth (Karlsruhe, Germany).

### *3.2. Strain Generation*

All strains in this study are based on the *P. pastoris* wildtype strain CBS7435. Generation of the Δoch1 strain and the single HRP variants are described in detail in our previous studies [26,27]. In short, the four Asn, representing glycosylation sites of HRP, were mutated by site directed

mutagenesis and subsequent digestion with *Dpn*I. The mutagenic PCR was performed as: 98 °C for 30 s; then 10 cycles of 98 °C for 10 s, 57 °C for 20 s, 72 °C for 1 min-10 cycles of 98 °C for 10 s, 60 °C for 20 s, 72 °C for 1 min-10 cycles of 98 °C for 10 s, 63 °C for 20 s, 72 °C for 1 min; with a final incubation at 72 °C for 10 min. Each reaction contained 1× HF buffer (Fermentas, Waltham, MA, USA), 0.01 μg of plasmid DNA, 2.5 U *Phusion* DNA polymerase (Fermentas), 10 μM of each dNTP and 5 pmol of each primer in a total volume of 50 μL. All primers are listed in Supplementary Table S1 and were purchased from Microsynth (Balgach, Sweden).

After PCR, the methylated template DNA was degraded by digestion with 10 U of *Dpn*I at 37 °C for at least three hours. The remaining PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and 5 μL of each purified PCR product were transformed into electro-competent *E. coli* TOP10 F′ cells. The successful introduction of the desired mutation and the absence of further mutations were confirmed by DNA sequencing (Microsynth). Transformation of approximately 2 μg *Swa*I-linearized pPpT4_S plasmid DNA harbouring the respective mutated HRP gene into the *P. pastoris* benchmark and the Δoch1 strain was done by electroporation. Stable transformants were generated via homologous recombination between the linearized plasmid DNA and genomic yeast DNA. Selection of successfully transformed clones was performed on Yeast Extract Peptone Dextrose medium (YPD; 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 20 g/L agar) supplemented with 100 mg/L Zeocin. In total, we generated and compared four recombinant *P. pastoris* strains producing four different HRP enzyme variants (Table 8).

**Table 8.** Recombinant *P. pastoris* strains and HRP enzyme variants in this study.

| ***P. pastoris* Chassis Strain** | **HRP Variant** | **Name of Recombinant *P. pastoris* Strain and Enzyme** |
|---|---|---|
| benchmark strain | wt HRP | $wt^{wt\ HRP}$ |
| | 4/8 HRP | $wt^{4/8\ HRP}$ |
| Δoch1 strain | wt HRP | $OCH1^{wt\ HRP}$ |
| | 4/8 HRP | $OCH1^{4/8\ HRP}$ |

### *3.3. Bioreactor Cultivations*

#### 3.3.1. Preculture

Precultures were grown in 100 mL YNB_Zeo medium (0.1 M potassium phosphate buffer, pH 6.0; 3.4 g/L YNB w/o amino acids and ammonium sulfate, 10 g/L $(NH_4)_2SO_4$, 400 mg/L biotin, 20 g/L glucose, 100 mg/L Zeocin) in 1000 mL baffled shake flasks at 30 °C and 230 rpm for 24 h.

#### 3.3.2. Dynamic Batch Cultivation

For dynamic batch cultivations, 3582.6 mL 2-fold concentrated basal salt medium (BSM; 26.7 mL/L 85% phosphoric acid, 1.17 g/L $CaSO_4 \cdot 2H_2O$, 18.2 g/L $K_2SO_4$, 14.9 g/L $MgSO_4 \cdot 7H_2O$, 4.13 g/L KOH, 0.3 mL/L Antifoam Struktol J650, 60 g/L glycerol) were sterilized in a 5 L working volume glass bioreactor (Infors, Molndal, Sweden). After sterilization, 4.35 mL PTM1 per litre medium were added (*i.e.*, 17.4 mL for 4 litres) and pH was set to 5.0 with concentrated ammonia solution. Pre-cultures were aseptically transferred to the respective vessel (10% inoculation volume)

and the batch phase on glycerol was carried out at 30 °C with the stirrer fixed at 1300 rpm. Aeration with compressed dry air was set to 1 volume per volume per minute (vvm). Dissolved oxygen ($dO_2$) was measured with a sterilizable VisiFerm DO 225 probe (Hamilton, ON, Canada). The pH was measured with a sterilizable electrode (Mettler Toledo, Greifensee, Switzerland) and maintained constant at pH 5.0. Reactor weight was continuously recorded by a precision balance (Sartorius, Göttingen, Germany). Batch and methanol adaptation were performed at 30 °C. After the complete consumption of glycerol, indicated by an increase of $dO_2$ and a drop in off-gas activity, the first methanol pulse at a final concentration of 0.5% volume per volume (*v*/*v*) was conducted with methanol supplemented with 12 mL/L PTM1. Following pulses were performed with 1% methanol/PTM1 (*v*/*v*) at different temperatures. At 30, 25 and 20 °C at least two pulses were applied, respectively. For each pulse, two samples were taken to determine the concentrations of substrate and product, as well as dry cell weight to calculate specific rates and yields.

#### 3.3.3. Analysis of Growth and Expression Parameters

Dry cell weight (DCW) was determined by centrifugation of 2 × 5 mL fermentation broth (4800 rpm, 10 min, 4 °C), washing the pellet with 5 mL water and subsequent drying to a constant weight at 105 °C. Optical density at 600 nm ($OD_{600}$) was determined in a spectrophotometer (Thermo Scientific, Waltham, MA, USA). Activity of HRP in the cell-free supernatant was determined using a previously described assay in a CuBiAn-XC enzymatic robot (Innovatis, Bielefeld, Germany) [27]. Protein concentration was determined using a Bradford protein assay kit (Thermo Scientific). All growth and protein expression parameters were determined in duplicates.

#### 3.3.4. Analysis of Substrate Concentration

Concentrations of methanol were determined in cell free samples by HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an ion-exchange column (Supelcogel C-610H, Sigma-Aldrich, St. Louis, MO, USA) and a refractive index detector (Agilent Technologies). The mobile phase was 0.1% $H_3PO_4$ with a constant flow rate of 0.5 mL/min and the system was run isocratic at 30 °C. Calibration was done by measuring standard points in the range from 0.1 to 10 g/L methanol. Measurements of biomass, product and substrate concentration were executed in duplicates.

#### 3.3.5. Calculation of Strain Physiological Parameters

The relevant parameters to physiologically characterize the recombinant yeast strains were: carbon dioxide evolution rate (CER; mmol/L/h), adaptation time to methanol (h), specific methanol uptake rate ($q_{MeOH}$; mmol/g/h), biomass yield ($Y_{X/MeOH}$; C-mol/C-mol), carbon dioxide yield ($Y_{CO2/MeOH}$; C-mol/C-mol) and C-balance. Details concerning the calculation of these parameters have been published before [20,21,27].

### *3.4. Protein Purification*

Cell-free cultivation broth was diafiltrated with a Centramate 500S TFF system (Pall) using a 10 kDa MWCO membrane. The buffer was HCIC-A (20 mM NaOAc, 1.0 M NaCl, pH 6.0) and the

protein solution was concentrated to a final volume of 100–120 mL. The HCIC resin MEP HyperCel™ was obtained from Pall (Port Washington, NY, USA) and HCIC was performed in flow-through mode [8,19]: a column containing 25 mL of MEP HyperCel™ resin was equilibrated with at least four column volumes (CV) buffer HCIC-A. The HRP solution in HCIC-A was loaded onto the column that was then washed with at least five CV of HCIC-A at a flow rate of 20 cm/h. Then a step elution to 100% buffer HCIC-B (50 mM Tris, pH 8.0) was performed. After elution, the column was washed with five CV 0.8 M NaOH before it was stored in EtOH 20%, 1.0 M NaCl. During all the steps, fractions were collected and analyzed for protein content and catalytic activity. Fractions showing HRP activity were pooled and concentrated to around 1.5 mL using Amicon Ultra-15 Centrifugal Filter Units with 10 kDa MWCO (Merck Millipore, Darmstadt, Germany).

