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Biotechnological advances towards an enhanced peroxidase production in *Pichia pastoris*

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ABSTRACT

Horseradish peroxidase (HRP) is a high-demand enzyme for applications in diagnostics, bioremediation, biocatalysis and medicine. Current HRP preparations are isolated from horseradish roots as mixtures of biochemically diverse isoenzymes. Thus, there is a strong need for a recombinant production process enabling a steady supply with enzyme preparations of consistent high quality. However, most current recombinant production systems are limited at titers in the low mg/L range. In this study, we used the well-known yeast *Pichia pastoris* as host for recombinant HRP production. To enhance recombinant enzyme titers we systematically evaluated engineering approaches on the secretion process, coproduction of helper proteins, and compared expression from the strong methanol-inducible P*AOX1* promoter, the strong constitutive P*GAP* promoter, and a novel bidirectional promoter P*HTX1*. Ultimately, coproduction of HRP and active Hac1 under P*HTX1* control yielded a recombinant HRP titer of 132 mg/L after 56 h of cultivation in a methanol-independent and easy-to-do bioreactor cultivation process. With regard to the many versatile applications for HRP, the establishment of a microbial host system suitable for efficient recombinant HRP production was highly overdue. The novel HRP production platform in *P. pastoris* presented in this study sets a new benchmark for this medically relevant enzyme.

1. Introduction

Horseradish peroxidase (HRP; EC 1.11.1.7) is a secretory plant peroxidase that has been studied for more than two centuries. Most commonly, HRP is employed as a reporter enzyme in diagnostic kits and histochemical assays (Krainer and Glieder, 2015; Krainer et al., 2016). More recent applications involve biocatalysis of polymerization reactions, biosensor and wastewater cleanup systems and enzyme-prodrug therapy for cancer treatment (Krainer and Glieder, 2015).

Despite the growing demand for a steady supply of considerable amounts of enzyme at consistent high quality, current HRP preparations are typically isolated from horseradish roots as isoenzyme mixtures. Up to 42 different isoenzymes have been detected by isoelectric focusing of commercial preparations (Hoyle, 1977). Recently, we published the sequences of 28 isoenzymes and found them to show diverging substrate profiles (Krainer et al., 2013a,b; Näätsaari et al., 2014). It has long been known that isoenzyme expression patterns vary and seem to depend on so far poorly understood environmental influences (Jermyn and Thomas, 1954), negatively affecting the consistency of the quality of isolates. Therefore, recombinant production of a single HRP isoform of choice with defined biochemical characteristics by a suitable host organism presents an attractive alternative. However, current recombinant production systems could not compete with the natural source so far, mainly due to limited enzyme titers. Already back in 1988,

Abbreviations: ABTS, 2,2′ azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; BSM, basal salts medium; BMD1%, buffered minimal dextrose medium; BMG1%, buffered minimal glycerol medium; BMGY, buffered minimal glycerol yeast medium; BMM, buffered minimal methanol medium; Ca-TBS, calcium-supplemented tris-buffered saline; DWP, 96-deep well plate; GRAS, generally recognized as safe; HRP, horseradish peroxidase; PTM1, trace element solution; *Rz, Reinheitszahl*; UPR, unfolded protein response; YE, yeast extract; YNB, yeast nitrogen base.

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production of recombinant HRP was reported in *Escherichia coli* (Chiswell and Ortlepp, 1988). Until now *E. coli* seems to be the preferred host for HRP production even though inactive enzyme requires inconvenient reactivation and refolding from inclusion bodies (Asad et al., 2013; Smith et al., 1990) and titers are low at <10 mg/L (Grigorenko et al., 1999). Owing to the presence of four disulfide bridges in active HRP (Gajhede et al., 1997 Welinder, 1976) and glycosylation of the native enzyme (Welinder, 1979), eukaryotic organisms might be more favorable production systems than bacterial ones. Apart from insect cell cultures (Segura et al., 2005) or horseradish hairy root cultures (Flocco et al., 1998) most prominent eukaryotic production hosts are yeast systems. HRP titers from *Pichia pastoris* were reported to be higher than from *Saccharomyces cerevisiae* but still in the lower mg/L range (Morawski et al., 2001, 2000), albeit under non-optimized production conditions. The lesser-known basidiomycete yeast strain *Cryptococcus* sp. S-2 however was shown to produce 110 mg/L (based on a volumetric activity of 171 U/mL) after 120 h of bioreactor cultivation (Utashima et al., 2014).

Major advantages of using *P. pastoris* as an expression platform are the well-established and fine-tuned handling and production protocols as well as the status of its products being generally recognized as safe (GRAS).[1] by the U.S. Food and Drug Administration. High titers of more than 18 g/L of secreted *Trichoderma reesei* cellulase were recently achieved in *P. pastoris* (Mellitzer et al., 2014) demonstrating the remarkable production capacities of this host. However, in order to adequately exploit these capacities for maximum yields of a target peptide, the system needs to be adjusted to meet the needs of the respective target: Regarding the nucleotide sequence of an expression target, codon optimization has been shown repeatedly to considerably affect production efficiency, *e.g.* (Abad et al., 2010b; Mellitzer et al., 2014).