The efficiency of the purification was evaluated by determining the purification factor (PF; Equation (1)) and the recovery yield of HRP activity in percentage (R%; Equation (2)). The suffixes "pre" and "post" indicate the respective values before and after the HCIC step.

$$PF = \frac{\text{specific activity}_{post}}{\text{specific activity}_{pre}} \quad (1)$$

$$R\% = \frac{\text{volumetric activity}_{post} * \text{volume}_{post}}{\text{volumetric activity}_{pre} * \text{volume}_{pre}} \quad (2)$$

Additionally, Reinheitszahl values (RZ; $A_{404}/A_{280}$) of the concentrated enzyme preparations were measured. Absorbance at 280 and 404 nm were determined in a quartz cuvette in a spectrophotometer (UV-1601; Shimadzu, Long Beach, CA, USA).

### *3.5. SDS-PAGE*

Apparent sizes and purities of produced HRP variants were followed by SDS-PAGE according to the Laemmli protocol [28]. Electrophoresis was done using an Amersham ECL Gel 8%–16% gel (GE Healthcare, Buckinghamshire, UK) in 1× Tris-glycine buffer. Before loading, the gel had to be pre-run at 160 V for 12 min. Protein separation was performed at 140 V for about 2 h. BLUeye Prestained Protein Ladder (GeneDirex, Taoyuan County, Taiwan) was used as protein mass standard. Gels were stained with Coomassie Blue sensitive stain.

### *3.6. Protein Identification and Peptide Analysis Using LC-ESI-MS*

The relevant protein bands were cut out from the SDS gel and digested in gel. S-alkylation with iodoacetamide and digestion with sequencing grade modified trypsin (Promega, Madison, WI, USA) were performed. The peptide mixture was analysed using a Dionex Ultimate 3000 system directly linked to an ion trap instrument (amaZon speed ETD, Bruker, Billerica, NA, USA) equipped with the standard ESI source in the positive ion, DDA mode (=switching to MSMS mode for eluting peaks). MS-scans were recorded (range: 400–1600 *m*/*z*; icc target: 100,000; max. accu time: 200 ms) and the 12 highest peaks were selected for fragmentation. Instrument calibration was performed using ESIcalibration mixture (Agilent). For separation of the peptides a Thermo BioBasic C18 separation column (5 µm particle size, 150 × 0.360 mm) was used. A gradient from 97% solvent A and 3% solvent B (Solvent A: 65 mM ammonium formiate buffer, B: 100% ACCN) to 32% B in 45 min

was applied, followed by a 15 min gradient from 32% B to 75% B, at a flow rate of 6 µL/min. The analysis files were converted using Data Analysis 4.0 (Bruker) to XML files, which are suitable to perform MS/MS ion searches with MASCOT (embedded in ProteinScape 3.0, Bruker) for protein identification. Only proteins identified with at least 2 peptides with a protein score higher than 80 were accepted. For the searches the SwissProt database was used. Peptide MS/MS data were evaluated against the target sequence using X! Tandem (www.thegpm.org/tandem/) with the following settings: reversed sequences no; check parent ions for charges 1, 2 and 3 yes; models found with peptide $\log_e$ lower −1 and proteins $\log_e$ lower −1; residue modifications: oxidation M, W and deamidation N, Q; isotope error was considered; fragment type was set to monoisotopic; refinement was used with standard parameters; fragment mass error of 0.3 Da and ± 50 ppm parent mass error; fragment types b and y ions; maximum parent ion charge of 3; missed cleavage sites allowed was set to 1; semi-cleavage yes.

### *3.7. Biochemical Enzyme Characterization*

We determined the basic kinetic parameters $K_m$ and $v_{max}$ for the substrates ABTS and $H_2O_2$ for the different HRP variants in a spectrophotometer UV-1601 (Shimadzu). The reaction mixture with a final volume of 1.0 mL contained 20 µL of HRP variant, 50 mM potassium phosphate buffer, pH 6.5, and either varying concentrations of ABTS (0.01–10 mM) and a saturating concentration of $H_2O_2$ of 1.0 mM or varying concentrations of $H_2O_2$ (0.001–1.0 mM) and a saturating concentration of ABTS of 10.0 mM, respectively. The increase in absorption was followed at 420 nm at 30 °C for 180 s. Absorption curves were recorded with a software program (UVPC Optional Kinetics software; Shimadzu). The maximum reaction rate ($v_{max}$) and the Michaelis constant ($K_m$) were calculated with the software Sigma Plot (Systat Software Inc., Chicago, IL, USA).

The thermal stability of individual HRP variants was tested at 60 °C. The residual activity with ABTS was measured after 1, 5, 10, 15, 30, 45, 60, 90 and 120 min of incubation at 60 °C in a PCR thermoblock. Protein concentrations were normalized to 0.1 mg/mL in Bis-Tris buffer (50 mM Bis-Tris, pH 6.5) to guarantee comparability. Residual activities were plotted *versus* the incubation time and the half-life times of thermal inactivation at 60 °C ($\tau$½) were calculated using Equation (3):

$$\tau_{1/2} = \frac{\ln 2}{k_{in}} \quad (3)$$

$k_{in}$: rate of inactivation (slope of the logarithmic residual activity).

## 4. Conclusions

In this study we combined protein and strain engineering to obtain an active and stable recombinant HRP variant with reduced surface glycosylation. We combined four mutations, which individually had shown beneficial effects on either catalytic activity or thermal stability before, and expressed this enzyme variant as well as the unmutated wildtype enzyme in both an unmodified *P. pastoris* benchmark strain and a Δoch1 strain. Our results can be summarized as:

- Production of the 4/8 HRP variant caused a physiological burden for the *P. pastoris* benchmark strain slowing down its metabolism. In contrast, production of the same enzyme variant did not

affect physiology of the Δoch1 strain. While $q_{s\ MeOH}$ increased with increasing temperature for the benchmark strain, the opposite was true for the Δoch1 strain. Based on strain physiological parameters identified in dynamic batch experiments, fed-batch strategies can be easily designed for future production of these enzyme variants.

- Reduced and missing surface glycosylation did not affect substrate affinity of HRP, but significantly reduced catalytic activity.
- Introducing the four mutations into HRP significantly boosted thermal stability. In fact, the 4/8 HRP variant produced in the *P. pastoris* benchmark strain even showed a 3.3-fold increased thermal half-life time compared to the HRP preparation from plant.
- Considering both, enzyme activity and thermal stability, the 4/8 HRP variant produced in the *P. pastoris* benchmark strain might be interesting for future applications in medical diagnostics. This enzyme variant can be efficiently produced in the bioreactor, shows considerable catalytic activity and high thermal stability and is less glycosylated, which might allow more controlled and efficient conjugation to antibodies and lectins.

In this study we show that enzymes can be modified not only by mutation, but that a combination of both strain and protein engineering is a useful way to obtain enzyme variants tailored to specific needs.

## Supplementary Materials

Supplementary materials can be found at http://www.mdpi.com/1422-0067/16/10/23127/s1.

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## Author Contributions

Simona Capone, Lejla Ćorajević, Günther Bonifert and Patrick Murth performed experiments. Oliver Spadiut was responsible for experimental design. Daniel Maresch and Friedrich Altmann conducted mass spectrometry analyses. Oliver Spadiut and Christoph Herwig initiated, planned and supervised the study. Simona Capone and Oliver Spadiut wrote the manuscript. All authors read and approved the manuscript.

## Conflicts of Interest

The authors declare no conflict of interest.

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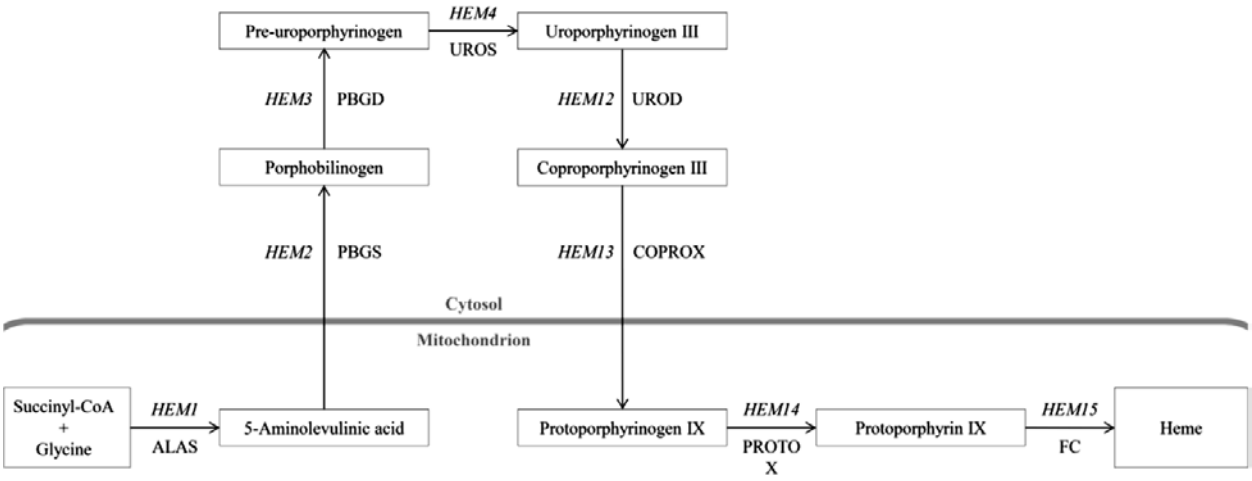
## Chapter IV – Increasing product specific cofactor availability

**"Optimizing cofactor availability for the production of recombinant heme peroxidase in *Pichia pastoris*"**

Krainer, F.W., et al., Microbial Cell Factories, 2015. 14: p. 4.



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MICROBIAL CELL FACTORIES

TECHNICAL NOTES Open Access

# Optimizing cofactor availability for the production of recombinant heme peroxidase in *Pichia pastoris*

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## Abstract

**Background:** Insufficient incorporation of heme is considered a central impeding cause in the recombinant production of active heme proteins. Currently, two approaches are commonly taken to overcome this bottleneck; metabolic engineering of the heme biosynthesis pathway in the host organism to enhance intracellular heme production, and supplementation of the growth medium with the desired cofactor or precursors thereof to allow saturation of recombinantly produced apo-forms of the target protein. In this study, we investigated the effect of both, pathway engineering and medium supplementation, to optimize the recombinant production of the heme protein horseradish peroxidase in the yeast *Pichia pastoris*.

**Results:** In contrast to studies with other hosts, co-overexpression of genes of the endogenous heme biosynthesis pathway did not improve the recombinant production of active heme protein. However, medium supplementation with hemin proved to be an efficient strategy to increase the yield of active enzyme, whereas supplementation with the commonly used precursor 5-aminolevulinic acid did not affect target protein yield.

**Conclusions:** The yield of active recombinant heme peroxidase from *P. pastoris* can be easily enhanced by supplementation of the cultivation medium with hemin. Thereby, secreted apo-species of the target protein are effectively saturated with cofactor, maximizing the yield of target enzyme activity.

**Keywords:** *Pichia pastoris*, Recombinant protein production, Plant peroxidase, Horseradish peroxidase, Metabolic engineering, Cofactor, Heme, Heme biosynthesis, Apo-protein

## Background

The methylotrophic yeast *Pichia pastoris* is a valuable host for the recombinant production of complex proteins. A considerable number of these proteins requires cofactors, amongst others heme, to form active biocatalysts. Heme biosynthesis (Figure 1) is tightly regulated and highly conserved throughout evolution [1-3].

To deepen our understanding on pathway regulation and improve cofactor availability for recombinant heme proteins, metabolic engineering of the heme biosynthesis pathway has been performed in *Aspergillus niger* [4,5], *Escherichia coli* [6] and *Saccharomyces cerevisiae* [7-9]. Despite the high conservation of the heme biosynthesis pathway, distinct differences were found among the different species. In *E. coli*, formation of 5-aminolevulinic acid (ALA) by the *HEM1*-encoded ALA synthase was described as rate-limiting step [10], whereas in *S. cerevisiae* the *HEM2*- and *HEM3*-encoded porphobilinogen synthase and deaminase, respectively, were described as rate-limiting [7]. Based thereon, overexpression of *HEM3* alone or in combination with *HEM2* and *HEM12* was described to be a valuable strategy to augment the production of recombinant heme proteins in *S. cerevisiae* [9,11]. Despite these promising results, there are some potential pitfalls connected to metabolic pathway engineering, such as the additional metabolic burden upon overexpression of multiple genes as well as an accumulation of free intracellular porphyrin intermediates which can lead to the formation of reactive oxygen species [12]. Thus, medium supplementation with iron, heme precursors or hemin (the ferric chloride species of heme) was assessed as alternative

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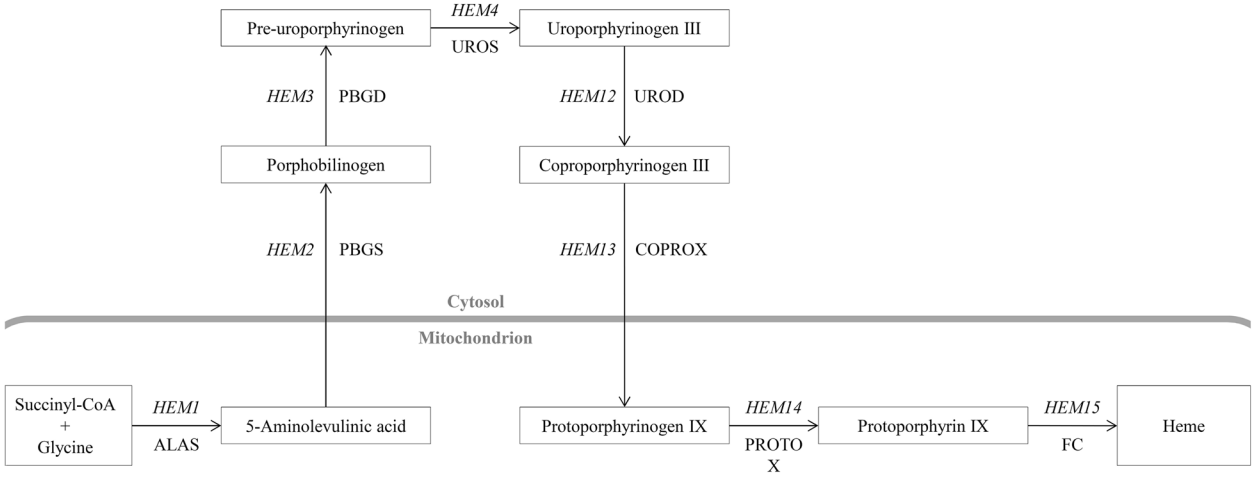


**Figure 1 Scheme of the heme biosynthesis pathway.** ALAS, 5-aminolevulinic acid synthase; PBGS, porphobilinogen synthase; PBGD, porphobilinogen deaminase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen III decarboxylase; COPROX, coproporphyrinogen III oxidase; PROTOX, protoporphyrinogen IX oxidase; FC, ferrochelatase.

strategy to effectively saturate recombinant apo-species of heme proteins [13-17].

In this study, we aimed at optimizing heme availability and thus boost the amount of recombinant active heme protein, namely horseradish peroxidase (HRP), in the yeast *P. pastoris* by evaluating pathway engineering and medium supplementation. In a systematic approach, we co-overexpressed HRP with genes of the endogenous heme biosynthesis pathway of *P. pastoris*, and assessed the effect of medium supplementation with iron, ALA and hemin on the yield of target enzyme activity.

## Results and discussion

### Heme biosynthesis pathway in *Pichia pastoris*

The heme biosynthesis pathway of several organisms has been described before and was found to be highly conserved [1-3]. Based on the *HEM* gene sequences from *S. cerevisiae*, we identified the corresponding homologs in the partially annotated genome of *P. pastoris* [18] *in silico* (Table 1). Sanger sequencing confirmed the correct nucleotide sequences of *HEM1*, *HEM2*, *HEM3*, *HEM4*, *HEM12*, *HEM13* and *HEM14*. For *HEM15*, nucleotide 918 was G in the GenBank database entries of the published genomes of *P. pastoris* strains CBS 7435 and GS155, but T in the Sanger-sequenced CBS 7435 *P. pastoris* strain used in the present study (Additional file 1). However, this single nucleotide polymorphism only resulted in a silent mutation and did not alter the amino acid sequence of the encoded protoheme ferro-lyase.