In terms of transcript levels, the choice of a suitable transcription regulation system is mandatory for maximum output (Vogl et al., 2015). The tightly regulated and strongly inducible promoter of the alcohol oxidase 1 gene, P*AOX1*, is most commonly used in *P. pastoris*. It is strongly repressed in the presence of carbon sources such as glucose or glycerol and greatly induced upon addition of methanol as the sole carbon source (Vogl and Glieder, 2013). Inducible promoters allow protein production which is only initiated when cell growth is not mandatory any more, which is particularly advantageous for the production of demanding (*e.g.* cytotoxic) target proteins. On the downside, P*AOX1*-driven transcription requires an initial cell growth phase with glucose or glycerol and an induction phase with hazardous methanol. Alternatively, strong constitutive expression of a target gene *e.g.* by use of P*GAP* might overload the protein production machinery during growth and impose a considerable metabolic burden on the cells causing cell death and lysis. This might be particularly true when additional recombinant target genes are constitutively coexpressed simultaneously.

Most commonly, recombinant proteins from *P. pastoris* are efficiently secreted by fusing the *S. cerevisiae* mating factor alpha prepro signal peptide to the N-terminus. The pre peptide is processed in the endoplasmatic reticulum, the pro peptide is further cleaved by the peptidases Kex2 and Ste13 at an Arg-Lys site and in Glu-Ala repeats, respectively (Bourbonnais et al., 1988; Brake et al., 1984). It has to be noted that processing by Ste13 is of limited specificity, resulting in heterogeneous N-termini (Bitter et al., 1984). Recently, a deletion mutant ΔN57-I70 of the mating factor alpha prepro signal peptide was reported to increase volumetric HRP titers by almost 60% (Lin-Cereghino et al., 2013).

[1] https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=204&sort=GRN_No&order=DESC&startrow=1&type=basic&search=pichia%20pastoris (last accessed May 2016)

Proteins passing through the secretory pathway can be subjected to several modifications. Peptides forming disulfide bridges can interact with the protein disulfide isomerase, Pdi1, which acts as a chaperone and promotes formation of correct disulfide bridges. Hence, coexpression of *PDI1* is a common approach to enhance the titers of functional secretory proteins and has been performed repeatedly, *e.g.* (Gasser et al., 2006; Inan et al., 2006). Alternatively, upregulation of the unfolded protein response (UPR) can be achieved by overexpression of an active form of the transcription factor Hac1 (Valkonen et al., 2003). Under non-stress conditions, no active Hac1 is translated from unspliced *HAC1* mRNA due to attenuation. Initiation of an unconventional splicing event is necessary to allow translation of active Hac1 and was described in detail for *Saccharomyces cerevisiae* (Chapman and Walter, 1997; Kawahara et al., 1997; Kimata et al., 2007). Inducible coexpression of the homologous spliced *HAC1* gene of *P. pastoris* has been shown previously to augment the production of some but not all recombinant proteins (Guerfal et al., 2010; Vogl et al., 2014).

Another aspect to be considered when producing secreted proteins in yeast is peptide glycosylation. Yeast-type glycosylation is typically characterized by heterogeneous hypermannosylation which causes proteins to appear as smear in SDS-PAGE (*e.g.* (Dietzsch et al., 2011; Morawski et al., 2000)) and impedes downstream processing. We recently dealt with this issue by generating a *P. pastoris* strain with an inactivated key mannosyltransferase (Och1) allowing for considerably more homogeneously glycosylated HRP (Krainer et al., 2013a,b).

The aim of this study was to systematically evaluate a set of strategies to increase the titers of recombinant HRP from *P. pastoris*. Based on initial data from high-throughput screenings we performed bioreactor cultivations of the most promising strains and for the first time achieved HRP titers in the 100 mg/L range from *P. pastoris*. Also, this is the first description of a recombinant HRP preparation from *P. pastoris* of high-end purity, *i.e.* at a *Reinheitszahl* (*Rz*) of 3.

2. Materials and methods

2.1. Chemicals

2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), D(+)-biotin and hemin were obtained from Sigma-Aldrich (Austria). Difco™ yeast nitrogen base w/o amino acids (YNB), Bacto™ tryptone and Bacto™ yeast extract (YE) were purchased from Becton Dickinson (Austria). Glanapon DG 160 defoamer was from Bussetti & Co (Austria). Zeocin™ was obtained from InvivoGen (France). Other chemicals were purchased from Carl Roth (Germany).

2.2. Strains and vectors

P. pastoris strains were based on the wildtype strain CBS 7435 (identical to NRRL Y-11430 and ATCC 76273). We previously found a strain with Mut^S phenotype to be superior over the Mut$^+$ phenotype for HRP production (Krainer et al., 2012). Therefore, all HRP production strains were based on a strain with *Δaox1:FRT* mutation (*Pp*MutS) (Näätsaari et al., 2012). In order to obtain homogeneously glycosylated HRP we used a strain with an additional *Δoch1:FRT* mutation (*Pp*FWK3) as described recently (Krainer et al., 2013a,b).

For P*AOX1*-regulated HRP production, a pPpT4_alpha_S-based vector (Näätsaari et al., 2012) was used (Lin-Cereghino et al., 2005), resulting in zeocin™ resistant transformants and HRP secretion with the full length *S. cerevisiae* mating factor alpha prepro signal peptide. Among the currently known 28 HRP isoenzymes (Näätsaari et al., 2014), the isoform C1A has been described as the