### Co-overexpression of *HEM* genes in microscale cultivations

In a recent study it was shown that metabolic engineering of the heme biosynthesis pathway allowed higher yields of active recombinant heme protein in *S. cerevisiae* [11]. Thus, we co-overexpressed either of the eight involved

**Table 1 *In silico* identification of *HEM* homologs in *P. pastoris***

| *S. cerevisiae* | *P. pastoris* | | | Sequence identity [%] |
|---|---|---|---|---|
| Gene annotation | Gene annotation | Chromosome | COG annotation | |
| *HEM1* | *HEM1* | II | 5-aminolevulinate synthase | 67.7 |
| *HEM2* | *HEM2* | IV | delta-aminolevulinic acid dehydratase | 75.3 |
| *HEM3* | *HEM3* | I | porphobilinogen deaminase | 54.0 |
| *HEM4* | *HEM4* | II | uroporphyrinogen-III synthase | 42.3 |
| *HEM12* | n/a | III | uroporphyrinogen decarboxylase | 73.2 |
| *HEM13* | *HEM13* | III | coproporphyrinogen III oxidase | 65.5 |
| *HEM14* | n/a | IV | protoporphyrinogen oxidase | 33.7 |
| *HEM15* | n/a | III | protoheme ferro-lyase (ferrochelatase) | 61.2 |

Genes *HEM12*, *HEM14* and *HEM15* were not annotated (n/a) for *P. pastoris* CBS 7435. Their chromosomal position in the genome, eukaryotic cluster of orthologous groups (COG; [19]) annotations and identities of the encoded amino acid sequences from *S. cerevisiae* and *P. pastoris* are shown.

*HEM* genes (Table 1) using the strong constitutive promoter P*GAP* [20] in a *P. pastoris* strain recombinantly producing the heme protein HRP. The screenings revealed trends of co-overexpressed *HEM1* and *HEM3* to be potentially beneficial for the production of active HRP (Figure 2). Surprisingly, there also seemed to be a negative trend upon co-overexpression of *HEM4*. We hypothesize, that *HEM4* co-overexpression might have led to the accumulation of a cytotoxic intermediate, which increased intracellular stress and ultimately caused a disadvantageous production environment for HRP under the tested conditions. At this point, we did not follow up on the effects of *HEM4* co-overexpression. Since co-overexpression of eGFP as negative control did not affect HRP productivity, the observed activity-enhancing trends seen for *HEM1* and *HEM3* were considered a consequence of the co-overexpressed *HEM* gene, although the standard deviation in these experiments was high. The high standard deviations in this screening (Figure 2) were caused by the transformant-to-transformant variation typically observed for *P. pastoris* [21]. Even the transformation of a single gene results in transformants showing different expression strengths. The majority of the transformants shaping the landscape behaved similar but a few transformants showed either no expression or elevated levels, leading to high standard deviations. Supplementation of the cultivation medium with 1 mM $FeSO_4$ to alleviate a potential iron limitation gave comparable results to non-supplemented medium and did not affect the observed trends upon *HEM* gene co-overexpression (data not shown).

In addition to strong constitutive co-overexpression of either *HEM1* or *HEM3* from P*GAP*, we also tested co-overexpression of these two genes from either P*AOX1* or P*CAT1*. Both promoters are strongly methanol-inducible, however P*CAT1* is already active in the absence of glucose or glycerol and then even further induced by addition of methanol, thereby allowing *HEM* co-overexpression already prior to, but also during HRP production [Vogl *et al.*, manuscript in preparation]. In order to indirectly assess the general functionality and applicability of the employed co-overexpression construct, we measured fluorescence of the eGFP co-overexpression control transformants and found this co-overexpression partner to be successfully produced (Additional file 2). Thus, we considered the endogenous *HEM* co-overexpression partners to be co-overexpressed in a comparable fashion. Ultimately, P*AOX1*-regulated co-overexpressions of *HEM1* and *HEM3* with HRP were found most promising (Additional file 3) and the best performing strains were used for further characterizations.

Since the data from microscale cultivations indicated merely trends for potential beneficial effects of co-overexpressed *HEM1* or *HEM3*, we aimed at obtaining more reliable data from controlled cultivations of the best performing strains in bioreactors. Prior to bioreactor cultivations, the strain producing HRP alone (hereafter called benchmark

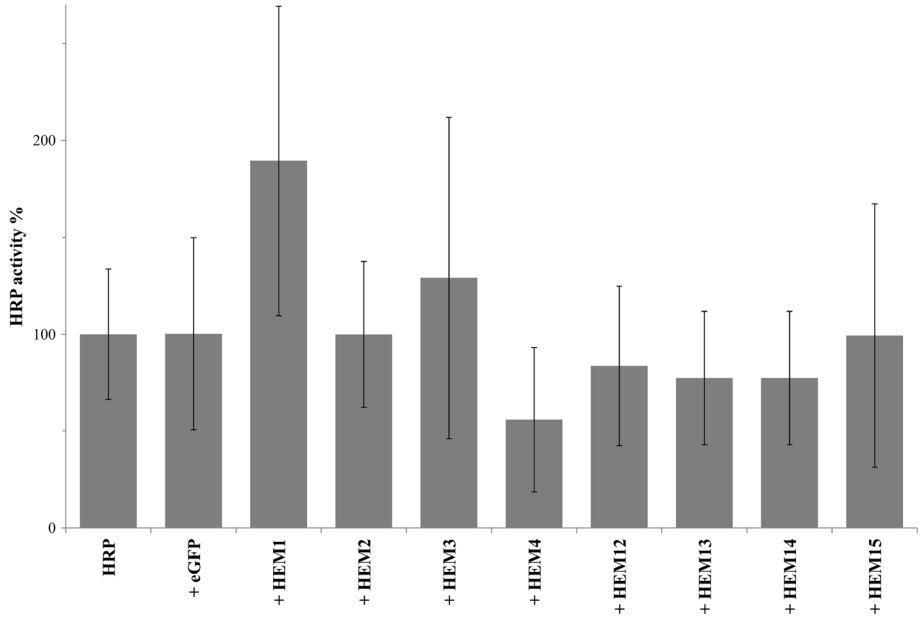


**Figure 2 Co-overexpressions of *HEM* genes with HRP.** HRP overexpression was regulated by P*AOX1*, co-overexpressions of eGFP and *HEM* were regulated by P*GAP*. Bars are average values of HRP production landscapes from microscale cultivations using 1 mM $FeSO_4$-supplemented medium. Error bars are standard deviations from all measured clones of a landscape. Average activity from strains producing HRP alone was set to 100%.

strain) as well as the *HEM1* and *HEM3* co-overexpressing strains (hereafter called HEM1 and HEM3 strains) were characterized by quantitative real-time PCR as strains with single copy integration of the HRP gene. Since these strains contained the same HRP gene dosage, genomic rearrangement of HRP gene copy number upon transformation of a *HEM* co-overexpression construct was excluded and the strains were regarded comparable in terms of target gene dosage.

### Co-overexpression of *HEM* genes in bioreactor cultivations

For physiological strain characterisation of the three yeast strains we employed a previously reported strategy of dynamic batch cultivations with methanol pulses [22-25]. The corresponding off-gas signals, specific uptake rates and yields are shown in Additional file 4. Physiological strain characteristic parameters of the three strains are summarized in Table 2. As shown in Table 2, C-balances for all cultivations closed to 1.0 indicating solid data quality. Apparently, the yeast strains were physiologically not impaired by co-overexpression of either *HEM1* or *HEM3* since adaptation time to methanol, yields and specific substrate uptake rates were comparable. However, co-overexpressing these genes did not boost the production of active HRP either. Whereas the benchmark and the HEM1 strain showed comparable production titres, the HEM3 strain even showed a 50% lower specific and volumetric productivity than the benchmark strain. Taken together, it turned out that the trends seen in microscale data could not be seen in the bioreactor. A possible explanation for these diverging findings could be found in the considerably different cultivation conditions. Microscale cultivations may challenge cell growth and productivity by phases of $O_2$ shortage or starvation, whereas a bioreactor provides optimal cultivation conditions.

Considering the complex and so far poorly understood regulation of heme biosynthesis, a multitude of single or combined factors may have either positive or negative effects on this pathway. Thus, additional studies will be required to unravel the regulation of heme biosynthesis, in order to allow non-speculative conclusions. Since our selection of *HEM* co-overexpression partners was based on trends from microscale cultivations, we cannot exclude, that either of the remaining six *HEM* genes or combinations thereof might yield more beneficial results than co-overexpressed *HEM1* or *HEM3* in bioreactor cultivations. Ultimately, in contrast to analogous studies in *S. cerevisiae* [9,11], metabolic pathway engineering in *P. pastoris* did not prove to be a useful strategy to allow higher titres of recombinant active heme protein, suggesting distinct differences of this pathway between the two yeasts. However, co-overexpression of two or more *HEM* genes from a library of promoters of varying strengths might still enhance endogenous heme biosynthesis in *P. pastoris* and will be assessed in future studies.

### Medium supplementation

#### *Medium supplementation in microscale cultivations*

We tested ALA, $FeSO_4$ and hemin as medium supplements in microscale cultivations of the benchmark strain. Supplementation with ALA at an excess concentration of 1 mM, as reported in literature [22,26,27], did not result in measurably more active product. However, addition of 1 mM $FeSO_4$ boosted the amount of active HRP up to 7-fold (Figure 3), hinting at a potential shortage of iron in heme biosynthesis. Upon supplementation of the minimal medium with different concentrations of hemin, we even measured 18-fold increased HRP activity compared to non-supplemented conditions. We also investigated the effect in case both, $FeSO_4$ and hemin, were supplemented concomitantly and observed that the beneficial effect of $FeSO_4$ became less pronounced with increasing concentrations of hemin (Figure 3). Apparently, as all apo-HRP was readily saturated with cofactor at a hemin concentration of 10 μM, additional excess of iron did not improve HRP activities any further and the cofactor bottleneck was opened up.

#### *ALA supplementation in the bioreactor*

We performed a comparative cultivation experiment in the bioreactor to confirm the results obtained in microscale cultivations that the commonly used supplement ALA [22,26,27] does not affect the production of a recombinant active heme protein in *P. pastoris*. We cultivated the benchmark strain in parallel dynamic batch cultivations with and without the presence of 1 mM ALA. Table 3 shows that both, strain physiology and productivity, were

**Table 2 Strain characteristic physiological parameters determined for the benchmark, HEM1 and HEM3 strain**

| Strain | $\Delta time_{adapt}$ [h] | $Y_{X/MeOH}$ [C-mol/C-mol] | $Y_{CO2/MeOH}$ [C-mol/C-mol] | C-balance | $q_{MeOH}$ [mmol/g/h] | $q_p$ [U/g/h] | $r_p$ [U/L/h] |
|---|---|---|---|---|---|---|---|
| Benchmark | 8.2 | 0.02 | 1.00 | 1.02 | 0.75 | 1.11 | 37.5 |
| HEM1 | 8.2 | 0.04 | 0.94 | 0.98 | 0.69 | 1.10 | 35.9 |
| HEM3 | 7.9 | 0.03 | 0.93 | 0.96 | 0.70 | 0.49 | 17.1 |

$\Delta time_{adapt}$, time for adaptation of the culture to methanol; $Y_{X/MeOH}$, $Y_{CO2/MeOH}$, yields of biomass or $CO_2$ per C-mol of substrate methanol; C-balance, sum of $Y_{X/MeOH}$ and $Y_{CO2/MeOH}$ which ideally should result in 1.0; $q_{MeOH}$, average specific uptake rate of methanol during consecutive methanol pulses; $q_p$, specific HRP productivity; $r_p$, volumetric HRP productivity calculated from the point of induction until the end of cultivation.

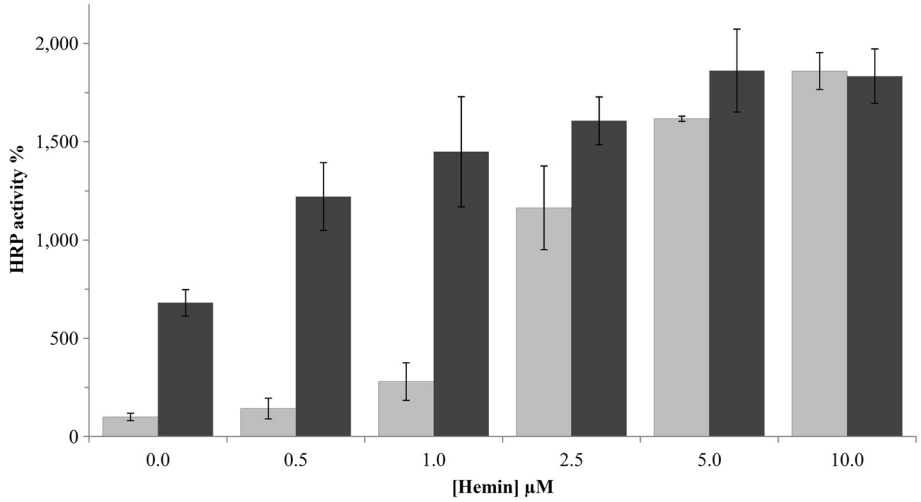


**Figure 3 Effect of medium supplementation with $FeSO_4$ and hemin on HRP activity in microscale cultivations.** Volumetric HRP activity from minimal medium without supplementation was set to 100%. Bars are average values ± SD from independent triplicate cultivations. Light gray, no $FeSO_4$; dark gray, 1 mM $FeSO_4$.

not affected by the presence of ALA. Since the amount of active HRP was the same for both conditions, we concluded that ALA was not beneficial for the activation of the produced apo-HRP. This observation is different from what was described previously by Morawski *et al*., where a 32% increase in HRP activity was measured in the culture supernatant after supplementation with ALA, trace elements and thiamine [17]. Thus, we speculate that the activation effect observed by Morawski *et al*. was rather caused by the addition of the iron-containing trace element solution than by ALA. Furthermore, our results conclusively showed that the intracellular production of ALA in the heme biosynthesis pathway of *P. pastoris* was not the rate limiting step in contrast to *E. coli* [10], which is also in agreement with the study of Arrese *et al.* [28].

### *Optimization of hemin supplementation in the bioreactor*

Based on the results obtained in microscale cultivations, we supplemented the cultivation broth of three parallel dynamic batch cultivations of the benchmark strain with 0.1, 1.0 or 10.0 μM hemin. As shown in Table 4, adaptation time, yields and uptake rates of the benchmark strain were not affected by the presence of hemin. In terms of productivity, both the specific and the volumetric productivity were doubled at a concentration of 10.0 μM hemin. Since the total extracellular protein content was comparable in all three cultivations, we concluded that not the productivity of the yeast was altered, but rather that the produced apo-protein was activated posttranslationally by hemin in the cultivation broth. Based on our previous experiences with this expression system [22,23,29,30], we estimated the amount of HRP in the cultivation broth to be < 3 μM. Thus, we concluded that an excess hemin concentration is required to effectively saturate secreted apo-HRP (Table 4).

### Activation studies with hemin

To prove our hypothesis of a posttranslational conversion of apo-HRP to holo-HRP by hemin, we incubated cell-free, sterile-filtered supernatant from a non-supplemented benchmark cultivation with different concentrations of hemin and analyzed activity over time. As shown in Table 5, already after 5 min of incubation a concentration dependent activation was observed and after 72 h of incubation the initial activity was even doubled. Based thereon,

**Table 3 Strain specific physiological parameters of the benchmark strain with and without ALA supplementation**

| ALA [1 mM] | $\Delta time_{adapt}$ [h] | $Y_{X/MeOH}$ [C-mol/C-mol] | $Y_{CO2/MeOH}$ [C-mol/C-mol] | C-balance | $q_{MeOH}$ [mmol/g/h] | $q_p$ [U/g/h] | $r_p$ [U/L/h] |
|---|---|---|---|---|---|---|---|
| + | 8.1 | 0.03 | 0.95 | 0.98 | 0.73 | 1.08 | 36.7 |
| - | 8.2 | 0.04 | 0.94 | 0.98 | 0.76 | 1.09 | 37.2 |

$\Delta time_{adapt}$, time for adaptation of the culture to methanol; $Y_{X/MeOH}$, $Y_{CO2/MeOH}$, yields of biomass or $CO_2$ per C-mol of substrate methanol; C-balance, sum of $Y_{X/MeOH}$ and $Y_{CO2/MeOH}$ which ideally should result in 1.0; $q_{MeOH}$, average specific uptake rate of methanol during consecutive methanol pulses; $q_p$, specific HRP productivity; $r_p$, volumetric HRP productivity calculated from the point of induction until the end of cultivation.

**Table 4 Strain characteristic physiological parameters determined for the benchmark strain cultivated in hemin-supplemented media**

| Hemin [μM] | $\Delta time_{adapt}$ [h] | $Y_{X/MeOH}$ [C-mol/C-mol] | $Y_{CO2/MeOH}$ [C-mol/C-mol] | C-balance | $q_{MeOH}$ [mmol/g/h] | $q_p$ [U/g/h] | $r_p$ [U/L/h] |
|---|---|---|---|---|---|---|---|
| 0.1 | 6.5 | 0.04 | 0.89 | 0.93 | 0.69 | 1.10 | 34.3 |
| 1.0 | 6.4 | 0.04 | 0.93 | 0.97 | 0.71 | 1.26 | 36.8 |
| 10.0 | 6.5 | 0.03 | 0.89 | 0.96 | 0.70 | 2.35 | 73.2 |

$\Delta time_{adapt}$, time for adaptation of the culture to methanol; $Y_{X/MeOH}$, $Y_{CO2/MeOH}$, yields of biomass or $CO_2$ per C-mol of substrate methanol; C-balance, sum of $Y_{X/MeOH}$ and $Y_{CO2/MeOH}$ which ideally should result in 1.0; $q_{MeOH}$, average specific uptake rate of methanol during consecutive methanol pulses; $q_p$, specific HRP productivity; $r_p$, volumetric HRP productivity.

we concluded that hemin should be present in excess to allow effective apo-protein activation.

## Conclusions

In this study we present a systematic approach to optimize heme availability and thus boost the amount of active heme protein produced in the yeast *P. pastoris* by evaluating metabolic pathway engineering and medium supplementation. The results can be summarized as:

- The heme biosynthesis pathway of *P. pastoris* was analyzed and corresponding genes were identified and annotated *in silico*.
- In contrast to previous studies, overexpression of single *HEM* genes did not result in enhanced activity or higher yield of the model heme protein HRP in bioreactor cultivations of recombinant *P. pastoris* strains. However, combinations of *HEM* genes co-overexpressed from a library of differently regulated promoters might still enhance endogenous heme biosynthesis of *P. pastoris*.
- Medium supplementation with the traditionally used and pricy heme precursor ALA did not increase the yield of active product and can be omitted in future cultivations. $FeSO_4$ and hemin on the other hand turned out to be useful medium supplements to increase the yield of active heme protein.
- Hemin was identified as the most effective supplement. It activated the secreted model heme protein posttranslationally in the cultivation broth and should be added in moderate excess to effectively saturate secreted apo-species of the target protein.

**Table 5 Posttranslational activation of apo-HRP with hemin**

| Hemin [μM] | Specific activity [U/mg] after 5 min of incubation | Specific activity [U/mg] after 72 h of incubation |
|---|---|---|
| - | 34.8 | 35.3 |
| 1.0 | 42.4 | 67.0 |
| 5.0 | 46.8 | 75.4 |
| 10.0 | 51.8 | 77.8 |

Cell-free cultivation supernatant was incubated with the indicated concentrations of hemin and volumetric HRP activity was measured after 5 min and 72 h.

The results shown in this study present a guideline for the successful recombinant production of active heme protein in the yeast *P. pastoris* and offer an easy-to-do solution to maximize the ratio of holo- over apo-protein resulting in a considerable increase of active target protein.

## Methods

### Chemicals

2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), D(+)-biotin and hemin were purchased from Sigma-Aldrich (Austria). Difco™ yeast nitrogen base (YNB) w/o amino acids, Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson (Austria). Zeocin™ was obtained from InvivoGen (France). Other chemicals were obtained from Carl Roth (Germany).

#### *Pichia pastoris strains*

All strains in this study were based on the *P. pastoris* wildtype strain CBS 7435 (identical to NRRL Y-11430 and ATCC 76273). The $Mut^S$ phenotype has been found previously to be superior over the $Mut^+$ phenotype for recombinant HRP production in terms of volumetric productivity and production efficiency [24]. Hence, an *aox1* deletion strain (*Pp*MutS) was used as starting strain [31]. Homologs of the *HEM* genes of *S. cerevisiae* were identified by BLAST searches [32] in the published genome sequence of *P. pastoris* CBS 7435 [18] and the sequences were verified by Sanger sequencing (Microsynth, Austria). Sequence identities of the *HEM* gene products from *S. cerevisiae* and *P. pastoris* were determined on the LALIGN server.

The HRP expression construct was based on the pPpT4_Alpha_S vector [31] where HRP C1A expression was under control of the promoter of the *AOX1* gene (P*AOX1*). Transformants were identified by Zeocin™ resistance. The *S. cerevisiae* MATα prepro signal peptide facilitated HRP secretion. All *HEM* co-overexpression constructs were based on the plasmid pPpKan_S [32], harboring a kanamycin resistance gene for selection and the strongly methanol-inducible P*AOX1* for gene expression. Also, the promoters P*GAP* and P*CAT1* [Vogl *et al.*, manuscript in preparation] were tested for co-overexpression. Transformation of the HRP expression cassette to *Pp*MutS and

of the co-overexpression constructs to the *Pp*MutS-based HRP production strain (called benchmark strain) was performed as described previously [24]. All eight *HEM* genes of the heme biosynthesis pathway were co-overexpressed separately and their influence on HRP production was studied by cultivation of approximately 80 transformants per co-overexpression construct in 96-deep well plates (DWP). As negative co-overexpression control, eGFP was co-overexpressed with HRP. The copy number of the HRP gene in selected strains was determined by quantitative real-time PCR according to a previous protocol [33] and as described recently [24].

### Microscale cultivations in 96-deep well plates

Microscale cultivations in 96-DWPs were performed similar to [34]: Strains were grown in 250 μL BMD1% (11 g/L alpha-D(+)-glucose monohydrate, 13.4 g/L YNB, 0.4 mg/L D(+)-biotin, 0.1 M potassium phosphate buffer, pH 6.0) at 28°C, 320 rpm, 80% humidity. After approximately 60 h, an induction pulse of 250 μL BMM2 (1% (v/v) methanol, 13.4 g/L YNB, 0.4 mg/L D(+)-biotin, 0.1 M potassium phosphate buffer, pH 6.0) was added, followed by three pulses of 50 μL BMM10 (5% (v/v) methanol, 13.4 g/L YNB, 0.4 mg/L D(+)-biotin, 0.1 M potassium phosphate buffer, pH 6.0) per well 12, 24 and 36 h after the first pulse. HRP activity was determined by mixing 15 μL supernatant with 140 μL assay solution (1 mM ABTS, 0.9 mM $H_2O_2$, 50 mM sodium acetate, pH 4.5) in a microtiter plate and following the increase in absorbance at 405 nm on a Spectramax Plus 384 platereader (MolecularDevices, Germany) at room temperature. Medium supplementation studies were performed by additions of ALA, $FeSO_4$ and hemin to BMD1% from a 100 mM ALA stock, a 100 mM $FeSO_4$ stock and a 500 μM hemin stock (in 10 mM KOH), respectively.

### Bioreactor cultivations

#### *Preculture*

Precultures were grown in YNB medium (0.1 M potassium phosphate buffer, pH 6.0; 3.4 g/L YNB w/o amino acids and ammonium sulfate; 10 g/L $(NH_4)_2SO_4$; 400 mg/L biotin; 20 g/L glucose) in 1 L shake flasks at 30°C and 230 rpm for 24 h.

#### *Dynamic batch cultivations*

For dynamic batch cultivations, 1.8 L of double concentrated basal salt medium (BSM; 26.7 mL/L 85% phosphoric acid; 1.17 g/L $CaSO_4 \cdot 2H_2O$; 18.2 g/L $K_2SO_4$; 14.9 g/L $MgSO_4 \cdot 7H_2O$; 4.13 g/L KOH; 0.3 mL/L Antifoam Struktol J650; 60 g/L glycerol) were sterilized in 3 L bioreactor vessels (DR03F; DASGIP, Switzerland). After sterilization, 4.35 mL/L trace element solution (PTM1; 6.0 g/L $CuSO_4 \cdot 5H_2O$; 0.08 g/L NaI; 3.0 g/L $MnSO_4 \cdot H_2O$; 0.2 g/L $Na_2MoO_4 \cdot 2H_2O$; 0.02 g/L $H_3BO_3$; 0.5 g/L $CoCl_2$; 20.0 g/L $ZnCl_2$; 65.0 g/L $FeSO_4 \cdot 7H_2O$; 0.2 g/L biotin; 5 mL/L $H_2SO_4$) were added and pH was set to 5.0 with concentrated ammonia solution. The precultures were transferred aseptically to the respective vessel (the inoculation volume was 10%) and a batch phase on glycerol was carried out at 30°C with the stirrer fixed at 900 rpm. Aeration with compressed dry air was set to 1 vvm. Dissolved oxygen ($dO_2$) was measured with a sterilizable VisiFerm DO 225 probe (Hamilton, Switzerland) and controlled to be higher than 20%. The pH was measured with a sterilizable electrode (Mettler Toledo, Switzerland) and maintained constant at pH 5.0. Reactor weight was continuously recorded by a precision balance (Sartorius, Germany). Following complete glycerol consumption as indicated by an increase in the offgas signal, temperature was lowered to 25°C and an adaptation pulse of 0.5% (v/v) methanol (containing 4.35 mL/L PTM1) was added. After adaptation, 1.0% (v/v) methanol was pulsed repeatedly (for an example see Additional file 4). Before and after each pulse samples were taken and analyzed for $OD_{600}$, dry cell weight, HRP activity, extracellular protein content and methanol concentration.

### Analysis of growth and expression parameters

Dry cell weight (DCW) was determined by centrifugation of 5 mL fermentation broth (4500 rpm, 10 min, 4°C), washing the pellet with 5 mL water and subsequent drying to a constant weight at 105°C. HRP activity in the cell-free supernatant was determined using a previously described assay [23] in a CuBiAn-XC enzymatic robot (OptoCell, Germany). Protein concentration was determined using a Bradford protein assay kit (Thermo Scientific, USA). All growth and protein expression parameters were determined in duplicates.

### Analysis of substrate concentration

Methanol concentration in the cell-free supernatant was determined by HPLC as described previously [22]. Glycerol concentration was measured from cell-free samples in the CuBiAn-XC enzymatic robot. The device was calibrated with water-diluted glycerol standards ranging from 0 to 0.25 g/L. Samples with higher glycerol concentrations were automatically diluted by the system.

### Calculation of strain physiological characteristics

Physiologically relevant parameters for characterization of the different yeast strains cultivated at different conditions and for quantification of the bioprocess were: Carbon dioxide evolution rate (CER; mmol/L/h), biomass yield ($Y_{X/MeOH}$; C-mol/C-mol), carbon dioxide yield ($Y_{CO2/MeOH}$; C-mol/C-mol), C-balance, specific methanol uptake rate ($q_{MeOH}$; mmol/g/h), specific productivity ($q_p$; U/g/h), volumetric productivity ($r_p$; U/L/h), specific activity (U/mg). All details on the calculation of these parameters have been published previously [34].

## Additional files

**Additional file 1: *Pichia pastoris* CBS 7435 *HEM* open reading frames.** The single nucleotide polymorphism of *HEM15*, T918, is marked in grey.

**Additional file 2: Co-overexpression of eGFP from P*AOX1*.** The principial applicability of the employed co-overexpression construct is demonstrated indirectly by eGFP fluorescence of the strains co-overexpressing HRP and eGFP. The first two bars represent the starting strain, *Pp*MutS, and the benchmark strain as controls. Fluorescence was determined at ex/em 488/507 nm, and normalized to the OD600 to account for potential growth differences.

**Additional file 3: Co-overexpressions of HRP with either *HEM1* or *HEM3* from either P*AOX1* or P*CAT1*.** 1, co-overexpression of *HEM1* or *HEM3* from P*AOX1*; 2, co-overexpression of *HEM1* or *HEM3* from P*CAT1*. White bars, eGFP co-overexpression control; black bars, *HEM1* co-overexpression; gray bars, *HEM3* co-overexpression. The benchmark strain was included in all cultivations and set to 100% as reference (first bars).

**Additional file 4: Batch cultivations of three recombinant *P. pastoris* strains.** 1, benchmark strain; 2, HEM1 strain; 3, HEM3 strain; a/c/e, CER signal (solid line) and specific methanol uptake rate (open circles); b/d/f, carbon dioxide yields ($Y_{CO2/S}$; grey squares) and biomass yields ($Y_{X/S}$; black circles).

### Abbreviations

ABTS: 2,2″-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; ALA: 5-aminolevulinic acid; *AOX1*: Alcohol oxidase 1 gene; CER: Carbon evolution rate; 96-DWP: 96-deep well plate; eGFP: Enhanced green fluorescent protein; HRP: Horseradish peroxidase; YNB: Difco™ yeast nitrogen base.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

FWK, SC, TV and OS designed the experiments, analyzed and interpreted the data. FWK, SC, MJ, MGE and TV performed the experiments. FWK, SC and OS wrote the manuscript. FWK, AG, CH and OS conceived of the study. AG, CH and OS supervised the research. All authors read and approved the final manuscript.

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# 6. Achievements and Novelties

In this section the different achievements derived by application of the described methodology are discussed.

In chapter I "The biopharmaceutical market" an overview of the biopharmaceutical market is provided encompassing blockbuster products and recent advances in terms of expression system, strain engineering, and production processes for the three main microbials *E. coli*, *S. cerevisiae* and *P. pastoris.* Therefore the main novelty of this paper consists in having provided an overview about the most recent advances for optimizing these three expression hosts for the expression of mAbs as alternative to mammalian cells.

In chapter II, "Avoiding methanol requirement" I describe a process engineering approach applied to replace methanol. Application of the designed mixed-feed strategy based on the concomitant feed of sorbitol and glycerol allowed not only to circumvent methanol requirement but it also proofed to be a valid approach to increase STY of rPLC. More than 2 folds increase in recombinant PLC productivity was achieved compared to what was obtained with glycerol as sole C-source. Therefore, the novelty of this study consists in having developed a mixed-feed strategy for a glycerol de-repressed pAOX promoter which allowed to circumvent methanol utilization without "sacrifying" final yields of the target protein. Additionally an innovative approach for the design of a mixed feed is provided based on dynamic experiments.

The third chapter of this Thesis "Reducing hyperglycosylation" consists of two different papers. In the first one an enzyme glycoengineering approach was applied with the main goal of reducing HRP hyperglycosylation. Two main achievements can be identified, first of all rational protein design proved to be a valid instrument to reduce HRP hyperglycosylation. Additionally this study allowed the identification of the most beneficial mutations to be introduced in a second round of site-directed mutagenesis. The novelty of this paper consists in having developed a glyco-variant library of the enzyme HRP and having generated a potentially unglycosylated enzyme variant by combining the "best" identified mutations for each N-glycosylation site. As described in the second paper the 4 most advantageous mutations, previously identified, were combined and the mutated HRP isoform was expressed in a *P. pastoris* glyco-engineered strain, thus integrating product and strain engineering. The so produced HRP isoform was less active and stable than both wild type enzyme and plant enzyme. However, when expressed in the benchmark strain the glycoengineered enzyme turned out to be significantly thermally stable. The novelty of this paper consists in having described an innovative approach based on the integration of product and strain engineering to produce a less glycosylated HRP isovariant with a more uniform glycosylation pattern.

In chapter IV, "Increasing product specific cofactor availability" two different approaches were applied, namely strain engineering and heme cofactor/precursor media supplementation with the aim of improving heme

availability as specific HRP cofactor. In contrast to previous studies, it was demonstrated that overexpression of single genes of the heme pathway (strain engineering) did not result in higher yields of recombinant active HRP. On the contrary a beneficial effect was found after hemin supplementation in the growth media, due to post-translational activation of the secreted apoHRP in the culture broth. The novelty of this paper consists in having identified an easy-to-do solution to maximize the conversion yield of the apo-enzyme to its holo form based on cofactor media supplementation.

Summarizing the major achievement of this Thesis consists in having tackled three main drawbacks associated to recombinant expression in *P. pastoris* by applying three different engineering approaches, namely process, strain and product engineering. Additionally integration of product and strain engineering represents an innovative approach for the production of a less hyperglycosylated HRP isoform with a more uniform glycan pattern, in *P. pastoris*.

# 7. Conclusions

Successful expression of many enzymes as well as of pharmaceutically relevant proteins has rendered the methylotrophic yeast *P. pastoris* an eminent expression host for recombinant protein production. However, although many basic elements of this expression system are now well developed there is still space for further optimization. Therefore in this Thesis I focused on three main drawbacks associated to recombinant expression in *P. pastoris* and applied three different engineering approaches to tackle them.

In order to avoid methanol requirement we speculated that design of a mixed feed strategy for a glycerol de-repressed pAOX1, as process engineering approach, could allow to circumvent methanol utilization and to reach final PLC productivity titers comparable the endogenous pAOX1 promoter driven expression.

- *Conclusion:* the applied methodological approach based on dynamic experiments for the design of a mixed-feed proved to be beneficial for increasing PLC final STY.

→ *Benefit*: the development of a bioprocess strategy based on the implementation of a de-repression strain in combination with a process engineering approach allowed avoiding methanol requirement.

In order to reduce hyperglycosylation, two different engineering approaches, namely product and strain glycoengineering, were applied firstly separated and then in an integrated mode. We speculated that both the approaches could be beneficial for the production of a less hyperglycosylated HRP isoform in *P. pastoris*.

- *Conclusion:* site-directed mutagenesis proved to be a fast method for generating a less glycosylated and more thermally stable HRP isovariant.

→ *Benefit:* the applied product (glyco)engineering approach proved to be a valid tool not only for reducing superficial hyperglycosylation but also for protein evolution.

In order to increase heme cofactor availability, we speculated that application of metabolic engineering and of cofactor media suplementation could result in an increased production of active protein.

- *Conclusion:* hemin addition allows to de-bottleneck heme cofactor availability and to increase final yields of active product.

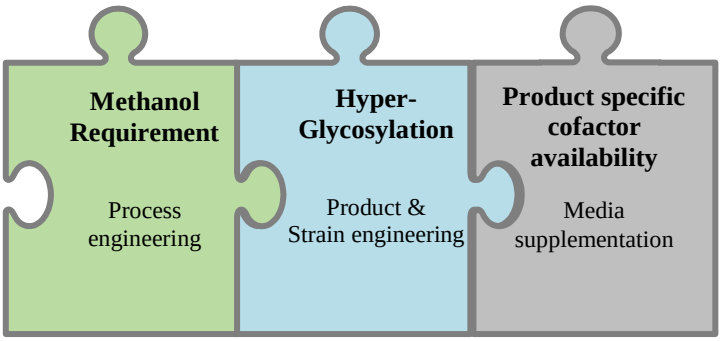
→ *Benefit:* description of an easy-to-do method for increasing successful recombinant production of active heme containing proteins in the yeast *P. pastoris*.

Therefore the main **benefit** of this Thesis consists in having provided three different strategies to be applied for respectively avoiding methanol requirement, reducing hyperglycosylation and increasing product specific cofactor availability.

Considering the mentioned benefits, the described methodology can be **extrapolated** in different directions. The described methodology for the design of a mixed-feed, as process engineering approach, could be easily applied for other de-repression strains. The applied protein glycoengineering approach could be used for a different glycosylated protein. We also proposed two different approaches to increase product specific cofactor availability therefore same strategy could be applied for a different cofactor.

# 8. Outlook

The applied methodology allowed to generate a knowledge foundation for the integration of the three described engineering approaches, namely process, product and strain engineering. Product and strain engineering were already integrated in this Thesis for the production of a more homogeneously and less glycosylated HRP isoform. Thus one first outlook would consist in the integration of the described process engineering approach with the main goal of replacing methanol as induction substrate, as shown in Scheme1.




Scheme1. Integration of the different described engineering approaches to tackle the previously identified hurdles.

In order to apply this integrated platform the most important aspect would be the goal definition. Assuming that the main goal would consist in the expression of an less hyperglycosylated heme containing protein displaying a more homogeneous glycosylation pattern by implementing a methanol-free bioprocess in *P. pastoris*, the following work-flow should be applied:

1. application of the described product (glyco)engineering approach, aimed at reducing surface glycan content but also at generating a mutated isovariant with increased activity and stability.
2. Expression of the mutated enzyme in a (glyco)engineered strain under a de-repressed promoter which does not requires methanol for induction.
3. Preliminary shakeflasks screening for checking strain viability and productivity.
4. Design of the most suitable fermentation strategy for the implemented de-repressed promoter. Therefore the same process engineering approach should be applied as described in this Thesis and implementation of dynamic experiments (batch with pulses and fed-batch) would allow a fast and reliable strain characterization in term of physiology and productivity.
5. Hemin supplementation in the growth media to increase final yields of the active secreted holoenzyme.

Finally although the described methodology allowed to achieve the main goal of this Thesis, different aspects could still be investigated such as

- the effect that different C-sources and different feeding regimes have effect on glycosylation;
- the effect that removal of all the HRP superficial N-glycosylation sites produced on the enzyme structure. Therefore crystallization of the unglycosylated enzyme would be highly desirable to verify possible changes at molecular level.

# Curriculum Vitae – Msc Simona Capone

**Personal Details**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**Academic Education**

| | |
|---|---|
| 12/08-02/12 | **Master study** in "Industrial Biotechnology", University of Milano-Bicocca, Milan. |
| 09/10-09/11 | **Master thesis** at the Institute for Biotechnology, University of Natural Resources and Life Sciences BOKU, Vienna. |
| 02/12 | **Master of Science** in "Industrial Biotechnology" graduated with distinction. |
| since 09/2012 | **Ph.D. Thesis** at the Research Division of Biochemical Engineering at the University of Technology VUT, Vienna, Austria. |

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**Training activities**

**Conferences**

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|---|---|
| 06/14 | Attendance at the international conference on "Bioprocess & Bioengineering" organized by OMICS Group as **speaker.** |
| 09/14 | Attendance at the "10th European Symposium on Biochemical Engineering Sciences & 6th International Forum on Industrial Bioprocesses (ESBES-IFIBIOP)" as **speaker.** |
| | Attendance at the "Microbiology & Biotechnology Meeting" organized by Pierre Fabre Research Institute in collaboration with School of Biotechnology KTH - Royal Institute of Technology, Stockholm as **speaker.** |

**Courses**

| | |
|---|---|
| 10/14 | Successful completion of the course **"Design of Experiments"** at the Umetrics Head Office, Basel, Swiss. |

**Academic Positions**

| | |
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| since 03/13 | **Teaching Assistant** in the laboratory course of "Chemical engineering" at the University of Technology VUT, Vienna, Austria. |

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**Summary of Technical Experience**

Biochemical, microbiological, standard techniques
Protein engineering
Bioprocess skills (planning, set up, data analysis)
Downstream processing
Design of Experiments (MODDE)

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**Scientific Projects**

2012 PhD student in the **Stand Alone Project** "Glycoengineered horseradish peroxidase for targeted cancer treatment" P24861-B19 funded by the Austrian Science Fund **FWF** (http://fwf.ac.at/asp/projekt_res.asp).

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**Publications**

Spadiut, O., et al., *Microbials for the production of monoclonal antibodies and antibody fragments.* Trends in Biotechnology, 2014. **32**(1): p. 54-60.

Capone, S., et al., *Development of a mixed feed strategy for a recombinant Pichia pastoris strain producing with a de-repression promoter.* Microbial Cell Factories, 2015. **14:101**.

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Krainer, F.W., et al., *Optimizing cofactor availability for the production of recombinant heme peroxidase in Pichia pastoris.* Microbial Cell Factories, 2015. 14: p. 4.